592. *Sporidesmins. Part I. Isolation and Characterisation of Sporidesmin and Sporidesmin-B.*

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Sporidesmin ($C_{18}H_{20(22)}ClN_3O_6S_2$) and sporidesmin-B ($C_{18}H_{20}ClN_3O_5S_2$), toxic metabolites of *Pithomyces chartarum*, have been isolated and characterised. Each contains two methoxyl, two *N*-methyl, and hydroxyl groups. The reactivity of the sulphur in sporidesmin is similar to that in gliotoxin. On alkaline degradation ammonia, methylamine, and a red ketone were isolated from both sporidesmins. It is concluded that sporidesmin is a hydroxysporidesmin-B.*

Sporidesmin [1,2] is a toxic metabolic product of the fungus *Pithomyces chartarum*,[3,4] and its implication in the ætiology of the animal disease " facial eczema " in New Zealand [5] has been discussed.[6] Higher yields of the metabolite from rye-grain (*Secale cereale*) cultures [7] were obtained by the modifications of the isolation procedures described in the Experimental section, and final purification of sporidesmin by adsorption chromatography on silica gel was less tedious than reversed-phase partition chromatography.[1,2]*

Stable crystalline solvates of sporidesmin with benzene, dibromomethane, methyl iodide, bromoform, and chloroform have been prepared in addition to the carbon tetrachloride solvate already reported.[1,2] The presence of the solvating entities in a 1 : 1 ratio was shown by gas–liquid chromatography, and bands assignable to benzene or carbon tetrachloride were seen in the infrared spectra of the appropriate solvates. Crystalline solvent-free sporidesmin was obtained from solutions in acetone or alcohols with or without addition of water. Analysis of sporidesmin, its solvates, and its diacetate indicated an empirical formula $C_{18}H_{20(22)}ClN_3O_6S_2$. The proton magnetic resonance spectrum showed clearly 19 protons and possibly 3 others. *X*-Ray crystallography, mass-spectra, and isopiestic molecular-weight determinations showed that the empirical formula was the molecular formula.

Aqueous solutions of sporidesmin were neutral, and potentiometric titration in the pH range 3—12 indicated the absence of ionisable groups. Insignificant differences were observed when ultraviolet spectra were measured in hexane, ether, alcohols, water, acid, and alkaline solutions. Sporidesmin did not dissolve in aqueous sodium carbonate or sodium hydrogen carbonate; it dissolved readily in solutions of pH > 12, but low recoveries were obtained on immediate acidification. Sporidesmin separated from solutions of its benzene solvate in concentrated hydrochloric acid.

A crystalline, biologically active diacetate showed no residual O–H stretching frequencies and had the same ultraviolet spectrum as sporidesmin. Evidence was obtained for the formation of a benzoate but we were unable to obtain other derivatives of a hydroxy-compound. The absence of bands in the proton magnetic resonance spectrum attributable to a methylene group also indicated the absence of a primary alcoholic function. Chelates were not obtained with cupric salts or ferric chloride, and sporidesmin was recovered after treatment with diazomethane. Two bands at τ 5·16 and

* In preliminary notes the degradation chemistry (Hodges, Ronaldson, Taylor, and White (*Chem. and Ind.*, 1963, 42) and *X*-ray crystallographic studies (Fridrichsons and Mathieson, *Tetrahedron Letters*, 1962, 1265) on sporidesmins are described.

[1] Synge and White, *Chem. and Ind.*, 1959, 1546.
[2] Synge and White, *New Zealand J. Agric. Res.*, 1960, **3**, 907.
[3] Ellis, *Mycol. Papers*, 1960, No. 76.
[4] Dingley, *New Zealand J. Agric. Res.*, 1962, **5**, 49.
[5] Done, Mortimer, and Taylor, *Res. Vet. Sci.*, 1960, **1**, 76.
[6] Mortimer and Taylor, *Res. Vet. Sci.*, 1962, **3**, 147.
Done, Mortimer, Taylor, and Russell, *J. Gen. Microbiol.*, 1961, **26**, 207

6·6, absent in the diacetate and after deuterium exchange, were assigned to hydroxylic protons and the displacement of the peaks at τ 4·7 and 5·42 to 3·91 and 4·22 in the diacetate suggested that the hydroxy-groups were secondary.[8] Zeisel analysis showed the presence of two alkoxy-groups. That they were methoxyl was shown by gas–liquid chromatography of the gases evolved during the determination and their conversion into tetramethylammonium iodide (see below). The 3-proton peaks at τ 6·13 and 6·18 are consistent with methoxy-substituents on an aromatic system.[8,9]

Identical infrared spectra were observed when all forms of sporidesmin were examined as films from acetone or in solution. The spectra of solvates in the solid state also showed two bands in the region 1715—1660 cm.$^{-1}$, but only one band (1695 cm.$^{-1}$) was observed in the spectra of sporidesmin and its diacetate in the crystalline state. These results suggested the presence of a ketonic group but we were unable to prepare typical derivatives. Hydrogen sulphide was the only volatile acid obtained after acid or alkaline hydrolysis, and the hydroxamic acid test for esters or lactones was negative.

