Plant Physiol. (1974) 54, 846–851

Enzymatic Esterification of Indole-3-acetic Acid to *myo*-Inositol and Glucose[1]

Received for publication April 26, 1974 and in revised form July 11, 1974

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ABSTRACT

Incubation of mature sweet corn kernels of *Zea mays* in dilute solutions of ^{14}C-labeled indole-3-acetic acid leads to the formation of ^{14}C-labeled esters of *myo*-inositol, glucose, and glucans. Utilizing this knowledge it was found that an enzyme preparation from immature sweet corn kernels of *Zea mays* catalyzed the CoA- and ATP-dependent esterification of indole-3-acetic acid to *myo*-inositol and glucose. The esters formed were 2-*O*-(indole-3-acetyl)-*myo*-inositol, 1-DL-1-*O*-(indole-3-acetyl)-*myo*-inositol, di-*O*-(indole-3-acetyl)-*myo*-inositol, tri-*O*-(indole-3-acetyl)-*myo*-inositol, 2-*O*-(indole-3-acetyl)-D-glucopyranose, 4-*O*-(indole-3-acetyl)-D-glucopyranose and 6-*O*-(indole-3-acetyl)-D-glycopyranose. An assay system was developed for measuring esterification of ^{14}C-labeled indole-3-acetic acid by ammonolysis of the esters followed by isolation and counting the radioactive indole-3-acetamide.

Kernels of sweet corn (*Zea mays*) contain 60 to 80 mg/kg of esterified indole-3-acetic acid and about 5% of that amount as free IAA (13). Of the esterified IAA, one-half consists of esters of IAA and *myo*-inositol or *myo*-inositol glycosides (13, 14, 15), and one-half consists of IAA esterified to cellulosic glucans (12, 13). There are, in addition, small amounts of esters of IAA and glucose (5).

We wish to report on the *in vivo* and *in vitro* synthesis of the esters of IAA. Dried, mature kernals of corn incubated with solutions of ^{14}C-IAA synthesize IAA esters. An enzyme preparation from immature kernels, in an ATP- and CoA-dependent reaction, synthesizes axial and equatorial IAA-inositols,[3] $(IAA)_2$-inositol, $(IAA)_3$-inositol, and the 2-*O*-, 4-*O*-, and 6-*O*-esters of IAA-glucose. There are no prior publications on the enzymatic synthesis of these compounds.

[1] This work was supported, in part, by the National Science Foundation (GB 18353-X and GB-40821-X). Scintillation counting equipment was provided by the United States Atomic Energy Commission, Contract AT-(11-1)-1338. Journal Article No. 6793 from the Michigan Agricultural Experiment Station.

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[3] Abbreviations: inositol: *myo*-inositol; BSTFA: N,O-Bis-(trimethylsilyl)-trifluoroacetamide; DMF: dimethylformamide; IAAEt: ethyl indole-3-acetate; IAM: indole-3-acetamide; MO: methoxime; TMCS: trimethylchlorosilane; TMS: trimethylsilyl.

MATERIALS AND METHODS

All materials were as previously described (1). Esters of IAA-glucose and IAA-inositol, used as standards, were isolated from *Zea mays* and had been characterized chemically and by GLC-mass spectrometry (4–6, 15). IAA-2-^{14}C was obtained from Schwarz/Mann, Orangeburg, New York 10962.

Samples were dried at 12 mm Hg at a bath temperature of 50 C. Solvent composition is v/v. TLC, detection of TLC spots, and elution of samples from TLC plates was as previously described (1, 5, 6). Silylation, GLC, and Dowex 50W-X2 (200–400 mesh) chromatography have been described (3). Radioactivity was determined by scintillation counting in Bray's solution. Protein was determined by the biuret reaction (16).

RESULTS

IN VIVO STUDIES

Ester Purification. Mature dry kernels of *Zea mays* var. Stowells' Evergreen hybrid sweet corn (50 g) were soaked 24 hr at 23 C in 15 ml of water containing 1.5 mg of ^{14}C-IAA (2.0×10^7 cpm). The kernels were washed (1.2×10^6 cpm were lost in the wash water) then ground in 70% acetone-water in a mortar and pestle. Three extractions and grindings were made with 70% acetone over a 24-hr period using a total volume of 650 ml. Following filtration, the acetone extract contained 1.4×10^7 cpm. This was evaporated to dryness and redissolved in 20 ml of water. The solution contained 1.1×10^7 cpm, and the residue contained 3×10^6 cpm. The soluble fraction was chromatographed on Dowex 50W-X2 (200–400 mesh) column (3). Esters of IAA emerged between 220 and 660 ml. These tubes were pooled, dried, and dissolved in 2 ml of 50% ethanol-water. The fraction was then chromatographed on a 48 ml bed volume Sephadex LH-20 column, using a flow rate of 5.5 ml/hr and 50% ethanol-water as eluent (4–6). The esters were eluted between 66 and 93.5 ml. The pooled, dried ester fraction was chromatographed on silica gel thin layer plates yielding a chromatogram similar to the guide strip of Figure 1 as determined by spraying a strip with Ehrlich's reagent.

