615. *Metabolic Products of* Stemphylium radicinum. *Part I. Radicinin.*

By John Frederick Grove.

Radicinin (stemphylone), a phytotoxic $C_{12}H_{12}O_5$ metabolic product of the plant pathogenic fungus *Stemphylium radicinum* (*Alternaria radicina*), is shown to have the pyrano[4,3-*b*]pyran structure (I; R = H). The isolation of an antifungal $C_8H_{10}O_3$ metabolic product of *S. radicinum* is recorded.

The mould *Stemphylium radicinum*, originally described under the name *Alternaria radicina*,[1] is a plant pathogen attacking the carrot at all stages of its growth, from the seed, germination of which is inhibited, to the mature plant.[2] Extraction of culture filtrates yielded [3,4] the keto-lactone, radicinin, $C_{12}H_{12}O_5$, m. p. 220° (decomp.). [Purified specimens have m. p. 238—240° (decomp.).] Stemphylone,[5] isolated from the same organism, was observed to inhibit germination of seeds of *Lepidium sativum* and is identical (see Experimental section) with radicinin, which is now shown to have structure (I; R = H).

S. radicinum culture filtrates, in common with those of several *Alternaria* species, also showed an antagonistic effect towards the fungus *Phytophthora erythroseptica* [6] and the physical and chemical properties of the crude active extract (A), m. p. 204—208°, seemed to exclude the possibility [6] that the antifungal activity might be due to the presence of alternaric acid,[7] a known antifungal and phytotoxic metabolic product of *Alternaria solani*. Examination of the product (A), kindly supplied by Dr. W. Newton, has confirmed the absence of alternaric acid; it consists, essentially, of a mixture, separable by chromatography, of two neutral compounds, radicinin (90%), which has negligible antifungal properties, and a second keto-lactone (6%), $C_8H_{10}O_3$, m. p. 176°, shown [8] to be (—)-7-hydroxy-4-oxo-oct-2-enoic acid lactone (II), which accounts for all the antifungal activity in *S. radicinum* broths and in the crude extract.

Chemical shifts (τ values) and coupling constants (c./sec.) of protons in radicinin and its acetyl derivative.

Compound	2-Me	2	3	8	1′	2′	3′	Ac	$J_{2,3}$	$J_{2\text{-Me}}$	$J_{1',2'}$	$J_{2',3'}$	$J_{1',3'}$
(I; R = H) †	8·25	5·5 *	5·68	3·90	3·7	2·9 *	7·95		12	6	16	7	1·5
	8·35		5·90		3·95		8·05						
(I; R = Ac)	8·40	5·35 *	4·60	4·08	3·8	3·0 *	7·96	7·78	11	7	16	7	1·5
	8·50		4·80		4·05		8·08						

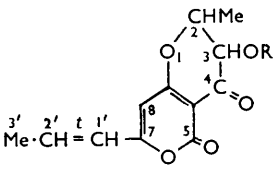
* Centre of multiplet (doublet of quartets). † In the presence of trifluoroacetic acid.

Purified specimens of radicinin are colourless, although the compound was originally described as a yellow pigment.[3,4] In this early work radicinin was shown to contain two *C*-methyl groups and three ethylenic double bonds, only one of which readily participated in electrophilic addition reactions. Four of the five oxygen atoms were shown to be present in hydroxyl, ketone, and lactone groupings; the fifth was assumed to be present as an ether linkage and the bicyclic partial structure (III) was proposed.

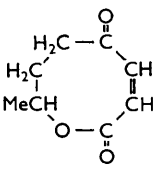
In the nuclear magnetic resonance (n.m.r.) spectrum of radicinin (see Table), a one-proton doublet (J = 12 c./sec.) centred at τ 5·79, which moved downfield to τ 4·70 in acetylradicinin (I; R = Ac), indicated that the hydroxyl group was secondary. Since

[1] Meier, Drechsler, and Eddy, *Phytopathology*, 1922, **12**, 157.
[2] Grogan and Snyder, *Phytopathology*, 1952, **42**, 215.
[3] Clarke and Nord, *Arch. Biochem. Biophys.*, 1953, **45**, 469.
[4] Clarke and Nord, *Arch. Biochem. Biophys.*, 1955, **59**, 269.
[5] Hansen, *Acta. Chem. Scand.*, 1954, **8**, 1332.
[6] Newton, *Canad. J. Bot.*, 1953, **31**, 423.
[7] Grove, *J.*, 1952, 4056; Bartels-Keith and Grove, *Proc. Chem. Soc.*, 1959, 398.
[8] Aldridge and Grove, Part II, following Paper.

