constant (in buffer at 20°) obtained from $\frac{1}{\sqrt{D}} = S \frac{\alpha}{\sqrt{D}}$ was 7.15×10^{-7} , while the application of the above method gave $\bar{D} = 6.94$ and 7.02×10^{-7} , from two intervals of time. The mean diffusion constant of a mixed solution of lactoglobulin and sucrose (Ogston, 1949) was found to be 10.82×10^{-7} , the expected value being 10.85×10^{-7} .

DISCUSSION

It is clear from the sedimentation diagrams that both polysaccharides are polydisperse, the dextrorotatory consisting of homogeneous and heterogeneous fractions and the laevorotatory being entirely heterogeneous. In addition, in neither case does all the refracting material appear in the sedimentation boundaries, which indicates that a proportion of the material may sediment too quickly or too slowly or may be too highly polydisperse to contribute to the boundary diagrams. Thus, while the mean sedimentation constants of the material can be regarded as established, in view of the uncertainties and errors discussed above neither the proportions of the components nor their diffusion constants should be regarded as more than rough estimates, and the same is true of the estimates of molecular weights,

given in Table 2. These were derived from the sedimentation and diffusion constants assuming a value of 0.62 for the partial specific volumes.

The value of the mean diffusion constant of the laevorotatory polysaccharide, calculated by the method described, would give a reliable estimate of its mean molecular weight if it were certain that the average applied to the same range of material as does the estimate of the sedimentation constant. The fact that only 0.64 of the refracting material appears in the sedimentation boundary shows that this may not be so.

SUMMARY

1. Measurements of the sedimentation and diffusion of two samples of polysaccharide from *Penicillium luteum* are described.

2. A new method is given for analysing the diffusion data obtained with the Gouy diffusimeter, so as to obtain a value of the arithmetic-mean diffusion constant which is comparable with the mean sedimentation constant.

3. Approximate values for the amounts and constants of the components of the polysaccharides have been deduced.

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Metabolic Products of *Trichothecium roseum* Link

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The work described in this paper was carried out independently at Stevenston and the London School of Hygiene and Tropical Medicine. When the separate investigations were communicated to the Biochemical Society (Freeman & Morrison, 1948; Michael, 1948) the results from the two laboratories were found to be so similar that it was decided to present a joint account of the work. This paper deals with the isolation of three crystalline metabolic products of *Trichothecium roseum* which had not hitherto been described. Two of these products which are present mainly in the mycelium, and only in smaller amounts in the culture fluid, were named

rosein I and rosein II, while the name rosein III is proposed for the third compound which has been found only in the culture fluid. The three new products are additional to trichothecin, the antifungal substance which was described by Freeman & Morrison (1949a).

It has been known for many years that fruits attacked by the pink rot caused by *T. roseum* Link contain a bitter principle. The latter was isolated in the form of a crude syrup and its solubility described by Iwanoff (1904). Antagonism between *T. roseum* and certain plant pathogenic fungi was reported by Whetzel (1909), Boning (1933), Koch (1934) and

Greaney & Machacek (1935). Koch (1934) stated that *T. roseum* actively parasitized the stroma of *Dibotryon morbosum* in the black knot disease of various hosts. Greaney & Machacek (1935) found that the fungus was antagonistic to *Helminthosporium sativum* when the two organisms were grown together in soil in the presence of cereal seedlings. The presence of an antifungal compound in culture filtrates of *Trichothecium roseum* was reported by Brian & Hemming (1947). The substance, trichothecin, responsible for the antifungal activity was isolated, and some of its chemical properties described by Freeman & Morrison (1949*a*). Its biological properties were described by Freeman & Morrison (1949*b*). Rosein I and crude rosein III were encountered as by-products in the isolation of trichothecin by Freeman & Morrison (1949*a*) and referred to in that paper as 'crystalline precipitate (II)' and 'precipitate (I)' respectively.

The mould was incubated for 3-4 weeks on Czapek-Dox medium which was enriched with corn-steep liquor. Extraction of the culture fluid with chloroform yielded a syrupy mixture from which rosein III was obtained as a colourless crystalline product by repeated precipitation with light petroleum from chloroform, followed by fractionation on an alumina column in chloroform-carbon tetrachloride. The petroleum mother liquors were evaporated and fractionated in ether by passing through an alumina column. The first fractions, on recrystallization from light petroleum containing some chloroform, yielded small amounts of roseins I and II; in the mother liquors of these products the bulk of the trichothecin was found.

Extraction of the dried and powdered mycelium with solvents yielded a mixture of two crystalline products, roseins I and II, the former in approximately double the amount of the latter. These products were accompanied in the extracts by considerable amounts of lipids.

In the London School of Hygiene and Tropical Medicine method, the extraction was carried out in a Soxhlet extractor with light petroleum. On concentrating and cooling the extract, a mixture of roseins I and II crystallized in yields of 2-3 % of the weight of mycelium. Separation of the two substances was effected by treatment with cold ethanol which leaves rosein I, m.p. 210°, undissolved, while rosein II, m.p. 186°, crystallizes from the mother liquors on addition of water.

In the Nobel Division method, boiling chloroform was used as solvent. Treatment of the concentrated extract with light petroleum afforded rosein I. On evaporation of the mother liquors and treatment with aqueous ethanol, rosein II was obtained.

