923. *Studies in Relation to Biosynthesis. Part XVII.* Sclerotiorin, Citrinin, and Citromycetin.*

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The routes of biosynthesis, in appropriate moulds, of the substances named in the title have been examined by means of feeding experiments with ^{14}C-labelled acetic and formic acids.

Some previous papers [1] dealt with a speculative examination of the biosynthetic origins of plant and mould products from acetic acid by head-to-tail linkage, or from acetic acid and methionine. These predictions have been confirmed by direct experiment with $CH_3{\cdot}^{14}CO_2H$ in two cases [2,3] where the molecule can arise from a straight chain of acetic acid units joined by head-to-tail linkage, and in one case (mycophenolic acid) [4,5] where methionine is also involved. We have now extended our researches to trace the origins of molecules which can only arise from *branched* carbon chains. From previous work there seem to be three likely modes of origin: (A) From acetic acid, by head-to-tail linkage in a straight chain with the introduction of C_1 units from the C_1 pool (choline, methionine etc.). (B) From acetic acid by production of branched chains employing head-to-tail linkage. (C) By the isoprene route formally similar to (B) but with a definite intermediate in the form of mevalonic acid.

Mycophenolic acid [4,5] is an interesting example of the working of routes (A) and (C) in one compound; no example of route (B) had, before the present work, been demonstrated. It cannot be excluded that *intermediates* in the production of griseofulvin or methylsalicylic acid, or any other compound involving the orcinol-type (aldol) of ring-closure, could be branched in the manner of route (B) but in the absence of experimental evidence no such intermediates need at present be postulated.

Sclerotiorin.—When the probable formula [6] (I) is considered the most likely route to sclerotiorin is of type (A) with three introduced methyl groups (or four if the carbon of the methinyloxy-group is derived from the C_1 pool). The " extra " acetic acid unit may also involve a branched main chain, but in view of the slight uncertainty in the structure we have confined ourselves to confirming its origin from acetic acid.

Sclerotiorin was produced from *Penicillium multicolor* in media containing, in separate experiments, $CH_3{\cdot}^{14}CO_2H$, $H{\cdot}^{14}CO_2H$, and $^{14}CH_3{\cdot}CO_2H$, the radioactivity being incorporated into the metabolite to the extent of 3, 20, and 8% respectively. The products were hydrolysed by alkali to 4 : 6-dimethylocta-1 : 4-dienoic acid (II) which was oxidised [8] to 2-methylbutanal (III) (examined as the 2 : 4-dinitrophenylhydrazone). The sclerotiorin and the 2-methylbutanal derivatives were subjected to Kuhn–Roth oxidation and the labelling in the resultant acetic acid was examined by pyrolysis of the lithium salt.[9] The results, set out in our usual fashion in Scheme 1, confirm the applicability of route (A) in showing the expected origin of sclerotiorin from nine molecules of acetic acid and three C_1 units.

The radioactive assay data (see Experimental section) are in quantitative agreement with the postulated distributions of radioactivity shown in (Ia), (Ib), and (Ic) in material

* Part XVI, *J.*, 1958, 2622.

[1] Birch and Donovan, *Austral. J. Chem.*, 1953, **6**, 360; Birch, Elliott, and Penfold, *ibid.*, 1954, **7**, 169; Birch, *Fortschr. Chem. org. Naturstoffe*, 1957, **16**, 186.
[2] Birch, Massy-Westropp, and Moye, *Austral. J. Chem.*, 1958, **8**, 539.
[3] Birch, Massy-Westropp, Rickards, and Smith, *J.*, 1958, 360.
[4] Birch, English, Massy-Westropp, Slaytor, and Smith, *J.*, 1958, 365.
[5] Birch, English, Massy-Westropp, and Smith, *ibid.*, p. 369.
[6] Fielding, Robertson, Travers, and Whalley, *J.*, 1958, 1814.
[7] Watanabe, *J. Pharm. Soc. Japan*, 1952, **72**, 807.
[8] Eade, Page, Robertson, Turner, and Whalley, *J.*, 1957, 4920.
[9] Cornforth, Hunter, and Popjak, *Biochem. J.*, 1953, **54**, 597.