IAA-inositols. The IAA-inositols (area E-F, Fig. 1) and IAA-inositol glycosides (area B-D) were eluted and dried. The dried esters were ammonolyzed, using 4 ml of 15 N NH_4OH for 20 min at 23 C, and the resultant IAM was extracted into ether (3×5 ml). These conditions result in a yield of 50% amide and 50% IAA from the esters hydrolyzed (5). Ether was removed *in vacuo*, and the amides were dissolved in 200 μl of 50% ethanol-water plus 1.2 ml of 0.1 N HCL. The acid-ethanol amide solution was applied to a 0.5 cm i.d. 3-ml bed volume Dowex 50W-X2 (200–400 mesh) column using a flow

rate of 0.1 ml/min. The column was washed with 1 to 2 ml of water and eluted with 2 M NH_4OH. IAM is eluted between 12 to 18 ml as monitored by TLC. The IAM containing tubes were pooled and dried, and the IAM was acetylated with 50 μl of acetic anhydride and 50 μl of pyridine for 1 hr at 100 C. The acetylated amide was then chromatographed on a 1.8 m × 6 mm glass column containing 5% SP-2401 on 100/120 mesh Supelcoport. At 180 C, and with a N_2 carrier gas flow of 60 ml/min, the retention time for IAM is 7.3 min. The IAM peak (see example in Fig. 3) was collected, and the amount of IAM was determined by a micro-modification of the Salkowski assay (1). For the IAA-inositol region, the amount of IAM collected was 0.8 μg and the radioactivity was 28 cpm and thus the specific radioactivity was 35 cpm/μg. Since the amount of IAA-inositols in 50 g of kernels is known to be about 750 μg, one may calculate that there has been a $2.6 \times 10^4 \times 10^2/2.0 \times 10^7 = 0.1\%$ incorporation of the applied radioactivity into the IAA inositol fraction.

IAA-inositol Glycosides. For the IAA-inositol-glycoside fraction (Sectors B to D of Fig. 1), 2.2 μg of amide were collected containing 21 cpm. The specific radioactivity is 10 cpm/μg, and since there is about 750 μg of IAA-inositol-glycosides in 50 g of kernels, there would be a 0.03% incorporation of radioactivity into the IAA-inositol-glycoside fraction.

IAA-glucans. Incorporation into the A fraction, which has previously been shown to be IAA esterified to a glucan (12), was determined in a similar manner. The IAA-glucan was prepurified by repeated solution in 70% acetone-water and precipitation by the addition of water. Upon drying, ammonolysis, acetylation, and chromatography, the specific activity of the acetylated amide was found to be 10 cpm/μg. Using analogous methods, the trimethylsilyl derivative of the amide was also prepared and subjected to GLC (3) and yielded a specific radioactivity of 13 cpm/μg. Since there would be about 1.5 mg of IAA-glucan in 50 g of kernels, one may calculate $11.5 \times 1500 \times 100 = 1.8 \times 10^6/2.0 \times 10^7 =$ approximately a 0.1% incorporation into the IAA glucan fraction.

Other Products. Several products of IAA metabolism that do not yield free IAA or IAM upon ammonolysis have been observed, but not characterized. They are, presumably, oxidation products of IAA. There are three major groups of compounds, A, B and C, eluting from the Dowex column between 121 to 176 ml, 220 to 660 ml, and 660 to 825 ml. The A group is not Ehrlich reactive and upon TLC yields radioactivity at ($R_{IAA} = 1$) 0 to 0.25, 0.45 to 0.65, and 0.8 to 1.0. The B group elutes at 66 to 93.5 ml from LH-20, is not Ehrlich reactive and on TLC has an R_{IAA} of 0.35 to 0.45. The C group yields an unstable pink color with Ehrlich and has an R_{IAA} of 0.60 to 0.75. These compounds have not been further characterized.

Extractability of ^{14}C-labeled IAA. Earlier work by Hemberg (7) indicated that incubation of corn kernels with IAA, under the conditions described above, led to extensive conversion of IAA into bound forms and it was this work which led us to the present experiments. As shown above, however, the bulk of the IAA remains unesterified and a small amount is oxidized. Thus Hemberg's "bound" IAA most likely represents IAA that has soaked into the kernel and is no longer extractable by ether. It is extractable by acetone-water and chromatographically appears to be IAA.

IN VITRO STUDIES

Enzyme Preparation. Ears of fresh sweet corn were purchased from local markets. Kernels were cut from the cob, chilled, and 50 g of kernels were homogenized in a cold mortar and pestle for 5 min with 50 ml of 0.05 M tris buffer at pH 7.6. The homogenate was strained through cheesecloth and centrifuged at 1 C for 5 min at 10,000*g*. The supernatant fluid, designated stage I enzyme, may be used immediately or stored frozen at −20 C. For certain experiments, stage I enzyme was centrifuged for 1 hr at 90,000*g* yielding enzyme at stage II. This, followed by dialysis for 24 hr against 0.05 M tris at 4 C, yielded stage III enzyme, 5.4 mg protein/ml (16).

