The Translocation of Antibiotics in Higher Plants

3. THE ESTIMATION OF GRISEOFULVIN RELATIVES IN PLANT TISSUE*

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Crowdy, Gardner, Grove & Pramer (1955) reported on the recovery of griseofulvin [*dd*-7-chloro-4:6:2'-trimethoxy-6'-methylgris-2'-en-3:4'-dione, (II; R = OMe)] from treated broad-bean plants and described methods of estimating griseofulvin in the treating solution and in the plant tissue extracts. These methods were used by Crowdy, Grove, Hemming & Robinson (1956) to study the rates of uptake and decay of griseofulvin in plant tissue. These studies have now been extended to include eleven compounds closely related chemically to griseofulvin, and the present paper describes analytical methods for estimating these compounds in bean tissue extracts and in the treating solutions. With the exception of the acidic trione (II; R = OH: enolic form) and the basic amino derivative (II; R = NH₂) the compounds studied were neutral. Reasons underlying the selection of these compounds and discussion of the results obtained with them form the subject matter of the next paper (Crowdy, Grove & McCloskey, 1959).

The analytical methods were essentially the same as those used for griseofulvin (Crowdy *et al.* 1955). Compounds remaining in the treating

solution after a given period of uptake were estimated spectrophotometrically after solvent extraction and chromatography of the extract. Solvent extracts of the roots and shoots were subjected to countercurrent distribution and the compounds estimated spectrophotometrically after further purification by chromatography. Special methods of estimation were necessary for some compounds and these are described in detail.

The identification and estimation in bean tissue of metabolites of the griseofulvin relatives studied is also described, together with details of a search for griseofulvin metabolites formed in the decay experiments recorded by Crowdy *et al.* (1956).

EXPERIMENTAL

Compounds. The compounds studied (Table 1) comprised the synthetic grisan-3:4'-dione (I) (McCloskey, 1958), the *ld*-diastereoisomer of griseofulvin (II; R = OMe) (MacMillan, 1959), the isomeric methyl enol ether (IV), and the dechloro analogue (VI; R = OMe) (MacMillan, 1953) of griseofulvin, three homologues of griseofulvin comprising

* Part 2: Crowdy, Grove, Hemming & Robinson (1956).

the *n*-propyl and *n*-butyl compounds (II; R = OPrⁿ and OBuⁿ respectively) (Duncanson, Grove & Jeffs, 1958) and the bisethoxy relative (V) (Duncanson, Grove, MacMillan & Mulholland, 1957), two neutral alcohols (III; R = H and OH), reduction products of the acidic trione (II; R = OH) (Grove, MacMillan, Mulholland & Rogers, 1952a), the trione (II; R = OH) and the basic amino compound (II; R = NH₂).

The melting point, 220°, of the alcohol (III; R = H) (cf. m.p. 198–200°, Grove *et al.* 1952a) is that of a dimorphic form obtained by crystallization from benzene–light petroleum (b.p. 60–80°).

MacMillan (1959) has shown that griseofulvin can be converted into a diastereoisomer in which the configuration at the spiran centre of asymmetry is inverted. The absolute configurations of these compounds have been deduced and can be expressed by using the convention of Cahn, Ingold & Prelog (1956). In this system correlation of configuration in a series of related compounds is often obscured; to overcome this difficulty in griseofulvin chemistry *d* and *l* are used to denote configuration at the asymmetric centres, the spiran centre being that first mentioned. Thus, griseofulvin is *dd*-7-chloro-4:6:2'-trimethoxy-6'-methylgris-2'-en-3:4'-dione and the above diastereoisomer the *ld* compound. With the exception of this compound all the derivatives of griseofulvin used in the present study have the *dd* configuration. For the grisan nomenclature see Grove, MacMillan, Mulholland & Rogers, 1952b).

Recovery techniques. All the compounds sublimed, unchanged, at 20–30° below the melting point at pressures between 10⁻¹ and 10⁻⁴ mm.; recovery was not quantitative. The neutral compounds (and the amine II; R = NH₂) were all recovered quantitatively from an alumina column (pH 4: activated at 250° for 3 hr.) by elution with benzene–methanol mixtures. The acidic trione (II; R = OH) was recovered quantitatively from a Celite 545 (Johns–Manville Co. Ltd.) column buffered at pH 7.0 (Serensen phosphate buffer) by elution with ether.

Griseofulvin was stable in aqueous solution at room temperature during 14 days, and hydrolysis to the trione (II; R = OH) was not appreciable: Griseofulvin (25 mg.) dissolved in acetone (5 ml.) was dispersed in water (1 l.). After standing with occasional stirring for 14 days at 25° (some crystallization took place) the solution was extracted with ether (4 × 300 ml.). The ethereal solution was concentrated to 200 ml. and extracted with 2*N*-sodium hydroxide (2 × 45 ml.). The aqueous alkaline layer was re-extracted with ether (2 × 50 ml.) and made up to 100 ml. The ultra-violet-absorption of this solution (*l*, 1 cm.) showed only a very weak peak at 290 mμ (trione content <0.1 mg.).

Some of the griseofulvin relatives were more susceptible to hydrolysis, particularly during recovery from a large volume of wet solvent after countercurrent distribution. Thus small quantities of the acidic trione (II; R = OH) were frequently obtained after recovery of (IV) and the griseofulvin propyl and butyl homologues; and the trione (VI; R = OH) was obtained from the dechloro compound (VI; R = OMe). The two triones were produced by hydrolysis during working up after countercurrent distribution, since they normally appear in tubes well removed from the series containing the parent compounds. In these cases the estimates of the amount of the particular compound present were corrected by addition of weights equivalent to the triones produced.

Table 1. *Properties of griseofulvin relatives*

Compound	Structure	Formula	M	M.p.
<i>ld</i> -7-Chloro-4:6:2'-trimethoxy-6'-methylgris-2'-en-3:4'-dione	(II; R = OMe)	C ₁₇ H ₁₇ O ₆ Cl	352.8	220–221°
4:6:2'-Trimethoxy-6'-methylgris-2'-en-3:4'-dione	(VI; R = OMe)	C ₁₇ H ₁₉ O ₆	318.3	179–181
7-Chloro-4:6:4'-trimethoxy-6'-methylgris-3'-en-3:2'-dione	(IV)	C ₁₇ H ₁₇ O ₆ Cl	352.8	200–201
7-Chloro-4:6-dimethoxy-6'-methyl-2'- <i>n</i> -propoxygris-2'-en-3:4'-dione	(II; R = OPr ⁿ)	C ₁₉ H ₂₁ O ₆ Cl	380.8	153–154
2'- <i>n</i> -Butoxy-7-chloro-4:6-dimethoxy-6'-methylgris-2'-en-3:4'-dione	(II; R = OBu ⁿ)	C ₂₀ H ₂₃ O ₆ Cl	394.8	156
7-Chloro-6:2'-diethoxy-4-methoxy-6'-methylgris-2'-en-3:4'-dione	(V)	C ₁₈ H ₂₁ O ₆ Cl	380.8	172
7-Chloro-4:6'-dihydroxy-4:6-dimethoxy-2'-methylgrisan-3-one	(III; R = OH)	C ₁₈ H ₁₉ O ₆ Cl	342.8	222–224
7-Chloro-6'-hydroxy-4:6-dimethoxy-2'-methylgrisan-3-one	(III; R = H)	C ₁₈ H ₁₉ O ₆ Cl	326.8	220
7-Chloro-4:6-dimethoxy-2'-methylgrisan-3:4':6'-trione	(II; R = OH)	C ₁₈ H ₁₉ O ₆ Cl	338.8	255–258 (decomp.)
2'-Amino-7-chloro-4:6-dimethoxy-6'-methylgris-2'-en-3:4'-dione	(II; R = NH ₂)	C ₁₈ H ₁₉ O ₆ NClH ₂ O	355.8	258 (decomp.)
Grisan-3:4'-dione	(I)	C ₁₈ H ₁₉ O ₃	216.2	89–90

Slight hydrolysis of griseofulvin homologues also took place during recovery from the treating solution, and this was allowed for by carrying out a control experiment similar to the one described above for griseofulvin. When the amount of trione isolated was significantly in excess of that found in the control, it was considered to be a metabolite (see p. 234).

