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BIOSYNTHESIS OF ECDYSTEROIDS IN *ZEA MAYS*

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Key Word Index—*Zea mays*; Poaceae; maize; phytoecdysteroids; phytosterols; ecdysteroid phosphates; 20-hydroxyecdysone; ecdysone.

Abstract—The incubation of several radiolabelled substrates with *Zea mays* plants demonstrated the incorporation of radiolabels into ecdysteroid conjugates. Radiolabelled [^{14}C]ecdysone and [^{14}C]20-hydroxyecdysone, were biosynthesized from [2-^{14}C]mevalonic acid (MVA) in *Z. mays*. These ecdysteroids can be released from their conjugates by treatment with wheat germ acid phosphatase or mild acid hydrolysis. The C-24 reduced side chain sterols, lathosterol and cholesterol were endogenously biosynthesized from [2-^{14}C]MVA and were identified by ^{1}H NMR. Lathosterol accounts for *ca* 0.5% of the total sterol composition, whereas cholesterol is present at 2.5%. However, lathosterol was consistently found to have a specific activity three to six times that of cholesterol. Incubation of [4-^{14}C]cholesterol with *Z. mays* leaves also demonstrated incorporation into a 20-hydroxyecdysone conjugate. Incubation of [22,23-^{3}H]ecdysone with *Z. mays* demonstrated incorporation into radiolabelled ecdysone diphosphate and ecdysone polyphosphate, which were cleaved upon acid phosphatase treatment. *Z. mays* biosynthesizes primarily ecdysteroid conjugates and does not appear to produce detectable levels of non-conjugated ecdysteroids.

INTRODUCTION

Ecdysteroid conjugates have been identified in numerous plant systems [1, 2] often associated with non-conjugated analogues [3, 4]. These compounds are usually reported as mono- or di-substituted side chain or nuclear conjugates [3]. Conjugates in plants are reported at C-22 as *O*-benzoates, sulphates, acetates [5–7] and C-25 glucopyranosides and 3-acetate glucopyranoside [8]. The di-conjugates are often reported as 2-, 3- or 20,22-monoacetonides and 3,22-*O*-digalactosides [9–11]. Recently, ecdysone-3-phosphate was demonstrated as an intermediate during biosynthesis of ecdysteroids in spinach [4]. The production of ecdysteroid polyphosphates was also demonstrated in spinach [4], and these polyphosphate conjugates appear to act as negative feedback regulatory compounds on the endogenous ecdysteroid biosynthesis in spinach [3, 12]. Since low levels of polyphosphorylated ecdysteroids apparently down-regulate the biosynthesis of ecdysteroids, any plant producing primarily polyphosphorylated ecdysteroids could be expected to biosynthesize only low levels of these products. Presumably, such plants would also biosynthesize little if any non-conjugated ecdysteroid. Zea mays possesses 20-hydroxyecdysone polyphosphate and ecdysone polyphosphate at the nanogram per gram fresh weight level and we were unable to identify any non-conjugated ecdysteroids produced by this plant.

RESULTS

Incorporation of [2-^{14}C] *mevalonic acid into ecdysteroid polyphosphates*

The incorporation of [2-^{14}C] mevalonic acid (MVA) into nine-day-old *Z. mays* plants (15 μCi total exposure) produced similar profiles at either 6, 12 or 24 hr post [^{14}C] incubation (Fig. 1A). The majority of the radiolabel chromatographed with an $\alpha_e = 0.50$ and was found in the early fractions (4 and 5) of the RP-HPLC chromatograph (Fig. 1A). The α_e values (K' sample/K' 20-hydroxyecdysone) were calculated by a standard method as previously described [13]. Since compounds found early in the chromatographic profile appeared to be polar conjugates of ecdysteroids in *Spinacia* [4], these early fractions were investigated for the presence of ecdysteroid phosphate conjugates in *Z. mays*. The peak of radioactivity observed in the RP-HPLC profile with an $\alpha_e = 0.50$ (fractions 4 and 5, Fig. 1A) was isolated by repeated RP-HPLC and treated with wheat germ acid phosphatase (WGAP). Levels of $0.9–1.4 \times 10^6$ cpm in fractions 4 and 5 were consistently isolated from the 24 hr incorporations of [2-^{14}C]MVA. The RP-HPLC isolated fractions ($\alpha_e = 0.50$) were subjected to WGAP treatment