There was no absorption in the N–H stretching, the *N*-acetyl, or the amide-II region for the diacetate, and sporidesmin was unaltered by treatment with nitrous acid; hence it was concluded that no N–H groups were present. Two *N*-alkyl groups were found analytically and confirmed by the two 3-proton absorptions at τ 6·7 and 6·93, typical of non-basic *N*-methyl groups.[10,11] Evidence that other *N*-alkyl groups were absent was also obtained from chemical studies of the type reported below. Later, we were informed that an interpretation of *X*-ray crystallographic results suggested the presence of one *N*-ethyl group; hence the *N*-alkyl groups were investigated in greater detail. A portion of the alkyl iodides from the alkylimide determination was examined by gas–liquid chromatography and the remainder estimated in the usual way. Results showed that 1·8 equivalents of alkyl iodide were evolved, that methyl and ethyl iodide were the only alkyl iodides formed, and that the latter accounted for less than 5% of the total. In other experiments the alkyl iodides were quantitatively converted into quaternary iodides by reaction with trimethylamine in nitrobenzene. Tetramethylammonium iodide (1·8 equivalents) was precipitated, and examination of the solutes by infrared spectroscopy showed the presence of bands characteristic of ethyltrimethylammonium iodide, absent in control determinations. This analysis showed that less than 10% of the alkyl iodides was ethyl iodide. Vigorous alkaline hydrolysis of sporidesmin, and the products of Kuhn–Roth oxidation, provided one equivalent of ammonia and one of methylamine. No ethylamine or other amine was obtained. The 3-proton peak at τ 7·97 is not due to a basic *N*-methyl group for the reasons stated above, and it is assigned to a *C*-methyl substituent on an unsaturated centre.[8] The low *C*-methyl value obtained in Kuhn–Roth determinations supports this assignment. It is concluded that tertiary amide links are present in sporidesmin and preliminary work showing the production of basic products from the reaction with lithium aluminium hydride supports this view.

Lead sulphide was obtained when sporidesmin was treated with alkaline plumbite,[12,13] alkaline solutions of sporidesmin did not react with nitroprusside, and sporidesmin gave no colour reactions characteristic of a thiol or thiocarbonyl (Grote) or a disulphide link easily opened by reduction. When sporidesmin was left on an alumina column, sulphur was obtained, and the yield was quantitative when the reactants were refluxed together in benzene. Hydrogen sulphide was collected quantitatively as antimony sulphide during the Zeisel determination and the volatile product from hydrogenation in methanol with Adams catalyst was shown to be hydrogen sulphide by passage of the gases through

Jackman, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry," Pergamon Press, London, 1959, pp. 55, 57.

[9] Pakrashi, Roy, Johnson, George, and Djerassi, *Chem. and Ind.*, 1961, 464.

[10] Roe and Gates, *Tetrahedron*, 1960, **11**, 148.

[11] Cohen, Daly, Kny, and Witkop, *J. Amer. Chem. Soc.*, 1960, **82**, 2184.

[12] Newton and Abraham, *Biochem. J.*, 1956, **62**, 655.

[13] Dutcher, Johnson, and Bruce, *J. Amer. Chem. Soc.*, 1945, **67**, 1736.

absorbents as described by Challenger.[14] Attempts at catalytic reduction with several catalysts in methanol were not successful: treatment of sporidesmin with Raney nickel or hydrogenation in methanol over Adams catalyst provided mixtures that did not contain sulphur; two methoxyl and two *N*-methyl groups were found on analysis of the total products from each reaction. Hence no *S*-methyl group is present in sporidesmin. These facts suggest the presence of a disulphide group similar to that present in gliotoxin [15] and certain β-keto-disulphides studied by Asinger *et al.*[16]

The presence of an aromatic ring in sporidesmin was indicated by its ultraviolet absorption spectrum (comparison is made with 2,3-dimethoxybenzaldehyde in Fig. 2), by the stability of the chlorine atom which was only slowly released by alkaline hydrolysis, by the retention of chlorine in the red ketone (see below), and by the single-proton peak

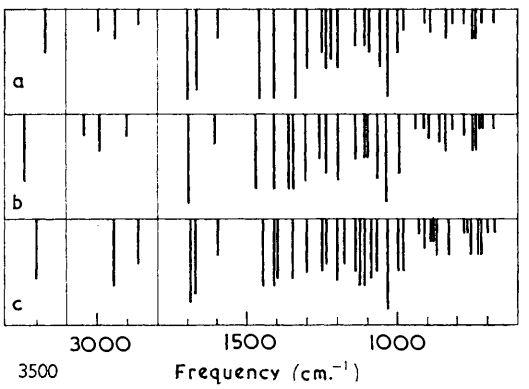

FIG. 1. Infrared spectra: (a) sporidesmin (film from acetone); (b) sporidesmin (in KBr); (c) sporidesmin-B (in KBr).

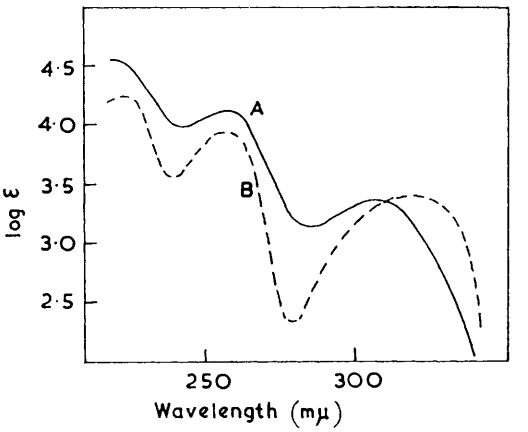

FIG. 2. Ultraviolet absorption spectra (in ether): A, sporidesmin, and solvates, sporidesmin-B, and acetates; B, 2,3-dimethoxybenzaldehyde.

at τ 2·92. No aromatic acids, however, were obtained on oxidative degradation. The red ketone, $C_{11}H_{10}ClNO_4$, was obtained in 15% yield after sporidesmin had been refluxed with dilute alkali, and in lower yield from a variety of oxidations, reductions, and by acid hydrolysis.