Plants and preparation of plant extracts

Where two experiments were carried out with the same compound, the letters *A* and *B* are appended in Table 2.

The conditions under which the plants, *Vicia faba* L., var. Sutton's Dwarf, were raised, treated and harvested have been described (Crowdy *et al.* 1955). Roots and shoots were weighed immediately after harvesting and the weights recorded as the fresh weights. The pH of all treating and control solutions was initially 5.6 ± 0.5 , and during the period of treatment rose to a final value of 7.0 ± 0.5 in the controls. In some cases (recorded in Table 2) the final pH of the treating solutions was outside this range.

With the propyl and butyl homologues of griseofulvin, 25 mg. of the compound was dissolved in a little acetone, and the solution was dispersed in 5 l. of water and supplied to 25 beakers each containing four plants: the solubility of the remaining compounds permitted the use of solutions having a concentration of 25 mg./l. (125 mg. in 5 l.), although this figure was sometimes considerably in excess of the solubility of the compound in pure water. With grisan-3:4'-dione one experiment (*B*) was carried out in which 400 mg. was dissolved in 16 l. of water and the solution supplied to 320 plants in 80 beakers.

The weight of water transpired during treatment was noted (Crowdy *et al.* 1956) and plants were harvested at the end of 3 days. The fresh weights of shoots and roots were determined separately and, with the exceptions detailed below, the tissues were then macerated in chloroform and extracted with the same solvent as previously described (Crowdy *et al.* 1955).

The trione (II; R = OH). In early experiments with the trione (II; R = OH) of which *A* (Table 2) is typical, roots and shoots were macerated in water and the acidified aqueous macerate was extracted with ether. In a later experiment (*B*) sodium hydrogen carbonate solution was used during maceration and the resulting solutions were acidified to Congo red with concentrated hydrochloric acid before extraction with ether. The low solubility of the trione in chloroform precluded the use of this solvent. The ultraviolet-absorption pattern of the tubes after counter-current distribution (see p. 233) of root tissue extract from untreated plants was approximately the same whether chloroform or ether was used for the extraction. With shoot tissue, however, the ether extract showed intense maxima at 370 and 270 m μ in tubes 38-48, which were absent from a similar chloroform extract. The material responsible for this absorption was subsequently identified (Abbot, Grove & McCloskey, 1958) as kaempferol, 3:5:7:4'-tetrahydroxyflavone, which is virtually insoluble in chloroform.

The amine (II; R = NH₂). The macerated roots and shoots which had already been extracted with chloroform were separately stirred for 24 hr. with 2N-hydrochloric acid (1 l.). Under these conditions any residual amine would probably have been converted into the trione (II; R = OH). After filtration the acid solution was extracted with ether

(acidic fraction) and the aqueous layer was then made alkaline by the addition of sodium hydroxide (cooling) and re-extracted with ether (basic fraction). The acidic and basic fractions were recovered and their trione and amine contents estimated as described below. Neither fraction from either shoots or roots showed any specific absorption maxima near 290 m μ , indicating the absence of both amine and trione.

Griseofulvin. In view of the failure to detect griseofulvin metabolic products in the decay experiments (pp. 238 and 239) the shoot and root macerates which had previously been extracted with chloroform were separately agitated for 24 hr. with sodium hydrogen carbonate. After acidification, ether extraction and recovery the extracts were separated into acidic and neutral fractions and compared with controls from untreated plants. No differences were seen and there was no specific absorption between 250 and 350 m μ in any of the fractions.

Calculation of uptake

Crowdy *et al.* (1956) showed that two processes were involved in the uptake of griseofulvin by broad bean: an initial rapid entry into the roots which quickly attained the griseofulvin concentration of the external solution, followed by a prolonged uptake linearly related to the transpiration. For treating solutions of concentration 25 μ g./ml. the mean of several experiments showed that transpiration uptake was 18 μ g./ml. of water transpired, although deviations from this mean were considerable. For lower concentrations, the transpiration uptake (μ g./ml. of water transpired) was taken as proportional to the concentration of the treating solution. In the present series of experiments with griseofulvin relative uptakes have been calculated from the fresh weight of roots and weight of water transpired, assuming that the same considerations hold.

If x is the fresh weight of roots, y the weight of solute taken, v the volume of the treating solution, t the volume of water transpired, and C a constant, then

$$\text{Initial uptake} = y \left(\frac{x}{v+x} \right).$$

$$\begin{aligned} \text{Transpiration uptake} &= \left[y \left(1 - \frac{x}{v+x} \right) \right] \frac{Ct}{v} \\ &= \frac{yCt}{v+x}. \end{aligned}$$

$$\text{Total uptake} = y \left(\frac{Ct+x}{v+x} \right).$$

For a 25 μ g./ml. solution, $C = 0.72$.

Uptakes calculated in this way are set out in Table 2; they are compared with the spectrophotometric estimations of uptake in Part 4 (Crowdy *et al.* 1959).

Spectrophotometric estimation and countercurrent distribution

With the exception of the trione (II; R = OH) all compounds obeyed Beer's law in the concentration range 10^{-3} – 10^{-6} M, both in ethanol and in the upper phase of the carbon tetrachloride-methanol-water system used for countercurrent distribution. Absorption maxima and the corresponding molecular extinction coefficients for the eleven compounds are recorded in Table 3. The form of the

Table 2. *Uptake of griseofulvin relatives by bean plants*

The transpiration data are for the 3-day treatment of 100 bean plants. For the calculation of uptake see text.

Compound	Expt.	Treating solution		Final pH	Water transpired (ml.) <i>t</i>	Fresh wt. of roots (g.) <i>x</i>	Calculated uptake (mg.) $y \left(\frac{Ct+x}{v+x} \right)$
		Vol. (ml.) <i>v</i>	Concn. (mg./l.) y/v				
Id-(II; R = OMe)		5000	25	—	2453	185	51
(VI; R = OMe)		5000	25	—	1167	255	28
(IV)		5000	25	—	1355	221	31
(II; R = OPr ⁿ)		5000	5	5.7	1858	202	10
(II; R = OBu ⁿ)	A	4000	20	—	1395	303	25
	B	5000	5	—	1498	426	9
(V)		5000	25	—	2351	228	46
(III; R = OH)		4600	25	—	2078	162	43
(III; R = H)		5000	25	—	2183	385	45
(II; R = OH)	A	5000	20	—	1655	490	34
	B	5000	25	—	4785	371	96
(II; R = NH ₂)		5000	25	6.3	1827	340	41
(I)	A	5000	15	—	1688	424	23
	B	16 000	25	4.85	6831	745	147

absorption curve for the acidic trione (II; R = OH) in ethanol, and in particular the intensity of the band near 290 mμ, varies with concentration due to dissociation of the compound in the ionizing solvent. For this reason the spectrophotometric estimation of the trione was carried out in 0.1N-sodium hydroxide in which dissociation was essentially complete and the anion might be assumed to be the only molecular species present. In this medium it was found that Beer's law held within about 3% for solutions in the range 10⁻⁴–10⁻⁵M.

The carbon tetrachloride-methanol-water (62:35:3, by vol.) system (Crowdy *et al.* 1955) was used for counter-current distribution of all the compounds studied. The mobile phase was the methanol-rich upper phase. In this system the partition ratios of most of the compounds were such as to bring them into a range of tubes uncontaminated by the carbon tetrachloride-soluble green pigments or methanol-soluble brown pigments. The more polar compounds, however, notably the trione (II; R = OH), the amine (II; R = NH₂) and the diol (III; R = OH), appeared in tubes heavily contaminated by phenolic and other brown pigments; this made estimation and recovery difficult, but the advantages of retaining the same solvent system throughout outweighed the disadvantages encountered with these three compounds.