derived from $CH_3 \cdot {}^{14}CO_2H$, $H \cdot {}^{14}CO_2H$, and ${}^{14}CH_3 \cdot CO_2H$, respectively. Further features of interest to emerge are:

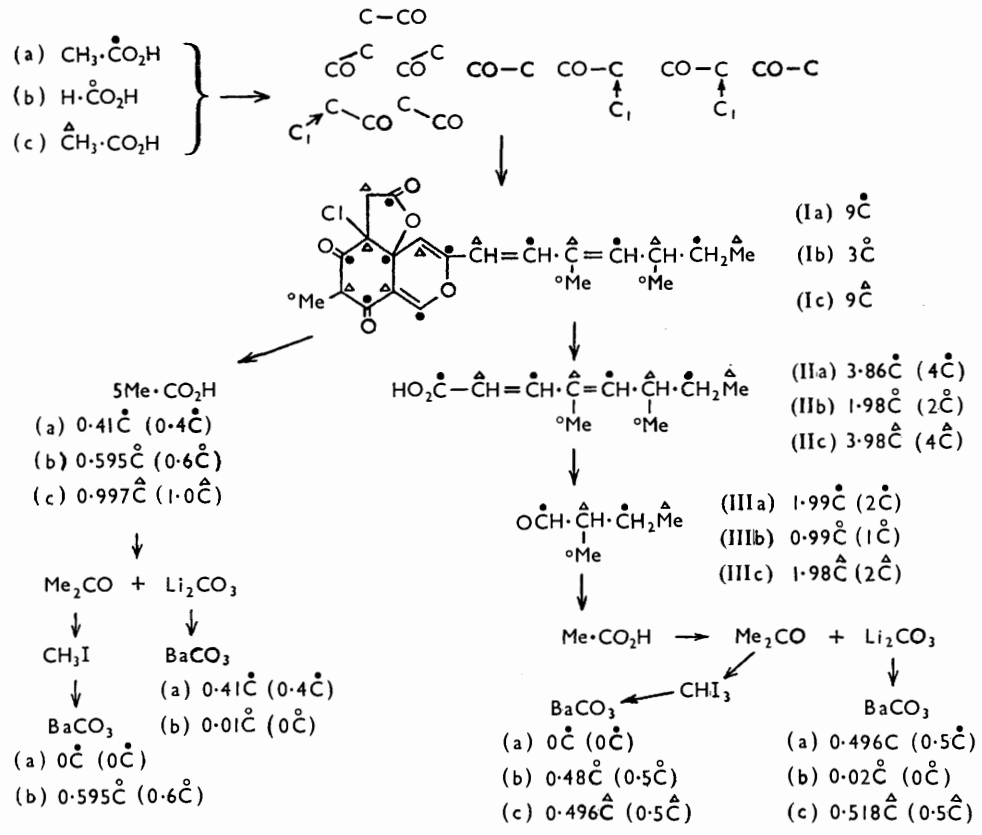
(i) The results of the Kuhn–Roth oxidations of sclerotiorin are consistent with the remaining degradations only if the production of *five* acetic acid units (in 90% yield) be assumed; *i.e.*, one arises from the " extra " acetic acid unit in the lactone ring which appears to be labelled to the same extent as those in the main chain.

(ii) The carbon of the methinyloxy-group must come from a reduced carboxyl group of acetic acid.

(iii) During the biosynthesis from ${}^{14}CH_3 \cdot CO_2H$ there is no appreciable redistribution of activity between the methyl- and the carboxyl-carbon atom, and no appreciable conversion into $H \cdot {}^{14}CO_2H$ or a biological equivalent.

(iv) The introduced methyl groups in the aliphatic side-chain are labelled to the same extent as that attached to the *cyclo*hexane-1 : 3-dione ring.

Scheme 1.