Incubation and TLC. Two incubation mixtures were prepared each containing, in μmoles, CoA, 1; ATP, 80; *myo*-inositol, 80; $MgCl_2$, 80; 2-^{14}C-IAA, 0.17 (containing 8×10^6 cpm) in a volume of 4.0 ml. To tube A were added 4 ml of enzyme, stage I, and to tube B, 4 ml of boiled enzyme. Incubation of both tubes was for 1 hr at 37 C. The reaction was stopped by adding 19 ml of acetone and precipitated protein was removed by centrifugation for 5 min at 10,000*g*. The precipitate was washed with 20 ml of 70% acetone-water and, following centrifugation, the supernatant fluids were combined and dried in a flash evaporator. The dried residue was dissolved in 0.5 ml of 50% ethanol-water, and 0.1 ml of this was chromatographed, as a band, on a 20 × 20 cm silica gel TLC plate. Following development, guide strips were sprayed with Ehrlich's reagent, and the plate was divided into the appropriate 1- to 3-cm sectors for elution. Figure 1 shows the distribution of radioactivity on the plate together with a semi-diagrammatic sketch of the position of the various IAA esters. As can be seen, the tube containing unboiled enzyme shows significant activity in the region of the IAA-inositols and the region containing $(IAA)_2$-, and $(IAA)_3$-inositols and the various IAA-glucoses. More ^{14}C-IAA remains in the boiled enzyme tube (shaded area).

Isolation of Enzymatically Synthesized ^{14}C-IAA-inositols. Silica gel from sector E to F of the plate was eluted and, ap-

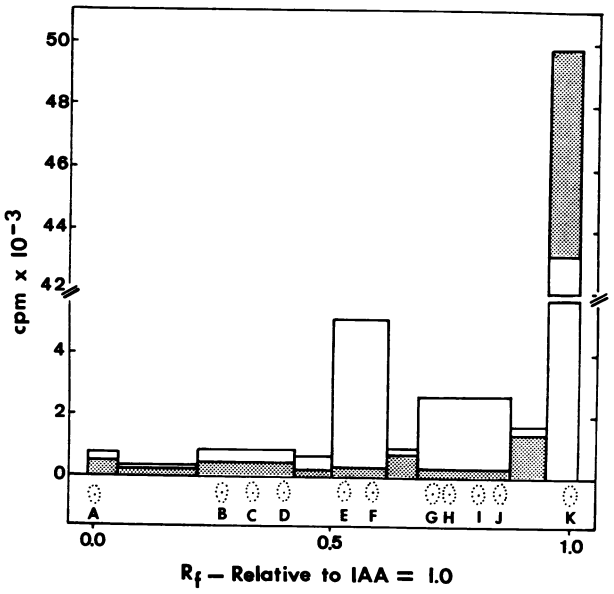

FIG. 1. Silica Gel G TLC of 70% acetone-water soluble products of enzyme reaction mixture. Clear areas represent radioactivity in the complete mixture and shaded areas that in the boiled enzyme control. Migration was in ethyl acetate-methylethylketone-ethyl alcohol-water (5:3:1:1) and is expressed relative to the migration of IAA = 1. Radioactivity was determined by scraping the gel from the plate and suspending in scintillation liquid. The guide spots are: A: IAA-glucan; B, C, D: IAA-inositol-glycosides; E, F: IAA-inositols; G, H, I, J: mixtures of 2-*O*-, 4-*O*-, and 6-*O*- esters of IAA-glucose and di-, and tri-IAA-inositols; K: IAA.