Data for 50 transfers with the pure compounds are included in Table 3. For the purposes of this paper *K* is defined as the partition ratio actually operating during the distribution, and was obtained from the equation

$$N = nK/(K+1),$$

where *n* is the total number of transfers and *N* the number of the equilibrium tube containing the maximum amount of the compound under investigation. *N* was determined experimentally in the usual way.

For all the compounds studied the experimentally determined distribution curve was found to be in good agreement with the theoretical distribution calculated (Williamson & Craig, 1947) from the value of *K* determined

in this way. The fraction (*T_N*) of the total solute appearing in tube *N* and the location of the batch of tubes containing 0.99 of the total solute are also given in Table 3. It was assumed that the constituents of the extracts of macerated root and shoot tissue would have a negligible effect on the distribution; this was shown to be true for griseofulvin (cf. Crowdy *et al.* 1955).

Estimation of griseofulvin relatives in treating solutions

Concentration of griseofulvin solutions *in vacuo* at 60° before extraction with chloroform was shown (Crowdy *et al.* 1955) to cause appreciable losses of griseofulvin, for which correction was made. In the present series of experiments the bulked root washings and treating solution were extracted without prior concentration. The residual aqueous solution was then examined spectrophotometrically (*l*, 4 cm.) to ensure that extraction was complete. Under these conditions griseofulvin (90 mg.) was recovered quantitatively from water (5 l.) by extraction with chloroform (3 × 2 l.). Chloroform was effective in extracting quantitatively all the neutral compounds with the exception of the diol (III; R = OH), where a final extraction with ether was necessary. In certain cases, notably the dechloro analogue (VI; R = OMe), 7-chloro-4:6:4'-trimethoxy-6'-methylgris-3'-en-3:2'-dione (IV) and the propyl and butyl homologues of griseofulvin, where the presence of metabolites was known or suspected, the recovered solute was dissolved in ether and separated into neutral, acidic and phenolic fractions by extraction with aqueous sodium hydrogen carbonate followed by 2N-sodium hydroxide and recovery. The neutral fraction was dissolved in benzene and chromatographed in ultraviolet light on alumina. After elution of coloured impurities with benzene, the blue fluorescent band was eluted with benzene-methanol and the compound under investigation estimated spectrophotometrically in the eluate after recovery and dissolution in ethanol. It was then recovered and identified chemically. The acidic and phenolic fractions were investigated

separately (see below). With the remaining neutral compounds, namely the griseofulvin *ld*-diastereoisomer, 7-chloro-6:2'-diethoxy-4-methoxy-6'-methylgris-2'-en-3:4'-dione (V), the alcohols (III; R = OH) and (III; R = H) and grisan-3:4'-dione, the chloroform extract was chromatographed without separation of acidic and phenolic fractions and the compound estimated spectrophotometrically. With the exception of grisan-3:4'-dione, all compounds were recovered and identified, and the weight recovered agreed reasonably well with that estimated spectrophotometrically. Special methods of extraction and estimation were necessary for the trione (II; R = OH) and the amine (II; R = NH₂).

The trione (II; R = OH). The treating solution and root washings were acidified (Congo red) with concentrated hydrochloric acid and continuously extracted with ether. The solute from the bulked ether extracts was dissolved in 0.1N-sodium hydroxide and the trione content estimated spectrophotometrically. After acidification and re-extraction with ether the trione was recovered and characterized chemically. The intensity of the absorption band at 290 m μ was such that interference from impurities exuded by the roots was negligible by comparison with the trione absorption as judged by the ratio of the band intensities at 290 and 330 m μ . Thus purification on a Celite column was unnecessary.

The amine (II; R = NH₂). In aqueous media the amine is hydrolysed to the trione (II; R = OH) and hence the treating solution was analysed for both components. It was impracticable to separate relatively large quantities of the amine from the other constituents by extraction with dilute hydrochloric acid, as decomposition to the trione (II; R = OH) is rapid under these conditions. The favoured procedure was to remove impurities from the amine; the treating solution was extracted with chloroform and the latter was extracted with aqueous sodium hydrogen carbonate. The acid fraction and the residual solute in the chloroform extract were recovered and the trione and amine contents estimated in 0.1N-sodium hydroxide and in ethanol respectively.

Detection and estimation of metabolites in the treating solutions

Weights estimated spectrophotometrically are preceded by $\equiv$, e.g. ($\equiv$ 1.5 mg.).

Compound (VI; R = OMe). The acidic fraction showed a sharp absorption maximum at 288 m μ (in 0.1N-sodium hydroxide). Chromatography of the solute in ether on Celite as described on p. 231 gave a gummy product (1.5 mg.) which contained the material with $\lambda_{\max}$ 288 m μ ($\equiv$ 1.5 mg.) but which could not be crystallized. It was assumed to be 4:6-dimethoxy-2'-methylgrisan-3:4':6'-trione (VI; R = OH) ($\lambda_{\max}$ 289 m μ , ϵ 45 750 in sodium hydroxide), produced by hydrolysis of (VI; R = OMe).

The phenolic fraction contained a trace of an unidentified metabolite with $\lambda_{\max}$ 285 m μ .

Compound (IV). The acidic fraction showed maxima at 272, 290 and $\sim$ 325 m μ ($\sim$ represents an inflexion) in ethanol, but the metabolite (3 mg.) could not be identified and the fraction was not examined further.

The phenolic fraction showed a weak maximum at 288 m μ , due possibly to a trace of a second metabolite.

Compound (I). Experiment (B) is described here and in subsequent sections.

Table 3. Molecular extinction coefficients and countercurrent-distribution data for griseofulvin relatives

Compound	Molecular extinction coefficients determined in						Countercurrent-distribution data			
	Ethanol			Upper phase			K	N	T _N	Tube numbers (range) containing 99% of total solute
	$\lambda_1^{\max}$ (m μ)	ϵ_1	$\lambda_2^{\max}$ (m μ)	$\epsilon_1 - \epsilon_2$	ϵ_1	ϵ_2				
<i>ld</i> -(II; R = OMe)	291	23 500	293	1-33	24 250	6175	1-56	30-5	0-114	22-39
(VI; R = OMe)	287	25 500	288	1-28	25 750	5610	1-38	29	0-114	21-38
(IV)	291	19 750	291	1-35	20 750	5220	1-70	31-5	0-114	24-40
(II; R = OP ⁿ)	292	22 000	294	1-30	23 250	5750	1-33	28-5	0-114	20-37
(II; R = OBu ⁿ)	292	23 500	294	1-29	24 000	5650	1-17	27	0-113	19-36
(V)	293	23 000	295	1-29	23 500	5400	1-22	27-5	0-112	19-36
(III; R = OH)	288	20 750	293	1-33	23 750	5420	5-41	42-2	0-151	35-49
(III; R = H)	289	19 500	290	1-30	20 500	4750	2-00	33-3	0-118	25-41
(II; R = OH)	290*	44 000*	294	1-17*	21 500†	5280†	3-20	38	0-131	31-45
(II; R = NH ₂)	290	47 250	294	1-17	46 000	6500	5-25	42	0-153	36-48
(I)	250	10 470	—	—	†	4940	1-10	26-2	0-112	18-35

† Inaccurate in this solvent.

* In 0.1N-sodium hydroxide.

† 1.6 μ M-solution.

Extinctions were measured in ethanol and in the upper phase of the countercurrent distribution system [carbon tetrachloride-methanol-water (82:35:3, by vol.)]. Fifty transfers were made in the distribution. For definition of symbols see text.

The neutral chloroform extract, a brown granular solid (360 mg.), was extracted with hot benzene and the soluble material, a brown gum (260 mg.), separated by chromatography on alumina in benzene-methanol (199:1 rising to 99:1, v/v) into two fractions:

(i) A gum (140 mg.) containing a component, $\lambda_{\max}$ 325 m μ , possibly grisan-3:4'-dione ($\approx$ 5 mg.). A positive identification was not obtained, however, despite further treatment, and only an intractable precipitate was obtained with Brady's reagent.