When the dienoic acid (II) labelled in the 4- and the 6-methyl group was added to the growth medium of *P. multicolor*, radioactive sclerotiorin was obtained and on degradation gave the dienoic acid (II) which was found to contain four-ninths of the activity of the precursor sclerotiorin. This result is consistent with the degradation of the acid (II) to ${}^{14}CH_3 \cdot CO_2H$ before incorporation and may be taken as evidence that this acid is not an intermediate in the biosynthesis.

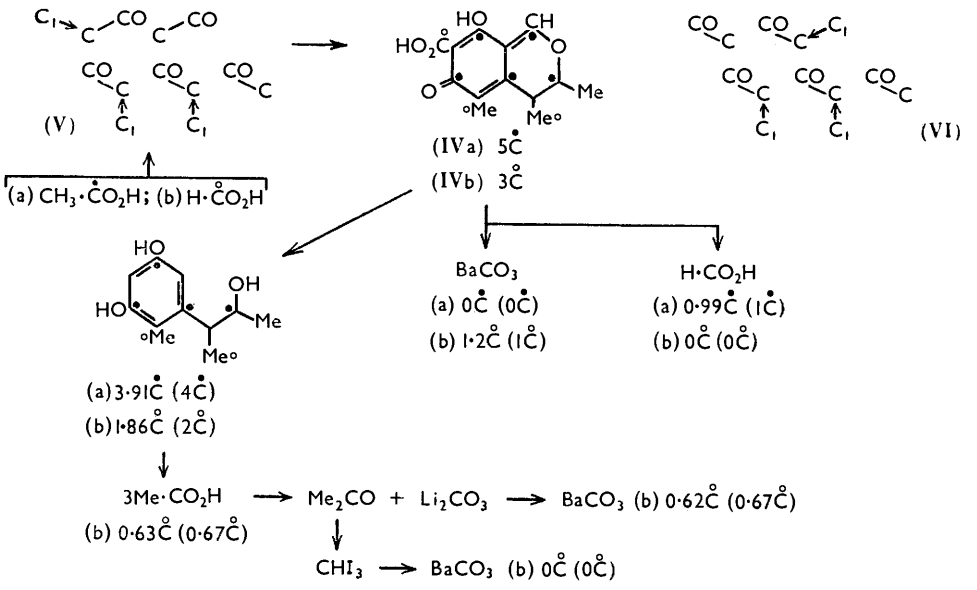
It is notable that in the present case, as with ergosterol,[10] introduced C_1 units are found

[10] Alexander, Gold, and Schwenk, *J. Amer. Chem. Soc.*, 1957, **79**, 2967; Alexander and Schwenk, *ibid.*, p. 4554.

in an aliphatic chain. This is of particular interest in view of the postulated biogenesis [4] of the macrolide antibiotics where methyl groups are similarly thought to be introduced into non-aromatic systems derived by head-to-tail linkage of acetic acid units.

Citrinin.—This substance [11] (IV) could arise from five acetic acid units and three C_1 units but it is not possible to decide from the formula whether the C_1 units comprise the two methyl groups and the carboxyl group introduced into a straight acetic-acid-derived chain as in (V), or whether the two methyl groups and the methinyloxy-group are introduced into a branched acetic-acid-derived chain, *e.g.*, as in (VI). Other less probable biosyntheses could be envisaged. In fact, feeding experiments with $CH_3{\cdot}^{14}CO_2H$ and $H{\cdot}^{14}CO_2H$ confirm the former route (V) in *Aspergillus candidus*. Its is particularly striking that the incorporation of radioactivity into citrinin with $H{\cdot}^{14}CO_2H$ as substrate is at least 66%: that from $CH_3{\cdot}^{14}CO_2H$ is lower (11%) but still high for acetate incorporation. The postulated distributions of radioactivity from $H{\cdot}^{14}CO_2H$ and $CH_3{\cdot}^{14}CO_2H$ are shown in the formulæ (IVa) and (IVb). They were confirmed by the degradations outlined in Scheme 2. The acid hydrolysis [12] of the labelled citrinins to carbon dioxide (from the carboxyl group) and formic acid (from the methinyloxy-group) was particularly instructive. With $CH_3{\cdot}^{14}CO_2H$ as substrate the former was inactive but the latter contained 0·99 atom of active carbon per mol. based on the distribution (IVa). With $H{\cdot}^{14}CO_2H$ as substrate