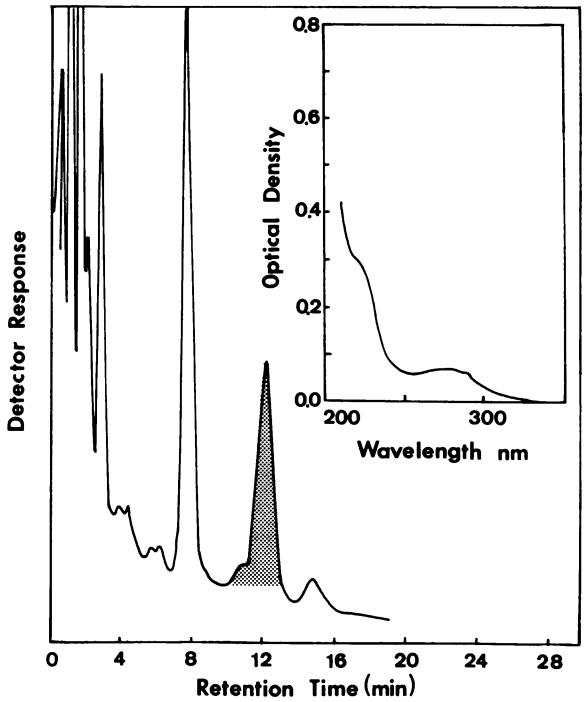

FIG. 2. Tracing of GLC chart showing hexa TMS-IAA-inositols. The compound emerging during the time indicated by shading was collected for counting. The poor, but recognizably indolylic, absorption spectrum is shown in the insert. The shaded peak is an equatorial ester, probably, 1-DL-1-*O*-(indole-3-acetyl)-*myo*-inositol, and the shoulder on that peak is another equatorial IAA-*myo*-inositol ester. The peak at about 8 min is the hexa TMS 2-*O*-(indole-3-acetyl)-*myo*-inositol and is also radioactive.

proximately, 20 μg of an isomeric mixture of IAA-inositols were added, as carrier. The mixture was evaporated, and the residue from tube A contained 144,000 counts, whereas that from tube B contained 6000 counts. The residues were silylated for GLC (3), in 20 μl of DMF plus 50 μl of BSTFA containing 1% TMCS for 0.5 hr at 50 C, and chromatographed on a 1.8 m $\times$ 6 mm glass column containing 5% SP-2401 on 100/120 mesh Supelcoport at 230 C with 60 ml/min of nitrogen as carrier gas. The retention profile is shown in Figure 2. Under these conditions the retention times for 2-*O*-(indole-3-acetyl)-*myo*-inositol is 7.9 min and for 1-DL-1-*O*-(indole-3-acetyl)-*myo*-inositol is 12.1 min. The material emerging in the peak indicated by shading was collected from tube A and found to contain 44,000 cpm, while that from tube B contained no radioactivity. The UV spectrum of the material collected in the peak is shown in the inset of Figure 2. Similar results are obtained with the 2-*O*-ester, as might be expected, since the esters interconvert by acyl migration (4, 9, 13).

Ammonolysis of ^{14}C-IAA-inositols. That the radioactive substances eluted from the E to F sector of the plate were IAA esters was demonstrated by ammonolysis and isolation of the amide. A TLC plate identical to that of Figure 1 was prepared, and the E to F sector again was eluted. To this was added 50 μg of IAM, as carrier, and the solution was evaporated to dryness. Ammonolysis was with 4 ml of 15 N NH_4OH, during 20 min, at 23 C. These conditions yield about 50% IAM and 50% IAA. The eluted sector of the plate contained 105,000 counts and extraction of the ammonolysis mixture with 3 $\times$ 5 ml of ether yielded 55,600 cpm from tube A and 24 cpm from tube B. The ether was evaporated to dryness, and the residue was dissolved in 200 μl of 50% ethanol-water plus 1.2 ml of 0.1 M HCl. The amides were absorbed to a 3 ml bed volume column of Dowex 50W-X2 (200–400 mesh) resin using a flow rate of 0.1 ml/min. The column was washed with 1 to 2 ml of water and developed with 2 M NH_4OH and the IAM eluted between 12 to 18 ml. This material was evaporated to dryness, and IAM was acetylated with 50 μl of pyridine plus 50 μl of acetic anhydride for 1 hr at 100 C. GLC was as described above at 180 C. The material emerging in the area of the peak indicated by shading was collected for counting. Radioactivity in these fractions is shown in Table I and the GLC and UV absorption spectrum of the collected amide is shown in Figure 3.

Experiments with Higher R_F Components. The ^{14}C-labeled IAA esters chromatographing in the G to J region of the plate of Figure 1 constitute a complex array of indoleacetyl esters including 2 isomeric $(IAA)_2$ inositols, 2 isomeric $(IAA)_3$ inositols (6) and the 2-*O*-, 4-*O*-, and 6-*O*-esters of IAA-glucose (5). The spot at the R_F of $(IAA)_2$ inositol would include the 6-*O*-ester of glucose and 1-*O*-ester, if present. The spot at the R_F of $(IAA)_3$ inositol includes the 4-*O*- and 6-*O*-esters of glucose. We first ammonolyzed the mixture to determine total esterified

Table I. *Radioactivity in Amides Prepared by Ammonolysis of IAA-inositols Found in Sector E to F of Thin Layer Plate of Fig. 1*

One fifth of the total incubation mixture was chromatographed.

