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THE BIOSYNTHESIS AND METABOLISM OF ANTHRAQUINONES IN *RUMEX OBTUSIFOLIUS*

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Abstract—Radioactive acetate was fed to *Rumex obtusifolius* plants and after 7 days aloe-emodin, chrysophanol and emodin were isolated and found to be radioactive Each was degraded to phthalic acids and the proportion of radioactivity present in the acids shown to be consistent with the acetate-malonate route earlier established for a related species of *Rumex*. The subsequent fate of the anthraquinones was studied by observing the changes in glycosidic and free anthraquinones in developing fruits and by feeding radioactive aglycones to the inflorescences The results indicate a rapid utilization of the anthraquinones in the early stages of fruit formation, followed by a more gradual use, and coincide well with similar work on the developing fruits of *Cassia acutifolia*

INTRODUCTION

GATENBECK,[1,2] Birch *et al* [3] and Shibata and Ikekawa[4] have shown that the anthraquinones of certain moulds are biosynthesized by the acetate-malonate route. In contrast, Leistner and Zenk[5,6] showed that in the higher plant *Rubia tinctorum* Ring A of the anthraquinone is derived from shikimate, and Ring C by the acetate-mevalonate route, thus confirming the theory of Burnett and Thomson.[7] *Rubia* anthraquinones, however, have no substituent in Ring A, but other higher plants produce compounds similar to those in the moulds (e.g emodin and chrysophanol) and have one or more hydroxyl groups in Ring A. It is quite possible, therefore, that these higher plants biosynthesize their anthraquinones by the acetate-malonate route. Some preliminary work by Maynard *et al* [8], however, suggests that in *Rheum rhaponticum* chrysophanol is formed from mevalonate, although in *Rhamnus frangula* emodin is formed from acetate-malonate

We therefore decided to feed labelled acetate to *Rumex obtusifolius* plants and to examine the emodin and other anthraquinones after a suitable interval. While carrying out this work, Leistner and Zenk[9] published a paper proving that chrysophanol is produced in *Rumex alpinus* via the acetate-malonate route Our results are consistent with this route and are based on a closely related species and include examination of emodin and aloe-emodin, as well as chrysophanol We have also investigated whether these compounds are further metabolized by the plant because of earlier work which indicated that the anthraquinone glycosides are associated with fruit development in *Cassia acutifolia* [10]

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[2] S GATENBECK, *Acta chem Scand* **14**, 102, 230, 296 (1960)
[3] A J BIRCH, A J RYAN and H SMITH, *J Chem Soc.* 4773 (1958)
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[7] A R BURNETT and R. H THOMSON, *Chem. Commun* 1125 (1967).
[8] A MAYNARD, A VILLE and H PACHECO, *Compt Rend* **266**, 1783 (1968)
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[10] J W FAIRBAIRN and A. B SHRESTHA, *Phytochem* **6**, 1203 (1967).

RESULTS

Feeding [^{14}C]acetate to the Plants

Separate batches of [1-^{14}C] and [2-^{14}C]acetate were fed to the stems of mature plants by the wick method. Seven days later the plants were harvested and their anthraquinones extracted and purified to constant activity. From the plants fed [1-^{14}C]acetate we obtained aloe-emodin (1007 × 10^3 dis/min/mM), chrysophanol (130 × 10^3) and emodin (460 × 10^3), and from the plants fed [2-^{14}C]acetate we obtained aloe-emodin (1673 × 10^3), chrysophanol (73 × 10^3) and emodin (783 × 10^3) Suitable quantities of the radioactive anthraquinones were converted into phthalic acids and, after purification, the total amount of radioactivity in the latter determined (Table 1) Work on inactive anthraquinones showed that conversion

Table 1. Proportion of radioactivity (dis/min) found in phthalic acids derived from radioactive anthraquinones

	Phthalic acid	
From [1-^{14}C]acetate fed plants	Radioactivity	Proportion
Aloe-emodin 1920	1012	52.7%
Chrysophanol 1388	692	50.0%
Emodin 3000	1600	53.3%
From [2-^{14}C]acetate fed plants		
Aloe-emodin 4812	2440	50.7%
Chrysophanol 3200	1408	47.1%
Emodin 18,668	9400	50.3%

