LXXIX. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXVII. (a) ON THE PRODUCTION OF HYDROXY- ANTHRAQUINONES BY SPECIES OF *HELMINTHO- SPORIUM*. (b) ISOLATION OF TRITISPORIN, A NEW METABOLIC PRODUCT OF *HELMINTHOSPORIUM TRITICI-VULGARIS* NISIKADO. (c) THE MOLECULAR CONSTITUTION OF CATENARIN.

BY HAROLD RAISTRICK, ROBERT ROBINSON
AND ALEXANDER ROBERTUS TODD.

*From the Division of Biochemistry, London School of Hygiene and
Tropical Medicine, University of London,
and the Dyson Perrins Laboratory, Oxford University.*

(Received February 27th, 1934.)

It has been previously shown [Charles *et al.*, 1933] that the mycelium of *Helminthosporium gramineum* Rabenhorst, when grown on a synthetic medium containing glucose as the sole source of carbon, contains surprisingly large amounts (30 %) of hydroxyanthraquinones, consisting of a mixture of helminthosporin, $C_{15}H_{10}O_5$ (I), and hydroxyisohelminthosporin, $C_{15}H_{10}O_6$ (III), approximately in the proportion of two to three parts of the former to one part of the latter. Further, we have shown [1933, 1] that the mycelium of *H. cynodontis* Marignoni and *H. euchlaenae* Zimmermann, when grown under the same conditions, contains in each case a third new hydroxyanthraquinone, cynodontin, $C_{15}H_{10}O_6$ (II). This hydroxyanthraquinone is the only one produced by *H. euchlaenae*, but it is accompanied in the case of *H. cynodontis* by small amounts of helminthosporin (I).

These observations have now been extended to many different species and strains of *Helminthosporium*, about 40 in all, most of which were obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland. While this further work has resulted in the discovery of only one hitherto undescribed hydroxyanthraquinone, it has led to a number of interesting observations which are recorded in this paper; numbers of the species examined gave no trace of hydroxyanthraquinones, but some of them gave considerable yields of one or more of the above-mentioned hydroxyanthraquinones.

The procedure followed was to grow the chosen species on a Czapek-Dox solution, filter off and dry the mycelium and extract it with a suitable solvent, *e.g.* chloroform or acetone. Under these conditions the results described have been obtained with the following species:

(a) *H. catenarium* Drechsler. Considerable yields of hydroxyisohelminthosporin (III) in an almost pure condition were isolated, and hence it is proposed to change the name of this hydroxyanthraquinone from that previously given

it to *catenarin* (III). *H. catenarium* was originally described by Drechsler [1923], who isolated it from wood reed grass (*Cinna arundinacea* L.) occurring near Brookline, New York, on the northern coast of Long Island. Drechsler gave the name *H. catenarium* to the species "because of its tendency toward the formation of chains of spores in nature as well as in pure culture."

(b) *H. tritici-vulgaris* Nisikado. Considerable yields of catenarin (III), together with smaller amounts of the new metabolic product *tritisorin* (see below) and helminthosporin (I) were isolated. Nisikado [1929, 1, 2] isolated and described *H. tritici-vulgaris* from diseased wheat (*Triticum vulgare* Vill.). This organism produces on wheat leaves a yellow spot disease which is of fairly common occurrence in Japan.

(c) *H. velutinum* Link. The only hydroxyanthraquinone isolated from this species was catenarin (III), but the mycelium also contained appreciable amounts of ergosterol. *H. velutinum* Link was first described by Link as long ago as 1809, and was found by him to be parasitic upon beech wood.

(d) *H. avenae* Eidam. *Cynodontin* (II) was isolated in small amounts and in almost pure condition. It was accompanied by small amounts of ergosterol. *H. avenae* was described originally by Eidam [1891], who found this organism on, and causing leaf spot disease of, oats occurring in Silesia. The disease on oats caused by *H. avenae* is widely distributed, having been recorded from almost all parts of the world.

Definite evidence was obtained by colour reactions of the production of hydroxyanthraquinones, although in amounts too small for isolation and identification, by the following species of *Helminthosporium*, which are arranged approximately in the order of decreasing intensity of the colour reactions given: *H. brizae* Nisikado, *H. pedicellatum* Henry, *H. geniculatum* Tracy and Earle, *H. leptochloae* Nisikado and Mke, *H. teres* Sacc., *H. filicicola* P. Henn, *H. torulosum* (Syd.) Ashby, *H. coicis* Nisikado, *H. gibberosporum* Curzi and *H. Yamadae* Nisikado.

No hydroxyanthraquinones could be detected by colour reactions from any of the following species: *H. setariae* Sawada, *H. interseminatum* Berk. and Rav., *H. anomalum* Abbott, *H. monoceras* Drechsler, *H. sacchari* Butler, *H. sativum* P.K. and B., *H. nodulosum* (B. and C.) Sacc., *H. hurae* P. Henn, *H. oryzae* Br. de Haan, *H. turcicum* Passer, *H. Kusanoi* Nisikado, *H. Leersii* Atkinson, *H. maydis* Nisikado and Mke, *H. panici-miliacei* Nisikado, *H. Ravenelii* Curtis and *H. zizaniae* Nisikado.