Fraction	Radioactivity	
	Enzyme	Boiled enzyme
	cpm	
Eluate from sector E to F	105,200	5960
In ether after ammonolysis	55,605	24
Dowex 50 amide fraction	24,360	0
GLC-IAM peak	21,280	0

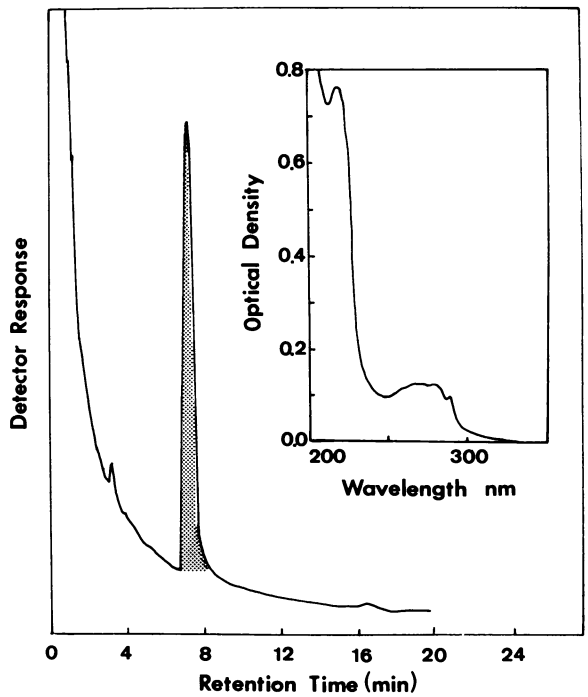

FIG. 3. Tracing of GLC chart showing acetylated indole-3-acetamide. The shaded area was collected for counting and the UV spectrum of the collected material is shown in the inset.

Table II. *Radioactivity in Amides Prepared by Ammonolysis of G to J Region of Thin Layer Plate of Fig. 1*

One-fifth of the incubation mixture described in text was utilized.

Fraction	Radioactivity	
	Enzyme	Boiled enzyme
	cpm	
Eluate from sector G to J	28,185	8580
In ether after ammonolysis	4,125	642
Dowex 50, amide fraction	3,040	0
GLC-IAM peak	2,490	0

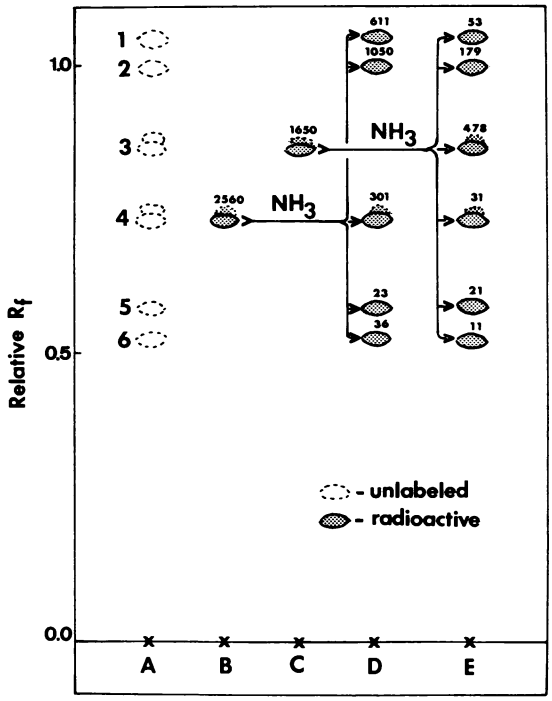

FIG. 4. Silica gel TLC of the $(IAA)_2$-inositols and $(IAA)_3$-inositols and the products formed upon partial hydrolysis. A: standard, 1 = IAA ethylester, 2 = IAA, 3 = 2 isomeric forms of $(IAA)_3$-inositol, 4 = 2 isomeric forms of $(IAA)_2$-inositol, 5 = 1-DL-1-*O*-(indole-3-acetyl)-*myo*-inositol, 6 = 2-*O*-(indole-3-acetyl)-*myo*-inositol; B: authentic 4 plus enzymatically produced putative 4; C: authentic 3 plus enzymatically produced putative 3; D: hydrolysis products of the material eluted from spot B; E: hydrolysis products of the material eluted from spot C. The R_F is relative to that of IAA = 1.0 in the solvent used for Fig. 1. Radioactivity (cpm) is indicated above each spot. Visualization is with Ehrlich's reagent.

IAA and then added unlabeled authentic standards of each of these compounds to permit reisolation as described below.

IAM Formation from High R_F Components. Utilizing the method described for the IAA-inositols, a dried aliquot of the eluate from G to J region was ammonolyzed, chromatographed on Dowex 50W-X2 (200–400 mesh), acetylated and subjected to GLC. As seen in Table II, radioactive IAM is formed from the products of tube A. Thus the (poly-IAA)-inositols and/or IAA glucose had been synthesized.

Radioactivity in $(IAA)_2$ inositol and $(IAA)_3$ inositol. Partial ammonolysis was used to demonstrate that at least part of the radioactivity in the $(IAA)_2$ inositol and the $(IAA)_3$ inositol was IAA esterified to a cyclitol. We have previously shown (6) that hydrolysis in weak NH_4OH results in the partial conversion of the (poly IAA)-inositols to a mixture of the monoacylated inositols, IAA, and IAAEt. Hydrolysis of an IAA-glucose ester would yield only glucose, IAA, and IAAEt.