Scheme 2.



the formic acid was inactive whilst the carbon dioxide contained 1·12 atoms of active carbon per mol. based on the distribution (IVb). In this case the inequality of labelling, which we consider to be outside the range of our usual experimental error, may imply a sequential rather than a simultaneous addition of C_1 units. In this biosynthesis, as with that of sclerotiorin, a notable step is the reduction of an acetic-acid carboxyl group to a lower oxidation state. Since this work was completed Dr. E. Schwenk (Worcester Foundation) has informed us of similar independent work arriving at the same conclusions: this is being published elsewhere.

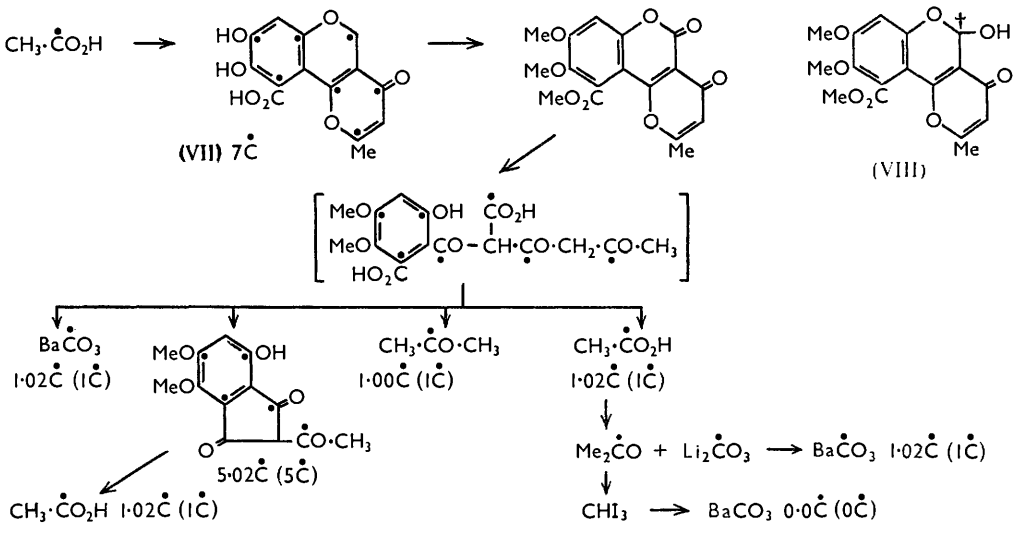
Citromycetin.—Attention has been drawn to the fact that citromycetin [13] (VII), fulvic

[11] Brown, Cartwright, Robertson, and Whalley, *J.*, 1949, 867.
[12] *Idem, ibid.*, p. 859.
[13] Robertson, Whalley, and Yates, *J.*, 1951, 2013.

acid, and fusarubin can all be derived from the same skeleton containing a branch involving one carbon atom [14] which we believed [4] to arise from the introduction of a C_1 unit in an acetate-derived chain. Earlier, however, Birch and Donovan had noted the possibility that fusarubin could be derived from a straight acetic acid chain.[14b] Feeding experiments with *P. frequentans* using $CH_3 \cdot {}^{14}CO_2H$ and $H \cdot {}^{14}CO_2H$ have shown that in citromycetin the carbon skeleton is entirely derived from acetic acid. Our first indication of this was that, whereas activity from $CH_3 \cdot {}^{14}CO_2H$ was incorporated into citromycetin to an expected extent (*ca.* 2%), only 0·8% of the activity of the added $H \cdot {}^{14}CO_2H$ was so recovered, an incorporation far lower than that so far encountered with any substance which incorporates C_1 units.