The results obtained point to an interesting correlation between the biochemical synthesis of hydroxyanthraquinones by species of *Helminthosporium* and their morphological characteristics. Nisikado [1929, 2] divides the genus *Helminthosporium* into two sub-genera on morphological grounds, (1) sub-genus *Eu-Helminthosporium*, (2) sub-genus *Cylindro-Helminthosporium*. Although Nisikado did not examine all the species which we have worked upon, and hence any generalisation may be somewhat inaccurate, it is interesting to find that four of those seven species of *Helminthosporium* which we have found to give good yields of hydroxyanthraquinones, i.e., *H. gramineum*, *H. catenarium*, *H. tritici-vulgaris* and *H. avenae*, are placed by Nisikado in the sub-genus *Cylindro-Helminthosporium*. The other three species, *H. cynodontis*, *H. euschlaenae* and *H. velutinum*, were not examined by Nisikado. On the other hand, twelve of the sixteen species giving entirely negative results, and four of the ten species which only give traces of hydroxyanthraquinones are placed by Nisikado in the sub-genus *Eu-Helminthosporium*. The remaining species were not examined by Nisikado with the exception of *H. teres* Sacc. which is placed by him in the

sub-genus *Cylindro-Helminthosporium*, and which we found to give only traces of hydroxyanthraquinones.

The molecular constitutions of helminthosporin (I) and of cynodontin (II) have already been satisfactorily elucidated [1933, 1, 2], but that of catenarin (III), formerly known as hydroxyisohelminthosporin, was left unsettled by previous work. Evidence is now presented, showing that catenarin must be a β -hydroxy-methyl-1:4:5-trihydroxyanthraquinone having one of the formulae (III), (IV) or (V). The evidence for this is briefly as follows.

Distillation of catenarin with zinc dust yields β -methylantracene in rather small quantities—relatively less than helminthosporin. As has been previously reported [Charles *et al.*, 1933], all efforts to oxidise the side-chain in catenarin acetate to carboxyl failed; in every case most of the starting material was recovered unchanged, the rest being completely destroyed. The oxidation of catenarin with concentrated nitric acid was also studied, but apart from a minute quantity of a brownish-yellow substance—apparently a tetranitro-catenarin—the only solid product which could be isolated was oxalic acid. The behaviour of catenarin towards such oxidising agents as nitric acid and chromic anhydride is strongly reminiscent of that of morindone [Simonsen, 1918], which has been shown by synthesis [Jacobson and Adams, 1925; Bhattacharya and Simonsen, 1927] to be 1:5:6-trihydroxy-2-methylantracene. Similarly, the methyl group in cynodontin acetate resists oxidation [Raistrick *et al.*, 1933, 1]. It is therefore reasonable to assume that catenarin contains a nuclear hydroxyl adjacent to a β -methyl or β -hydroxymethyl group. Further evidence for the structure of catenarin may be stated briefly as follows:

(1) Catenarin is unaffected by heating for 8 hours at 100° with methyl iodide and sodium methoxide. Thus, despite its solubility in aqueous sodium carbonate, catenarin appears to contain no β -hydroxyl groups, as these are methylated under such conditions.

(2) Some support for the view that catenarin contains a hydroxymethyl group is afforded by the fact that its acetate, on heating with a saturated solution of hydrogen bromide in acetic acid, yields a product containing bromine. As this product is not homogeneous and the bromine content is low, this evidence is inconclusive.

(3) On oxidation with manganese dioxide and concentrated sulphuric acid, catenarin gives a hydroxycatenarin (VI), $C_{15}H_{10}O_7$, which is but a feeble mordant

dyestuff. Any anthraquinone derivative containing five nuclear hydroxyls is of necessity a derivative of alizarin and it follows that hydroxycatenarin can contain only four, and catenarin, consequently, only three, nuclear hydroxyl groups. The colour of its solution in concentrated sulphuric acid—blue, with a red fluorescence—taken in conjunction with its feeble dyeing properties, indicate that hydroxycatenarin is probably a derivative of 1:4:5:8-tetrahydroxy-anthraquinone. Its production from catenarin in the manner described limits the possible formulae for the latter substance to the three expressions III, IV and V.

(4) That catenarin, like helminthosporin and cynodontin, contains nuclear hydroxyls in the 1 : 4 : 5-positions, is further borne out by the similarity of the absorption curves of the three substances in concentrated sulphuric acid solution (see Fig. 1).

Fig. 1. I. Catenarin. II. Helminthosporin. III. Cynodontin. IV. Hydroxycatenarin.

Constitution of tritisporin. This substance, of which only a small amount has been available for investigation, appears to have the formula $C_{15}H_{10}O_7$; it has been analysed as its pyridine salt and as a penta-acetyl derivative. It is only a weak adjective dyestuff and cannot be an alizarin derivative. Furthermore, although readily soluble in aqueous sodium carbonate, its acetate is insoluble in alkaline solutions. Hence the substance does not contain a carboxyl group. On distillation with zinc dust it affords β -methylantracene.

The colour reactions and fluorescent properties are generally similar to those of catenarin and we are of the opinion that tritisporin must be a β -(hydroxy-methyl)-derivative of 1:3:5:7-, 1:3:6:8-, 1:4:5:8- or 1:3:5:8-tetrahydroxy-anthraquinone. The 1:4:5:8-orientation is at once excluded because this arrangement is required for hydroxycatenarin (VI), which is isomeric with, but very different from, tritisporin.