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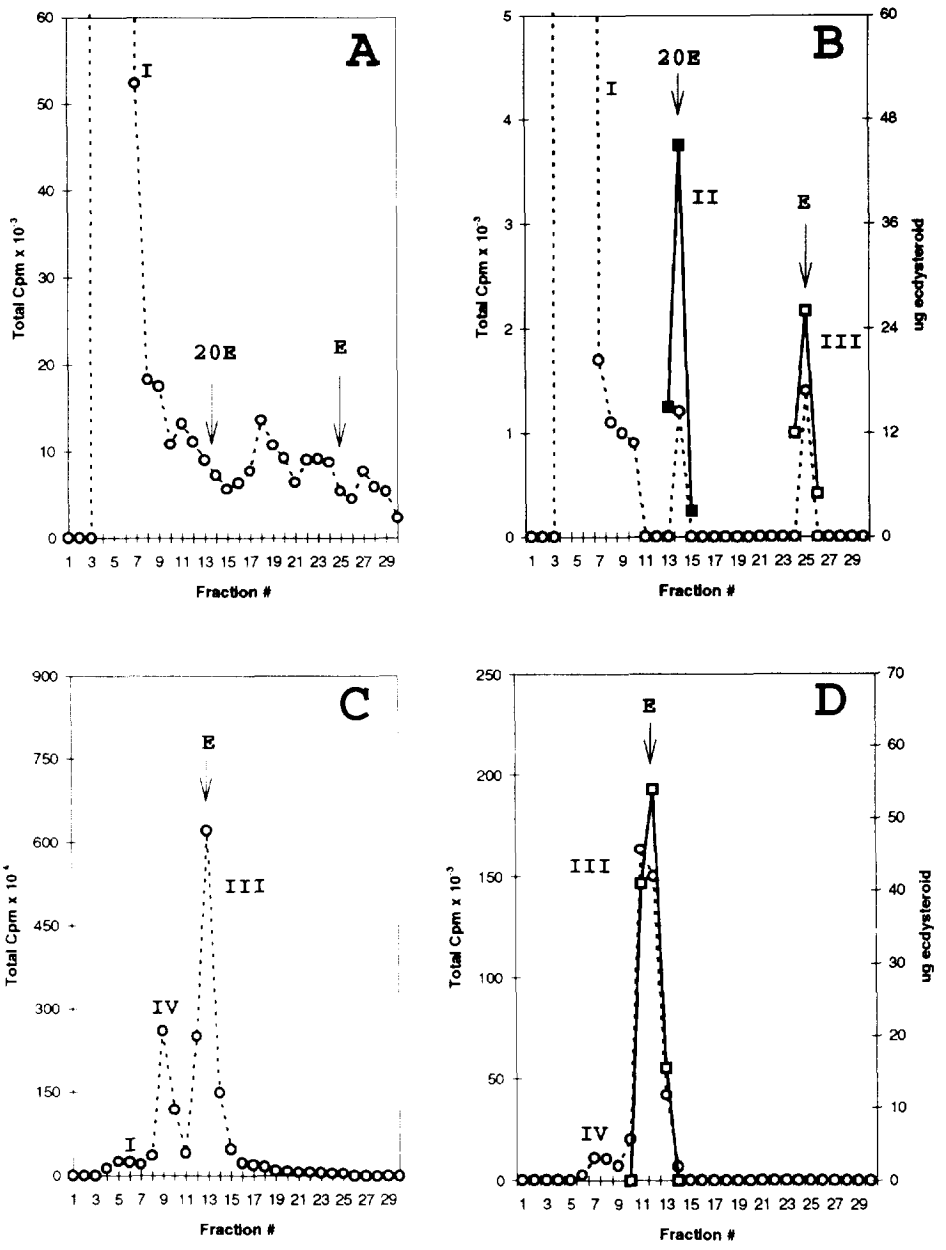