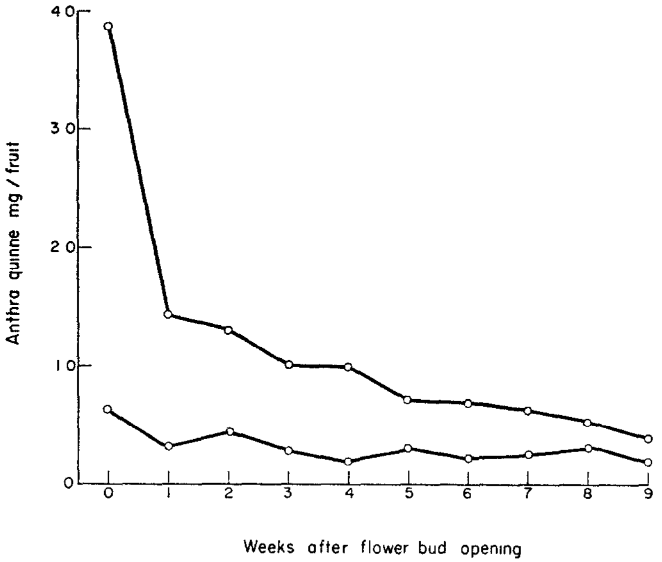

Fig 1 Changes in anthraquinone glycoside (upper curve) and free anthraquinone (lower curve) content during development of the fruit

Both calculated as μg aglycone per fruit Ordinate anthraquinone (μg/fruit), Abscissa weeks after flower bud opening to phthalic acid gave yields of 90–95 per cent in the conditions used. The proportions formed from Rings A and C, however, varied with the anthraquinones used, for example, aloe-emodin and chrysophanol derived acids were mainly from Ring A; the rhein derived acid mainly from Ring C (about 70 per cent in one experiment).

Metabolism of Anthraquinones in the Developing Inflorescences

The following approaches were used

(a) *Variation in anthraquinone content at weekly intervals.* Samples of flowers were collected just after bud opening and at weekly intervals afterwards, from a large number of *R obtusifolius* plants of equal age Free anthraquinones, *O*-glycosides and *C*-glycosides were determined in each sample on a fresh weight, dry weight and weight per fruit basis. The results given in Fig 1 are based on weight per fruit, no significant difference in this pattern occurred when fresh weight or dry weight figures were used It can be seen that there is a marked drop in the glycosidal anthraquinone followed by a gradual fall, but no corresponding increase in free compounds

(b) *Feeding radioactive anthraquinones to the developing inflorescences.* Radioactive anthraquinones were separated from plants which had been grown in $^{14}CO_2$ purified and made into glucosides with non-radioactive glucose The glucosides (124×10^3 dis/min total) were fed to 20 young inflorescences (Week 0). Representative samples of the developing fruits from all the plants were collected at 3, 5, 8, 11 and 16 days after feeding, counted and weighed, and the radioactivity of a methanol extract and of the total anthraquinones derivatives were determined on each sample. Table 2 shows the results

TABLE 2 ANALYSES OF SAMPLES OF DEVELOPING FRUITS COLLECTED AT INTERVALS AFTER FEEDING RADIOACTIVE ANTHRAQUINONES (124×10^3 dis/min) TO THE BASE OF THE INFLORESCENCES

Time interval	Fresh weight of sample (g)	Radioactivity of MeOH extract (dis/min $\times 10^{-3}$)	Proportion of radioactivity in anthraquinones (%)
W_0 + 3 days	24 5	1 45	12 2
W_0 + 5 days	26 7	1 70	12 3
W_0 + 8 days	34 9	1 54	9 6
W_0 + 11 days	33 1	1 80	9 1
W_0 + 16 days	35 3	1 48	8 7
		Total. 7 97	

W_0 = time of injection, when the flower buds had just opened

DISCUSSION

Biosynthetic pathways

In view of the varying proportions of Ring A and Ring C phthalic acids produced on oxidation of the anthraquinones, our results are definitely consistent with the acetate-malonate route of biosynthesis If the acetate-mevalonate route were involved, only Ring C would be radioactive (there is a slight possibility that some radioactivity would be in Ring A because of metabolism of acetate to $^{14}CO_2$ and subsequent incorporation of some of this into sugar, then into shikimate, but the dilution factor would be considerable) In such circumstances the radioactivity of the phthalic acids would vary markedly according to whether they came from Ring A or C The two sets of results in Table 1 show a value of about 50 per cent for all three compounds and this is consistent with an approximately even distribution of radioactivity in the two rings when derived by the acetate-malonate route

Metabolism of the Anthraquinones

The rapid fall in glycosidic anthraquinone with no corresponding increase in aglycone (Fig 1) during the first week of fruit development, indicates that at this stage the anthraquinone moiety of the glycoside is being metabolized This is confirmed by the results of feeding glucosides with radioactive aglycone to the developing inflorescences (Table 2) Obviously the weight of fruits collected represents only a proportion of the total weight of the inflorescence and stem into which the radioactive glycosides had been injected, this very largely explains why the total amount of radioactivity in the collected fruits only represents about 8 per cent of the fed activity However, the important fact emerges that after 3 days 88 per cent of the radioactivity was due to non-anthraquinone substances The continued steady, though slower, rate of conversion during the next 13 days is consistent with the picture presented in Fig 1

The combined results, therefore indicate a rapid use of the anthraquinones in the early stages of fruit development, followed by a slow utilization later This is exactly similar to the situation found with the anthraquinones in the developing fruits of *Cassia acutifolia* L [10] and suggests a similar useful role for these compounds in both plants