A second azide–iodine [17] positive substance, named sporidesmin-B, was eluted before sporidesmin on partition chromatography of fungal extracts. It was isolated after chromatography on silica gel and purified by further partition chromatography. It was biologically active. Analysis and molecular-weight determination indicated a formula $C_{18}H_{20}ClN_3O_5S_2$, and the proton magnetic resonance spectrum showed 20 protons. Its physical properties were similar to those of sporidesmin (Figs. 1c and 2). A single C=O stretching mode (1705 cm.$^{-1}$) was observed in solution spectra and two bands (1697, 1666 cm.$^{-1}$) in solid-state spectra. Two methoxyl, two *N*-methyl, and one *C*-methyl group were found on analysis of sporidesmin-B; and the red ketone, ammonia, and methylamine were produced on alkaline hydrolysis. No solvates were obtained. Sporidesmin-B reacted slowly with alkaline plumbite, but no sulphur was released when solutions were refluxed with alumina. The formation of a monoacetate and the appearance of an

[14] Challenger, " Aspects of the Organic Chemistry of Sulphur," Butterworths Scientific Publns., London, 1959, p. 18.
[15] Bell, Johnson, Wildi, and Woodward, *J. Amer. Chem. Soc.*, 1958, **80**, 1001.
[16] Asinger, Thiel, and Schäfer, *Annalen*, 1960, **637**, 146.
[17] Russell, *Nature*, 1960, **186**, 788.

exchangeable proton band at τ 6·47 showed that a single hydroxyl group was present. The proton magnetic resonance spectrum also showed the presence of a methylene group, absent in sporidesmin. Thus sporidesmin is regarded as a hydroxysporidesmin-B, and its greater solubility in water supports this view.

Experimental

M. p.s are corrected. Infrared spectra were determined on a Perkin-Elmer "Infracord" 137 spectrophotometer; large-scale extractions and concentrations were carried out in industrial glassware (Q.V.F. Ltd., catalogue numbers given); proton magnetic resonance spectra in $CDCl_3$ were measured on a Varian A-60 instrument; ultraviolet spectra were measured for ether solutions. Diethyl ether was treated with ferrous sulphate, dried ($CaCl_2$), and distilled. Silica gel (L. Light and Co.; 200—300 mesh, chromatographic) was washed with benzene and methanol and dried at 140°. Paper chromatography of column fractions was done on Whatman No. 1 paper (disc method, water as developing solvent, detection by the azide–iodine reaction [17]) as an additional means of locating sporidesmins [sporidesmin R_F (circular) 0·54, sporidesmin-B R_F 0·37].[2] Acetyl determinations were done by the *C*-methyl technique to avoid interference from hydrogen sulphide. All solvent mixtures are by volume.

A proton magnetic resonance spectrum of sporidesmin carbon tetrachloride solvate was kindly run by Dr. L. M. Jackman, and the isopiestic molecular weight was determined by Dr. A. D. Campbell, and the *X*-ray molecular weight by Mrs. D. C. Hodgkin.

Isolation of Sporidesmin.—The methods of cultivation of *P. chartarum* were those described previously;[7] high yields on bran media were not reproducible. The mixture of culture and 2 : 3 water–methanol[7] was stirred with a stainless-steel stirrer (240 rev./min.) in a large glass vessel fitted at its lower end with a stopcock of 1" bore. After 24 hours' stirring, the vessel was drained, and methanol (5 l.) was added to the residual rye grain or bran and allowed to drain through; 2 : 3 Water–methanol (30 l.) was added and the mixture stirred as before (48 hr.). The first methanol extract and washing were combined and filtered, and the filtrate was concentrated to 5 l. in a cyclone evaporator (CY2) operating at 10 mm. The heating heat-exchanger (HEB 6/5, effective area 5 sq. ft.) was supplied with water at 80° (flow rate 1 l./min.), and the condensing heat-exchanger (HE 2/3, effective area 7 sq. ft.) was supplied with coolant at −40° (flow rate 250 ml./min.). The extract was run into the heating heat-exchanger at such a rate that the fluid entering the cyclone did not exceed 30°. The second aqueous methanol extract was similarly processed, and the two concentrates were bulked, diluted with water (2 l.), and concentrated to 6 l. The concentrate was extracted with di-isopropyl ether (4 × 750 ml.), and the extracts were bulked and evaporated at 20°/10 mm. The residue was dissolved in methanol (100 ml.), filtered, and evaporated to dryness (5 g. of 40% sporidesmin [17]) on a rotary evaporator at 20°/0·5 mm. Alumina (British Drug Houses; 750 g.) was stirred intermittently for 1 hr. with a solution (1225 ml.) of acetic acid (24·5 ml.) in ether; the supernatant layer was then decanted. The residual slurry was divided into 3 equal parts, each of which was transferred to a glass column (5 cm. internal diameter), then each was washed with ether (2·4 l.). The above concentrate and carotene (6 mg.) were dissolved in ether (60 ml.) and 20 ml. of this solution were applied to each column. The eluates (400 ml. each) were collected from the first appearance of carotene in the eluate, bulked, and evaporated. The residue was dissolved in methanol (160 ml.), water (40 ml.) added, and the solution extracted with light petroleum (b. p. 60—80°; 4 × 200 ml.). The methanol phase was evaporated to dryness *in vacuo*. Purified[2] Hyflo Super-cel (255 g.) was blended in 6 portions (5 × 45 g., and 30 g.) with the top phase (5 × 30 ml., and 20 ml.) of the solvent system carbon disulphide–methanol–water (50 : 12 : 3). After mixing, each portion was added to a column (40 × 7 cm.) fitted with a stopcock at its lower end, plugged with cotton-wool, and charged with bottom phase (600 ml.). Each portion of Hyflo Super-cel was dispersed and packed under pressure with a packing tool.[18] The concentrate (containing *ca.* 1·5 g. of sporidesmin), carotene (6 mg.), Hyflo Super-cel (6 g.), and top phase (4 ml.) were evenly mixed and applied to the top of the column. The column thus obtained was 20—22 cm. long. The tube above the column was filled with bottom phase, which was run through at 12—22° until carotene appeared in the eluate. Fractions were then collected as the surface of the liquid in the column descended each 2 cm. The following weight distribution was obtained: fractions