(ii) A gum (100 mg.) containing a component, $\lambda_{\max}$ 282, 289 m μ . After sublimation at 150°/10⁻¹ mm. the product was combined with a similar fraction obtained by sublimation, at 150°/10⁻¹ mm., of the benzene-insoluble material, and the total sublimate (83 mg.) crystallized from benzene, yielding colourless needles (67 mg.), m.p. 168-188°; $[\alpha]_D^{25} + 68.5^\circ$ in ethanol (c, 1.1) (Found: C, 71.3; H, 7.3. C₁₅H₁₆O₈ requires C, 70.9; H, 7.3%), light-absorption in ethanol, $\lambda_{\max}$ 282, 290 m μ , ϵ 3240, 2870 respectively, of a mixture of α - and β -grisan-3:4'-diols in almost equal proportion, identified by comparison of the infrared spectrum (Nujol mull) with that of a synthetic mixture prepared from ($\pm$)- α - and ($\pm$)- β -grisan-3:4'-diols (McCloskey, 1958). The specific rotation of the natural mixture indicates that only the (+)-diols are present, since (+)- α -grisan-3:4'-diol has $[\alpha]_D^{25} + 73^\circ$ in ethanol (c, 1.0).

The unsublimed residue (33 mg.) of the benzene-insoluble material (above) was extracted twice with hot ethanol and once with boiling benzene, and the final insoluble residue (5 mg.), m.p. >300°, was rejected. Evaporation of the combined extracts yielded a gum which was separated by treatment with warm ethanol into a soluble gum (8 mg.; $\approx$ 6 mg. of grisan-3:4'-diol), and an insoluble solid (20 mg.), m.p. 186-194° [Found: C, 75.8; H, 7.2%; *M* (Rast) 1970], light-absorption in ethanol, $\lambda_{\max}$ 282, $\approx$ 289 m μ , $E_1^{1\%}$ 110, 95 respectively. The infrared spectrum showed bands attributable to an aromatic ring, but no hydroxyl or carbonyl bands.

The amount of grisan-3:4'-diol present in the residual treating solution was estimated spectrophotometrically as 93 mg.

Estimation of griseofulvin relatives in plant-tissue extracts

In the early work on the determination of griseofulvin in shoot-tissue extracts (Crowdy *et al.* 1955) it was possible to estimate the griseofulvin content directly from the values of the extinctions in the equilibrium tubes after counter-current distribution. In contrast with these observations, more recent work on extracts of untreated plants has shown that the background absorption at 291 and 326 m μ in tube 33 does not always have the same value, and that with certain extracts the background absorption begins to rise steeply with increasing tube number well in advance of tube 33. This was first noticed in decay experiments (Crowdy *et al.* 1956), but was subsequently found to be of fairly general occurrence. The reason for these variations in background absorption is not known, although seasonal influences may be involved. Acidic and particularly phenolic components are mainly responsible for the background absorption in untreated plant extracts.

Where the simple procedure outlined by Crowdy *et al.* (1955) was unsatisfactory, it was generally found that the

background absorption in tube 33 was linear over the wavelength range 270-320 m μ ; the extinction due to griseofulvin was then calculated by using the three-point correction procedure of Morton & Stubbs (1946).

Alternatively, the solute in tubes 25-41 was recovered and separated into neutral, phenolic and acidic components and the griseofulvin content of the neutral fraction estimated spectrophotometrically after chromatography on alumina. This last general method of analysis of shoot-tissue extracts was adopted for all the neutral compounds in the present study and was satisfactory. An identical procedure was adopted for root-tissue extracts.

Extinctions E_1 and E_2 were measured at two wavelengths, λ_1 and λ_2 (Table 3) and the ratio E_1/E_2 compared with ϵ_1/ϵ_2 for the pure compound. Agreement was usually good; but where appreciable background absorption was suspected the observed extinctions were corrected by the procedures of Crowdy *et al.* (1955) (constant background) or Morton & Stubbs (1946) (linear variation with wavelength) as appropriate. In the former method the corrected extinction at λ_1 is given by $\epsilon_1(E_1 - E_2)/(\epsilon_1 - \epsilon_2)$. Values of $\epsilon_1/(\epsilon_1 - \epsilon_2)$ are given in Table 3.

Special methods of estimation were again necessary for the trione (II; R = OH) and the amine (II; R = NH₂).

The trione (II; R = OH). The solute from tubes 31-45 was dissolved in ether and was separated into acidic, phenolic and neutral fractions in the usual way. The trione content of the acidic fraction was estimated spectrophotometrically in 0.1N-sodium hydroxide either directly or after chromatography on Celite. Thus in a trial estimation the trione (10.0 mg.) added to shoot tissue extract (2.84 g.) from untreated plants was subjected to 50 transfers. The solute from tubes 31-45 was dissolved in ether (200 ml.) and extracted with aq. sodium hydrogen carbonate (2 $\times$ 50 ml.). The acid fraction on recovery was redissolved in ether (200 ml.) and chromatographed on Celite (5 g.), buffered at pH 7.0, by the fractional elution technique (eluent: ether, 200 ml. portions). Eluates III-VI contained the trione together with some impurities: spectrophotometric estimation of the trione in 0.1N-sodium hydroxide gave a high value ($\approx$ 12.0 mg.).

Similar, but slightly more accurate, results were obtained with root-tissue extract in place of shoot-tissue extract.

The amine (II; R = NH₂). The solute from tubes 36-48 was dissolved in ether and the basic fraction separated by extraction at 0° with 2N-hydrochloric acid. This procedure, by which the amine is separated from other constituents, is the reverse of that found most suitable for analysis of the amine in the treating solution, but was adopted as the quantities involved were small. It led to some decomposition of the amine, and the acid extract (made alkaline) after recovery of the basic fraction by ether extraction contained some of the trione (II; R = OH). This was estimated separately in the usual way. The amine content of the basic fraction was estimated spectrophotometrically in ethanol. In a trial estimation, the amine (5.0 mg.) added to shoot-tissue extract (1.0 g.) from untreated plants was subjected to 50 transfers and tubes 36-48 were analysed as described above with the following results: (II; R = NH₂) (basic fraction), 1.5 mg.; (II; R = OH) (acid fraction), 2.0 mg., (by decomposition during isolation of basic fraction), 0.8 mg.; total 4.3 mg.

There is no decomposition of the amine to the trione during the actual countercurrent distribution: the trione

appearing in the acid fraction must therefore arise by decomposition of the amine during the extraction with 2N-hydrochloric acid. The amount of decomposition was not strictly reproducible.

A similar almost quantitative estimation of the amine (5.0 mg.) in root-tissue extract (500 mg.) was also achieved.

In the following sections weights of griseofulvin relatives or metabolites estimated spectrophotometrically in individual fractions after countercurrent distribution are preceded by ($\equiv$). To obtain the total weight present in the tissue extract a correction has sometimes to be applied to allow for the weight of the compound, calculated to be present from the distribution data (Table 3), in adjacent batches of tubes. This allowance has been made in the balance sheets presented in the next paper (Part 4).

Extraction and identification of compounds from shoot tissue and search for metabolites

In order to establish the validity of the spectrophotometric methods of estimation it was necessary to recover the compounds in question and identify them chemically (Crowdy *et al.* 1955). In the majority of cases this had been done and the experimental procedures are described below. The weight of each compound recovered has been recorded and may be compared with the weight believed to be present from spectrophotometric estimation. The recovery of some compounds proved to be more difficult than the recovery of griseofulvin (Crowdy *et al.* 1955) and only a fraction of the weight believed to be present from the spectrophotometric estimation was actually recovered. In

no case, however, was a second component with similar absorption maxima in the ultraviolet isolated from the same fraction.