Fig. 1. Incorporation profiles by RP-HPLC versus total cpm (---) from incubations with [2-^{14}C]MVA and [22,23-^{3}H] ecdysone with 9-day-old *Z. mays*. The arrow designation 20E (20-hydroxyecdysone) or E (ecdysone) indicates the fractions in which authentic standards chromatograph for these elution profiles. See text for α_e description. (A) Incorporation of [2-^{14}C]MVA into the butanol fraction 24 hr post stem uptake of radiolabel. Total cpm in the offscale peak I ($\alpha_e \leq 0.50$) is 1.4×10^6. (B) Profile of the butanol fraction obtained post wheat germ acid phosphatase treatment of the isolated peak I from panel A. Peak II (■) is cold carrier 20-hydroxyecdysone mass and peak III (□) is cold carrier ecdysone mass. Total cpm in the offscale peak I is 2×10^5. RP-HPLC solvent MeOH–H_2O (2:3) at 0.75 ml min^{-1} for panels A and B. (C) Incorporation of [22,23-^{3}H]ecdysone into the butanol fraction 48 hr post stem uptake. Radiolabel peak III corresponds to ecdysone, peak IV to ecdysone diphosphate, and peak I to ecdysone polyphosphate. Altered solvent mix for panels C and D compared to panels A and B. (D) Profile of the butanol fraction obtained post wheat germ acid phosphatase treatment of the isolated peak IV ($\alpha_e = 1.14$) from panel C. Peak III cpm chromatographs with cold carrier ecdysone mass (□). RP-HPLC solvent MeOH–H_2O (9:11) at 0.75 ml min^{-1} for panels C and D.

after the addition of 100 μg each of 20-hydroxyecdysone and ecdysone as carrier ecdysteroids. Following WGAP treatment the RP-HPLC profile was consistently observed to produce two peaks of radiolabel in the [^{14}C] profile, which co-chromatographed with 20-hydroxyecdysone and ecdysone (Fig. 1B, peaks II and III). The fractions corresponding to peak II (Fig. 1B) were subjected to TLC, and the region corresponding to 20-hydroxyecdysone ($R_f = 0.45$) was extracted and rechromatographed by RP-HPLC. This 20-hydroxyec-

dysone isolated by TLC retained the radiolabel. Similar data could be obtained from surface application of [2-^{14}C]MVA to *Z. mays* leaves. However, stem uptake of the radiolabel gave the most consistent results in nine-day-old plants.

Analysis of sterol composition

Since the Δ^7-analogue of cholesterol, lathosterol, is the preferred sterol precursor to the ecdysteroids in spinach [14], the sterols biosynthesized by corn were examined. Several studies on *Z. mays* sterols [15–18] report the presence of sitosterol, stigmasterol, campesterol, cholesterol, avenasterol and 22-dihydrospinasterol. The sterols from 40 g of dried six-week-old leaves were subjected to TLC to separate the 4-desmethylsterols ($R_f = 0.18$), the steryl esters ($R_f = 0.84$) and polar steryl conjugates ($R_f = 0.0$–0.10). The composition of the 4-desmethylsterol content of *Z. mays* leaves, as determined by GLC, RP-HPLC and ^{1}H NMR analyses, are presented in Table 1. The previously reported sterols sitosterol, stigmasterol, 24-methylcholesterol and cholesterol were confirmed by these analyses and isofucosterol was identified. The 24-methylsterol in this *Z. mays* strain is 22-dihydrobrassicasterol (24β-methyl) as compared to the campesterol (24α-methyl) previously reported [15]. The mixed 24-desalkylsterol fraction was isolated by repeated separation by RP-HPLC and analysed by GLC and ^{1}H NMR. The GLC, RP-HPLC and ^{1}H NMR spectroscopy confirmed the presence of lathosterol (cholest-7-en-3β-ol) as well as cholesterol (cholest-5-en-3β-ol). Post TLC the steryl esters were likewise analysed by GLC and RP-HPLC after saponification. The same sterols were present in the steryl ester pool. When the steryl ester mass is compared to the total 4-desmethylsterol present in a saponified aliquot of corn extract, which possessed originally free sterol and steryl conjugates, the percentage of sterol conjugated is determined and is presented in Table 1. Thus, 83% of the sitosterol was non-conjugated free sterol and 17% of the sitosterol was present as sitosteryl ester (Table 1). The sitosterol, however, makes up only 39% (Table 1) of the total sterol composition of the plant as conjugated and non-conjugated sterol. The large percentage of lathosterol occurring as fatty acid ester (94%, Table 1) is substantially different from that of the other sterols found in *Z. mays*. No identifiable sterols could be isolated from the steryl polar conjugate region of the TLC chromatogram ($R_f = 0.0$–0.10) in these studies.