The data of Figure 4 demonstrate that partial hydrolysis of the radioactive esters co-chromatographing with $(IAA)_2$ inositol and $(IAA)_3$ inositol leads to the formation of IAA-inositol, thus proving that $(IAA)_2$ inositol and $(IAA)_3$ inositol are labeled. Authentic standards were mixed with the unknown in amounts sufficient for detection with Ehrlich's reagent.

Labeling of 2-*O*-, 4-*O*-, and 6-*O*-IAA-glucose. To an aliquot of ^{14}C-labeled esters from the high R_F region were added approximately 2 μg each of the 2-*O*-, 4-*O*-, and 6-*O*-esters of IAA-glucose, and the mixture was dried. The dried residue was dissolved in 50 μl of pyridine, and 100 μg of methoxylamine-HCl were added. The solution was reacted at 80 C for 2 hr in a Teflon-lined, screw-capped vial, thus converting glucose esters with a free reducing group to the methoxime derivatives (5, 10). BSTFA (80 μl) was then added to the mixture and heating was continued for 20 min at 80 C to form the penta TMS methoximes of IAA-glucose (5). As seen in Figure 5, the methoximes of 2-*O*-, 4-*O*-, and 6-*O*-IAA-glucose can now be separated from each other by GLC and from the 1-*O*-IAA-glucose ester. The shaded areas of the peaks, as well as the area where the 1-*O*-IAA-glucose would have emerged, were it present, were collected. The radioactivity of areas 1, 2, 3, and 4 were 48, 32, 26, and 1 cpm. Thus the 2-*O*-, 4-*O*-, and 6-*O*-esters have been labeled.

Experiments with IAA-inositol Glycosides. Figure 1 (B–D) shows a small amount of radioactivity in the IAA-inositol glycoside fraction in excess of that in the boiled enzyme control. The B to D region would contain the 3 isomeric IAA-inositol arabinosides and the 3 isomeric IAA-inositol galactosides (6, 15). The difference in radioactivity appears significant, but amide formation from esters in this region of the plate has not been studied.

Factors Affecting IAA Esterification *in vitro*. The data of

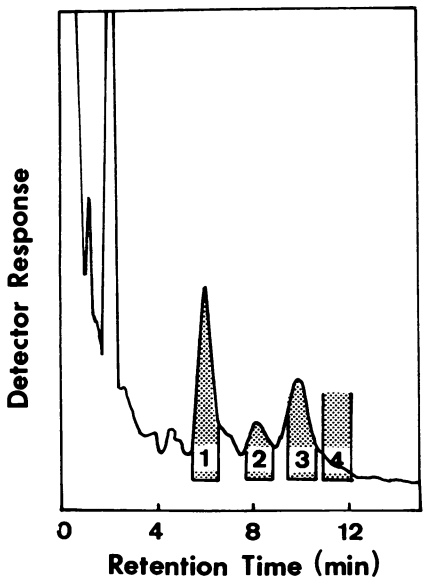

FIG. 5. Tracing of GLC chart of derivatives of IAA-glucose. The column is 3 mm × 1.3 m containing 0.65 m of OV-1, 2% and 0.65 m of 5% SP-2401 on Supelcoport. Carrier gas flow is 45 ml/min at 212 C. The shaded areas are those collected for counting. Peaks 1, 2, and 3 are the MO-TMS derivatives of 4-*O*-, 2-*O*-, and 6-*O*-IAA glucose, respectively. The area marked 4 is where TMS-1-*O*-IAA-glucose would have emerged.

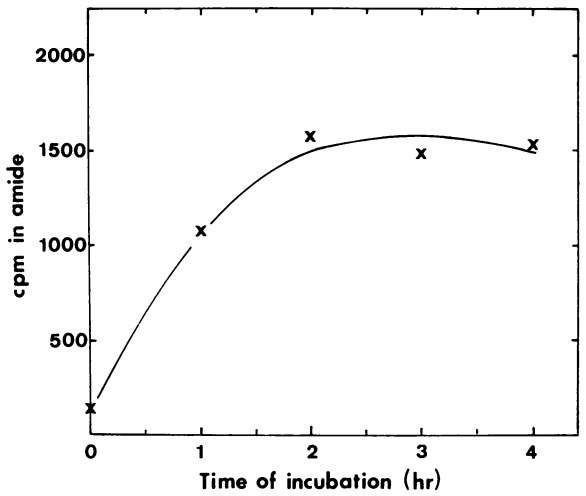

FIG. 6. Synthesis of IAA esters as a function of time.