Rosein I ($C_{19}H_{26}O_3$, m.p. 210°), which is slightly soluble in cold ethanol, crystallizes from this solvent in colourless tetrahedra. It is optically active, its

specific rotation in chloroform being $[\alpha]_D -112.5^\circ$. It is a neutral compound of ketonic character, as shown by the formation of a 2:4-dinitrophenylhydrazone and an oxime. Under the influence of hot alkali, it is rearranged to a lactone, *isorosein* I ($C_{19}H_{26}O_3$, m.p. 144°), which is no longer ketonic, but which possesses a hydroxyl group which can be benzoylated. Potassium permanganate at room temperature oxidizes rosein I to a monobasic acid, $C_{16}H_{24}O_4$, m.p. 242°.

Rosein II ($C_{19}H_{28}O_3$, m.p. 186°) crystallizes from aqueous ethanol or from toluene in colourless fibrous needles. It possesses weak optical activity, its specific rotation $[\alpha]_D$ in chloroform being $+5.9^\circ$. It has no ketonic or hydroxyl groups, but its properties correspond to those of a lactone. It is soluble in hot alkali and is recovered unchanged on acidification. Oxidation with potassium permanganate in acetone at room temperature gave a monobasic acid $C_{16}H_{26}O_5$. Rosein II was obtained from cultures on the enriched Czapek-Dox medium, but was not detected in cultures on Raulin-Thom medium.

Rosein III ($C_{20}H_{28}O_4$, m.p. 221°) crystallizes from toluene in colourless plates. It has a ketonic character as shown by the formation of a 2:4-dinitrophenylhydrazone. Hot alkali converts rosein III into an isomeric lactone, *isorosein* III, $C_{20}H_{28}O_4$, m.p. 155°.

During the isolation of one of the strains used in this work (BB 105*a*) it was observed that an apple infected with the mould developed an intensely bitter taste, which permeated the whole of the fruit even when only a small area was infected. Filtrates from cultures of the mould on liquid media also possessed a bitter taste. An amorphous solid, similar in properties to Iwanoff's (1904) bitter principle, was isolated by ether extraction of the culture filtrate; trichothecin was shown to be one of its principal bitter components.

Antibiotic properties of the metabolic products. While roseins I and III were devoid of antibiotic activity against the test organisms, rosein II inhibited the growth of *Bacillus subtilis* at a concentration of 5 mg./l. It inhibited *Mycobacterium phlei* at 1:81,000, was only slightly active against *M. tuberculosis* and had no effect on *M. butyricum*.

EXPERIMENTAL

Organisms and cultural conditions

The organisms used for most of the work in Nobel Division were strains of *Trichothecium roseum* Link isolated from mouldy dead wood, laboratory catalogue numbers F 109 and F 227. They are described in detail by Freeman & Morrison (1949*b*). The strains used at the London School of Hygiene and Tropical Medicine were L.S.H.T.M., catalogue number BB 105*a*, isolated from an apple and identified by Mr G. Smith in 1945 and L.S.H.T.M., catalogue number 95, isolated from a tomato by Mrs Marcus in 1947.

The strains all grew well on Czapek-Dox agar and beer-wort agar and rapidly developed the typical pink conidial areas. Preliminary experiments showed that on liquid Czapek-Dox or Raulin-Thom media growth and sporulation were slow and sparse. Czapek-Dox solution (350 ml.) in 1 l. conical flasks was inoculated with a heavy spore suspension and incubated at 24°. After 14 days, a thin mycelium had formed and pink conidial areas began to appear; at this stage the culture filtrate still contained approximately 4% glucose and its pH was 6.4. After incubation for 31 days the mycelium was thin, but well covered with spores. The pale yellow culture filtrate contained 3.2% glucose and its pH was 7.3.

After incubation for 7 days cultures on Raulin-Thom solution developed a white mycelium which had a tendency to sink below the surface of the liquid. Weak and patchy sporulation appeared after 24 days.

Czapek-Dox medium, which was enriched by the addition of 1% corn-steep liquor (50% total solids), sustained more rapid and abundant growth and spore formation. Czapek-Dox solution (350 ml.) + corn-steep liquor (3.5 ml.) in 1 l. conical flasks was inoculated with a heavy spore suspension and incubated at 24°. After 6 days a thick mycelium had formed and spore formation was beginning. After 14 days the mycelium was completely covered by pink conidial areas. The cultures were harvested after incubation for 21 days, when the pale yellow culture filtrate contained 0.7% glucose and its pH was 7.6.

The culture medium used in Nobel Division was based on the Czapek-Dox formula in which NaNO_3 was replaced by the equivalent quantity of ammonium tartrate and with the addition of 1% corn-steep liquor. The medium used at L.S.H.T.M. was Czapek-Dox solution with the addition of 1% corn-steep liquor.

Isolation of roseins I and II

Nobel Division method

Rosein I. The medium used contained ammonium tartrate (2 g.); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g.); K_2HPO_4 (1.0 g.); KCl

(0.5 g.); $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01 g.); crude commercial glucose (50 g.); corn-steep liquor (10 ml.) in water to 1 l. It was based on Czapek's formula (as modified by Dox, 1910), in which ammonium tartrate was substituted for NaNO_3 and corn-steep liquor was added as a supplementary nutrient. Crude commercial glucose ('glucose chips') was found to be a satisfactory source of sugar.

The medium (300 ml.) in 'Glaxo' bottles was sterilized by autoclaving. The reaction was adjusted to pH 5.0. The bottles were inoculated with a heavy spore suspension and incubated at 25° for 28 days in darkness.