Incorporation of [2-^{14}C]MVA into sterols

The incorporation of [2-^{14}C]MVA into the *Z. mays* sterols is presented in Table 1. The aerial portions of 32 plants which were nine days old were incubated with 21 μCi of [2-^{14}C]MVA for 24 hr and 48 hr. The sterols were isolated by RP-HPLC, through repeated injection and collection of RP-HPLC fractions. The specific activity of lathosterol was higher than that of cholesterol and the 24-alkylsterols at both 24 and 48 hr post MVA incorporation, but appeared to decrease over the time course (Table 1). The other sterols were all radiolabelled at three- to six-fold less than lathosterol, with a slight increase in specific activity observed over the time course for cholesterol and the 24-alkylsterols with the exception of isofucosterol. Isofucosterol is a proposed biosynthetic precursor to other C-24 ethylsterols [19] and, therefore, this decrease would be expected.

Incorporation of [4-^{14}C]cholesterol into ecdysteroid polyphosphates

In order to determine if the 24-desalkylsterols serve as biosynthetic intermediates to the ecdysteroid polyphosphates, [4-^{14}C]cholesterol was incubated with *Z. mays*

Table 1. 4-Desmethylsterols in *Zea mays* From mass analysis* and following [2-^{14}C]MVA incorporation†

Sterol‡	Total sterol (mg)*	Percentage of total sterol*	Percentage of sterol conjugated§	Specific activity (cpm μg^{-1})†	
				24‖	48‖
Δ^7-Sterol					
Lathosterol	0.032	0.5	94	19 000	14 700
Δ^5-Sterols					
Cholesterol	0.16	2.5	24	3670	5000
22-Dihydrobrassicasterol	1.0	16.0	10		
Stigmasterol	1.4	21.0	11	2210¶	3600¶
Sitosterol	2.6	39.0	17	3650	5780
Isofucosterol	1.4	21.0	11	5970	4540

* 4-Desmethylsterols from 40 g sample of 6-week-old *Z. mays* leaves separated by TLC post-saponification.

† Radiolabel incorporated into 32 9-day-old *Z. mays* plants.

‡ Compounds identified by GLC, RP-HPLC and ^{1}H NMR.

§ Conjugated sterol esters determined by separating esterified sterols on TLC plates, saponification, and analysis by GLC and RP-HPLC. Compared to total free sterols for percentage of conjugation (see text).

¶ 22-Dihydrobrassicasterol and stigmasterol reported as combined specific activity.

‖ Duration radiolabel was incubated with the plants (in hr).

leaves. Since the 24-desalkylsterol in spinach, lathosterol, serves as a biosynthetic percursor to the 24-desalkyl ecdysteroids in spinach [14], experiments incubating [4-^{14}C]cholestrol with *Z. mays* were performed to determine if the ecdysteroid polyphosphates could be radiolabelled from a 24-desalkylsterol precursor. [4-^{14}C]Cholesterol (6 μCi per leaf) was applied to the surface of leaves of nine-day-old *Z. mays* in 0.001% Tween 20 solution and allowed to incubate for either 24, 48 or 60 h. At 48 and 60 hr 82 and 87% of the total radiolabel recovered in the butanol fraction, which contained ecdysteroids and ecdysteroid conjugates, was present on the RP-HPLC chromatogram with an $\alpha_e = 0.50$ (fractions 4 and 5) similar to the profile observed in Fig. 1A. No incorporation of [^{14}C]cholesterol was observed in the butanol fraction at 24 hr. The [^{14}C]-labelled fractions 4 and 5 ($\alpha_e = 0.50$) from the 60 hr incubation were isolated by repeated RP-HPLC and subjected to wheat germ acid phosphatase. The subsequent products of WGAP treatment were subjected to TLC and the region which co-chromatographed with 20-hydroxyecdysone and ecdysone was analysed by RP-HPLC. The [^{14}C] profiles so obtained were similar to those observed in Fig. 1B. Analysis of the 48 hr incubation in a similar manner using WGAP for one analysis and a mild acid hydrolysis (Experimental) for a second analysis also demonstrated release of [^{14}C] labelled 20-hydroxyecdysone from this RP-HPLC polar peak $\alpha_e = 0.50$ from the butanol fraction.