[18] Howard and Martin, *Biochem. J.*, 1950, **46**, 535.

1—3, 0·45 g.; 4—9, 1·50 g., sporidesmin-B and a substance, m. p. 244°; 10—19, 1·35 g., sporidesmin; 20—22, 0·25 g. Fractions 10—19 were combined after each had been checked for the presence of sporidesmin by paper chromatography, dissolved in benzene (28 ml.), and seeded with sporidesmin benzene solvate; then light petroleum (b. p. 60—80°; 28 ml.) was added during 1 hr. Batches where the sporidesmin content of the concentrate was more than 30% before the partition stage could be crystallised directly. Crude sporidesmin benzene solvate (1·5 g.) was dissolved in benzene and run on a silica gel column (20 × 2·5 cm.). The chromatogram was developed with 1 : 4 ether–benzene. Fractions were collected from the appearance of ether in the eluate, as the liquid in the column descended each 4 cm. Fractions were bulked on the basis of their infrared spectra. Recovery of sporidesmin from side-fractions was achieved by further partition chromatography with the systems, carbon tetrachloride–light petroleum (b. p. 40—60°)–ethanol–water (138 : 120 : 33 : 9) or nitromethane–light petroleum (b. p. 100—120°)–chloroform (66 : 275 : 93·5). *Sporidesmin benzene solvate* separated from benzene on addition of light petroleum as colourless needles, m. p. 110—120° (decomp.), $[\alpha]_D^{23}$ −33·5° (*c* 1·1 in MeOH), +6·9° (*c* 1·4 in $CHCl_3$) [Found: C, 51·7, 52·2; H, 4·9, 5·0; Cl, 5·6, 6·3; N, 7·7; O, 17·9; S, 11·4; *C*-Me (Kuhn–Roth), 0·53; C_6H_6 (see below), 15·0. $C_{18}H_{20}ClN_3O_6S_2,C_6H_6$ requires C, 52·2; H, 4·7; Cl, 6·4; N, 7·6; O, 17·4; S, 11·6; *C*-Me, 2·7; C_6H_6, 14%], $\lambda_{max.}$ 218·5, 254, 302 mμ (log ε 4·60, 4·12, 3·45), $\nu_{max.}$ (in paraffin) 1715, 1664 (C=O), 3558, 3355w (O–H) cm.$^{-1}$, modified peaks near 1040 and 690 cm.$^{-1}$ consistent with the presence of benzene. This solvate is particularly stable and benzene is lost only just before melting. The solvate (0·153 g.) was dissolved in acetone and the solution evaporated to dryness. This process was repeated, the residue dissolved in methanol (0·75 ml.), and water (0·05 ml.) added. *Sporidesmin* separated from aqueous methanol as colourless needles with a faint green sheen, m. p. 179° (dependent on rate of heating), $[\alpha]_D^{20}$ −45° (*c* 0·98 in MeOH) (Found: C, 45·8; H, 4·42; N, 9·0. For a sample prepared in an analogous way from ethanol: C, 45·6; H, 4·55; N, 8·9. $C_{18}H_{20}ClN_3O_6S_2$ requires C, 45·6; H, 4·22; N, 8·9%), $\nu_{max.}$ (in KBr) see Fig. 1b, $\nu_{max.}$ (in paraffin) 1695 cm.$^{-1}$ (C=O), τ 2·92, 4·70, 5·16, 5·42, 6·13 (intensity 3), 6·18 (intensity 3), 6·6, 6·70 (intensity 3), 6·93 (intensity 3), 6·9(?), 7·97 (intensity 3), 8·0(?) (intensities 1 unless specified). Sporidesmin is very slightly soluble in water and light petroleum but readily in most solvents. Evaporation of acetone solutions leaves sporidesmin as a colourless resin, $[\alpha]_D^{20}$ −25° (*c* 0·75 in MeOH), $\nu_{max.}$ (cf. Fig. 1a) 1701, 1666 (C=O) cm.$^{-1}$, (in chloroform) 1701, 1670.