The colour reactions of tritisporin are also very divergent from those of anthrachryson as described in the literature. For further confirmation we prepared a substance which was undoubtedly 1:3:5:7-tetrahydroxy-2:6-

dimethylantraquinone (dimethylantrachrysone) by the action of sulphuric acid at 100° on 2:6-dihydroxy-*p*-toluic acid. The product was sublimed in orange needles and gave a yellow non-fluorescent solution in acetic acid, an orange-yellow solution in aqueous sodium carbonate, a reddish orange solution in aqueous sodium hydroxide and a deep cherry-red solution in sulphuric acid. This behaviour is very unlike that of tritisporin, and it is improbable that the latter can be a derivative of anthrachrysone. It seems that 1:3:6:8-tetrahydroxyanthraquinone is unknown, but it would probably resemble anthrachrysone, much as chrysazin resembles anthrarufin.

For these reasons we consider that tritisporin is a derivative of 1:3:5:8-tetrahydroxyanthraquinone. It happens that a pure, analysed specimen of 1:3:5:8-tetrahydroxy-2-methylantraquinone (VII) was available (unpublished synthesis by Dr E. H. Charlesworth) and this was found to be generally

similar and yet somewhat different from tritisporin in its reactions. In fact the substance VII resembled catenarin more closely in respect of the colour of its solution in sulphuric acid.

All the pigments isolated from species of *Helminthosporium* give fluorescent solutions in acetic acid, a property dependent on the quinizarin grouping. The differences existing between catenarin and VII on the one hand and tritisporin on the other suggest that catenarin should be represented by the formula III and one of the constitutions VIII or IX should be attributed to tritisporin.

EXPERIMENTAL.

A quantity of Czapek-Dox medium of the following composition was made up: glucose, 50.0 g.; NaNO_3 , 2.0 g.; KH_2PO_4 , 1.0 g.; KCl , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water, 1000 ml. 350 ml. quantities of this medium were distributed in each of a number of 1-litre conical flasks and sterilised. A measured quantity, previously determined, of sterile *N* sodium hydroxide solution was added to each flask to bring the p_{H} of the medium to 8.0. Five flasks were then each sown with a suspension in sterile Czapek-Dox medium of a pure culture of the species of *Helminthosporium* under investigation. The inoculated flasks were incubated at 24° for about 7 weeks, filtered, the mycelium dried in a vacuum-oven, ground to a fine powder and exhaustively extracted with chloroform in a Soxhlet apparatus. The chloroform extract was evaporated to dryness and portions of the dry extract were tested with strong sulphuric acid, aqueous sodium hydroxide solution, and concentrated ammonia. As mentioned in the introduction, a large number of species gave either entirely negative colour reactions, or where positive gave such small yields of hydroxyanthraquinones as to render isolation impracticable. In these cases no further investigation was carried out. In a few cases however considerable yields of

hydroxyanthraquinones were produced and these have been more exhaustively studied. The species chosen for further investigation were:

(a) <i>H. gramineum</i> Rabenhorst.	(e) <i>H. tritici-vulgaris</i> Nisikado.
(b) <i>H. cynodontis</i> Marignoni.	(f) <i>H. velutinum</i> Link.
(c) <i>H. euchlaenae</i> Zimmermann.	(g) <i>H. avenae</i> Eidam.
(d) <i>H. catenarium</i> Drechsler.	

The results obtained with species (a), (b) and (c) have already been reported, and those obtained with (d), (e), (f) and (g) form the subject of the present communication.

In each case the cultural conditions, methods of separation and isolation of the crude pigment were similar and were as follows: 100 one-litre conical flasks, each containing 350 ml. Czapek-Dox medium, adjusted to p_H 8.0, were sown with the species under investigation and incubated at 24°. At the end of the incubation period the mycelium was filtered off, washed, pressed, dried in a vacuum-oven and ground to a fine powder in a coffee mill. The powdered mycelium was extracted in a large metal Soxhlet apparatus with chloroform until the chloroform extracts were colourless. The extraction usually occupied several days, and a series of solid fractions separating from the chloroform was obtained. These fractions were then examined for their hydroxyanthraquinone content. The final chloroform mother-liquors were evaporated to dryness and treated with light petroleum, by which means a considerable quantity of "fat" was isolated. The experimental details for each of the species examined are as follows.

(A) *Helminthosporium catenarium* Drechsler.

The culture used was purchased from Baarn and had been received by them in 1929 from Nisikado, who isolated it from *Beckmannia*. Incubation period, 65 days. Residual glucose, 0.65 %. The surface of the mycelium was flame-coloured and slightly buckled with a chocolate-brown reverse. Weight of mycelium from 97 flasks, 204 g. The chloroform extract was bright orange in colour and gave the following fractions:

- (a) Fraction 1. First day's extraction. Weight, 9.96 g.; m.p. 220–226°.
- (b) Fraction 2. Second and third day's extraction. Weight, 3.72 g.; m.p. 244–245°.
- (c) Fraction 3. Fourth, fifth and sixth day's extraction. Weight, 6.10 g.; m.p. 193–205°.
- (d) Fraction 4. *Ex.* mother-liquors from fractions 1, 2 and 3. Weight, 2.35 g.; m.p. 193–198°.

The extraction in the Soxhlet proved very tedious, and hence the mycelium was now re-extracted by boiling with a large volume of chloroform giving:

- (e) Fraction 5. Weight, 11.29 g.; m.p. 244–247°, and
- (f) Fraction 6. Weight, 9.30 g.; m.p. 244–246°.

Even now the pigment was not completely extracted, and hence the incompletely extracted mycelium was dried and re-extracted with a large volume of boiling acetone and there were thus obtained:

- (g) Fraction 7. Weight, 4.72 g.; m.p. 238–241°, and
- (h) Fraction 8. Weight, 1.55 g.; m.p. 179–181°.