Incorporation of [22,23-^{3}H]ecdysone into ecdysteroid polyphosphates

To examine further the production of ecdysteroid polyphosphates in *Z. mays*, [22,23-^{3}H]ecdysone was incubated with nine-day-old plants via stem uptake. The RP-HPLC profile of the radiolabel in the butanol fraction is presented in Fig. 1C. The radiolabelled peak III (Fig. 1C) co-chromatographed with carrier ecdysone and was unmetabolized substrate. The radiolabelled peaks I ($\alpha_e = 0.50$, fraction 5) and IV ($\alpha_e = 1.14$, fraction 9) in Fig. 1C were isolated by repeated injection and collection from the RP-HPLC column. The isolated peak IV was subjected to WGAP treatment and subsequently re-analysed by RP-HPLC. The WGAP hydrolysis of peak IV released most of the radiolabel as the [^{3}H]ecdysone peak III (Fig. 1D), which co-chromatographed with carrier ecdysone. Some radiolabel remained associated with peak IV (Fig. 1D) post WGAP. The chromatographic evidence concerning peak IV suggests that it was an ecdysone di-phosphate since the chromatographic behaviour of the monophosphate of ecdysone (ecdysone-3-phosphate, $\alpha_e = 1.40$) is known [4]. Thus, the $\alpha_e = 1.14$ observed for peak IV is correct for the monophosphorylation of two hydroxyl groups on the ecdysone. However, a pyrophosphate analogue cannot be excluded at this juncture.

Several isolations and hydrolyses were performed on peak IV and peak I isolated from the RP-HPLC profile in Fig. 1C. Some of the results of these hydrolyses are presented in Table 2. Typically, when WGAP treatment of peak IV was performed the level of radiolabelled [^{3}H]ecdysone released was 50–75% of the total radiolabel recovered post-hydrolysis. The remainder resided in unconverted peak IV (Table 2). When peak I (Fig. 1C) was isolated and subjected to hydrolysis either by WGAP or mild acid the level of [^{3}H]ecdysone recovered was substantially less (2–11%) (Table 2) than that recovered by hydrolysis of peak IV. Interestingly, acid hydrolysis of peak I ($\alpha_e = 0.50$) produced a radiolabelled peak which chromatographed as peak IV ($\alpha_e = 1.14$) as well as [^{3}H]ecdysone (Table 2). The WGAP treatment of peak I ($\alpha_e = 0.50$), however, only released [^{3}H]ecdysone (Table 2). The lower conversion

Table 2. Wheat germ acid phosphatase and acid hydrolysis of conjugates of [22,23-^{3}H]ecdysone from *Z. mays*

Hydrolysis type	Cpm in fraction before hydrolysis: $\alpha_e = 0.50 \pm 0.10$ (peak I)‡	Cpm in fraction before hydrolysis: $\alpha_e = 1.14 \pm 0.05$ (peak IV)‡	Total cpm recovered post-hydrolysis§	Cpm recovered post-hydrolysis (% of cpm recovered post-hydrolysis): $\alpha_e = 0.50 \pm 0.10$	Cpm recovered post-hydrolysis (% of cpm recovered post-hydrolysis): $\alpha_e = 1.14 \pm 0.05$	Cpm recovered post-hydrolysis (% of cpm recovered post-hydrolysis): (ecdysone)¶
WGAP*		3.6×10^6	1.21×10^6		0.34×10^6 (28%)	0.86×10^6 (71%)
WGAP*		1.5×10^6	1.12×10^6		0.57×10^6 (51%)	0.55×10^6 (49%)
WGAP*	3.7×10^5		0.94×10^5	0.92×10^5 (98%)		0.02×10^5 (2%)
Acid hydrolysis†	1.2×10^5		0.09×10^5	0.05×10^5 (56%)	0.03×10^5 (33%)	0.01×10^5 (11%)

* WGAP – Wheat germ acid phosphatase.

† Acid hydrolysis performed with 0.5 M HCl. Sample incubated at room temperature for 24 hr.