The mycelium (strain F 109) was harvested and separated from the culture fluid by filtration through fluted filter paper, washed with cold water and dried in a current of air at 20°. Air-dried, powdered mycelium (159.2 g. from 15 l. of medium) was extracted three times under reflux for 0.5 hr. with boiling CHCl_3 (500 ml.). The combined extracts were concentrated, yielding an orange-yellow oily residue (36.3 g., 22.8% of mycelium). The residue was treated with light petroleum (b.p. 60–80°, 250 ml.) and a crystalline precipitate of crude rosein I (6.58 g., 4.13% of mycelium) slowly formed. The precipitate was collected, washed with light petroleum and extracted with cold ether. The mother liquor (A) and the ether extract (B) were retained. The crude rosein I was dissolved in CHCl_3 (5 ml.) and the solution passed through a column (20 × 1.5 cm.) of activated alumina in CHCl_3 . The solvent was evaporated from the eluate and the residue (5.18 g.) was dissolved in the minimum volume of CHCl_3 (5 ml.) and diluted with ether (30 ml.). Slightly impure rosein I (m.p. 206°, 4.53 g.) crystallized out. The pure substance was obtained after one recrystallization from ethanol (4.11 g., 2.58% of mycelium, m.p. 210°) in the form of colourless tetrahedra.

Rosein II. The mother liquor (A), consisting mainly of a solution of lipids in light petroleum, was evaporated to dryness and the orange-coloured residue (29.71 g.) extracted for a few minutes with boiling 75% (v/v) aqueous ethanol (100 ml.). The extract was cooled, shaken with light petroleum (b.p. 60–80°, 30 ml.) and the ethanol layer separated and combined with the ether extract (B). On evaporation of

Fig. 1. Isolation of roseins I and II from mycelium.

the combined ethanol and ether solutions nearly to dryness crude rosein II crystallized (2.50 g.). It was dissolved in CHCl_3 , boiled with a little activated charcoal and filtered. The residue, formed on evaporation of the filtrate, was recrystallized first from light petroleum (b.p. 60–80°) and then from 50% (v/v) aqueous ethanol giving pure rosein II (2.01 g., 1.26% of mycelium, m.p. 186°) in the form of colourless fibrous needles. Passage of an ether solution through a column of activated alumina was used in purification of some batches of crude rosein II.

The residue remaining after isolation of roseins I and II consisted mainly of a mixture of lipids, partially crystalline at 20°.

The fractionation of the mycelial products is summarized in Fig. 1.

London School of Hygiene and Tropical Medicine method

In several experiments 1 l. conical flasks (50), each containing Czapek-Dox solution (350 ml.) and corn-steep liquor (3.5 ml.) were inoculated and incubated for 21–26 days at 24°. At the end of this period the metabolism solution was separated from the mycelium by filtration and the latter well pressed, washed with water, dried *in vacuo* at 35° and finally ground to a fine powder. The yield from 50 flasks was 200–210 g. dry mycelium.

The culture filtrate was a clear, pale yellow liquid, pH 7.6. It contained 0.5–0.7% residual glucose. It gave no reaction with FeCl_3 . The solution had an intensely bitter taste. After cooling to 5° for 24 hr., small amounts (0.1–0.2 g. from 17 l.) of colourless crystals had separated. They were filtered off, washed with water and dried *in vacuo*. The crystalline product consisted of a mixture of needles and tetrahedra identical with specimens of roseins I and II isolated from the mycelium.

The culture filtrate was three times extracted with $\frac{1}{3}$ vol. of ether. The extract was filtered through a dry filter, evaporated to small volume, and then allowed to evaporate slowly. Small amounts (0.1–0.2 g.) of the same mixture of crystals separated. They were filtered off, washed with a little ether and dried *in vacuo*. The filtrate on evaporation to dryness gave an amorphous, buff-coloured powder (2 g.), having an intensely bitter taste. The product was slightly soluble in water, soluble in dilute NaOH giving a frothy solution and soluble in ethanol, acetone and ether.

Isolation and separation of metabolic products from mycelium. The dried, powdered mycelium was extracted in a Soxhlet extractor with light petroleum (b.p. 40–60°). After 3–4 hr. colourless crystals began to appear in the receiver. After 7 hr. the extract was yellow and had a strong smell of a fatty or oily character. At this stage the receiver was changed and extraction continued for four to five periods of 7 hr. The extracts were separately concentrated to small volume, kept at 0° for 16 hr. and the crystalline deposit filtered off, washed well with cold light petroleum and dried *in vacuo*. The product consisted of varying mixtures of colourless needles and tetrahedra. On evaporation of the combined mother liquors to small volume a further crop of crystals was obtained.

A typical extraction of dry mycelium (42 g.) gave from successive 7 hr. extractions 0.8 g. crystals, mainly needles; 0.3 g. crystals, a mixture; 0.1 g. crystals, mainly tetrahedra; and 0.05 g. crystals, mainly tetrahedra (total 1.25 g.; 2.98% of mycelium). The average yield, throughout the work, of the mixed crystalline product was 2–3% of the dry mycelium,

equivalent to 0.3–0.4 g./l. of culture medium. The final mother liquors of the crystalline products, when freed from solvent, were semi-solid yellow oils.

The mixed crystalline product was separated into its components by extraction with cold ethanol in which rosein II was readily soluble, whilst rosein I was only slightly soluble. The mixture (2.85 g.) was suspended in ethanol (30 ml.) and stirred for 2 min. at room temperature. The colourless extract was filtered off and the insoluble residue washed with two 5 ml. portions of ethanol. The insoluble fraction was dried *in vacuo*; it consisted of rosein I, colourless tetrahedra (1.67 g., m.p. 210°).

The ethanolic mother liquor and washings of rosein I were combined, warmed to 60° and diluted with an equal volume of water at 60°. On cooling, colourless needles of rosein II separated. After standing at 0° for 16 hr., the product was filtered off, washed with ice-cold 50% (v/v) aqueous ethanol and dried *in vacuo*. Yield of rosein II, 0.8 g., m.p. 183°. The melting point of the product was raised to 186° on recrystallization from toluene.