the adjacent proton at τ 5·5 appeared as a doublet of quartets (J = 12, 6 c./sec.), and was coupled to the protons in a *C*-methyl group at τ 8·3, the partial structure (IV; RR′ ≠ H) is present in radicinin. The existence of a *trans*-crotonoyl group [$\nu_{max.}$ (C=O) 1657 cm.$^{-1}$], or a vinylogue of this system, was deduced [4,9] from the isolation of αβ-dibromobutyric acid after permanganate oxidation of radicinin dibromide, and is supported by the n.m.r. spectrum in which the characteristic ABX_3 pattern associated with the propenyl radical, conjugated (from the chemical shift values: see Table) with a carbonyl group, is clearly seen, and the coupling constant ($J_{1',2'}$ = 16 c./sec.) is consistent with the *trans*-configuration. Similar data have recently been briefly reported [9] but were interpreted in terms of structure (V). On vigorous acid hydrolysis, radicinin gives carbon dioxide (1 mol.) and a ketone, $C_{11}H_{12}O_3$ (VI), $\nu_{max.}$ 1660, 1642 cm.$^{-1}$, $\lambda_{max.}$ 234, 275 mμ, which forms only a mono-dinitrophenylhydrazone and has the physical and chemical properties of a γ-pyrone. The n.m.r. spectrum of the ketone reveals, in addition to the crotonoyl (or vinylogous) grouping of radicinin which is retained at τ 8·0, 3·8, and 3·2, the presence of an ethyl group [A_2B_3 system at τ 7·05 (quartet) and 8·80 (triplet), J = 7 c./sec.] resulting from dehydration of



(I)



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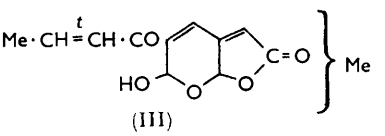

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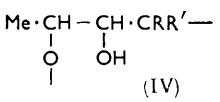

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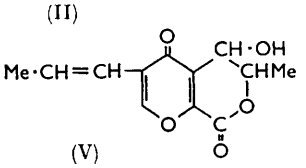

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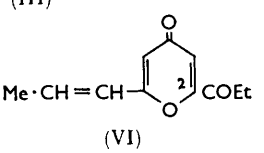

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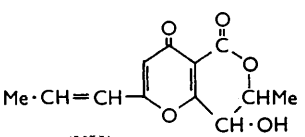

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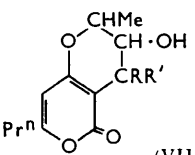

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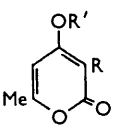


(IX)

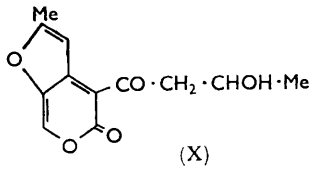
the α-glycol derived from partial structure (IV; RR′ ≠ H). The ketone contains two olefinic hydrogens at τ 3·10 (at position 3) and 3·80, $J_{3,5}$ = 2·5 c/sec., instead of the isolated single olefinic hydrogen at τ 3·90 in radicinin. The chemical shifts of these protons indicate the absence of =CH–O– groups in both compounds [10] and are consistent only with the formulation of the ketone as a 2,6-disubstituted γ-pyrone. The 6-propenyl-2-propionyl structure (VI), as opposed to the alternative formulation with crotonoyl and ethyl substituents, follows from the simultaneous formation of pentane-2,3-dione, isolated as its bisdinitrophenylhydrazone, as a minor hydrolysis product. Pentane-2,3-dione is also formed as a product of the alkaline hydrolysis of radicinin.