Total weight of crude pigment, 48.99 g.

There were also obtained from the light petroleum extract 16.10 g. of crude "fat."

The colours developed by the various fractions on dissolution in conc. sulphuric acid indicated the probable presence in the crude pigmented material of hydroxyisohelminthosporin—henceforth called catenarin, as it is the characteristic product of *H. catenarium*—and helminthosporin. On this basis fractions 1 and 2 consist of catenarin contaminated with a small quantity of helminthosporin; fraction 3 is similar, but contains rather more helminthosporin, whilst fractions 4 and 8 are almost entirely composed of this pigment. Fractions 5 and 6

consist of nearly pure catenarin, and fraction 7 is somewhat similar but contains a trace of helminthosporin. For the isolation of the two pigments in a pure state, fractions 4 and 5 were employed.

Catenarin. The crude material was recrystallised from alcohol, when it formed red, slender plates containing alcohol of crystallisation which it lost slowly in air. It had m.p. 246° and agreed in all its properties with "hydroxyisohelminthosporin" (m.p. 246°) from *H. gramineum*; a mixed m.p. with the latter substance showed no depression. (Found in material dried at 110°: C, 63.1; H, 3.7 %. $C_{15}H_{10}O_8$ requires: C, 62.9; H, 3.5 %.)

Catenarin acetate. The crude pigment was acetylated in the usual way with acetic anhydride containing a trace of concentrated sulphuric acid. After recrystallisation from glacial acetic acid, the acetate was obtained as small lemon-yellow rods, m.p. 234–235° alone or mixed with "hydroxyisohelminthosporin" acetate (m.p. 234–235°). (Found: C, 61.1; H, 4.1 %. $C_{23}H_{18}O_{10}$ requires: C, 60.8; H, 4.0 %.)

Isolation of helminthosporin. Helminthosporin acetate. The crude pigment, on acetylation with acetic anhydride containing a trace of concentrated sulphuric acid, yielded an acetate, which, after three recrystallisations from glacial acetic acid, formed a mass of fluffy yellow needles, m.p. 225°. (Found: C, 63.6; H, 4.1 %. $C_{21}H_{16}O_8$ requires: C, 63.6; H, 4.0 %.) It had all the properties of helminthosporin acetate, and the mixed m.p. with this substance showed no depression.

Helminthosporin. The above acetate was hydrolysed by warming on the water-bath with 15 % sodium hydroxide during 90 minutes. The deep violet solution was acidified with hydrochloric acid and, after boiling for a few minutes to ensure coagulation, the red-brown precipitate was collected, washed with water, dried and twice recrystallised from pyridine. The product formed flat, maroon-coloured needles with a bronze lustre, m.p. 226–227°. (Found: C, 66.8; H, 3.8 %. $C_{16}H_{10}O_5$ requires: C, 66.7; H, 3.7 %.) It had all the properties of helminthosporin (m.p. 226–227°), and showed no alteration in m.p. when mixed with a sample of the latter substance.

(B) *Helminthosporium tritici-vulgaris* Nisikado.

The culture used was purchased from Baarn and was received by them in November 1929 from Nisikado, strain number 285, who isolated it from diseased wheat, *Triticum vulgare*. Incubation period, 39 days. Residual glucose, 0.88 %. The surface of the mycelium was buff-coloured, somewhat crumpled and with a chocolate-coloured reverse. Weight of mycelium from 100 flasks, 204 g.

The chloroform extract gave the following fractions:

(a) Fraction I. Separating from the combined chloroform extracts after 6 days' extraction. Weight, 9.04 g.; m.p. 216–225°.

(b) Fraction II. Separating from mother-liquors from fraction I after evaporation to low bulk. Weight, 0.30 g.; m.p. 152–176°.

(c) Fraction III. The mother-liquors from fraction II were evaporated to dryness and extracted with light petroleum giving an insoluble residue, fraction III, weight 1.85 g.; m.p. 128–140°.

Total weight of crude pigment, 11.19 g.

There were also obtained from the light petroleum extract 39.3 g. of crude "fat."

Preliminary examination of the colours produced on dissolving samples of the three fractions in concentrated sulphuric acid indicated that fraction I probably consisted largely of catenarin, but the reddish tinge of the solution suggested that some other substance was also present; fractions II and III gave red-violet solutions with weak red fluorescence and appeared to contain some helminthosporin.

The method of isolation of the pigments was the following:

Fraction I was extracted with alcohol in a Soxhlet apparatus for about 3 hours, after which time the liquid siphoning over became distinctly lighter in colour, and the extract was allowed to crystallise. A brownish material (A) was left as unextracted residue. The reddish-brown crystals separating from the alcohol extract were ground and again extracted with alcohol in a Soxhlet, the process being interrupted when a small amount (*ca.* 0.5 g.) remained in the thimble; this residue was combined with (A). The alcohol extract deposited on standing brownish-red plates (B).

Isolation of tritisporin from (A). The crude material had an indefinite m.p. 250–260° and contained appreciable quantities of a greyish impurity which was not readily removed by simple recrystallisation. Accordingly the crude substance was acetylated in the usual way with acetic anhydride containing a trace of sulphuric acid, and after several recrystallisations from glacial acetic acid *tritisporin acetate* was obtained as a mass of very fine pale yellow needles, m.p. 215°. (Found: C, 58.5; H, 3.8 %. $C_{25}H_{20}O_{12}$ or $C_{15}H_5O_2(OAc)_5$ requires: C, 58.6; H, 3.9 %.) The substance was insoluble in cold dilute sodium carbonate solution.