At the same time a search was made for metabolites with the aromatic ring intact by scanning the tubes after countercurrent distribution and all fractions derived therefrom for specific ultraviolet absorption above 250 m μ , and by comparing the spectra obtained with the spectra of similar fractions derived from untreated plants. Major deviations from the absorption pattern found in the control experiment are summarized in Table 4. Details are also given of the search for metabolites after the griseofulvin-decay experiments (Crowdy *et al.* 1956). Except where stated, all fractions were examined in ethanol solution. With the exception of metabolites of grisan-3:4'-dione the amount of unidentified major metabolites present has been estimated roughly, assuming a molecular weight of 350 and $\epsilon = 25,000$.

Compound 1d-(II; R = OMe). Tubes 0-25 and 37-50 from the countercurrent distribution were similar to the control and were not investigated. Tubes 26-36 showed specific absorption at 285 m μ and the solute was separated into acidic, phenolic and neutral fractions in the usual way.

The phenolic fraction was similar to the control but the acidic fraction had absorption maxima at 282 and 325 m μ . The amount present was very small. The neutral fraction, showing $\lambda_{\max}$ 285 m μ , was dissolved in benzene and chromatographed on alumina in the usual way. Benzene: methanol (200:1, v/v) eluted material with $\lambda_{\max}$ 288 m μ ($\equiv 2$ mg.) and this was sublimed at 190°/0.01 mm. Crystallization of the sublimate from ethanol afforded

Table 4. *Recovery of griseofulvin relatives from bean plants and detection of metabolites*

Griseofulvin relatives and metabolites were detected by differences in the ultraviolet-absorption spectra of extracts of treated and untreated plants. Tube numbers refer to a 50-transfer countercurrent distribution (for details see text).

Compound	Shoots		Roots	
	Recovery	Metabolites	Recovery	Metabolites
1d-(II; R = OMe)	++	Acidic, tubes 26-36, $\lambda_{\max}$ 282, 325 m μ	++	—
(VI; R = OMe)	++	Acidic, tubes 24-33, $\lambda_{\max}$ 280, 325 m μ	++	—
(IV)	+	—	—	3-Chloro-2-hydroxy-4:6-dimethoxy-benzoic acid Neutral, tubes 24-35, $\lambda_{\max}$ 291 m μ Acidic, tubes 24-35, m.p. 284-286°, $\lambda_{\max}$ 287, ~330 m μ Neutral, tubes 36-50, $\lambda_{\max}$ 262, 290 m μ Acidic, tubes 36-50, $\lambda_{\max}$ ~285 m μ
(II; R = OPr ⁿ)	+	—	++	—
(II; R = OBu ⁿ)	+	—	++	Acidic, tubes 34-50, $\lambda_{\max}$ 287 m μ
(V)	++	Acidic, tubes 20-33, $\lambda_{\max}$ 282, 325 m μ	++	—
(III; R = OH)	+	Acidic, tubes 35-50, $\lambda_{\max}$ 282, 320 m μ	++	—
(III; R = H)	++	—	++	—
(II; R = OH)	—	—	—	—
(II; R = NH ₂)	+	Acidic, tubes 33-45, $\lambda_{\max}$ 282, 320 m μ	+	Neutral, tubes 28-36, $\lambda_{\max}$ 285 m μ Acidic, tubes 28-47, $\lambda_{\max}$ 290 m μ
(I)	—	Acidic, tubes 20-32, $\lambda_{\max}$ 280, 322 m μ α - and β -4'-Hydroxygrisan-3-ones (+)- α -Grisan-3:4'-diol Neutral, tubes 33-50, $\lambda_{\max}$ 325 m μ	—	Neutral, tubes 21-31, $\lambda_{\max}$ 281, ~288 m μ α - and β -Grisan-3:4'-diols

++ = Present; positive identification.

+ = Presence inferred from strong evidence.

— = Absent.

(1) needles, m.p. 236–40° raised to 256° by repeated crystallization from ethanol, and (2) an amorphous powder, m.p. 129–190°. The compound (m.p. 256°) is a normal constituent of the broad-bean shoot and more especially of the root; it was obtained in this fraction in all experiments and was identified as betulin (Abbot *et al.* 1958).

The amorphous powder (2) was separated into ketonic and non-ketonic fractions by treatment with Girard's reagent P. The ketonic fraction ($\lambda_{\max}$ 292 m μ) crystallized from ethanol in needles (1 mg.), m.p. and mixed m.p. with 4:7-chloro-4:6:2'-trimethoxy-6'-methylgris-2'-en-3:4'-dione, 218–220°.

In subsequent descriptions in this and the following sections, only data for tubes and fractions which differed significantly from the control will be described. Fractions not so mentioned were similar to the control.

Compound (VI; R = OMe). Tubes 24–33: the acid fraction contained a trace of an acidic substance with $\lambda_{\max}$ 325 and 280 m μ .

The neutral fraction ($\equiv$ 8 mg.) was extracted with boiling benzene and the solution chromatographed, elution being effected with benzene:methanol (99:1, v/v). Eluates showing absorption maxima at 287 m μ were combined and the solute, after washing with ether, crystallized from methanol in needles (4.5 mg.) of 4:6:2'-trimethoxy-6'-methylgris-2'-en-3:4'-dione, m.p. 180–181°, undepressed on admixture with authentic material.

Compound (IV). Tubes 25–37: the neutral fraction showed a maximum at 288 m μ with an inflexion at 265 m μ . After alumina chromatography a gummy eluate was obtained showing maxima at 261 and 290 m μ ($\equiv$ 1 mg.), characteristic of 7-chloro-4:6:4'-trimethoxy-6'-methylgris-3'-en-3:2'-dione (Grove *et al.* 1952a), but a positive identification could not be obtained.

Compound (II; R = OP⁺). Tubes 21–35: chromatography of the neutral fraction yielded a very impure eluate with $\lambda_{\max}$ 291 m μ ($\equiv$ 0.3 mg.). The solute was recovered and separated into ketonic and non-ketonic portions by treatment with Girard's reagent P. The ketonic fraction did not crystallize from ether and the 291 m μ -absorbing component was not formally identified.

Compound (II; R = OBu⁺). Expt. A is described here and in the following section.

Tubes 22–30: the neutral fraction showed weak specific absorption at 290 m μ and was chromatographed on alumina. Elution of a blue fluorescent band with benzene-methanol (99:1, v/v) afforded some concentration of this material, but only a trace (<0.5 mg.) was present and precluded a formal identification.

Compound (V). Tubes 20–33: the neutral fraction, $\lambda_{\max}$ 290 m μ ($\equiv$ 3 mg.), was chromatographed on alumina and the eluates containing the 290 m μ -absorbing material were combined and the solute sublimed at 115°/10⁻³ mm. The sublimate was treated with ether and the more soluble portion crystallized from ether-light petroleum (b.p. 40–60°) in small prisms (0.5 mg.), identified as 7-chloro-6:2'-diethoxy-4-methoxy-6'-methylgris-2'-en-3:4'-dione by mixed m.p. (165–170°) and comparison of infrared spectra.

The acidic fraction contained a trace of a substance with $\lambda_{\max}$ 282 and 325 m μ .

Compound (III; R = OH). Tubes 35–50: the acidic fraction had specific absorption maxima at 282 and 320 m μ .

The neutral fraction showed a maximum at 288 m μ . The

ether soluble portion was chromatographed on alumina in ether and eluates exhibiting maxima at 288 m μ ($\equiv$ 0.1 mg.) were sublimed giving an intractable solid, m.p. 130–160°.

Compound (III; R = H). Tubes 27–37: the neutral fraction showed $\lambda_{\max}$ 287 m μ ($\equiv$ 5 mg.), and after separation of insoluble amorphous material was chromatographed on alumina in ultraviolet light. Elution of the blue fluorescent band with benzene-methanol (99:1, v/v) gave a yellow gum which was sublimed at 170°/0.1 mm. The sticky yellow sublimate was treated with methanol, insoluble material rejected, and the solute, after recovery, crystallized from benzene:light petroleum (b.p. 60–80°). Three crystallizations gave 7-chloro-6'-hydroxy-4:6-dimethoxy-2'-methylgris-3-one (1 mg.), m.p. and mixed m.p. 200–203°.