‡ Isolated from incubations similar to those reported in Fig. 1C (see text).

§ Total cpm recovered post enzyme and chemical hydrolysis, post Sep-Pak separation and post RP-HPLC.

¶ Free ecdysone released from conjugates post WGAP and acid hydrolysis.

rate of peak I into [^{3}H]ecdysone with WGAP and the production of radiolabelled peak IV and [^{3}H]ecdysone upon acid hydrolysis suggest that peak I ($\alpha_e = 0.50$) was a polyphosphate of [^{3}H]ecdysone. This polyphosphate was apparently not as easily cleaved by WGAP as by acid. However, the WGAP treatment did not cause as great a loss of radiolabel when the pre-assay versus post-assay recovery (Table 2) of radiolabel was compared between the two treatments. Since some moieties of the ecdysteroid structure are acid labile, this decreased recovery is not unexpected. If a similar chromatographic analysis of the α_e value for peak I ($\alpha_e = 0.50$) is performed using the chromatographic fractions generated from ecdysone, ecdysone-3-phosphate and peak IV (ecdysone diphosphate), then peak I ($\alpha_e = 0.50$) must minimally be a tetraphosphorylated ecdysone. It could, however, be a mixture of various phosphate analogues, since ecdysone has five hydroxyl groups available for phosphorylation or pyrophosphorylation.

Incorporation of non-conjugated ecdysteroids into conjugated ecdysteroids

The [22,23-^{3}H]ecdysone was incubated for 48 hr with *Z. mays* plants that were one to five weeks of age, as well as with-six-week-old leaves. In all cases, 57–75% of the radiolabel recovered post-incubation was present as unmetabolized [^{3}H]ecdysone. The [^{3}H]ecdysone diphosphate ($\alpha_e = 1.14$) contained 17–30% of the radiolabel at all six ages and a component with $\alpha_e = 1.40$, that was chromatographically similar to ecdysone-3-phosphate, was produced at 1–8% of total radiolabel recovered from aerial portions of plants during weeks one to five. The polyphosphorylated [^{3}H]ecdysone ($\alpha_e = 0.50$) was present at detectable levels during weeks 1, 4, 5 and 6 at levels of 4, 3, 1 and 2% of the total radiolabel recovered, respectively. Analysis of the six-week-old plants was also performed by TLC. Four major bands of radiolabel were detected on the TLC plate and these were subsequently chromatographed by RP-HPLC. The TLC band with $R_f = 0.60$ chromatographed with ecdysone on RP-HPLC and possessed 57% of the radiolabel recovered from these plants. All of this [^{3}H]-label chromatographed with ecdysone. In addition, three major bands of radiolabel were discovered from these plants with R_f values of 0.41, 0.30 and 0.19, and these bands possessed 7, 15 and 15% of the total radiolabel recovered, respectively. Of the radiolabel recovered for each of these bands, *ca* 75–82% of the radiolabel chromatographed on RP-HPLC with an $\alpha_e = 1.14$. This suggests that the ecdysone diphosphate RP-HPLC peak $\alpha_e = 1.14$ may have been composed of a mixture of diphosphate isomers of ecdysone. Similarly, these same three bands also possessed radiolabelled components which chromatographed on RP-HPLC with peak 1 ($\alpha_e = 0.50$). This also suggests that the RP-HPLC peak I ($\alpha_e = 0.50$) was a mixture of polyphosphates of ecdysone. These data from six-week-old *Z. mays* adds support to the evidence that the components isolated by RP-HPLC as peak 1 ($\alpha_e = 0.50$) and peak IV ($\alpha_e = 1.14$) were most probably mixtures of di- and polyphosphates of ecdysone. In a similar experiment, [^{14}C]-radiolabelled 20-hydroxyecdysone, biosynthesized in spinach from [2-^{14}C]MVA and subsequently purified, was incubated with nine-day-old *Z. mays* plants. After 24 hr incubation only [^{14}C]-labelled 20-hydroxyecdysone could be re-isolated from the plants. However, at 48 hr post-incubation both [^{14}C]-labelled 20-hydroxyecdysone and a peak I chromatographing with $\alpha_e = 0.50$ were detected. This peak I ($\alpha_e = 0.50$) did not have sufficient radiolabel incorporated to process the sample further. The majority of the evidence to date suggests that this peak is also a mixture of polyphosphates of 20-hydroxyecdysone.