This acetate was hydrolysed by heating on the water-bath with aqueous sodium hydroxide (15 %) during 90 minutes. The blue-violet solution was then acidified and boiled for a few minutes to coagulate the reddish-brown precipitate which was collected, washed with water and dried. The product was sparingly soluble in acetic acid and alcohol but the best solvent for recrystallisation was pyridine from which the *tritisporin* separated as long narrow reddish-brown plates with a bronze lustre, apparently as a pyridine salt. (Found: C, 63.6; H, 4.0; N, 3.8 %. $C_{15}H_{10}O_7 \cdot C_5H_5N$ requires: C, 63.0; H, 3.9; N, 3.7 %.) *Tritisporin* separates from alcohol or acetic acid in flat needles slightly browner in colour than the pyridine salt; it has m.p. 260–262° but the m.p. is difficult to determine accurately as considerable sublimation occurs from 250° onwards.

Tritisporin dissolves very readily in cold sodium carbonate solution to a violet-red solution and in caustic soda to a blue-violet solution which is slowly decolorised in the air. Its solution in concentrated sulphuric acid is reddish violet, redder in tone than *catenarin* solutions, and on addition of boric acid it becomes blue in thin layers and violet in thick layers. Solutions of *tritisporin* in acetic acid are salmon-pink with a yellowish green fluorescence.

2-Methylantracene from tritisporin. *Tritisporin* (120 mg.) was subjected in portions of 20 mg. to distillation with electrolytic zinc dust according to the micro-zinc dust distillation method of Kögl [1924]. The product of each experiment was deposited in the cooler parts of the tube as a mass of yellowish leaflets with a greenish fluorescence. The combined distillates were extracted from the tubes with ether, the solution filtered and the ether removed on the water-bath. The crystalline residue (*ca.* 10 mg.) was now recrystallised from alcohol. After three crystallisations it was obtained as glittering almost colourless leaflets, m.p. 200–201°, which showed all the properties of 2-methylantracene. The mixed m.p. with a specimen of synthetic 2-methylantracene (m.p. 203–204°) was 203° while a mixture with anthracene melted at 188–190°.

Isolation of catenarin from (B). The crude brownish red material was acetylated in the normal way by heating with acetic anhydride containing a trace of sulphuric acid. After three recrystallisations from acetic acid the acetate was obtained as small lemon-yellow rods, m.p. 234–235° alone or mixed with *catenarin acetate*.

The above acetate was hydrolysed by heating with aqueous sodium hydroxide (15 %) on the steam-bath during 90 minutes. The deep violet solution was acidified, boiled for a few minutes and the red-brown precipitate collected, washed with water, dried and recrystallised from alcohol. The recrystallised product formed slender red plates containing alcohol of crystallisation and had m.p. 246°. (Found in material dried at 110°: C, 63.0; H, 3.2 %. $C_{15}H_{10}O_6$ requires: C, 62.9; H, 3.5 %.) It had all the properties of catenarin and the mixed m.p. with a specimen of the latter substance showed no depression.

Preparation of helminthosporin acetate from fractions II and III. Fractions II and III showed a weak red fluorescence when dissolved in concentrated sulphuric acid, and the presence of helminthosporin was suspected. As the quantity present appeared to be small, however, the direct isolation of helminthosporin by crystallisation was deemed impracticable. Accordingly the combined material of the two fractions was extracted with alcohol for a short time to remove any readily soluble impurities and then acetylated in the usual way with acetic anhydride containing sulphuric acid. The yellow acetate was now separated into two portions by fractional crystallisation from acetic acid. The less soluble part, which consisted mainly of catenarin acetate, was discarded. The more soluble fraction which contained the helminthosporin acetate was subjected to repeated crystallisation from acetic acid, and a small amount was finally obtained in pale yellow fluffy needles, m.p. 224–225°, which had all the properties of helminthosporin acetate (m.p. 225°). The mixed m.p. with a sample of helminthosporin acetate was unchanged (a mixture with catenarin acetate melted at ca. 200°).

The presence of helminthosporin in the products from *H. tritici-vulgaris* is thus demonstrated.

Owing to unavoidable losses entailed in the separation of the three pigments an accurate analysis cannot be submitted; the coloured material from *H. tritici-vulgaris* is however estimated to consist of 75–80 % catenarin, 20–25 % tritisorin and 2–4 % helminthosporin.

Comparison of colours and fluorescences of solutions of various natural and synthetic pigments of the anthraquinone group in acetic acid.

Quinizarin	Orange-yellow; green fluorescence
Alizarin	Orange-yellow; no fluorescence
Anthrarufin	Yellow; no fluorescence
Chrysazin	Yellow; no fluorescence
1-Hydroxyanthraquinone	Pale yellow; no fluorescence
Hydroxyanthrarufin	Pure yellow; no fluorescence
Purpurin	Deep orange-yellow; weak green fluorescence
Emodin	Greenish yellow; no fluorescence
Morindone	Yellow; no fluorescence
2-Methyl-1:3:5:8-tetra-hydroxyanthraquinone	Orange-yellow; strong green fluorescence
Helminthosporin	Brighter orange-yellow, red shade in thin layers; green fluorescence
Catenarin	
Tritisorin	
Cynodontin	
Hydroxycatenarin	Salmon-red; yellowish-green fluorescence
	Bluish red, crimson in thin layers; weak greenish yellow fluorescence

It will be observed that fluorescence is characteristic of quinizarin derivatives.