The mother liquor of rosein II was freed from ethanol by evaporation and cooled to 0° for 16 hr. A deposit (0.3 g.) of a mixture of roseins I and II separated and was worked up with a subsequent batch. The initial crystalline mixture contained approximately two parts of rosein I and one part of rosein II.

Isolation of rosein III from culture filtrate

Rosein III was not obtained from the mycelium. It was extracted, together with trichothecin and small amounts of roseins I and II, by CHCl_3 from the culture filtrate (strain 227) and isolated by the following procedure.

The culture filtrate in lots of 1 l. was twice extracted with CHCl_3 (200 ml.). The combined extracts were evaporated to dryness under reduced pressure and the dried extract from 80 l. of culture filtrate was obtained as a brown gummy solid (21.23 g.). The latter was dissolved in CHCl_3 (20 ml.) and the solution diluted with ether (450 ml.). The resultant precipitate was rejected and the supernatant solution evaporated to dryness. The residue was redissolved in CHCl_3 (20 ml.) and the solution diluted with light petroleum (b.p. 60–80°, 450 ml.). The gummy precipitate which formed was separated by decantation, redissolved in CHCl_3 (10 ml.) and reprecipitated by addition of light petroleum as above. The extracts were combined and on evaporation to dryness gave 11.65 g. of an amorphous residue (A). The precipitate (B, 7.68 g.) was dissolved in 50% (v/v) $\text{CCl}_4/\text{CHCl}_3$ mixture and fractionated chromatographically on an alumina column (30 × 3.8 cm.) which was developed with a mixture of CCl_4 and CHCl_3 . The eluate was collected in 50 ml. fractions, which after evaporation of the solvent were treated with a little ether. The crystalline fractions were combined (4.72 g.) and dissolved in a little CHCl_3 . On addition of ether, rosein III crystallized out. The pure compound (3.68 g., m.p. 221°) was obtained after recrystallization from toluene as colourless plates.

The petroleum ether-soluble portion (A, 11.65 g.) containing trichothecin was dissolved in ether and fractionated chromatographically on an activated alumina column. The eluate was collected in 100 ml. fractions, the first three of which contained the bulk of the trichothecin. After evaporation of the solvent (ether) the fractions were dissolved in light petroleum (b.p. 60–80°) containing 5% (v/v) of CHCl_3 . After standing at 5° for 16 hr., fraction II gave a crystalline deposit of rosein I (0.25 g.) and fraction III gave rosein II

(0.11 g.). After separation of these crystalline precipitates, the combined mother liquors were used for preparation of trichothecin. Fractions IV, V, VI and VII gave crystalline deposits of crude rosein III, totalling 2.11 g. The pure product (m.p. 221°) was obtained on recrystallization.

It was found to be most convenient and efficient to separate the fractions of (A) which were free from trichothecin (as determined by antifungal tests) and combine them with (B), for chromatographic purification of rosein III, as shown in Fig. 2. The total yield of rosein III was about 60 mg./l. of culture filtrate.

in CHCl_3 (c, 1). Nitrogen, halogens and sulphur were absent. (Found: (Weiler and Strauss): C, 75.4, 75.4; H, 8.9, 8.8; mol. wt. (cryoscopic in benzene), 302; (Rast), 316; methoxyl, nil; active hydrogen, nil. $\text{C}_{19}\text{H}_{26}\text{O}_3$ requires C, 75.5; H, 8.6%; mol. wt. 302.)

Rosein I dissolved in conc. H_2SO_4 to give a pale orange-yellow solution. The compound gave no colour reactions for sterols. A solution in aqueous ethanol was neutral, gave no reaction with FeCl_3 and did not reduce Fehling solution or Tollens ammoniacal AgNO_3 reagent. There was no reaction with Br_2 in CCl_4 . An acetone solution of KMnO_4 was slowly

Fig. 2. Isolation of rosein III.

Properties and reactions of metabolic products

Rosein I

Rosein I crystallized from hot ethanol in colourless tetrahedra, m.p. 210°. It was readily soluble in CHCl_3 or acetone and was fairly soluble in benzene, slightly soluble in ether or ethanol, very slightly soluble in cold light petroleum and practically insoluble in water, cold dilute NaOH and dilute HCl . It was optically active: $[\alpha]_D^{24} - 112.5^\circ$, $[\alpha]_{5461}^{24} - 140^\circ$

decolorized at room temperature. Alkaline KMnO_4 was not decolorized at room temperature, but was rapidly decolorized on warming. Rosein I gave no benzoyl derivative on treatment with benzoyl chloride and pyridine.

Rosein I 2:4-dinitrophenylhydrazone. Rosein I (100 mg.) was dissolved in glacial acetic acid (5 ml.) and mixed with 2:4-dinitrophenylhydrazine (150 mg.) in glacial acetic acid (10 ml.) After 18 hr., the mixture was diluted with 2N-HCl and the crystalline precipitate filtered off and washed first with 2N-HCl and then with water until neutral. The crude

product (147 mg.) was dissolved in CCl_4 and purified chromatographically on activated alumina. The main band and several minor bands were developed and eluted by gradually increasing the proportion of CHCl_3 in the solvent. The main fraction (120 mg.) crystallized from aqueous ethanol in the form of yellow needles, m.p. 239°.