Radicinin, therefore, has either structure (VII), resembling that, (V), advanced by the American workers [9] but consistent with the n.m.r. data, or the derived α-pyrone structure (I; R = H). The infrared absorption ($\nu_{max.}$ 1762 cm.$^{-1}$) of the lactone carbonyl group renders (VII) unlikely: in addition, the following evidence has been obtained which conclusively establishes structure (I; R = H). Catalytic hydrogenation [4] of radicinin gave a substance, m. p. 156°, assumed to be the dihydro-derivative since the ultraviolet spectrum

[9] Clarke, Nord, and Bhacca, *Arch. Biochem. Biophys.*, 1963, **102**, 473.
[10] Beak and Abelson, *J. Org. Chem.*, 1962, **27**, 3715; Terahara, *Bull. Chem. Soc. Japan*, 1960, **33**, 1310.

was similar to that of radicinin dibromide. Repetition of this work confirmed this conclusion and the dihydro-derivative (VIII; RR′ = O) is reduced further by sodium borohydride to a diol, $C_{12}H_{16}O_5$ (VIII; RR′ = H, OH), $\lambda_{max.}$ 286 mμ. Partial structure (IV; RR′ = O) and the propenyl radical account for seven of the twelve carbon atoms of radicinin. The ultraviolet absorption of the diol (VIII; RR′ = H, OH) requires that the remaining five, including the lactone carbonyl group, form the (linear) chromophoric system which, by comparison with sorbic acid, $\lambda_{max.}$ 254 mμ, β-methoxysorbic acid, $\lambda_{max.}$ 265 mμ,[11,12] and " triacetic acid lactone " methyl ether (IX; R = H, R′ = Me), $\lambda_{max.}$ 280 mμ,[13] must carry an oxygen substituent in the δ-position with formation of an α-pyrone ring. Since the partial structure (IV; RR′ = O) is part of a six-membered ring, the coupling constant ($J_{2,3}$ = 12 c./sec.) indicates that the protons are *trans*-diaxial and the hydroxyl substituent therefore lies in the plane of the dihydropyran ring.

Structure (I; R = H), in contrast to structures (III),[4] (V),[9] and (X) [14] proposed earlier, accounts satisfactorily for the known chemical and spectroscopic properties of radicinin. The ultraviolet absorption of dihydroradicinin, $\lambda_{max.}$ 310 mμ, agrees well with that of 3-acetyl-4-ethoxy-6-methyl-2-pyrone (IX; R = Ac, R′ = Et), $\lambda_{max.}$ 312 mμ,[15] and further conjugation with the 7-propenyl substituent causes an additional bathochromic shift of 32 mμ in radicinin, $\lambda_{max.}$ 342 mμ. Weak intramolecular hydrogen bonding observed [4] in the infrared spectrum of radicinin, and confirmed during the present investigation, is attributed to the α-ketol grouping. Contributions to the resonance hybrid from ionic structures of type (XI), in place of the more usual α-pyrone type (XII), provide an explanation for the lactone carbonyl frequencies close to 1765 cm.$^{-1}$ in the 3-acyl-2-pyrones radicinin and dihydroradicinin. These frequencies are unusually high for α-pyrones ($\nu_{max.}$ 1700—1740 cm.$^{-1}$) [16] and were ascribed,[4] not unreasonably, to the presence of a 5-membered-ring lactone. The formation of acetaldehyde on alkaline hydrolysis of radicinin [4] is attributed to a retro-aldol reaction and, as expected, dihydroradicinin also gives acetaldehyde under the same conditions. The reducing properties of radicinin arise



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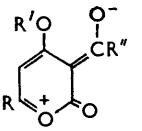

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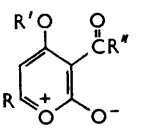

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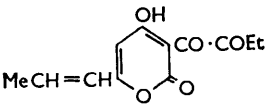

(XIII)

from the α-ketol grouping or from the liberation of acetaldehyde. Acetaldehyde and pentane-2,3-dione are the products of competing reactions in which the retro-aldol fission predominates. The intermediate (XIII) in the formation of pentane-2,3-dione can be isolated from the acidic fraction after mild acid hydrolysis of radicinin. By the classical 3-acyl-2-pyrone ⟶ 2-alkyl-4-oxopyran-3-carboxylic acid rearrangement, with simultaneous decarboxylation, (XIII) then gives the γ-pyrone (VI).