Isolation of Sporidesmin-B.—The bulked fractions 5—9 (13·7 g.) from the partition column collected during the production, on wheat grain or bran, of about 25 g. of sporidesmin, was triturated with ether (50 ml.); the colourless crystals were collected (7·5 g.) and had m. p. 244° [2] (Found: C, 69·0; H, 6·4; N, 4·8%). The filtrate was evaporated and the resulting syrup (8·2 g.) applied in 1 : 3 ether–benzene to a silica gel column (35 × 5 cm.) which was developed with the same solvent mixture. The following fractions were collected: 470 ml. of colourless eluate; 50 ml. from a yellow band on the column; a small yellow eluate of apparently colourless material on the column; and finally a yellow eluate (600 ml.) arising from the first yellow-brown zone on the column. The last fraction, on evaporation, gave a yellow resin (2·3 g.) which was separated into 2 equal parts. Each was applied as described above to a partition column of similar dimensions and the preceding solvent system was used. Fractions 5—9 of each run (later fractions contained traces of sporidesmin) were combined and evaporated, and the resulting dark resin was taken up in acetone. Small volumes of water were added to the solution at 2° as long as a crystalline precipitate was formed on storage. *Sporidesmin-B* separated from aqueous acetone as colourless needles (1·5 g.), m. p. 183°, $[\alpha]_D^{21}$ −27° (*c* 1·0 in MeOH), +12° (*c* 0·75 in $CHCl_3$) [Found: C, 47·3; H, 4·5; Cl, 8·1; N, 9·0; O, 17·0; S, 14·3; MeO, 12·8; *N*-Me, 5·0, 6·3; *C*-Me (Kuhn–Roth), 0·3%; *M* (Rast), 380. $C_{18}H_{20}ClN_3O_5S_2$ requires C, 47·2; H, 4·4; Cl, 7·7; N, 9·2; O, 17·5; S, 14·1; 2MeO, 13·5; 2*N*-Me, 6·1; *C*-Me, 3·3%; *M*, 457·6], $\lambda_{max.}$ 218, 256, and 307 mμ (log ε 4·50, 4·08, 3·41), $\nu_{max.}$ (cf. Fig. 1c), (in paraffin) 1697, 1666 cm.$^{-1}$, (in $CHCl_3$) 1705 cm.$^{-1}$ (C=O), τ 2·93, 4·6, 6·14 (intensity 3), 6·19 (intensity 3), 6·47, 6·76 (intensity 3), 6·97 (intensity 3), 7·97 (intensity 3), and 6·8, 7·2 with J_{AB} = 16·1 c./sec. Sporidesmin-B is more soluble in light petroleum and less soluble in water, ether, and benzene than sporidesmin.

Sporidesmin Carbon Tetrachloride Solvate.—A concentrated sporidesmin solution in ether (2 ml.) was treated with an excess of carbon tetrachloride. The *solvate* separated as colourless plates, m. p. 109—126° (decomp.), $[\alpha]_D^{24}$ −23° (*c* 0·96 in MeOH), +6·1° (*c* 0·52 in $CHCl_3$) [Found: C, 37·0, 37·8; H, 3·25, 3·4; Cl, 25·5, 27·8; N, 6·5, 6·9; O, 15·9; S, 10·2, 10·3; MeO, 9·7, 9·9;

N-Me, 5·5, 6·1; *C*-Me (Kuhn–Roth), 0·7; CCl_4 (see below), 20%; *M*, 693 (isopiestic in chloroform), 615 (*X*-ray). $C_{18}H_{20}ClN_3O_6S_2$,0·9CCl_4 requires C, 37·0; H, 3·6; Cl, 26·7; N, 6·8; O, 15·6; S, 10·4; 2MeO, 10·1; 2*N*-Me, 4·9; *C*-Me, 2·4; CCl_4, 22%; *M*, 612], $\lambda_{max.}$ 218·5, 252, and 305 mμ (log ε 4·65, 4·15, and 3·41) (very similar values in ethanol and water with flattening of the 252 mμ peak), $\nu_{max.}$ (in paraffin) 1672 and 1688 cm.$^{-1}$ (C=O), modifications in the 760—790 cm.$^{-1}$ region attributed to CCl_4. Some solvent is lost when this solvate is kept for months at 20°.

Sporidesmin Dibromomethane Solvate.—Sporidesmin was dissolved in dibromomethane and the solution treated with light petroleum (b. p. 40—60°). The *solvate* separated from these solvents as colourless prisms, m. p. 112—120° (Found: C, 35·6; H, 3·7; Br, 24·6; Cl, 5·3; N, 6·5; O, 14·9; S, 9·6; CH_2Br_2, 20. $C_{18}H_{20}ClN_3O_6S_2$,CH_2Br_2 requires C, 35·2; H, 3·4; Br, 24·5; Cl, 5·5; N, 6·5; O, 15·0; S, 9·9; CH_2Br_2, 27%), $\nu_{max.}$ (in paraffin) 1688 and 1664 cm.$^{-1}$ (C=O).