The same product was obtained by treatment of rosein I (100 mg.) in absolute ethanol with 1.5 ml. Brady reagent (1 g. 2,4-dinitrophenylhydrazine in 2 ml. conc. H_2SO_4 and 15 ml. ethanol). Yellow needles began to crystallize after 30 min., and the product was collected after 20 hr. at room temperature. The hydrazone in ethanolic solution gave an intense red colour with strong alkali. (Found: C, 62.7, 62.3; H, 6.3, 6.3; N, 11.3, 12.5. $\text{C}_{25}\text{H}_{30}\text{O}_8\text{N}_4$ requires C, 62.2; H, 6.2; N, 11.6%.)

Rosein I oxime. Rosein I (100 mg.) dissolved in warm ethanol (5 ml.) was treated with hydroxylamine hydrochloride (30 mg.) and anhydrous sodium acetate (200 mg.). The mixture was kept at 60° for 1 hr. and most of the ethanol distilled off. The residue was diluted with water and extracted with ether. The extract was washed with water, dried over Na_2SO_4 and evaporated to dryness. The residue (96 mg.) was recrystallized from aqueous ethanol and formed colourless prisms, m.p. 215°. (Found: C, 71.7; H, 8.5; N, 4.3, 4.5. $\text{C}_{19}\text{H}_{27}\text{O}_8\text{N}$ requires C, 71.9; H, 8.5; N, 4.4%.)

isoRosein I. Rosein I (220.8 mg.) was heated under reflux for 6 hr. with 0.1N-ethanolic KOH (20 ml.). The solution was cooled, diluted with water and titrated with 0.1N-HCl. (Found: 220.8 mg. rosein I equiv. to 7.56 ml. 0.1N-HCl; saponification value, 292 (theory, 302).) The bulk of the ethanol was distilled off and the residue made acid to congo red indicator by addition of conc. HCl. The resultant colourless precipitate was extracted with ether. The extract crystallized slowly on evaporation of the solvent giving colourless crystalline isorosein I (190 mg., 86.1% yield). The product was recrystallized from light petroleum (b.p. 60–80°) and then from 50% (v/v) aqueous ethanol in the form of colourless prisms, m.p. 144°. isorosein I was readily soluble in cold ethanol, ether, CHCl_3 , acetone and benzene, slightly soluble in light petroleum and insoluble in water. It dissolved slowly in hot 2N-NaOH. No precipitate appeared on cooling. On addition to the cold alkaline solution of an equal volume of phosphate buffer, pH 6, no precipitate appeared. On acidifying with 2N-HCl, isorosein I, m.p. 143°, crystallized. It did not dissolve in NaHCO_3 solution and had no acidic properties. (Found: C, 75.7, 75.4; H, 8.5, 8.9; mol. wt. (cryoscopic in benzene), 280. $\text{C}_{15}\text{H}_{26}\text{O}_8$ requires C, 75.5; H, 8.6%; mol. wt. 302.) $[\alpha]_{5461}^{20} + 40^\circ$ (c, 1 in ethanol). isorosein I did not react with hydroxylamine or with 2,4-dinitrophenylhydrazine.

Monobenzoyl isorosein I. isorosein I (200 mg.), dissolved in pyridine (5 ml.), was treated with benzoyl chloride (500 mg.). After standing at 20° for several days, the mixture was poured into ice-cold dilute HCl and extracted with ether. The extract was washed first with water, then with dilute NaOH and finally with water until neutral. On evaporation of the solvent, the residue crystallized slowly. It was decolorized by solution in ether and passage through a column of activated alumina. Recrystallization from aqueous ethanol gave colourless needles of monobenzoyl isorosein I, m.p. 156° (180 mg.). (Found: C, 75.7; H, 7.6. $\text{C}_{26}\text{H}_{30}\text{O}_4$ requires C, 76.9; H, 7.4%.)

Hydrolysis of monobenzoyl isorosein I. Monobenzoyl isorosein I (81 mg.) was dissolved in ethanol (10 ml.) and

heated under reflux with 0.1N-KOH (10 ml.). Titration with 0.1N-HCl showed that roughly 2 equiv. of alkali had been taken up, which was in accordance with the hypothesis that 1 equiv. was taken up in hydrolysis of the benzoate and 1 equiv. in hydrolysis of a lactone ring. The solution was made acid to congo red and allowed to stand for 24 hr. A slight excess of NaHCO_3 was then added and the solution extracted with ether. The extract was washed with water, dried over Na_2SO_4 and the solvent evaporated off. The crystalline residue was recrystallized from ethanol giving colourless crystals, m.p. 213–214°, in insufficient quantity for analysis.

Permanganate oxidation of rosein I. Rosein I (50 mg.) was dissolved in a 1% solution of KMnO_4 in acetone (5 ml.). After 1 hr. the purple colour had disappeared and MnO_2 had been precipitated. A further 5 ml. of KMnO_4 solution was added; after standing for 16 hr. the purple colour had again been discharged. The MnO_2 was filtered off, washed well with acetone and dried. On evaporating the filtrate and washings, rosein I (37 mg.) was recovered. The MnO_2 was twice extracted with 10 ml. portions of boiling water. On acidification of the colourless extract with HCl, sheaves of colourless needles soon crystallized out. They were collected after 16 hr. at 0°, m.p. 242° (7.5 mg.).

In a second experiment, rosein I (50 mg.) was treated with 1% KMnO_4 in acetone (25 ml.). After 3 days at room temperature, the permanganate was nearly decolorized and the solution was decolorized by passage of SO_2 . The mixture was worked up as described above and the same product isolated (8.5 mg.). (Found on product dried to constant weight *in vacuo* at 60°: C, 68.2; H, 8.1; acid equiv.: 5.4 mg. required 0.18 ml. 0.1N-NaOH (cold or hot) giving equiv. for one acidic group, 300. $\text{C}_{16}\text{H}_{24}\text{O}_4$ requires C, 68.6; H, 8.6%; equiv. 280.)