The co-occurrence of the C_8 lactone (II) and radicinin in *S. radicinum* broths provides circumstantial evidence that the biosynthesis of radicinin involves condensation at the activated methylene group of the terminal unit of a C_8 moiety, made up by head-to-tail linkage of four " acetate " units, with the biochemical equivalent of acetoacetate. Tracer studies to investigate this point are in hand: a similar condensation involving " acetoacetate " takes place in the biosynthesis of alternaric acid,[17] and, possibly, citromycetin.[18]

[11] Jones and Whiting, *J.*, 1949, 1423.
[12] Heilbron, Jones, and O'Sullivan, *J.*, 1946, 866.
[13] Bu'Lock and Smith, *J.*, 1960, 502.
[14] Murray, *Chem. and Ind.*, 1963, 1347.
[15] Berson, *J. Amer. Chem. Soc.*, 1952, **74**, 5174.
[16] Dean, " Naturally Occuring Oxygen Ring Compounds," Butterworths, London, 1963, p. 82.
[17] Turner, *J.*, 1961, 522.
[18] Gatenbeck and Mosbach, *Biochem. Biophys. Res. Comm.*, 1963, **11**, 166.

Experimental

Melting points were taken on a Kofler hot-stage apparatus and are corrected. Unless otherwise stated, infrared and ultraviolet spectra were determined for Nujol mulls and ethanol solutions, respectively. Light petroleum had b. p. 60—80°. N.m.r. spectra were determined for $CDCl_3$ solutions (tetramethylsilane as internal reference) with a Varian Associates A.60 spectrometer (60 Mc.).

Examination of the Product (*A*).—A portion (50 mg.) of the product [6] (supplied by Dr. W. Newton), m. p. 204—208°, in ether (200 ml.) was extracted with sodium hydrogen carbonate. The acid fraction (1 mg.), on recovery, did not give a chloroform-soluble blue complex on treatment with cupric acetate.[7] The neutral fraction (49 mg.) was combined with the rest of the material (350 mg.) and fractionally crystallised from acetone giving (i) prisms (320 mg.), m. p. 236—240° (decomp.), and (ii) a brown semi-solid residue (80 mg.).

Fraction (i) was recrystallised from ethanol giving long colourless needles, m. p. 238—240° (decomp.), of radicinin, 3,4-*dihydro*-3-*hydroxy*-2-*methyl*-7-*propenyl*-2H,5H-*pyrano*[4,3-b]*pyran*-4,5-*dione*, (I; R = H) [Found: C, 61·1; H, 5·3%; Equiv. (lactone titration) 248. $C_{12}H_{12}O_5$ requires C, 61·0; H, 5·1%, *M* 236], $[\alpha]_D^{22}$ −187° (*c* 1·02 in $CHCl_3$), $\nu_{max.}$ 3450 (OH), 3100 (=CH), 1761, 1657 (C=O), 1605 (C=C), 1522 cm.$^{-1}$ (=C–O); (in carbon tetrachloride) 3495 cm.$^{-1}$ (OH), unaltered by dilution; (in chloroform) 1762, 1655, 1605 cm.$^{-1}$, identical with that of an authentic specimen of radicinin kindly supplied by Prof. F. F. Nord. The decomposition point, which varied with the rate of heating, was not depressed on admixture with authentic material {lit.,[4] m. p. 220° (decomp.) $[\alpha]_D^{22}$ −208° (*c* 1·25 in $CHCl_3$)}. The physical constants of the acetate and dinitrophenylhydrazone were identical with those of radicinin acetate and dinitrophenylhydrazone.[4] A specimen of radicinin submitted for comparison with stemphylone [m. p. 220° (decomp.), $[\alpha]_D$ −125° (*c* 1·26 in ethanol–chloroform)] [5] was also identical.[19]

Fraction (ii), in benzene (25 ml.) was chromatographed on alumina (pH 3·0, activated at 180°, 6 × 0·7 cm.) in ultraviolet light giving the following fractions (eluant in parentheses): (i) blue fluorescent band (benzene, 80 ml.), 25 mg. (ii) pale blue interband (benzene, 150 ml.), 3 mg. intractable, (iii) violet band [benzene–methanol (100 : 1), 100 ml.], 30 mg., yielding radicinin (11 mg.) m. p. 238° (decomp.). Fraction (i) gave (−)-7-*hydroxy*-4-*oxo*-*oct*-2-*enoic acid lactone* (II),[8] needles, m. p. 176° (from ethanol or light petroleum), $[\alpha]_D^{20}$ −47° (*c* 0·36 in acetone) [Found: C, 62·4, 62·2; H, 6·5, 6·4%; Equiv. (lactone titration), 147; *M* (*X*-ray), 154. $C_8H_{10}O_3$ requires C, 62·3; H, 6·5%; *M*, 154.].