Table III. *Radioactivity in Amides Prepared by Ammonolysis of Esters Produced during 1 Hr Incubation*

Reaction Mixture	Radioactivity			
	Expt. I		Expt. II	
	cpm	% control	cpm	% control
Complete, undialyzed enzyme	3106		2620	
Complete, dialyzed enzyme	1602	100	1428	100
Without ATP	210	13	268	19
Without CoA	230	14	244	17
Without inositol	428	27	336	24
Without inositol plus glucose	1138	71	816	57
Complete, zero time	124	8		
Complete, boiled enzyme	0	0		

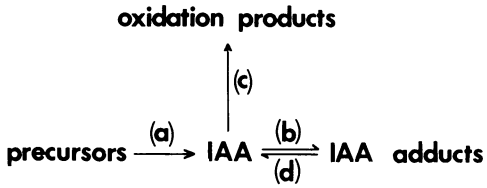

FIG. 7. Metabolic reactions that might regulate *in vivo* hormonal concentrations.

Figure 1 and the sections following demonstrate that the esterified ^{14}C-IAA is present in an array of IAA-inositol and IAA-glucose esters. They show further that a boiled enzyme control does not form compounds from IAA that yield ^{14}C-IAM upon ammonolysis. Thus, a convenient assay for IAA esterification may be based upon formation of ^{14}C-IAM and this assay is used in the following experiments. Incubation mixtures, unless otherwise indicated, contained in μmoles: CoA, 0.065; ATP, 5; *myo*-inositol, 5; $MgCl_2$, 5; glutathione, 5; and ^{14}C-IAA, 0.01 (5.0×10^5 cpm) in a volume of 0.3 ml. To this was added 0.3 ml of enzyme, and incubation was for 1 hr at 37 C. Reactions were terminated by the addition of 3 ml of acetone, and the precipitate formed upon centrifugation for 5 min at 10,000*g* was washed with 2 ml of 70% acetone-water. The dried supernatant fluids were combined and taken up in 50% ethanol-water. Ammonolysis and purification of the amide were exactly as described above. The amide containing fractions from the Dowex column, as detected by TLC, were pooled, dried, and counted. The radioactivity then represents about one-half of the total esterified ^{14}C-IAA in the incubation mixture.

Effect of Age of Kernels. An attempt was made to determine whether stage I enzyme, prepared from the relatively younger small kernels at the silk end of the ear, was more active than that prepared from full size kernels, at the stalk end of the ear. Enzyme was prepared as described above. A zero time control, in which acetone was added immediately after enzyme was added to the incubation mixture yielded 82 cpm in IAM. Enzyme from younger kernels gave 3910 cpm in IAM and enzyme from older kernels gave 4320 cpm so, over this age interval, there is no difference in enzyme activity.

Enzyme Solubility. A stage I enzyme preparation was centrifuged at 90,000*g* in an SW-25.1 rotor for 1 hr resulting in separation into a yellow lipid layer, a clear solution, and a white pellet. The pellet was resuspended in 30 ml of buffer and centrifuged again as above. The pellet was then again resuspended in the original volume of buffer so that a comparison might be made of activity in the lipid layer, the clear solution layer, and particulate material. Upon incubation and isolation of ^{14}C-IAM, 3650, 5470, 880, and 540 cpm were found, respectively, in IAM formed by uncentrifuged enzyme, the clear solution layer, the yellow lipid layer, and the particulate preparation. Thus, the enzyme does not sediment at 90,000*g* during 1 hr.

Dependency upon Enzyme Concentration. Stage III enzyme was used to establish the dependency of ester formation upon enzyme concentration. Incubation was with no enzyme, 0.15 ml, or 0.3 of enzyme. Radioactivity in IAM was 0, 810, and 1400 cpm, respectively.

Ester Synthesis as a Function of Time. Enzyme of stage III was used and incubation was at 37 C for time intervals of up to 4 hr. The data of Figure 6 show that the amount of ester increases during the first 2 hr of incubation and then remains essentially constant.

Cofactor Dependency of Ester Formation. Enzyme of stage III was used to determine the cofactor dependency of the reaction. As can be seen in the data of Table III, dialysis results in an approximately 50% loss of enzyme activity. Omission of ATP or CoA results in about an 85% decrease in ester formation. Omission of inositol results in a 70% loss of ester formation and addition of glucose, instead of inositol, restores activity to 70% of that of the dialyzed control.

CONCLUSIONS

Our prior studies (1, 13, and R.S. Bandurski, unpublished) have demonstrated that "bound" auxins are the predominant form in both seeds and vegetative tissue of *Zea, Avena,* and *Pisum.* Thus, the mechanism of biosynthesis and of hydrolysis of these compounds becomes of importance in regulating hormone concentrations. Our concept of reactions that might regulate hormonal levels is shown in Figure 7. Both hydrolysis (d) and synthesis (b) of IAA adducts might be regulated. Evidence for enzymatic hydrolysis of IAA-esters has previously been presented (1).

The stimulation of IAA ester formation by ATP and CoA suggests that the reaction proceeds by formation of adenyl-IAA, followed by formation of CoA-IAA, and then acylation of the cyclitol or glucose. Monoacyl inositol could then be diacylated by a second mole of IAA-CoA, the reactions being analogous to those involved in acylation of glycero-3-phosphate. Earlier work by Leopold (11) produced evidence for IAA-CoA thiol ester formation. Zenk has studied the chemical synthesis of IAA-CoA and adenyl-IAA (19) and the biosynthesis of IAA-glycine (17).