The degradations illustrated in Scheme 3 conclusively show the presence of seven almost equally labelled carbon atoms in the citromycetin derived from $CH_3 \cdot {}^{14}CO_2H$, and it is notable that the activity of the branch carbon atom is the same as that of the labelled carbon atoms in the main chain. The explanation of the results cannot therefore be merely that the formic acid is not biologically activated for incorporation. Degradation of the material derived from $H \cdot {}^{14}CO_2H$ indicated a small general distribution of radioactivity. Thus the carbon dioxide obtained by decarboxylation of citromycetin to citromycin contained 13% of the total activity and the acetone (representing three carbon atoms of the main chain) and formic acid obtained by alkaline degradation of citromycetinol (VIII) contained 24 and 0·5% respectively of this activity. Had citromycetin been formed by route (A) then all of the activity from $H \cdot {}^{14}CO_2H$ would have been localised on the carbon atom marked † in formula (VIII) and thus recovered in the formic acid above.

Scheme 3.



Experimental

General directions are as for Part XIV.[4] Relative molar activities (r.m.a.'s) (a), (b), and (c) refer to the labelled metabolites and their degradation products derived from $CH_3 \cdot {}^{14}CO_2H$, $H \cdot {}^{14}CO_2H$, and ${}^{14}CH_3 \cdot CO_2H$ respectively. Theoretical numbers of carbon atoms in degradation products are based on the appropriate postulated distributions in the labelled metabolites. Samples of barium carbonate denoted (Me) or (CO_2H) are from the methyl and the carboxyl groups respectively of acetic acid produced by Kuhn–Roth oxidation.

[14] (*a*) Dean, Eade, Mombasher, and Robertson, *Nature*, 1957, **179**, 366; (*b*) Birch and Donovan, *Chem. and Ind.*, 1954, 1047.

[^{14}C]*Sclerotiorin.*—*P. multicolor* Grigorieva-Manoilova and Poradielova was cultured as described earlier [15] except that on the tenth day labelled sodium formate or sodium acetate (0·1—0·4 mc) in water was distributed equally between three flasks (750 c.c. of culture medium per flask) under aseptic conditions. After a further 5 days the cultures were harvested in the usual manner. After a preliminary purification by crystallisation from methanol the crude sclerotiorin was diluted with inactive material and brought to radiochemical purity by a combination of processes involving further crystallisations from methanol, percolation in benzene through a column of Florisil and high-vacuum sublimation [Found: r.m.a. $\times 10^{-5}$, (a) 4·41; (b) 6·27; (c) 3·28].

Degradations of [^{14}C]*Sclerotiorin.*—(i) The labelled metabolite was hydrolysed with N-sodium hydroxide as previously described.[7] The resulting 4 : 5-dimethylocta-2 : 4-dienoic acid was purified by recrystallisation from 50% aqueous methanol and then by vacuum-sublimation. It had m. p. 91—92° (lit.,[8] m. p. 92°) [Found: r.m.a. $\times 10^{-5}$, (a) 1·89 (4C, 1·96); (b) 4·13 (2C, 4·18); (c) 1·45 (4C, 1·46)].

(ii) Kuhn–Roth oxidation of the labelled sclerotiorin gave acetic acid (isolated as lithium acetate). An aliquot part was converted into the *p*-bromophenacyl ester, m. p. 86—87° [Found: r.m.a. $\times 10^{-5}$, (a) 0·195 (0·4C, 0·196); (b) 1·24 (0·6C, 1·25); (c) 0·363 (1C, 0·364)]. The remainder of the lithium salt was pyrolysed, giving $BaCO_3(Me)$ [Found: r.m.a. $\times 10^{-5}$ (a) 0; (b) 1·24 (0·6C, 1·25)] and $BaCO_3(CO_2H)$ [Found: r.m.a. $\times 10^{-5}$, (a) 0·199 (0·4C, 0·196); (b) 0·003].

(iii) The [^{14}C]sclerotiorin was ozonised as previously described.[8] The resultant 2-methylbutanal 2 : 4-dinitrophenylhydrazone was percolated in benzene through bentonite–kieselguhr (1 : 1; 15 g.) and twice recrystallised from methanol. It had m. p. 133—134° and was identical with the derivative from the synthetic (±) aldehyde [Found: r.m.a. $\times 10^{-5}$ (a) 0·976 (2C, 0·980); (b) 2·07 (1C, 2·09); (c) 0·716 (2C, 0·730)].