(C) *Helminthosporium velutinum* Link.

The culture used was purchased from Baarn, where it was isolated in July 1929 from *Salix* spec. Incubation period, 83 days. Residual glucose, 0.77 %. This species gave a thick, glutinous orange-red mycelium with an orange-red reverse. Weight of mycelium from 95 flasks, 297 g.

The chloroform extract had an orange-red colour indistinguishable from that given by *H. catenarium*, and gave the following fractions:

(a) Fraction 1. Separating from the combined chloroform extracts after 6 days' extraction. Weight, 11.88 g.; m.p. 244–245°.

(b) Fraction 2. Separating from mother-liquors from fraction 1 after evaporation to low bulk. Weight, 0.67 g.; m.p. 115–140°.

(c) Fraction 3. The mother-liquors from fraction 2 were evaporated to dryness and extracted with light petroleum giving an insoluble residue, fraction 3, weight 2.13 g.; m.p. 126–130°.

Total weight of crude pigment, 14.68 g.

There were also obtained from the light petroleum extract 64.9 g. of crude "fat."

Fractions 1 and 2 of the crude material dissolved in concentrated sulphuric acid to give solutions with the characteristic blue-purple colour shown by catenarin under these conditions. Fraction 3 was lighter in colour than the others, and its sulphuric acid solution was slightly redder and had a weak greenish fluorescence. Fraction 1 furnished, on recrystallisation from alcohol, catenarin, whose identity was established by analysis, m.p. and mixed m.p., and by preparation of its acetate. Fraction 3 contained a colourless substance, which was isolated and shown to be ergosterol (m.p., mixed m.p., colour reactions, and formation of acetate). The remainder of fraction 3, after removal of the ergosterol, consisted essentially of catenarin.

Catenarin. Fraction 1 crystallised from alcohol in red, slender plates containing alcohol of crystallisation, which was lost slowly in air. It had m.p. 246°, alone or mixed with catenarin (m.p. 246°; from *H. catenarium*), and showed all the properties of the latter. (Found in material dried at 110°: C, 63.0; H, 3.8 %. $C_{15}H_{10}O_6$ requires: C, 62.9; H, 3.5 %.)

The above material, on acetylation with acetic anhydride containing a trace of concentrated sulphuric acid, yielded an acetate which crystallised from glacial acetic acid in small, lemon-yellow rods, m.p. 234–235°. The m.p. was not depressed by admixture with catenarin acetate.

Examination of fraction 3. The material (2 g.) was extracted in a Soxhlet with light petroleum (b.p. 40–60°) during several hours.

(A) *Residue*. The residue in the thimble was red in colour, and on heating softened about 200° and melted finally *ca.* 230°. On acetylation, followed by repeated recrystallisation of the product from acetic acid, it gave catenarin acetate, m.p. 234–235°.

(B) *Extract*. The light orange-red light petroleum extract deposited, on cooling, a quantity of faintly pink crystals (0.5 g.). After several recrystallisations from alcohol this product was obtained as colourless, glittering leaflets, m.p. 161–162°. The mixed m.p. with an authentic specimen of ergosterol (m.p. 160–161°) was 161°, and a comparison of the colour reactions (Salkowski, Tortelli-Jaffé, Liebermann-Burchard, Rosenheim) of the two specimens revealed no divergences.

For further proof that the above product was indeed ergosterol, a portion of it (0.1 g.) was acetylated by boiling for one hour with acetic anhydride (0.5 ml.). On cooling, the acetate separated in glistening plates and, after recrystallisation from alcohol, had m.p. 178–179°.

The peculiar colour of the crude fraction 3 in sulphuric acid is explained by the presence of ergosterol. The colour could be artificially produced by adding ergosterol to solutions of catenarin in sulphuric acid.

(D) *Helminthosporium avenae* Eidam.

The culture used was isolated in 1925 by Mr F. T. Brooks, School of Botany, Cambridge, from oats (*Avena sativa* L.) showing signs of leaf blight disease. As the yield of pigment given by this organism was very small, three separate experiments using a total of 252 flasks were carried out. The average incubation period was 12 weeks. The residual glucose varied from 0.05 to 0.40 %. The mycelium of this species was greyish-white on the surface with a red-black reverse. The total weight of mycelium from 252 flasks was 941 g. The pigment was readily extracted with chloroform, and the chloroform extracts, which had a brilliant eosin-red colour reminiscent of cynodontin, were evaporated to moderate bulk and from this there separated:

(a) Fraction 1. Weight, 3.60 g.; m.p. about 250°.

(b) Fraction 2. The mother-liquors from fraction 1 were evaporated to low bulk, giving fraction 2, weight 1.92 g.; m.p. 245–260°.

(c) Fraction 3. The mother-liquors from fraction 2 were evaporated to dryness and extracted with cold light petroleum. The insoluble portion, representing fraction 3, weighed 0.66 g.; m.p. 135–145°.

Total weight of crude pigment from 262 flasks, 6.18 g.

There was also obtained from the light petroleum solution a total weight of crude "fat" of 165.9 g.