Compound (II; R = OH). In Expt. A in which the tissues were macerated in water a careful investigation of all tubes after countercurrent distribution, and of all fractions derived therefrom, failed to reveal the presence either of the trione or of any metabolites. Accordingly, in experiment B a very high uptake was arranged (Table 2), and the tissues were macerated in aq. sodium hydrogen carbonate (p. 232). All tubes and fractions were carefully examined, but there was no trace either of the trione or of metabolites.

Compound (II; R = NH₂). Tubes 33–45: the acidic fraction showed strong peaks at 282 and 320 m μ (293 and 342 m μ in 0.1 N-sodium hydroxide) ($\equiv$ 9 mg.). The material responsible for this absorption was not identified.

Tubes 46–50: the acidic fraction had a weak peak at 290 m μ in 0.1 N sodium hydroxide. The compound responsible for this absorption could not be obtained in crystalline form: if it was the trione (II; R = OH) less than 1 mg. was present. The trione is not found in this batch of tubes, and if present must have been produced by decomposition of the amine during recovery of the solute or separation of the fractions. On this admittedly slender evidence a trace of 2'-amino-7-chloro-4:6-dimethoxy-6'-methylgris-2'-en-3:4'-dione may have been present in the shoots.

Compound (I). Tubes 20–32: the acidic fraction (6 mg.) showed peaks at 280 and 322 m μ , but purification was not attempted.

The neutral fraction (500 mg.), $\lambda_{\max}$ 325 m μ , was extracted with ether and the recovered extract chromatographed on alumina in benzene. Elution with benzene-methanol afforded a gum (59 mg.), containing a component $\lambda_{\max}$ 327 m μ ($\equiv$ apparent 7 mg. of gris-3:4'-dione) which passed into the non-ketonic fraction on treatment with Girard's reagent P, but further purification by fractional sublimation up to 150°/0.1 mm. was ineffective and the gums could not be crystallized. However, the infrared spectrum (Nujol mull) of the main fraction was fairly similar, in the carbonyl and hydroxyl regions, to the spectra of both α - and β -4'-hydroxygris-3-one (McCloskey, 1958), and it seems likely that both these products are present as metabolites.

Tubes 33–50: the neutral fraction, a partly crystalline gum (141 mg.), showed a strong peak at 282, ~290 m μ ($\equiv$ 33 mg. gris-3:4'-diol) and was chromatographed on alumina in benzene. Fractional elution with benzene-methanol (199:1, v/v) gave a component, $\lambda_{\max}$ 325 m μ ($\equiv$ 6 mg., apparent gris-3:4'-dione), which was not examined, and a component, $\lambda_{\max}$ 282, ~290 m μ ($\equiv$ 34 mg. gris-3:4'-diol), a portion of which (30 mg. $\equiv$ 16 mg. of

diol) was sublimed at $165^{\circ}/10^{-1}$ mm. The sublimate was washed with ether and then crystallized from benzene, yielding needles (12 mg.) of α -grisan-3:4'-diol, m.p. 187 – 198° , $[\alpha]_D^{25} + 79^{\circ}$ in ethanol (c, 0.6; microdetermination on 1.5 mg.). (Found: C, 70.4; H, 7.3. $C_{13}H_{18}O_3$ requires C, 70.9; H, 7.3%). The infrared spectrum (Nujol mull) was identical with that of authentic (+)- α -grisan-3:4'-diol, $[\alpha]_D^{25} + 73^{\circ}$ in ethanol (c, 1.0) (McCloskey, 1958). Counter-current distribution ($n = 50$) of ($\pm$)- α -grisan-3:4'-diol gave $K = 3.35$ and $N = 38.5$; tubes 30–46 contained 0.99 of the total diol distributed.

Compound dd-(II; R = OMe). The 14-day decay experiment (Crowdy *et al.* 1956) is described here but the 6.5-day decay experiment was almost identical. There was little difference between the controls corresponding to the 3-day uptake and the decay experiments.

Tubes 26–36: the acidic fraction contained a small amount of a substance with $\lambda_{\max}$ 290 and 320 μ .

The neutral fraction had $\lambda_{\max}$ 285 μ ($\equiv$ 4 mg.) and was chromatographed on alumina. Elution of the blue-fluorescent band with benzene-methanol (99:1, v/v) gave, after recovery, sublimation *in vacuo*, treatment with light petroleum and crystallization from ethanol, griseofulvin (2 mg.) prisms, m.p. 212 – 215° , undepressed by admixture with an authentic specimen.

Tubes 37–50: the acidic fraction showed $\lambda_{\max}$ 285 and 320 μ ($\equiv$ 13 mg.) ($\lambda_{\max}$ 293 and 342 μ in 0.1 N-sodium hydroxide). Considerable purification was effected (accompanied by appreciable losses of material) by molecular distillation, chromatography on Celite and fractional precipitation from solvents, but the substance absorbing at 285 and 320 μ was not obtained in crystalline form. A quantitative determination of halogen in the final purified fraction (5 mg.) gave Cl, 0.84%. A corresponding determination on the control fraction gave Cl, 1.2%.

Extraction and identification of compounds from root tissue and search for metabolites

Compound ld-(II; R = OMe). Tubes 24–36: the neutral fraction showed $\lambda_{\max}$ 290 μ ($\equiv$ 13 mg.) and was separated into non-ketonic and ketonic fractions in the usual way. The ketonic material crystallized from ethanol in needles (5 mg.), m.p. and mixed m.p. with *ld*-7-chloro-4:6:2'-trimethoxy-6'-methylgris-2'-en-3:4'-dione, 210 – 215° .

Compound (VI; R = OMe). Tubes 23–35: the neutral fraction had a sharp peak at 286 μ ($\equiv$ 8 mg.). Repeated sublimation of the material at 120 – $150^{\circ}/0.1$ mm. and treatment of the sublimate with ether, followed by crystallization of the more soluble component from ether-methanol, afforded 4:6:2'-trimethoxy-6'-methylgris-2'-en-3:4'-dione (0.5 mg.; m.p. and mixed m.p. 177 – 180°).

Compound (IV). The tubes, after countercurrent distribution, showed a well-defined peak at 290 μ with a shoulder at 285 μ , first obvious in tube 24 and rising to maximum intensity in tube 31. Thereafter the intensity of the peak declined, eventually merging with the shoulder to form a single broad maximum at 275 μ which persisted almost to tube 50.

Tubes 24–35: the acidic fraction showed $\lambda_{\max}$ 286, ~ 332 μ ($\equiv$ 9 mg.) ($\lambda_{\max}$ 294, ~ 335 μ in 0.1 N-sodium hydroxide). Treatment of the crude material (17 mg.) with benzene-ether yielded a phenolic acid (3 mg.), m.p. 284 – 288° (decomp.) (gas evolution), ($\lambda_{\max}$ 287, ~ 330 μ , $E_{1\text{cm}}^{1\%}$ 625, 122 respectively), soluble, with evolution of CO_2

in sodium hydrogen carbonate. There was insufficient material for further purification. The infrared spectrum showed C=O bands at ~ 1709 and 1685 cm^{-1} in chloroform solution, and this together with the ultraviolet spectrum suggested the presence of a 4-hydroxycoumaranone chromophore (Duncanson *et al.* 1957).

Sublimation at $215^{\circ}/0.0001$ mm. of the gummy residue obtained by evaporation of the mother liquors gave a sublimate which, on treatment with ether, afforded a solid (1 mg.), m.p. and mixed m.p. with 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid (VII), 213 – 218° (decomp.). Under the standard conditions used for countercurrent distribution ($n = 50$), 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid has $K = 2.03$ and $N = 33.5$. The ultraviolet-absorption curve shows maxima at 221, 265, and 306 μ and in the crude acidic fraction these peaks were presumably masked by the much greater amount of the component, m.p. 284 – 288° (decomp.), $\lambda_{\max}$ 287 μ .