Rosein II

Rosein II crystallized from light petroleum (b.p. 60–80°), toluene or 50% (v/v) aqueous ethanol in long fibrous needles, m.p. 186°. It was practically insoluble in water and cold 2N-NaOH, only slightly soluble in light petroleum, but readily soluble in most other organic solvents, such as ether, acetone, ethanol and CHCl_3 . Its solution in conc. H_2SO_4 was deep orange yellow. There was no reaction with ethanolic FeCl_3 . The Salkowski and Liebermann-Burchard reactions for sterols were negative. A solution of rosein II in aqueous ethanol was neutral and had no reducing properties. A 2,4-dinitrophenylhydrazone could not be prepared. Rosein II was weakly dextrorotatory; $[\alpha]_D^{25} + 5.9^\circ$ in CHCl_3 (c, 2); $[\alpha]_{5461}^{20} + 7.5^\circ$ in ethanol (c, 1). Nitrogen, sulphur and halogens were absent. (Found: C, 75.2, 75.3, 75.4; H, 9.3, 9.5, 9.3; mol. wt. (Rast) 278, 337; methoxyl, nil; active hydrogen, nil. $\text{C}_{18}\text{H}_{28}\text{O}_8$ requires C, 75.0; H, 9.2%; mol. wt., 304.)

Action of alkali. Rosein II (0.15 g.) was dissolved in ethanol (50 ml.) and 10N-NaOH (3 ml.) added. The clear solution was heated under reflux on a steam bath for 2 hr. After cooling and removing about half the ethanol *in vacuo*, the solution was diluted with water (30 ml.) and acidified with 2N-HCl; rosein II (0.135 g., m.p. 186°) crystallized as colourless needles.

Permanganate oxidation. Rosein II (1.008 g.) was dissolved in a 1% solution of KMnO_4 in acetone (200 ml.). After 16 hr. at 25° the purple colour had almost disappeared. The precipitated MnO_2 was filtered off, washed well with

acetone and dried. On evaporating the acetone filtrate and washings unchanged rosein II (0.3157 g., m.p. 186°) was recovered.

The dried MnO₂ was twice extracted with boiling water (200 ml.). On cooling there was a small deposit (7.5 mg.) from the first aqueous extract. The filtrate was acidified by addition of conc. HCl (2.5 ml.) giving an immediate amorphous colourless precipitate of oxidation product (0.2672 g.). The latter was crystallized from 50% (v/v) aqueous ethanol (17 ml.) yielding colourless hexagonal plates (0.098 g., m.p. 296°). (Found: C, 64.1, 64.2; H, 8.5, 8.7. C₁₆H₂₆O₅ requires C, 64.5; H, 8.8%.) Acid equiv.: 32.6 mg. required 2.14 ml. 0.05N-NaOH (hot or cold) in aqueous ethanolic solution giving equiv. for one acidic group, 305 (theory 298).

Rosein III

Rosein III crystallized from toluene in colourless plates, m.p. 221°. It was only very slightly soluble in water or light petroleum (b.p. 60–80°), slightly soluble in ether and readily soluble in CHCl₃ or ethanol. $[\alpha]_D^{19} - 124^\circ$ in CHCl₃ (c, 1). Nitrogen, sulphur and halogens were absent. (Found: C, 72.1, 72.5, 72.5; H, 8.3, 8.5, 8.5; methoxyl, nil; mol. wt. (Rast) 339. C₂₀H₂₈O₄ requires C, 72.3; H, 8.4; mol. wt., 332.)

In aqueous ethanolic solution, rosein III was neutral, had no reducing properties and gave no reaction with FeCl₃. On treatment with acetic anhydride in glacial acetic acid, the compound (m.p. 221°) was recovered unchanged.

Rosein III 2,4-dinitrophenylhydrazones. Rosein III (0.110 g.) in ethanol (2 ml.) was treated with Brady reagent (1.5 ml.) as described above. The crude hydrazone (0.167 g.) was purified chromatographically from ether solution on activated alumina. The main fraction was crystallized from ethanol as yellow fibrous needles (0.107 g., m.p. 280° decomp.). (Found: C, 60.2; H, 6.2; N, 11.3. C₂₈H₃₂O₇N₄ requires C, 60.8; H, 6.2; N, 11.0%.)

isoRosein III. Rosein III (0.5 g.) dissolved in 6% methanolic KOH (25 ml.) was heated under reflux for 4 hr. The solution was cooled, diluted with water and the methanol distilled off *in vacuo*. The residue was acidified with HCl and extracted with CHCl₃. The extract was washed, dried over Na₂SO₄ and evaporated almost to dryness. The residue was a pale yellow syrup which crystallized slowly. The crude product was recrystallized from toluene and obtained as colourless microcrystalline needles (0.135 g., m.p. 155°). (Found: C, 71.9; H, 8.3; mol. wt. (Rast) 272. C₂₀H₂₈O₄ requires C, 72.3; H, 8.4%; mol. wt., 332.)

Biological properties of metabolic products

Roseins I, II and III were tested for antibiotic activity against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Penicillium digitatum*. The metabolic products in 0.1% solution in ethanol were placed in porcelain cylinders embedded in suitable media in Petri dishes, the media having been seeded with one of the test organisms. The plates were examined for zones of inhibition after 24 hr. incubation at 32° in the case of the bacterial cultures and incubation for 5 days at 25° for *P. digitatum*. There was no inhibition of growth except that of *B. subtilis* by rosein II, which gave a zone of partial inhibition of 2.5 cm. diameter. In serial dilution tests *B. subtilis* grew normally in nutrient broth medium containing 2.5 mg. rosein II/l., but there was complete inhibition at 5.0 mg./l.