Zenk (20) has suggested, without published data, that biosynthesis of the 1-*O*-ester between IAA and glucose involves UDPG in a reaction analogous to esterification of nicotinic acid and glucose as described by Jacobelli *et al.* (8). We observed formation of only the 2-*O*-, 4-*O*-, and 6-*O*-esters of IAA and glucose. Thus, it is likely that biosynthesis of the 1-*O*-ester (18) is a mechanistically different reaction. Since acyl migration occurs readily in the case of the IAA-inositols and IAA-glucose (5, 6, 9), we are uncertain which isomeric forms are synthesized first.

LITERATURE CITED

1. Bandurski, R. S. and A. Schulze. 1974. Concentrations of indole-3-acetic acid and its esters in *Avena* and *Zea*. Plant Physiol. 54: 257–262.
2. Corner, J. J. and T. Swain. 1965. Enzymatic synthesis of the sugar esters of hydroxy-aromatic acids. Nature (London) 207: 634–635.
3. Ehmann, A. and R. S. Bandurski. 1972. Purification of indole-3-acetic acid *myo*-inositol esters on polystyrene-divinylbenzene resins. J. Chrom. 72: 61–70.
4. Ehmann, A. 1973. Indole compounds in seeds of *Zea mays*. Ph.D. thesis. Michigan State University, East Lansing.
5. Ehmann, A. 1974. The isolation of 2-*O*-(indole-3-acetyl)-D-glucopyranose, 4-*O*-(indole-3-acetyl)-D-glucopyranose and 6-*O*-(indole-3-acetyl)-D-glucopyranose from mature sweet corn kernels of *Zea mays*. Carbohyd. Res. 34: 99–114.
6. Ehmann, A. and R. S. Bandurski. 1974. The isolation of di-*O*-(indole-3-acetyl)-*myo*-inositol and tri-*O*-(indole-3-acetyl)-*myo*-inositol from mature kernels of *Zea mays*. Carbohyd. Res. In press.
7. Hemberg, T. 1955. Studies on the balance between free and bound auxin in germinating maize. Physiol. Plant. 8: 418–432.
8. Jacobelli, G., M. J. Tabone, and D. Tabone. 1958. La synthèse enzymatique de l'ester β glucosidique de l'acide anthranilique: Ses relations avec les processus de synthèse et de dégradation de l'uridine diphosphate glucose. Bull. Soc. Chim. Biol. 40: 955–963.
9. Labarca, C., P. B. Nicholls, and R. S. Bandurski. 1965. A partial characterization of indoleacetylinositols from *Zea mays*. Biochem. Biophys. Res. Commun. 20: 641–646.
10. Laine, R. S. and C. C. Sweeley. 1973. *O*-Methyl oximes of sugars. Analysis as *O*-trimethylsilyl derivatives by gas-liquid chromatography and mass spectrometry. Carbohyd. Res. 27: 199–213.
11. Leopold, A. C. and F. S. Guernsey. 1953. A theory of auxin action involving coenzyme A. Proc. Nat. Acad. Sci. U.S.A. 39: 1105–1111.
12. Piskornik, Z. and R. S. Bandurski. 1972. Purification and partial characterization of a glucan containing indole-3-acetic acid. Plant Physiol. 50: 176–182.
13. Ueda, M. and R. S. Bandurski. 1969. Quantitative estimation of alkali-labile indole-3-acetic acid compounds in dormant and germinating maize kernels. Plant Physiol. 44: 1175–1181.
14. Ueda, M., A. Ehmann, and R. S. Bandurski. 1970. Gas-liquid chromatographic analysis of indole-3-acetic acid myoinositol esters in maize kernels. Plant Physiol. 46: 715–719.
15. Ueda, M. and R. S. Bandurski. 1974. Structure of indole-3-acetic acid *myo*-inositol esters and pentamethylmyo-inositols. Phytochemistry 13: 243–253.
16. Zamenhof, S. 1957. Preparation and assay of deoxyribonucleic acid from animal tissue. Methods Enzymol. 3: 700.
17. Zenk, M. H. 1960. Enzymic activation of auxins and their conjugation with glycine. Z. Naturforsch. 15: 436–441.
18. Zenk, M. H. 1961. 1-*O*-(Indole-3-acetyl)-beta-D-glucose, a new compound in the metabolism of indole-3-acetic acid in plants. Nature (London) 191: 493–494.
19. Zenk, M. H. 1961. Darstellung und Eigenschaften aktivierter Verindungen der Indol-3-essigsaure. Naturwissenschaften 48: 455–456.
20. Zenk, M. H. 1964. Isolation, biosynthesis and function of indole acetic acid conjugates. Colloq. Intern. Centre Natl. Rech. Sci. Paris. 123: 241–249.