Fermentations with Stemphylium radicinum (Alternaria radicina [1]).—Production of antifungal activity was followed by a spore germination assay with *Botrytis allii*.[20] The culture filtrate [4,6] was treated with activated carbon and the carbon was extracted with acetone as previously described.[6] The total crude gummy extract was separated by fractional crystallisation and chromatography of the residues, as described above, into radicinin (78%) and the keto-lactone (II) (16%; 30 mg./l.) which accounted for all the antifungal activity of the fermentation broth.

Antifungal activity of the metabolic products of *S. radicinum*.*

	Concn. (μg./ml.) causing inhibition of germination	Concn. (μg./ml.) causing stunting of germ tubes
Product (A) [6]	>100	6·25
Radicinin (I; R = H)	>100	25·0
Keto-lactone (II)	100	0·4

* Measured at pH 3·5 against *B. allii*.[20]

Dihydroradicinin (VIII; RR′ = O).—Radicinin (500 mg.) in ethanol (200 ml.) was hydrogenated in the presence of palladium–charcoal (10%; 100 mg.). After uptake of 1·3 mol. (15 min.), the mixture was worked up in the usual way giving *dihydroradicinin*, needles (366 mg.), m. p. 158° (from ethanol) (Found: C, 60·3; H, 6·1. $C_{12}H_{14}O_5$ requires C, 60·5; H, 5·9%), $[\alpha]_D^{20}$ −149° (*c* 0·5 in EtOH), $\lambda_{max.}$ ~250, 310 mμ (log ε 3·46, 4·07), $\nu_{max.}$ 3494 (OH), 3115 (=CH), 1767, 1662 (C=O), 1636, 1528 cm.$^{-1}$. The substance obtained by Clarke and Nord,[4] in a similar reduction of radicinin, had m. p. 156°, $\lambda_{max.}$ 310 mμ (log ε 4·04).

Reduction of Dihydroradicinin with Sodium Borohydride.—The dihydro-compound (50 mg.)

[19] Dr. O. R. Hansen, personal communication.
[20] Brian and Hemming, *Ann. Appl. Biol.*, 1945, **32**, 214.

in methanol (2 ml.) at 0° was treated with sodium borohydride (50 mg.) in methanol (1 ml.). After 1 hr. the residual solution (0·5 ml.) obtained on concentration *in vacuo* at room temperature was neutralised with dilute acetic acid and extracted with ethyl acetate yielding a gum (34 mg.) which distilled at 120°/10^{-1} mm. (bath temp.). The oily *diol* (VIII; RR′ = H, OH) (24 mg.) (Found: C, 60·1; H, 6·9. $C_{12}H_{16}O_5$ requires C, 60·0; H, 6·7%), $\lambda_{max.}$ 213, 286 mμ (log ε 4·19, 4·02), $\nu_{max.}$ 3300vbr, 1700, 1635, 1580 cm.$^{-1}$, crystallised from ether to give felted needles, m. p. 97—103°, of an etherate which regenerated the diol in drying *in vacuo*.

Acid Hydrolysis of Radicinin.—(a) *Heterogeneous reaction.* Finely powdered radicinin (100 mg.) was heated under reflux in N-sulphuric acid (10 ml.) for 3 hr. in a stream of nitrogen and the effluent gas was passed through traps containing, successively, 2,4-dinitrophenylhydrazine in N-sulphuric acid, and barium hydroxide (0·85 mol. carbon dioxide liberated). The radicinin slowly dissolved and, simultaneously, a brown tar was thrown out of solution. A small amount of red precipitate formed in the dinitrophenylhydrazine trap and gave deep red needles (1 mg.), m. p. 277—279° (decomp.) (from ethyl acetate–light petroleum). These were combined with identical material from other hydrolyses and recrystallised from ethyl acetate to give pentane-2,3-dione bisdinitrophenylhydrazone,[21] deep red rosettes or orange needles (stable form), m. p. and mixed m. p. (with an authentic specimen,[22] kindly provided by Dr. C. E. Stickings) 280—281° (decomp.) (Found: C, 44·6; H, 3·75. Calc. for $C_{17}H_{16}O_8N_8$ C, 44·3; H, 3·5%).