DISCUSSION

The endogenous biosynthesis of ecdysteroids as polyphosphates by *Z. mays* is supported by several lines of evidence. Primary among these is the ability to isolate radiolabelled ecdysone and 20-hydroxyecdysone from incubations of [2-^{14}C]MVA with *Z. mays*. These components are isolated following hydrolyses of the radiolabelled polar fractions ($\alpha_e = 0.50$, RP-HPLC) (Fig. 1A and B). The [2-^{14}C]MVA also radiolabelled lathosterol (Table 1), a known ecdysteroid precursor sterol in *Spinacia* [14]. The high specific activity observed in lathosterol in *Z. mays* decreasing with time post-incorporation, suggests that lathosterol is either being metabolized to some other steroidal components such as the ecdysteroid polyphosphates or that the decrease is due simply to a dilution effect from the increasing mass of lathosterol with time. It is unlikely that much lathosterol is being converted into cholesterol, a very possible fate for lathosterol, since the cholesterol specific activity was substantially lower than that for lathosterol (Table 1). If lathosterol were being converted into cholesterol at an appreciable rate the specific activity of cholesterol should increase with time accompanied by a concomitant drop in specific activity for lathosterol. Lathosterol is also unlikely to proceed to the 24-alkylsterols since lathosterol already possesses a reduced side chain. [^{14}C]Cholesterol is known to proceed to the ecdysteroids in spinach in spite of the fact that spinach does not biosynthesize any cholesterol [14]. In *Z. mays* [4-^{14}C]cholesterol can be metabolized to 20-hydroxyecdysone polyphosphate. If lathosterol were the preferred substrate to ecdysteroid biosynthesis in corn, as it is in spinach, and cholesterol is only converted after longer time periods of incorporation as a less than optimal substrate as previously suggested [14], then corn may also convert cholesterol into its ecdysteroid polyphosphates. The less than optimal incorporation of [4-^{14}C]cholesterol into the ecdysteroid polyphosphates in corn is supported by the fact that cholesterol can only be converted into these products after a substantial incubation time (48–60 hr). This is similar to the time observed in spinach for cholesterol conversion into ecdysteroids [14]. Thus, lathosterol could be the preferred substrate to the ecdysteroid polyphosphates in *Z. mays* but cholesterol could also serve this function. This slow decrease in radiolabel content in

lathosterol from 24 to 48 hr (Table 1), the observation that 94% of lathosterol is present as steryl fatty acid esters, and the knowledge that corn makes primarily ecdysteroid polyphosphates, which are known negative feedback inhibitors in spinach [4], suggest that the rate of biosynthesis of ecdysteroid polyphosphates in corn is slow because of the down-regulatory effect of the end products produced. This down-regulation could produce the sequestration of lathosterol into the steryl ester pool and subsequently the slow release of this lathosteryl ester for additional biosynthesis of ecdysteroid polyphosphates. This type of regulation could explain the high level of [^{14}C] incorporation observed in lathosterol from [2-^{14}C]MVA. The biosynthesis of low levels of ecdysteroid polyphosphates is also supported by the low levels of incorporation of [2-^{14}C]MVA into corn ecdysteroids as demonstrated by their release from ecdysteroid polyphosphates (Fig. 1B).

The metabolism of [22,23-^{3}H]ecdysone by *Z. mays* suggests that a mixture of diphosphate isomers as well as polyphosphate isomers are biosynthesized (Fig. 1, C and D, and Table 2). The levels of these polyphosphorylated components biosynthesized by *Z. mays* is very low (ng g^{-1} fr. wt), and proof of the radiolabelled incorporation into these compounds can only be concluded after extensive chromatographic isolation and purification of the carrier ecdysteroids utilized. This low level of ecdysteroid polyphosphate biosynthesis is expected. In spinach the polyphosphorylated ecdysteroids appear to be involved in a negative feedback loop which down-regulates ecdysteroid biosynthesis from [2-^{14}C]MVA [4, 12]. Since no free ecdysteroids could be detected in *Z. mays* either by mass or radiolabelled methods it is suggested that these polyphosphates also have a similar regulatory role in corn. Thus, only very low levels of biosynthesis of phosphorylated ecdysteroids would be expected since a high level of production would be inhibitory to the pathway. These data also suggest that the enzymology or regulation of the biosynthetic pathway is different in *Z. mays* and *Spinacia*, since the latter produces high levels of non-conjugated ecdysteroids (40 μg g^{-1} fr. wt) and *Z. mays* produces no detectable quantities of these. This regulatory inability to produce free ecdysteroids might help to explain the apparent dissimilarities observed in closely related species to produce uniformly the same types of secondary defence compounds.