The phenolic fraction showed $\lambda_{\max}$ 288 μ ($\equiv$ 6 mg.) and was sublimed at $200^{\circ}/0.0001$ mm. The sublimate crystallized from acetone in needles (1 mg.) m.p. 218 – 224° (decomp.) of 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid (see above). From the ultraviolet absorption this was a minor component only of the phenolic fraction; the major component ($\lambda_{\max}$ 288, ~ 330 μ) could not be identified, but it was possibly the same as the major component, m.p. 284 – 288° (decomp.) of the acidic fraction.

The neutral fraction showed a single maximum at 289 μ ($\equiv$ 7 mg.). Sublimation at $200^{\circ}/0.1$ mm. and chromatography of the sublimate afforded a gum (12 mg.) which had a sharp absorption band at 291 μ but could not be solidified. The infrared spectrum showed a broad OH band and a broad C=O band at 1725 cm^{-1} .

Tubes 36–50: the acidic fraction had a weak maximum at 285 μ .

The neutral fraction contained a trace of material absorbing at 290 and 262 μ , but the quantity was too small for further investigation.

Compound (II; R = OPrⁿ). Tubes 21–35: the neutral fraction had $\lambda_{\max}$ 290 μ ($\equiv$ 3 mg.). Chromatography on alumina and elution of the blue fluorescent band with benzene-methanol (200:1, v/v) yielded a solid which was separated into ketonic and non-ketonic fractions. The ketonic fraction crystallized from ether in micro-needles (< 0.2 mg.), identified as 7-chloro-4:6-dimethoxy-6'-methyl-2'-*n*-propoxygris-2'-en-3:4'-dione by comparison of the infrared spectrum with that of an authentic specimen.

Compound (II; R = OBuⁿ). Tubes 20–33: the neutral fraction showed $\lambda_{\max}$ 292 μ ($\equiv$ 6 mg.). Chromatography followed by fractional crystallization of the solute from light petroleum (b.p. 60 – 80°) afforded 2'-*n*-butoxy-7-chloro-4:6-dimethoxy-6'-methylgris-2'-en-3:4'-dione (3 mg.), identified by comparison of the infrared spectrum with an authentic specimen.

Tubes 34–50: the acidic fraction had $\lambda_{\max}$ 287 m μ [$\equiv$ 1.5 mg. of the trione (II; R = OH)]. The gummy fraction was extracted with benzene, but the benzene-insoluble portion was intractable and a positive identification was not obtained. Compound (II; R = OH), if present, would have been produced before countercurrent distribution, and possibly during maceration.

Compound (V). Tubes 20–35: the neutral fraction showed $\lambda_{\max}$ 290 m μ ($\equiv$ 26 mg.) and was separated into ketonic and non-ketonic portions. The ketonic material crystallized from a small volume of methanol in needles (16 mg.), m.p. and mixed m.p. with 7-chloro-6:2'-diethoxy-4-methoxy-6'-methylgris-2'-en-3:4'-dione, 163–170°.

Compound (III; R = OH). After countercurrent distribution an absorption peak ($\lambda_{\max}$ 290 m μ) was observed in tubes close to tube 42.

Tubes 36–50: the neutral fraction had $\lambda_{\max}$ 288 m μ ($\equiv$ 4 mg.) and was chromatographed on alumina in ether. Eluates containing 288 m μ -absorbing material were bulked and the solute repeatedly sublimed at 150–180°/0.0001 mm. The final sublimate, a colourless gum, could not be obtained in crystalline form, but the infrared spectrum showed bands due to –OH and C=O and was generally similar to that of 7-chloro-4:6'-dihydroxy-4:6'-dimethoxy-2'-methylgris-3-one.

Compound (III; R = H). Tubes 27–37: the neutral fraction had $\lambda_{\max}$ 287 m μ ($\equiv$ 8 mg.) and was chromatographed in benzene on alumina. Elution with benzene:methanol (99:1, v/v) of the band fluorescing blue in ultra-violet light afforded a colourless solid (36 mg.) which was fractionally crystallized from ethanol. The more soluble fraction (18 mg.) was sublimed at 170°/0.1 mm. and the sublimate (11 mg.) repeatedly crystallized from benzene-light petroleum (b.p. 60–80°). Identification of the purified product (4 mg.), m.p. 203–205°, undepressed by admixture with 7-chloro-6'-hydroxy-4:6'-dimethoxy-2'-methylgris-3-one, was confirmed by comparison of the infrared spectra.

Compound (II; R = OH). As with the shoot tissue extracts (see p. 237), examination of the extracts from both experiments A and B failed to disclose the presence either of the trione or of metabolites.

Compound (II; R = NH₂). Tubes 28–36: the acidic fraction had $\lambda_{\max}$ 290 m μ [$\equiv$ 0.5 mg. of the trione (II; R = OH): see below].

The neutral fraction had a weak peak at 285 m μ . This metabolite was not identified but not more than 0.3 mg. could have been present. It was not eluted by benzene-methanol (98:2, v/v) from an alumina column and was not therefore 2'-acetamido-7-chloro-4:6'-dimethoxy-6'-methylgris-2'-en-3:4'-dione ($\lambda_{\max}$ 289 m μ , ϵ 29 400) which is quantitatively eluted from such a column by benzene-methanol (99:1, v/v). The acetamido-compound was thought to be a possible metabolite in view of the work of Jones & Wignall (1955).

Tubes 37–47: the basic fraction showed specific absorption at 290 m μ ($\equiv$ 2 mg.). Attempts to crystallize and identify this substance as the amine (II; R = NH₂) were unsuccessful, but it is unlikely that a basic metabolite of the amine would be present.

The acidic fraction showed specific absorption at 290 m μ in 0.1N-sodium hydroxide [$\equiv$ 0.9 mg. of the trione (II, R = OH)]. The equivalent amount of trione, calculated from the theoretical distribution, was found in tubes 28–36

(which contain no amine), and the latter trione must have been present as such before countercurrent distribution. It seems probable therefore that both trione fractions were produced during maceration and extraction of the macerate.

Compound (I). Tubes 21–31: the neutral fraction (40 mg.) showed $\lambda_{\max}$ 281, ~288 m μ ($\equiv$ 10 mg. of apparent gris-3:4'-diol) and was chromatographed on alumina in benzene. Elution with benzene:methanol (99:1, v/v) afforded a gum which showed only a fraction ($\equiv$ 4 mg.) of the initial absorption at 282 m μ . Further attempts at purification failed.

Tubes 37–49: the neutral fraction (40 mg.) showed $\lambda_{\max}$ 280, ~290 m μ ($\equiv$ 29 mg. of gris-3:4'-diol) and was chromatographed as described above. Fractions showing $\lambda_{\max}$ 282 m μ were combined and sublimed at 150–160°/0.03 mm. pressure; the sublimate was treated with ether, furnishing a solid which was crystallized from benzene, yielding prisms (1 mg.), m.p. 171–186° of a mixture of α - and β -gris-3:4'-diols in approximately equal proportions as shown by the infrared spectrum.

Compound dd-(II; R = OMe). No differences were observed between the griseofulvin-treated and control extracts in the decay experiments.

DISCUSSION

The spectrophotometric methods of analysis outlined above proved satisfactory in practice; on plant-tissue extracts containing 1–20 mg. of the compounds they were accurate to $\pm$ 1 mg. (Crowdy *et al.* 1955) considered adequate for work of this type. Estimations on treating solutions were probably rather less accurate, as the elaborate series of control experiments performed with griseofulvin (Crowdy *et al.* 1955) was not carried out.

The griseofulvin *ld* diastereoisomer, dechloro-griseofulvin, 7-chloro-6:2'-diethoxy-4-methoxy-6'-methylgris-2'-en-3:4'-diol. (V) and the alcohol (III; R = H) were recovered, unchanged, from both shoots and roots of treated plants; and the propyl and butyl homologues of griseofulvin and the diol (III; R = OH) were recovered from the roots. In addition there was strong presumptive evidence that the last three compounds, and also 7-chloro-4:6:4'-trimethoxy-6'-methylgris-3'-en-3:2'-dione, were present unchanged in the shoots; and that the amine (II; R = NH₂) existed unchanged in both roots and shoots. The trione (II; R = OH) and gris-3:4'-dione were absent from both roots and shoots.