Sporidesmin Methyl Iodide Solvate.—This was obtained as described for the dibromomethane solvate and had m. p. 124—140° (decomp.) (Found: C, 37·8; H, 4·0; Cl, 5·9; I, 19·6; N, 6·9; O, 15·6; S, 10·2. $C_{18}H_{20}ClN_3O_6S_2$,CH_3I requires C, 37·0; H, 3·7; Cl, 5·9; I, 20·6; N, 6·8; O, 15·6; S, 10·5%), $\nu_{max.}$ (in paraffin) 1688 and 1664 cm.$^{-1}$. Treatment with acetone produced sporidesmin and refluxing with methyl iodide did not produce a methiodide.

Acetylation of Sporidesmin.—Sporidesmin benzene solvate (0·248 g.) was dissolved in pyridine (dried over BaO; 0·75 ml.) and treated with acetic anhydride (0·09 ml.). After 72 hr. at 2° the mixture was evaporated in a vacuum-desiccator (H_2SO_4 and soda lime). The residual gum was treated with acetone, and the volatile components were again evaporated *in vacuo*. The gummy residue was treated with iced water (10 ml.) and extracted with ether (3 × 10 ml.). The extract was evaporated and the residue run in benzene on a silica gel column (11·5 × 1·9 cm.). The column was developed with benzene (80 ml.), then with 7 : 93 ether–benzene. The first 28 ml. of eluate were discarded and the next 170 ml. collected and evaporated. The residual gum (0·256 g.) was dissolved in a minimum volume of ethanol, and water was added as long as crystals (m. p. 165—167°; 0·22 g.) separated from the cooled solution. *Sporidesmin diacetate* separated from aqueous ethanol as colourless needles, m. p. 170—171°, $[\alpha]_D^{22}$ −25° (*c* 1·2 in MeOH), +7·8° (*c* 0·51 in $CHCl_3$) (Found: C, 47·7; H, 4·6; Cl, 6·2; N, 7·5; S, 11·2; Ac, 17·1. $C_{22}H_{24}ClN_3O_8S_2$ requires C, 47·3; H, 4·4; Cl, 6·3; N, 7·5; S, 11·5; 2Ac, 15·5%), $\lambda_{max.}$ 220, 255, and 310 mμ (log ε 4·55, 4·10, and 3·41), $\nu_{max.}$ (in paraffin) 1751, 1230 (OAc), 1695 (C=O) cm.$^{-1}$, no absorption in the O–H stretching region, τ 3·23, 3·91, 4·22, 6·16 (intensity 3), 6·18 (intensity 3), 6·62 (intensity 3), 7·00 (intensity 3), 7·88 (intensity 3, acetate C*H*$_3$), 7·9, 7·97 (intensity 3, acetate C*H*$_3$), 8·37 (intensity 3), 8·4. Other acetylation conditions gave lower yields and other ill-defined products.

Sporidesmin-B acetate. Sporidesmin-B (58 mg.), in pyridine (0·3 ml.), with acetic anhydride (0·09 ml.) as above, gave an amorphous *monoacetate* that, separating from aqueous ethanol, had m. p. 93—114° (Found: C, 48·8; H, 4·8; N, 8·0; S, 13·6; Ac, 9·5. $C_{20}H_{22}ClN_3O_6S_2$ requires C, 48·0; H, 4·4; N, 8·4; S, 12·8; Ac, 8·6%), $\lambda_{max.}$ 220, 256, 308 mμ (log ε 4·55, 4·17, 3·55), $\nu_{max.}$ (in paraffin) 1748, 1222 (OAc), 1689, 1701 (C=O) cm.$^{-1}$. No modification of procedure produced a crystalline acetate; the propionate was also amorphous.

Ketone, m. p. 145°.—(*a*) Sporidesmin benzene solvate (0·21 g.) was heated at 90° for 2 hr. in 0·25N-sodium hydroxide (10 ml.). The sporidesmin dissolved and the yellow solution became greenish and became finally orange. The solution was extracted with chloroform, and the aqueous phase acidified and extracted with chloroform. The aqueous phase was made 0·25N with sodium hydroxide solution, and the whole process repeated twice. Analogous extracts were bulked (extract from alkaline solutions, 6 mg.), and the orange gum (113 mg.) obtained from the extracts of acidified solutions was triturated with 1 : 49 ether–benzene (3 × 2 ml.), filtered (residue 40 mg.), and run on a silica gel column (16 × 1·25 cm.). The first orange zone to be eluted with 1 : 49 ether–benzene gave an orange gum (20 mg.). This was run in benzene on an alumina (activity IV) column (9 × 0·5 cm.). Elution with benzene gave a leading blue zone which was discarded and a following orange zone that was collected. The residue (12 mg.) after evaporation of the eluate recrystallised from 95% aqueous methanol. Further elution with 1 : 19 acetic acid–ether provided a little more of the ketone (m. p. 144°; 14 mg.). The *ketone* separated from aqueous methanol as waxy red needles, m. p. 145° (Found: C, 53·0, 52·2; H, 4·6, 4·4; Cl, 13·6, 13·5; N, 5·4, 5·3; O, 24·4. $C_{11}H_{10}ClNO_4$ requires C, 51·7; H, 3·9; Cl, 13·9; N, 5·5; O, 25·1. $C_{12}H_{12}ClNO_4$ requires C, 53·4; H, 4·5; Cl, 13·2; N, 5·2; O, 23·7%). Its 2,4-*dinitrophenylhydrazone*, m. p. 321°, was prepared in aqueous ethanol and recrystallised

from chloroform–ethanol (Found: C, 46·9; H, 3·3; N, 15·9. $C_{17}H_{14}ClN_5O_7$ requires C, 46·9; H, 3·2; N, 16·1. $C_{18}H_{16}ClN_5O_7$ requires C, 48·0; H, 3·5; N, 15·6%). Its *oxime*, prepared in aqueous ethanol, separated from ethanol as yellow needles, m. p. 240° (decomp.) (Found: N, 9·7. $C_{11}H_{11}ClN_2O_4$ requires N, 10·4%).