The mixture was steam-distilled and 10 ml. fractions were titrated against 0·1N-sodium hydroxide. The first 40 ml. contained 0·05 mol. of a volatile acid, which was not identified. After extraction of the cooled mixture with ethyl acetate, the organic layer was separated into neutral (30 mg.) and intractable acidic (34 mg.) brown gummy fractions by extraction with sodium hydrogen carbonate and recovery. The neutral fraction was chromatographed on alumina (pH 4, grade II, 3 g.) in benzene and after gums (3 mg.) had been eluted with benzene (65 ml.), benzene–methanol (200 : 1; 15 ml.) eluted a brown band which furnished plates (1 mg.), m. p. 100°. Fluorescent bands eluted with benzene–methanol (100 : 1) and ethyl acetate yielded intractable gums.

The plates were combined with similar material (17 mg.) from another experiment using 500 mg. of radicinin and sublimed at 90°/10^{-3} mm. to give 6-*propenyl*-2-*propionyl*-4-*pyrone* (VI), plates (12 mg.), m. p. 100° (from ethyl acetate–light petroleum) (Found: C, 69·0; H, 6·5. $C_{11}H_{12}O_3$ requires C, 68·7; H, 6·3%), $\lambda_{max.}$ 234, 275, ~310 mμ (log ε 4·31, 4·03, 3·80), $\nu_{max.}$ (OH absent), 1712w, 1660, 1642 (C=O), 1608, 1572 cm.$^{-1}$. The *dinitrophenylhydrazone* formed needles, m. p. 240—241° (decomp.) (from ethyl acetate) (Found: N, 16·6. $C_{17}H_{16}N_4O_6$ requires N, 15·9%).

The ketone (VI) was appreciably soluble in water. It gave no colour with concentrated sulphuric acid or with ferric chloride but gave a deep yellow solution with sodium hydroxide.

(b) *Homogeneous reaction.* Concentrated hydrochloric acid (10 ml.) was added to radicinin (500 mg.) in ethanol (40 ml.), and the mixture was refluxed for 4 hr. in an atmosphere of nitrogen. After concentration of the solution *in vacuo* to 10 ml., water (10 ml.) was added, and the mixture was extracted with ethyl acetate. The extract was separated as before into neutral (161 mg.) and acidic (184 mg.) fractions. Alumina chromatography and sublimation of the neutral fraction yielded the ketone (VI) (34 mg.). The acid fraction gave 3-(1,2-*dioxobutyl*)-4-*hydroxy*-6-*propenyl*-2-*pyrone* (XIII), prisms (66 mg.), m. p. 164—165° (from ethyl acetate) (Found: C, 61·2; H, 5·2; O, 33·8. $C_{12}H_{12}O_5$ requires C, 61·0; H, 5·1; O, 33·9%), $\nu_{max.}$ 3355 (OH), 1757, 1688 (C=O), 1642, 1584, 1540 cm.$^{-1}$. It sublimed at 140°/10^{-2} mm. It was insoluble in water but soluble in sodium hydrogen carbonate with liberation of carbon dioxide. It reacted with diazomethane in ether but the product was intractable. It gave an orange-brown colour with ferric chloride and a precipitate with Brady's reagent.

Alkaline Hydrolysis of Radicinin.—Radicinin (1 g.) was heated under reflux with potassium hydroxide in a stream of nitrogen as previously described [4] and volatile carbonyl compounds were absorbed from the effluent gases by a trap containing 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid. The residues from the crystallisation of the crude acetaldehyde dinitrophenylhydrazone [4] were chromatographed on alumina in benzene. After acetaldehyde dinitrophenylhydrazone (yellow band) had been eluted, ethyl acetate removed a red band which

[21] Heilbron, Jones, Smith, and Weedon, *J.*, 1946, 54.
[22] Birkinshaw, Oxford, and Raistrick, *Biochem. J.*, 1936, **30**, 394.

furnished red needles (1 mg.), m. p. 276—279° (decomp.), of pentane-2,3-dione bisdinitrophenylhydrazone. The alkaline reaction mixture, on acidification and extraction with ether, afforded an intractable brown gum.

Alkaline Hydrolysis of Dihydroradicinin.—Dihydroradicinin (91 mg.) in 0·3N-potassium hydroxide was treated as described for radicinin. The acidic product was an intractable brown gum (48 mg.). The crude dinitrophenylhydrazone formed from the volatile products was separated, as before, into acetaldehyde dinitrophenylhydrazone and pentane-2,3-dione bisdinitrophenylhydrazone.

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Imperial Chemical Industries Limited, Akers Research Laboratories,
The Frythe, Welwyn, Herts.

London School of Hygiene and Tropical Medicine,
London W.C.1. (Present address.)

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