Fractions 1 and 2, from their appearance, and the colour of their sulphuric acid solutions—pure blue with a red fluorescence—appeared to consist almost entirely of cynodontin or of hydroxycatenarin. The presence of the latter was however ruled out by the insolubility of the material in cold, aqueous sodium carbonate. Recrystallisation of the crude pigment from pyridine yielded cynodontin, identified by m.p., mixed m.p., and by preparation of its acetate. In order to verify that the colourless fraction 3 was indeed ergosterol, it was further purified and compared with an authentic specimen of the latter compound; its acetate was also prepared.

Cynodontin. The crude material was recrystallised several times from pyridine, when it formed brown leaflets with a fine bronze lustre, m.p. 260°. (Found: C, 62.9; H, 3.4 %. $C_{15}H_{10}O_6$ requires: C, 62.9; H, 3.5 %.) This product had all the properties of cynodontin, and its m.p. was not depressed on mixing with the latter substance.

The above material, on acetylation with acetic anhydride containing a trace of concentrated sulphuric acid and recrystallisation from glacial acetic acid, gave an acetate forming bright yellow needles or rods, m.p. 224–225° alone or mixed with cynodontin acetate (m.p. 224–225°).

Fraction 3. Ergosterol. Recrystallisation of this, first from light petroleum, then from alcohol, gave colourless, glittering leaflets, m.p. 161–162°, showing no divergence in reactions from a specimen of ergosterol (mixed m.p., colour reactions of Salkowski, Tortelli-Jaffé, Liebermann-Burchard, Rosenheim). On boiling with acetic anhydride for one hour, it gave an acetate crystallising from alcohol in colourless, glistening plates, m.p. 178–179°.

Further experiments with hydroxyisohelminthosporin (catenarin).

It has been found that the m.p. of hydroxyisohelminthosporin, previously given as 238° [Charles *et al.*, 1933], can be raised to 246° by conversion into its acetate, followed by hydrolysis with 15 % sodium hydroxide and recrystallisation from alcohol. (Found in material dried at 110°: C, 63.2; H, 3.3 %. $C_{15}H_{10}O_6$ requires: C, 62.9; H, 3.5 %.) As this substance only occurs in very small amount in the products from *H. gramineum*, but is the main coloured product of *H. catenarium*, it has been re-named *catenarin*.

2-Methylanthracene from catenarin. Catenarin (0.5 g.) was distilled with zinc dust at a dull red heat in a current of hydrogen. 2-Methylanthracene was deposited in the cooler part of the tube as colourless leaflets with a green fluorescence; the yield of product was low (total weight from three such experiments, 50 mg.). After recrystallisation from alcohol the substance had m.p. 200° and had all the properties recorded for 2-methylanthracene; a mixture with the latter compound (m.p. 203–204°) had m.p. 202–203°.

Oxidation of catenarin with manganese dioxide and sulphuric acid. Preparation of hydroxycatenarin. Catenarin (2 g.) was dissolved in concentrated sulphuric acid (15 ml.) and finely powdered manganese dioxide (1.5 g.) added gradually with constant stirring, the temperature being maintained at 60–70°. After all had been added, the mixture was kept at 60–70° during a further 15 minutes and then poured into water. A brownish violet precipitate formed, which, on boiling for a short time with water, became dark brown by hydrolysis. The crude product was recrystallised from pyridine and was thus obtained as dark red-brown crystals. For further purification this material was converted into its acetate by boiling for a few minutes with acetic anhydride containing a trace of sulphuric acid. After four recrystallisations from glacial acetic acid hydroxycatenarin acetate was obtained as small greenish-yellow rods, m.p. 233–234° (mixed m.p. with catenarin acetate, ca. 210–213°). (Found: C, 58.6; H, 4.0 %. $C_{25}H_{20}O_{12}$ requires: C, 58.6; H, 3.9 %.) Yield, 0.55 g. The above acetate (0.4 g.) was warmed on the water-bath for 90 minutes with 15 % sodium hydroxide solution (30 ml.), when it dissolved slowly to a deep blue solution. After acidifying with hydrochloric acid and boiling for a few minutes to coagulate the dark brown precipitate, the latter was collected and twice recrystallised from pyridine. It separated as small brownish leaflets with a bronze lustre; on heating it underwent partial sublimation above 280° and melted at about 320°. (Found in material dried at 110° in a high vacuum over P_2O_5 : C, 59.2; H, 3.5 %. $C_{15}H_{10}O_7$ requires: C, 59.6; H, 3.3 %.)

The crystals separating from pyridine solutions of hydroxycatenarin contain pyridine which is only partly lost on drying at room temperature. In appearance and in many of its properties hydroxycatenarin resembles cynodontin. Thus, it dissolves in concentrated sulphuric acid to a pure blue solution with a red fluorescence, and it possesses very feeble dyeing properties—much feebler than catenarin, although it is admittedly less soluble than the latter substance. This renders it highly probable that hydroxycatenarin is a derivative of 1:4:5:8-tetrahydroxyanthraquinone, while its fifth hydroxyl group must be in the side-chain as it would otherwise be an alizarin derivative and consequently a powerful mordant dyestuff. In view of its properties hydroxycatenarin must be 1:4:5:8-tetrahydroxy-2-(hydroxymethyl)anthraquinone (VI).

Oxidation of catenarin with nitric acid. Catenarin (1.5 g.) was warmed on the water-bath with nitric acid (20 ml. sp. gr. 1.42). After the initial reaction had subsided the mixture was heated on a boiling water-bath till evolution of brown fumes had practically ceased, then evaporated to small bulk and poured into water, a slight brownish precipitate being formed. The mixture was filtered.