In the 3-day-uptake experiments described in this paper it was not expected, by analogy with griseofulvin (Crowdy *et al.* 1955), that metabolites of the compounds studied would be detected or isolated. This expectation proved to be correct for the compounds more closely related to griseofulvin, namely the *ld*-diastereoisomer, the dechloro analogue and three homologues of griseofulvin and also for the alcohols (III; R = H and OH). On the

other hand, both compound (IV) and grisan-3:4'-dione gave rise during 3 days to relatively stable metabolites in amounts permitting their detection and identification. With the compound (IV) additional metabolites were detected, though not identified, in both roots and treating solution. Of the metabolites identified 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid may be regarded as an oxidation product of compound (IV), whereas the grisan-3:4'-diols and 4'-hydroxygrisan-3-ones are reduction products of grisan-3:4'-dione. Although it was not possible to estimate accurately the relative amounts of the α - and β -grisan-3:4'-diols present in the whole plant system, including the treating solution, the diol mixture actually isolated from the various sites (some 54% of the total, estimated spectrophotometrically) was shown to contain not less than 46% of the β (less stable) isomer. Thus the proportion of the β -diol present in the entire system was at least 25%.

Root extracts of plants treated with the amine (II; $R = NH_2$) and with the butyl homologue of griseofulvin contained traces of an acidic substance believed to be the trione (II; $R = OH$); a trace of an unidentified neutral substance was also detected in roots treated with the amine. The trione (II; $R = OH$) is unstable in bean roots, none having been recovered from the roots of treated plants, and it follows that the acidic substance was probably produced by hydrolysis of the amine (II; $R = NH_2$) and butyl homologue during maceration and extraction.

With these two compounds and with grisan-3:4'-dione the final pH of the treating solution was abnormally low (Table 2), but there was no correlation between low pH and the production in the treating solution of acidic metabolites detectable by the spectrophotometric techniques employed. Acidic metabolites were only detected in the residual solution from plants treated with the dechloro analogue of griseofulvin and with (IV). In the former case the acidic substance was believed to be the corresponding trione (VI; $R = OH$).

No metabolites of griseofulvin were detected in the shoots of treated plants following the 3-day-uptake experiment (Crowdy *et al.* 1955). Griseofulvin is nevertheless slowly broken down in the shoots of broad bean (Crowdy *et al.* 1956), and the shoot-extracts from two decay experiments, in which griseofulvin was estimated chemically, were examined for the presence of metabolites. An acidic fraction, absent from the control, with strong absorption peaks at 285 and 320 $m\mu$, showing a characteristic shift to 293 and 345 $m\mu$ in alkaline solution, was obtained in both experiments. A similar acidic fraction, absent from the control, and showing identical ultraviolet-absorption characteristics, was obtained from the shoots

of plants treated for 3 days with the amine (II; $R = NH_2$; Table 4). Traces of acidic substances with a similar absorption spectrum in ethanol were also found in the shoots of plants treated with the dechloro analogue, the homologue (V), the *ld*-diastereoisomer and the diol (III; $R = OH$). The acidic substance, first detected in the griseofulvin-decay experiments, was for some time believed to be a metabolite of griseofulvin; the possibility that it might be a metabolite common to the breakdown of all the above chlorine-containing griseofulvin relatives was also considered. The observed absorption shift in alkali is characteristic of the 4:6-dihydroxycoumaranone chromophore (VIII) (Duncanson *et al.* 1957). The substance was labile, being particularly sensitive to atmospheric oxidation, and although some purification was effected by chromatographic and distillation techniques it remained unidentified. The halogen contents of purified fractions were nevertheless no greater than those of controls from untreated plants.

An acidic fraction with identical ultraviolet-absorption maxima was eventually obtained from the shoot-extract control to the large-scale grisan-3:4'-dione experiment (B); the corresponding fraction from the treated plants showed an identical absorption. The substance must therefore be a normal plant constituent, a conclusion in agreement with the halogen determinations; it seems unlikely that the griseofulvin molecule would lose halogen without disruption of the aromatic ring (Wright & Grove, 1957). The absence of the acidic substance from the appropriate controls in all the cases listed in Table 4 is most striking; the most likely explanation seems to be that the substance, a normal plant constituent, is produced in much greater quantity in shoots treated with griseofulvin relatives. The partial structure (VIII), suggested by the ultraviolet-absorption data, is common to many known plant constituents.

(VIII)

The spectrophotometric techniques employed in this work are well suited to the routine estimation of the compounds examined; they are not so well suited to metabolic studies which could more conveniently be carried out on labelled compounds with tracer techniques.

SUMMARY

1. Analytical methods, similar to those used for griseofulvin (Crowdy *et al.* 1955), are described for the estimation in bean tissues and the treating

solutions of eleven compounds chemically related to griseofulvin. The compounds studied comprised a diastereoisomer of griseofulvin, dechlorogriseofulvin, three homologues of griseofulvin, 7-chloro-4:6:4'-trimethoxy-6'-methylgris-3'-en-3:2'-dione, the parent acidic trione, its basic amino derivative, two neutral alcoholic reduction products of the trione, and grisan-3:4'-dione.

2. The compounds were recovered and characterized chemically in the majority of cases where the analytical methods indicated their presence in the roots and shoots of treated plants. 3-Chloro-2-hydroxy-4:6-dimethoxybenzoic acid, a metabolite of 7-chloro-4:6:4'-trimethoxy-6'-methylgris-3'-en-3:2'-dione and mixtures of 4'-hydroxygrisan-3-ones and optically active grisan-3:4'-diols, metabolites of grisan-3:4'-dione, were also recovered and identified. Other metabolites of these two compounds, and a substance, increased production of which is associated with the degradation of certain griseofulvin relatives in shoot tissue, were detected but not identified.

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The Translocation of Antibiotics in Higher Plants

4. SYSTEMIC FUNGICIDAL ACTIVITY AND CHEMICAL STRUCTURE IN GRISEOFULVIN RELATIVES

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Griseofulvin causes a characteristic distortion ('curling') of the hyphae of most fungi with chitinous cell walls, and the capacity to produce this hyphal distortion seems to be specific to griseofulvin and its near relatives (J. F. Grove, unpublished work). By suitable modification of the molecule it is possible to produce a range of compounds in which the antifungal activity is varied widely. With suitable biological testing it is possible to show how far the systemic fungicidal activity of these compounds is related to their ability to induce hyphal distortion. In tracing this relationship systemic fungicidal activity must be assessed in relation to the amount of the compound actually present in the plant tissues. To this end analytical methods, similar to those used for griseofulvin (Crowdy, Gardner, Grove & Pramer, 1955), were worked out (Crowdy, Green, Grove, McCloskey & Morrison, 1959) for estimating the griseofulvin relatives in plant tissue and in the

treating solutions. Balance sheets were then drawn up relating the amount of material actually entering the plant to that found in the roots and shoots.

EXPERIMENTAL

Analytical methods have been described in detail in the preceding paper (Crowdy *et al.* 1959).

Antifungal tests. The antifungal activity *in vitro* was assessed against germinating spores of *Botrytis allii* (Brian, Curtis & Hemming, 1946). Concentrations of griseofulvin causing hyphal 'curling' and stunting of *Alternaria solani* are similar to those having a like effect on *B. allii*.

Details of the test for systemic fungicidal activity, measured by the reduction of leaf infection of tomato by *A. solani* after treatment of the roots with 0.05% dispersions of the compounds, have been given by Brian, Wright, Stubbs & Way (1951), and Stubbs (1952). Reproducibility in this test was not high and the results have been graded for convenience on an arbitrary scale (Table 1).

Compounds giving 0-49% control of disease were considered unlikely to be of importance in practice and were