EXPERIMENTAL

Radioactive acetate feeding To ten plants of approximately equal age and in flower was fed 0 1 mCi of [1-^{14}C]acetate in H_2O by the wick method, a similar group of plants was fed with 0 1 mCi of [2-^{14}C]-acetate Seven days later, the entire plants were harvested and each group processed separately The fresh plants were macerated in 70% MeOH to exhaustion, the extract concentrated to small volume, diluted with H_2O and the free anthraquinones removed with $CHCl_3$ The glycosides (*O*- and *C*-) were hydrolysed by refluxing with aq $FeCl_3$ (4%) and HCl (4N) for 4 hr,[11] the liberated anthraquinones were extracted into $CHCl_3$ The latter was rinsed with H_2O, extracted with N NaOH, and the combined NaOH extracts acidified, extracted with $CHCl_3$ and the latter dried and evaporated to dryness Individual anthraquinones were separated from this residue by preparative TLC [12] Chrysophanol was separated from the chrysophanol-physcion mixture by demethylating the physcion to emodin (by refluxing for 2 hr in HOAc–HBr (sp gr 1 5), equal vols) and further chromatography in the same TLC system Both free and glycosidal anthraquinones were radioactive (the former more active than the glycosides) and were combined for further work

All the compounds were recrystallized to constant specific activity and their authenticity checked by TLC (4 systems) and UV spectra [12] Because of their intense yellow colour their radioactivities were determined in a gas-flow counter (Nuclear-Chicago Model 1105)

Phthalic acid derivatives About 50 mg of each anthraquinone were dissolved in 15 ml N NaOH, heated in a boiling H_2O bath and 30% aq H_2O_2 added dropwise till the pink colour was discharged After cooling and acidifying, the phthalic acids were extracted with ether, the latter evaporated off and the residue purified by preparative TLC (silica gel G, *n*-BuOH–HOAc–H_2O (4 1 1)) The quantity of phthalic acids formed was determined by dissolving in ether, shaking with excess 0 1 NaOH and back titrating with 0 1 N HCl Thus 100 mg aloe-emodin (0 37 mM) yielded 0 35 mM phthalic acids, 44 mg rhein (0 16 mM) yielded 0 14 mM phthalic acids, 100 mg chrysazin (0 41 mM) yielded 0 30 mM Paper chromatography of the phthalic acids (Whatman No 1, BAW (4 1 1) room temp overnight) showed that varying amounts of two major acids had been formed From chrysazin (1′8-dihydroxyanthraquinone) only one spot of R_f 0 57 occurred and this was concluded to represent the hydroxyphthalic acid from Ring A, as in this compound Ring C would give an identical acid Chrysophanol derived acids showed a major spot R_f 0 57 and a minor one R_f 0 63, aloe-emodin derived acids a major one at R_f 0 57 and two minor ones at R_f 0 48 and R_f 0 63 On the other hand, rhein derived phthalic acids had a major spot R_f 0 47 and a minor one at 0 57 In this instance the two acids were separated by band chromatography and titrated as before The phthalic acid of R_f 0 47 represented

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66% of the total phthalic acids Emodin showed three spots of fairly similar size, R_f values 0 46, 0 57 and 0 63.

Radioactive glucosides Potted plants of *R obtusifolius* and *R alpinus* were exposed to $^{14}CO_2$ for 48 hr in a special chamber The anthraquinones were then separated and purified to constant specific activity and their diglucosides formed by the method of Muhtadi and Moss [13]

Collection and treatment of samples of developing fruits From a sufficiently large number of plants of similar age and development, growing in open ground, those inflorescences whose flower buds had just opened were labelled ‘Week 0’ At this stage, and at suitable intervals afterwards, a representative sample of flowers or young fruits from all the inflorescences was collected The number of fruits in, and the fresh and dry weights of, each sample was determined Each sample was then extracted with 70% MeOH and the radioactivity determined in plants fed with radioactive precursors The free anthraquinones were extracted into $CHCl_3$ after diluting the methanolic extract with H_2O The remaining aqueous phase was heated with 4 N HCl and 4% $FeCl_3$ for 4 hr under reflux, and the liberated aglycones extracted into $CHCl_3$ The free anthraquinones in the first $CHCl_3$ solution were extracted with N NaOH and the amount present determined as emodin at 520 nM (E 1 % 1 cm in NaOH = 340) The glycosidic anthraquinones in the second $CHCl_3$ extract were determined similarly as chrysophanol at 500 nM (E 1%, 1 cm in NaOH = 330) Where appropriate, the radioactivity of these anthraquinones was also determined, as previously described

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Key Word Index—Rumex obtusifolius, Polygonaceae, anthraquinones, biosynthesis, metabolism, aloe-emodin, chrysophanol, emodin, radioactive acetate