(iv) The above radioactive 2 : 4-dinitrophenylhydrazone was diluted with synthetic material [Found r.m.a. $\times 10^{-4}$ (a) 2·38; (b) 2·15; (c) 1·98]. Kuhn–Roth oxidation gave acetic acid (1·92 mol.) obtained as lithium acetate which was pyrolysed, giving $BaCO_3(Me)$ [Found: r.m.a. $\times 10^{-4}$, (a) 0; (b) 1·02 (0·5C, 1·07); (c) 0·491 (0·5C, 0·495)] and $BaCO_3(CO_2H)$ [Found: r.m.a. $\times 10^{-4}$, (a) 0·590 (0·5C, 0·595); (b) 0·039; (c) 0·513 (0·5C, 0·495)].

[^{14}C]*Citrinin.*—*Aspergillus candidus* was cultured as described by Wyllie [16] except that on the seventh day $CH_3{\cdot}^{14}CO_2Na$ or $H{\cdot}^{14}CO_2Na$ (0·1 mc) was added to each of three culture flasks (750 c.c. of medium). After a further 3 days the citrinin was harvested in the usual fashion, diluted with inactive material, and brought to radiochemical purity by repeated recrystallisation from methanol. It had m. p. 169—171° [Found: r.m.a. $\times 10^{-6}$ (a) 2·727; (b) 1·319].

Degradations of [^{14}C]*Citrinin.*—(i) Labelled citrinin was hydrolysed with sulphuric acid as previously described.[12] The resulting carbon dioxide was converted into barium carbonate [Found: r.m.a. $\times 10^{-6}$, (a) 0; (b) 0·490 (1C, 0·430)]. The formic acid was collected as sodium formate which was converted by Whalley's method [17] into *NN'*-diphenylformamidine, m. p. 188—190° (lit.,[17] m. p. 193°) [Found: r.m.a. $\times 10^{-6}$, (a) 0·541 (1C, 0·545); (b) 0·009].

(ii) Radioactive citrinin was hydrolysed with aqueous ammonia as described by Schwenk *et al.*[18] The crude 5-(2-hydroxy-1-methylpropyl)-4-methylresorcinol was purified by percolation of its solution in ether through Florisil and recrystallisation from chloroform. Obtained thus, the product was a chloroform solvate, m. p. 129—130° (Found: C, 53·9; H, 6·6. Calc. for $2C_{11}H_{16}O_3,CHCl_3$: C, 54·0; H, 6·5%), and since the chloroform was difficult to remove completely was best assayed as such [Found: r.m.a. $\times 10^{-6}$, (a) 2·13 (4C, 2·18); (b) 0·815 (2C, 0·879)]. Kuhn–Roth oxidation gave acetic acid (2·25 mol.), collected as lithium acetate. An aliquot part was converted into *p*-bromophenacyl acetate [Found: r.m.a. $\times 10^{-6}$, (b) 0·276 (0·67C, 0·293)]. The remainder was pyrolysed, giving $BaCO_3(Me)$ [Found: r.m.a. (b) 0], and $BaCO_3(CO_2H)$ [Found: r.m.a. $\times 10^{-6}$, (b) 0·273 (0·67C, 0·293)].

[^{14}C]*Citromycetin.*—*P. frequentans* Westling L.S.H.T.M. No. Ad. 7 was cultured by Hetherington and Raistrick's method.[19] On the seventh day $CH_3{\cdot}^{14}CO_2Na$ or $H{\cdot}^{14}CO_2Na$ (0·1 mc) was added to each of four flasks and after a further 7 days the citromycetin was

[15] Curtin and Reilly, *Biochem. J.*, 1940, **34**, 1419; 1943, **37**, 36.
[16] Wyllie, *Canad. J. Publ. Health*, 1945, **36**, 477.
[17] Whalley, *J.*, 1948, 1015.
[18] Schwenk, Schubert, and Stahl, *Arch. Biochem. Biophys.*, 1949, **20**, 220.
[19] Hetherington and Raistrick, *Phil. Trans.*, 1931, **220**, *B*, 209.

isolated. An aliquot part of the material was purified in the usual manner [Found: r.m.a. × 10^{-6}, (a) 1·29; (b) 0·159].