EXPERIMENTAL

Corn (*Z. mays*) line B-73 was supplied by CIBA Agricultural Biotechnology Research Laboratories and was grown in the laboratory on a 12 hr light–12 hr dark cycle for 9 days. Stem uptake of [2-^{14}C]MVA soln was performed by cutting the plants at the soil line and allowing dissolution by uptake through evapotranspiration. The metabolism of [22,23-^{3}H]ecdysone was similarly tested with excised plants at 1-week intervals from 9 days to 5 weeks. The 6-week-old [22,23-^{3}H]ecdysone metabolism study was performed with excised 4th leaves, which are the 2nd youngest leaves on the plants. The [2-^{14}C]MVA DBED salt was purchased from Amersham (CFA 484). The [22,23-^{3}H] α-ecdysone and [4-^{14}C]cholesterol were purchased from New England Nuclear (NEC-018 and NET-621). Radiolabel substrates were exposed to cut aerial portions of the plant in H_2O for MVA and C_7H_8–H_2O (1:5) for incorporation of ecdysone. Radiolabel cholesterol was applied to the surface of corn 1st leaves in EtOH–H_2O (1:1) with 0.001% Tween 20. Plant material was extracted in MeOH for 24 hr, pulverized and re-extracted. Extracts were evapd *in vacuo* at 40°, redissolved in 70% aq. MeOH, and partitioned (×4) against equal vols of pre-equilibrated hexane. The aq. MeOH residue was redissolved in H_2O and partitioned (×4) against equal vols of BuOH; ecdysteroid polyphosphates and ecdysteroids were analysed only from the BuOH extract. Analyt. RP-HPLC with a Supelco C_{18} column, MeOH–H_2O (9:11) at 1 ml min^{-1} at 33° was used with a Perkin Elmer LC-75 spectrophotometric detector and an LCI-100 integrator to quantitate ecdysteroids. Standard curves with correlation coefficients of 0.99 were determined at 254 nm over 4 orders of magnitude of ecdysteroid (25 ng–25 μg). Radiolabel counting was performed on a Beckman LS 1801 liquid scintillation counter using Bio-Safe cocktail [20, 21]. For the mass extraction used to identify the sterols in corn, the 4-desmethylsterols were isolated in pure form by prep. TLC and repeated injection and collection by RP-HPLC, from the hexane fr. isolated from 100 g sample of 6-week-old corn. These purified sterols where characterized by RP-HPLC, GLC and ^{1}H NMR (200 MHz) in $CDCl_3$. Structural and chromatographic values (α_e–K' sample/K' cholesterol) [13] agree with those for authentic standards and previous reports for cholesterol, lathosterol [22], 22-dihydrobrassicasterol [23], stigmasterol, sitosterol [24] and isofucosterol [25]. GLC was performed on a SE-30 fused silica glass capillary column. TLC was performed with neutral Silica gel G with toluene–EtOAc (9:1). Wheat germ acid phosphatase (EC 3.1.3.2, Sigma P-3627) treatments, with conditions as previously reported [4], were used for hydrolytic cleavage of phosphate moieties from the RP-HPLC frs possessing ecdysteroid polyphosphates. Sepn of freed ecdysteroid from any unconverted ecdysteroid di- or poly-phosphates post-WGAP treatment or acid hydrolysis was performed by silica Sep-pak (Waters Associates) as previously reported [26]. Acid hydrolysis of ecdysteroid di- or poly-phosphates was performed with 0.5 M HCl for 24 hr at room temp. in the dark. These conditions for acid hydrolysis are considered to be mild as 20-hydroxyecdysone did not dehydrate to any appreciable extent (< 1%). Elevating the temp. and acid concn increases dehydration of the C-14 hydroxyl group.

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