Weak antibacterial activity against *B. subtilis* was observed in filtrates of cultures of *T. roseum* F 109, grown on Czapek-Dox medium, by the cylinder-plate technique at an early stage in the work. This is now shown to be due to the presence of rosein II. Dilutions of the culture filtrates were only feebly active since the solubility of rosein II in water at 20° does not greatly exceed its limiting antibacterial concentration.

The effect of rosein II on the growth of three species of acid-fast bacteria was investigated by Dr A. R. Martin. The test against *Myc. tuberculosis* was carried out by incorporating the substance in an agar medium and inoculating the organism on to the surface. After incubation for 14 days the organism grew poorly in medium containing a concentration of 1:3000 of rosein II and some inhibition was still apparent at a concentration of 1:81,000. *Myc. phlei* and *Myc. butyricum* were grown on liquid media in which rosein II had been dispersed and the observations made after incubation for 2 days. Growth of the former was completely inhibited at 1 in 81,000, but normal growth took place at 1 in 243,000. Normal growth of the latter took place in the presence of 1 part rosein II in 1000.

Isolation of trichothecin as a component of the bitter principle of *Trichothecium roseum*

Iwanoff (1904) first reported that the metabolic products of *T. roseum* contained a very bitter substance. This was also noticed by the present authors and by Yasue (1948, private communication), but the bitter components have not hitherto been characterized.

The culture filtrate from 50 cultures of strain BB 105a (17.5 l.) was three times extracted with 2 l. lots of ether. On evaporation, an amorphous, buff-coloured powder (2 g.) of intensely bitter taste was obtained. A sample (36.2 mg.) was dissolved in ethanol (1 ml.) and diluted to 100 ml. with water giving an opalescent solution. After suitable dilution, the solution was assayed for antifungal activity by the *Penicillium digitatum* spore-germination technique which showed that a powerful antifungal compound was present. The ether extract (1.48 g.) was fractionated by the method described by Freeman & Morrison (1949a) for the isolation of trichothecin. The combined light petroleum (b.p. 60–80°) extracts (0.80 g.) were dissolved in ether (10 ml.) and fractionated on a column of activated alumina (25 × 2.0 cm.). The chromatogram was developed with ether and the eluate collected in 100 ml. fractions. These were evaporated to dryness and treated with light petroleum containing 5% (v/v) CHCl₃ (5 ml.). Fraction 2 gave crystalline trichothecin (57.5 mg.), m.p. and mixed m.p. 118°. Fractions 5 + 6 gave crystalline rosein II (14.2 mg.), m.p. 186°.

The fraction insoluble in light petroleum was fractionated as described in the preparation of rosein III, but only traces of uncharacterized crystalline fractions were obtained. No rosein III was detected.

Tasting tests by four independent observers showed that the water-insoluble compounds, roseins I and II, were tasteless; rosein III was slightly bitter, and trichothecin and isotrichothecin (hitherto referred to as the alkaline hydrolysis product of trichothecin by Freeman & Morrison, 1949a) were very bitter.

The available evidence suggests that trichothecin is responsible for part, at least, of the bitter taste of *T. roseum* culture filtrates and the bitterness of fruits attacked by the fungus.

*Carbon balance of metabolic products of
Trichothecium roseum*

In order to determine whether trichothecin and roseins I, II and III, isolated in yields of 50, 3, 1 and 61 mg./l. respectively from modified Czapek-Dox medium, represented the main soluble metabolic products of *T. roseum*, partial carbon balance sheets of four strains grown on Czapek-Dox medium were prepared. The results (Table 1) showed that of the carbon in the initial glucose, 7–15% remained in the culture filtrate in the form of non-volatile components other than glucose and 11–18% was found in the mycelium. There was no production of acidic substances as shown by a steady rise of pH during incubation, until a final value of pH 7–9 was reached. The observations were based on the bulked filtrates from two 'Glaxo' bottle cultures (350 ml.), of each strain, which were diluted to 1 l. for analysis; initial pH 6.6. Portions (100 ml.) of the diluted filtrates were twice extracted with CHCl_3 (50 ml.), the aqueous fraction freed from CHCl_3 by evaporation and carbon determined by the wet combustion method of Houghton (1945). The carbon extracted from the culture filtrate by CHCl_3 represented less than half of the non-volatile carbon of the metabolic products. The carbon accounted for by the four known metabolic products, i.e. 81 mg./l. of the culture filtrate, was small compared with the total CHCl_3 -soluble carbon of the filtrate (mean for the four strains, 792 mg./l.) and it is clear that the greater part of the soluble metabolic products of the fungus remain to be identified.

The carbon balance of a culture of *T. roseum* (*Cephalothecium roseum* Corda) was determined by Birkinshaw, Charles, Hetherington & Raistrick (1931). It was shown that 'this species grew well on Czapek-Dox solution, but was only able to metabolize very slowly the glucose supplied, since, even after 67 days, only about 65% of the glucose had been utilized'. The fungus was shown to have a respiration coefficient of 1.19.

Properties of the lipids

The CHCl_3 extract of the mycelium consisted mainly of a liquid mixture of esters, which amounted to 20% of the weight of the dry mycelium. The crude oil, dark brown in colour, was decolorized by passage through a column of charcoal. The almost colourless filtrate was liquid at 20° and crystallized partially below 15°. It had the following constants: saponification value, 194; iodine value, 57.