(*b*) Sporidesmin-B (6·7 mg.) was treated with 5N-sodium hydroxide (2 ml.), and the red ketone isolated by chromatography on alumina. The infrared spectrum of the product was identical with that of the above ketone and its mixed m. p. was not depressed.

Isolation of Ammonia and Methylamine from Sporidesmins.—(*a*) Sporidesmin benzene solvate (41 mg.) was treated with a solution (5 ml.) of chromic acid [5N-aqueous, 4 ml., and sulphuric acid (*d* 1·84), 1 ml.], refluxed for 2 hr., basified with sodium hydroxide solution, and steam-distilled. The volatile base was titrated with standard acid (12·4%, as methylamine), and the solution basified and treated with yellow mercuric oxide as described by Werle.[19] The filtrate was steam-distilled and titration of the distillate gave 1·3 mg. of volatile base. The titrated solution was evaporated, the residue dissolved in a minimum volume of water, and an excess of aqueous flavianic acid added (*A*). Recrystallisation of the precipitate from water gave needles, m. p. 272° (decomp.), undepressed on admixture with methylamine flavianate. No bands (909, 798 $cm.^{-1}$) characteristic of ethylamine flavianate were observed in the infrared spectrum (paraffin mull) of the flavianate from sporidesmin. The mercury complex was decomposed with formic acid, and the liberated ammonia distilled and estimated (1·3 mg.) in the usual way; the flavianate (prepared as described at *A*) had m. p. 288° (decomp.), undepressed on admixture with ammonium flavianate. Sporidesmin-B (39·1 mg.) gave in the same way 11·2% base separated into methylamine (1·9 mg.) and ammonia (1·0 mg.). Small-scale (5 mg.) experiments gave, with both sporidesmins, about 11% of these bases in an approximately 1 : 1 ratio.

(*b*) Sporidesmin benzene solvate (0·2030 g.) was shaken with 4N-sodium hydroxide (30 ml.) until dissolved, then it was slowly distilled for 45 min. in a Kjeldahl apparatus, into standard acid. The total bases (1·9 equiv.) were estimated by titration and the proportion of ammonia present (52%) was determined as described previously.[20]

(*c*) Sporidesmin or sporidesmin-B (5—15 mg.) was refluxed with 5N-sodium hydroxide (5 ml.) for 1 hr., then the solution was distilled. The volume in the distillation flask was kept constant by addition of water, and the volatile base (*ca.* 1 equiv.; smaller quantities were obtained with more dilute alkali) that distilled was titrated. Infrared spectroscopy of the hydrochlorides and flavianates of the bases showed no bands consistent with the presence of ethylamine. Determination of the proportion of ammonia in the mixture of bases gave 26% for sporidesmin and 35% for sporidesmin-B.

(*d*) *Paper chromatography of hydrochlorides of ammonia, methylamine, and ethylamine.* Standard solutions (5 μl.) of the hydrochlorides (4 mg./ml.) were applied to Whatman No. 3MM paper, and the chromatograms were developed (descending) with a solution (100 ml.) of 2·5N-hydrochloric acid (17 ml.) in t-butyl alcohol for 50 hr. at 20°. The papers were dried at 20° in a stream of air for at least 4 hr. and then sprayed with a 0·2% solution of 1,2-naphthaquinone-4-sulphonic acid in 2N-sodium carbonate, or with ninhydrin. Chromatography of a mixture of methylamine hydrochloride (72 μg.) and ethylamine hydrochloride (8 μg.) resulted in clearly defined spots of both bases. The hydrochlorides (180 μg.) of the volatile bases from sporidesmin-B were chromatographed as above; only ammonia and methylamine were detected. A similar result was obtained with the hydrochlorides (120 μg.) of the volatile bases from sporidesmin. Ethylamine hydrochloride (8 μg.) was added to the hydrochlorides (120 μg.) of the volatile bases from sporidesmin, and the mixture was chromatographed. A spot of correct R_F for ethylamine was clearly resolved from the other two bases. More rapid separations were obtained by chromatography on discs of Whatman No. 1 paper with the developing solvent described above, or, less efficiently, with butan-1-ol saturated with water. Pink spots were not observed when the chromatograms were sprayed with the Folin reagent, indicating the absence of secondary amines. No spots were observed when the papers were sprayed with Dragendorff's reagent.[21]

Isolation of Methyl Iodide from Alkoxyl and Alkimide Determinations on Sporidesmin.—

[19] Werle, in "Moderne Methoden der Pflanzenanalyse," ed. Paech and Tracey, Springer, Berlin, 1955, Vol. IV, p. 533.
[20] Atkinson and Taylor, *J.*, 1955, 4241.
[21] Munier and Macheboeuf, *Bull. Soc. Chim. biol.*, 1951, **33**, 846.