Recrystallisation of the solid from acetic acid afforded fine brownish yellow needles (7 mg.); on heating the substance charred at about 250° without melting. It may be a tetranitrocatenarin. (Found: N, 9.4 %. $C_{15}H_6O_{14}N_4$ requires: N, 9.9 %.)

The only solid product which could be isolated from the filtrate was oxalic acid.

Attempted methylation of catenarin with methyl iodide. Catenarin (0.4 g.) was heated at 100° for 8 hours in a sealed tube with methyl iodide (1 g.) and sodium methoxide (1 g. sodium dissolved in 10 ml. methyl alcohol). After cooling the contents of the tube were acidified and the red precipitate collected; it consisted entirely of unchanged catenarin.

Action of hydrobromic acid on catenarin acetate. A mixture of catenarin acetate (0.5 g.) and a saturated solution of hydrobromic acid in acetic acid (15 ml.) was heated in a sealed tube at 100° during 8 hours. The contents of the tube, which were red in colour owing to hydrolysis, were poured into water, and after boiling for a few minutes to coagulate the precipitate the latter was collected and recrystallised from alcohol. The product had an indefinite m.p. 215–220° and was clearly a mixture. (Found: Br, 6.3 %. $C_{15}H_9O_5Br$ requires: Br, 22.9 %.) It was found impossible to isolate a homogeneous bromo-compound from this product by crystallisation. In a second experiment an unsuccessful attempt was made to purify the crude reaction product by acetylation and fractional crystallisation of the acetate; no definite separation was effected in six recrystallisations.

Efforts to improve the method by varying the conditions met with no success.

Absorption spectra.

The absorption of catenarin, hydroxycatenarin, helminthosporin¹ and cynodontin in the visible region of the spectrum is shown in Fig. 1 (p. 562). The curves refer to solutions in concentrated sulphuric acid, the concentration in each case being approximately $0.5 \times 10^{-4} M$. The close similarity of the curves in their essential features, indicates the close relationship existing between these compounds and may be regarded as strong support for the formulae advanced for catenarin and hydroxycatenarin. (The absorption spectra of 1-methyl-3:5:8-trihydroxyanthraquinone and the related 1:4:6-trihydroxyanthraquinone described by Dimroth and Fick [1916] differ widely from any of those here described.)

SUMMARY.

1. Hydroxyanthraquinones have been found to be produced by various species of *Helminthosporium*, in addition to those previously examined, when grown in a culture medium containing glucose as the sole source of carbon. The mycelium of *Helminthosporium catenarium* Drechsler contains hydroxyisohelminthosporin as the chief constituent of hydroxyanthraquinone nature. This substance is more appropriately named *catenarin* and its constitution as a β -(hydroxymethyl)-1:5:8-trihydroxyanthraquinone is supported by its oxidation to a hydroxycatenarin closely resembling cynodontin (1:4:5:8-tetrahydroxy-2-methylantraquinone).

2. *Helminthosporium velutinum* Link also produces catenarin but in this case the mycelium was found to contain appreciable amounts of ergosterol.

¹ Graves and Adams [1923] condensed 3:6-dimethoxyphthalic acid and *m*-cresol to a dimethoxyhydroxy-2-methylantraquinone which was demethylated to a substance, m.p. 227°, described as 1:5:8-trihydroxy-3-methylantraquinone. The compound was obtained in small quantity and was not analysed. However, it should have been identical with helminthosporin, and through the courtesy of Prof. Adams, to whom we are greatly indebted, we have been able to compare the substance with natural and synthetic helminthosporin with the result that the three specimens have been found to be identical. Owing to the confusion caused by the different system of numbering, we overlooked this important work of Graves and Adams in our first communication on helminthosporin.

3. *Helminthosporium avenae* Eidam was found to produce cynodontin and ergosterol in small relative amount.

4. *Helminthosporium tritici-vulgaris* Nisikado yielded catenarin, a little helminthosporin and a new pentahydroxy-2-methylanthraquinone termed *tritisorin*. The constitution of this substance is discussed and it is regarded as a 1:3:5:8-tetrahydroxy-6- (or 7-) (hydroxymethyl)anthraquinone.

We desire to express our sincere thanks to Mr W. K. Anslow for much technical assistance during the preparation of the crude pigments.

REFERENCES.

- Bhattacharya and Simonsen (1927). *J. Ind. Inst. Sci.* **10**, A, 6.
Charles, Raistrick, Robinson and Todd (1933). *Biochem. J.* **27**, 499.
Dimroth and Fick (1916). *Liebig's Ann.* **411**, 334.
Drechsler (1923). *J. Agric. Res.* **24**, 670.
Eidam (1891). *Der Landwirth.* **27**, 509.
Graves and Adams (1923). *J. Amer. Chem. Soc.* **45**, 2439.
Jacobson and Adams (1925). *J. Amer. Chem. Soc.* **47**, 283.
Kögl (1924). *Liebig's Ann.* **440**, 33.
Link (1809). *Mag. Ges. Naturf. Fr.* Berlin, **3**, 10.
Nisikado (1929, 1). *Ber. Chara Inst. landw. Forsch.* **4**, 103.
—— (1929, 2). *Ber. Chara Inst. landw. Forsch.* **4**, 111.
Raistrick, Robinson and Todd (1933, 1). *Biochem. J.* **27**, 1170.
—— ——— (1933, 2). *J. Chem. Soc.* 488.
Simonsen (1918). *J. Chem. Soc.* **113**, 766.