The degradations of both types of labelled material are based on procedures already described.[13, 20]

Degradations of Citromycetin derived from $CH_3{\cdot}^{14}CO_2Na$.—(i) The labelled citromycetin was decarboxylated and the carbon dioxide converted into barium carbonate (Found: r.m.a., 985).

(ii) The crude citromycetin was converted into di-*O*-methylcitromycetin methyl ester by treatment with ethereal diazomethane. The ester was purified by repeated high-vacuum sublimation. It had m. p. 176—178° (Found: r.m.a. × 10^{-6}, 1·290) and was converted into di-*O*-methylcitromycetinone methyl ester (1·3 g.), m. p. 240° (decomp.) (r.m.a. × 10^{-6}, 1·29), which was degraded with hot aqueous 2N-sodium hydroxide (65 c.c.). The resultant acetone was collected as the 2 : 4-dinitrophenylhydrazone (200 mg.) which was purified by chromatography in chloroform on alumina and by recrystallisation from methanol. It had m. p. 124—126° [Found: r.m.a. × 10^{-6}, 0·184 (1C, 0·184)]. The precipitate from the neutralised and cooled hydrolysate was recrystallised from methanol, to give 2-acetyl-7-hydroxy-4 : 5-dimethoxyindane-1 : 3-dione (310 mg.), m. p. 156°, previously thought to be 3-acetyl-4-hydroxy-6 : 7-dimethoxycoumarin-5-carboxylic acid [Found: r.m.a. × 10^{-6}, 0·926 (5C, 0·922)]. The filtrate was refluxed in a stream of nitrogen for 1 hr. and the effluent gases were trapped in aqueous barium hydroxide. The barium carbonate was washed and dried [Found: r.m.a. × 10^{-6}, 0·187 (1C, 0·184)]. The residual solution was distilled and the acetic acid collected as lithium acetate. An aliquot part was converted into the *p*-bromophenacyl ester which was chromatographed on Florisil in mixtures of light petroleum (b. p. 60—80°) and benzene, and recrystallised twice from light petroleum (b. p. 60—80°). It had m. p. 84—86° [Found: r.m.a. × 10^{-6}, 0·188 (1C, 0·184)]. The residual lithium acetate was pyrolysed, giving $BaCO_3(Me)$ (Found: r.m.a., 0) and $BaCO_3(CO_2H)$ [Found: r.m.a. × 10^{-6}, 0·187 (1C, 0·184)].

(iii) The above indane-1 : 3-dione was fused with potassium hydroxide. The melt was dissolved in water, and the resulting solution was acidified and distilled, to give acetic acid (2·0 mol.) which was collected as lithium acetate and converted into *p*-bromophenacyl acetate, m. p. 84—86° (Found: r.m.a. × 10^{-6}, 0·187 (1C, 0·184)].

Degradations of Citromycetin derived from $H{\cdot}^{14}CO_2H$.—(i) Decarboxylation as above gave barium carbonate (Found: r.m.a. × 10^{-4}, 2·14).

(ii) Di-*O*-methylcitromycetin methyl ester was oxidised with lead tetra-acetate in acetic acid, to give (from methanol) di-*O*-methylcitromycetinol methyl ester (VIII) which we obtained as a *solvate*, m. p. 156—158°· [Robertson *et al.*[13] gave m. p. 234° (decomp.) for the unsolvated material] (Found: C, 572; H, 5·8%; r.m.a. × 10^{-4}, 15·8. $C_{17}H_{16}O_8,CH_2{\cdot}OH$ requires C, 56·8; H, 5·3%). This ester (200 mg.) was degraded with aqueous sodium hydroxide in a stream of nitrogen. Acetone 2 : 4-dinitrophenylhydrazone (35 mg.) was obtained from the effluent vapours and purified as before. It had m. p. 122—124° (Found: r.m.a. × 10^{-4}, 3·88).

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[20] Robertson and Whalley, *J.*, 1949, 848.