These experiments were done in a Friedrich apparatus with a small anhydrone tube inserted after the washer (10% aqueous potassium antimonyl tartrate). The issuing gases were passed into a 0·5N-solution of trimethylamine in nitrobenzene (4 ml.).[22] A standard alkoxyl determination was carried out on sporidesmin (12·5 mg.) and provided only tetramethylammonium iodide (10 mg.). Two distillations were done in the following alkimide determination, and the alkyl iodides evolved were collected in fresh trimethylamine solution. The precipitate (8·2 mg.) of tetramethylammonium iodide was collected and the filtrate evaporated to dryness. The water-soluble residue (2·0 mg., of which the solubility of tetramethylammonium iodide accounts for *ca.* 1 mg.) on infrared spectroscopy gave weak bands at 826, 877, 1014, 1172, and 1241 cm.$^{-1}$ consistent with the presence of trimethylethylammonium iodide. Such absorptions were not observed when the process was repeated with omission of sporidesmin.

The alkyl iodides evolved from an alkoxyl determination on sporidesmin benzene solvate (17·7 mg.) were condensed in dichloromethane (0·05 ml.) at −80° in a trap similar to that described by Haslam *et al.*[23] The liquid in the trap was removed quantitatively and an aliquot part injected into the carrier gas (argon, 100 ml./min.) of a gas–liquid (Apiezon " L ") chromatographic column (Perkin-Elmer Corp., 2 m. × 8 mm.) operating at 65°. Two peaks with retention times 10 min. (dichloromethane) and 12·5 min. (methyl iodide) were observed. Three distillations were carried out in the subsequent alkimide determination, the iodides were condensed in dichloromethane and, in addition, the effluent gas from the trap was passed into a tube serving as the bromine absorber used in standard alkoxyl determinations. At the end of the reaction the dichloromethane solution (0·044 ml.) was removed from the trap and an aliquot part (15 μl.) injected into the carrier gas of the chromatographic column. The remainder of the dichloromethane solution was transferred quantitatively to bromine solution, and the liberated iodine was titrated. Similarly the iodine liberated from the bromine absorber was titrated. The results showed a total recovery of 4·4% of *N*-CH_3 (Calc., 5·4%); almost all the iodides evolved were condensed in the dichloromethane. The gas–liquid chromatogram showed 3 peaks of retention times, 10 (dichloromethane), 12·5 (methyl iodide), and 19 min. (ethyl iodide). By running mixtures of known composition a calibration curve was constructed and the proportion (<5%) of ethyl iodide in the alkyl iodides from sporidesmin calculated.

Desulphurisation of Sporidesmin.—Sulphide was not detected when the products from (*a*) and (*b*) below were fused with sodium, and only traces of antimony sulphide appeared in the washer during the MeO and *N*-Me determinations.

(*a*) Hydrogen was passed over a stirred solution of sporidesmin benzene solvate (20·1 mg.) in methanol (4 ml.) containing Adams catalyst (5 mg.). After 4 hr. the mixture was filtered and more catalyst (6 mg.) added. After a further 4 hr., when only traces of hydrogen sulphide were being evolved, the solution was filtered and the filtrate evaporated giving 18·2 mg. of residue (Found: MeO, 11·6; *N*-Me, 5·6%).

(*b*) Sporidesmin benzene solvate (20 mg.) was refluxed in ethanol (10 ml.) for 24 hr. with 1 g. of W4-Raney nickel.[24] The mixture was filtered and the filtrate evaporated, giving a colourless resin (13 mg.) (Found: MeO, 13·6; *N*-Me, 6·5%).

Isolation of Sulphur from Sporidesmin.—Sporidesmin benzene solvate (290 mg.) was refluxed for 8 hr. in benzene (25 ml.) with alumina (activity IV; 13 g.). The mixture was filtered and the residual alumina extracted with benzene. Evaporation of the filtrate and washings left a gum (122 mg.) that was run in benzene on an alumina column (activity IV; 11·5 × 1·8 cm.). The column was eluted with benzene, and the eluate was collected up to the first pink zone on the column. Evaporation of the eluate gave colourless prisms (21 mg.) that, recrystallised from benzene, had m. p. 115° (Found: S, 100·1%). A maximum of 10% of sulphur (Calc., for 2S, 11·4%) was obtained in other experiments, lower yields were obtained when sporidesmin was left on an alumina column (activity IV) for several days.

Estimation of Solvents in Sporidesmin Solvates.—The solvate (10 mg.) was dissolved in ether (0·2 ml.), and the solution (20 μl.) injected into the carrier gas (argon, 25 ml./min.) of a gas–liquid (Apiezon " L ") chromatographic column (Perkin-Elmer Corp., 2 m. × 8 mm.) operating at 126°. The solvents had the following retention times under these conditions: diethyl ether, 4 min.; benzene, 10 min.; carbon tetrachloride, 13 min.; dibromomethane,

[22] Makens, Lothringer, and Donia, *Analyt. Chem.*, 1959, **31**, 1265.
[23] Haslam, Hamilton, and Jeffs, *Analyst.*, 1958, **83**, 66.
[24] Pavlic and Adkins, *J. Amer. Chem. Soc.*, 1946, **68**, 1471.

19 min. The peaks obtained on the chromatograms were cut out and weighed. Known amounts of the solvents were run under identical conditions and from the results a calibration curve was determined. Replicate analyses indicated the accuracy to be $\pm 5\%$.

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