After saponification, the fatty acids were separated into saturated and unsaturated fractions by the lead salt method (Twitchell, 1921). The mixed fatty acids (151 g.) yielded 47 g. (31%) of saturated and 95 g. (63%) of unsaturated acids. The latter contained a small proportion of linoleic acid, but no linolenic acid was detected. The major component was probably oleic acid. A portion of the unsaturated acids (0.5 g.) was dissolved in CHCl_3 and excess bromine added. When the reaction was complete, the excess bromine and CHCl_3 were distilled off. The residue was completely soluble in ether indicating the absence of linolenic acid. After distillation of the ether, the residue was dissolved in hot light petroleum (b.p. 60–80°). On cooling colourless needles of linoleic acid tetrabromide (0.05 g.) separated. Recrystallization from light petroleum gave soft needles, m.p. 115° (literature value, m.p. 114–115°).

Table 1. *Partial carbon balance of metabolic products of four strains of Trichothecium roseum*

Strain no.	Initial glucose carbon (g./100 ml.)	Period incubation (days)	Residual glucose carbon (g./100 ml.)	Total carbon in solution (g./100 ml.)	Carbon in volatile neutral compounds (g./100 ml.)	Carbon accounted for by difference (as % of original glucose carbon)	Carbon extracted from culture filtrate by CHCl_3 (g./100 ml.)	CHCl_3 -extracted carbon (% of original glucose carbon)	Mycelium dry weight (g.)	Carbon content of mycelium (%)	Mycelial carbon (% of original carbon)	Fungistatic activity as trichothecin after incubation for 42 days (mg./l.)	Final pH at time of harvest
109	1.317	58	0.012	0.169	0.009	11.24	0.070	5.31	4.81	49.89	18.2	4.5	7.2
198	1.317	71	0.044	0.194	0.006	10.93	0.029	2.20	3.87	53.77	15.8	27.1	7.1
227	1.317	42	0.056	0.153	0.006	6.90	0.022	1.67	4.38	51.17	17.0	28.9	7.8
271	1.317	42	0.024	0.178	0.002	14.53	0.088	6.68	3.14	48.54	11.6	18.7	8.9

SUMMARY

1. The isolation and properties of roseins I and II from the mycelium of *Trichothecium roseum* and of rosein III from the culture filtrate are described.

2. Rosein I, colourless tetrahedra, m.p. 210°, $[\alpha]_D^{24} - 112.5$ in chloroform (c, 1) has the molecular formula $C_{19}H_{26}O_3$. It is a neutral ketone, which reacted with one equivalent of hot ethanolic alkali and yielded a lactone, *isorosein* I, m.p. 144°, on acidification. The latter has an hydroxyl but no ketonic group. On oxidation with potassium permanganate, rosein I gave a monobasic acid, $C_{16}H_{24}O_4$, m.p. 242°.

3. Rosein II, $C_{19}H_{28}O_3$, crystallized in colourless fibrous needles, m.p. 186°, $[\alpha]_D^{23} + 5.9^\circ$ in chloroform (c, 2). It has no ketonic or hydroxyl groups, but its properties correspond to those of a lactone. Oxidation with potassium permanganate gave a monobasic acid, $C_{16}H_{26}O_5$, m.p. 296°.

4. Rosein III, $C_{20}H_{28}O_4$, crystallized from toluene in colourless plates, m.p. 221°, $[\alpha]_D^{19} - 124^\circ$ in chloroform (c, 1). It was a neutral ketone which, on

treatment with hot alkali, yielded *isorosein* III, m.p. 155°.

5. Rosein II inhibited growth of *Bacillus subtilis* at 5 p.p.m. It was slightly active against *Mycobacterium tuberculosis*, inhibited *Myco. phlei* at 1 in 81,000 and had no effect on *Myco. butyricum* at 1 in 1000. Roseins I and III had no effect on three test bacteria and on *Penicillium digitatum*.

6. The bitter principle of *Trichothecium roseum* observed by Iwanoff (1904), and others has been shown to contain trichothecin.

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Note added in proof. A paper has recently appeared by Robertson, A., Smithies, W. R. & Tittensor, E. (*J. chem. Soc.* 1949, p. 879) describing the isolation of rosenonolactone and rosonolactone from *Trichothecium roseum* Link. It appears probable that these two substances are identical with roseins I and II respectively.

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Acid-soluble Pigments of Molluscan Shells

2. PIGMENTS OTHER THAN PORPHYRINS

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The object of the study described in this paper was to identify, if possible, the acid-soluble shell pigments which accompany shell porphyrins in several groups of marine molluscs (Comfort, 1948, 1949a) or which occur in related genera, and to examine their chromatographic behaviour and phylogenetic distribution. Work was concentrated on the shell pigments of the bivalve genus *Pinctada*, in the hope of relating them to the metabolism of porphyrins. This paper contains a summary of their chromatographic behaviour and general properties, compared with those of other acid-soluble molluscan shell pigments.

Previous work on shell pigments contains descriptions of a number of substances, but no clear picture of their chemical and phylogenetic relationships. Linear tetrapyrroles are known to occur in shells (Krukenberg, 1883; Schultz, 1904). Tixier (1947) isolated a number of fractions by chromatography of acid methanol extracts of *Turbo regenfussi* and *T. marmoratus*, and identified the main crystallizable shell pigment as a glaucobilin (Turboglucobilin). Dhéré & Baumeler (1929) regarded the red pigment of *Haliotis refuscens* as a pyrrole derivative. The blue pigment of *H. californiensis* has been variously described as an indigoid