CLVII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXII. CYNODONTIN (1:4:5:8-TETRAHYDROXY-2-METHYLANTHRAQUINONE), A METABOLIC PRODUCT OF *HELMINTHOSPORIUM CYNODONTIS* MARIGNONI AND *HELMINTHOSPORIUM EUCHLAENAE* ZIMMERMANN.

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HELMINTHOSPORIUM CYNODONTIS Marignoni was first described by Marignoni [1909] as occurring on dry leaves of *Cynodon Dactylon* Persoon, found near Schio in Northern Italy. The species was apparently not reported again until Drechsler [1923], in a monograph on the graminicolous species of *Helminthosporium*, refers to it in considerable detail. *Cynodon Dactylon*, the host plant, is the so-called "creeping dog's-tooth grass" which is found on sandy shores in the south-west of England, the Bermuda grass of the southern United States, and the "durba" grass of India. It forms an important forage grass in many parts of America and India. Drechsler reports that "the parasite *Helminthosporium cynodontis* is exceedingly common throughout the south-eastern section of the United States where the host is everywhere present in the fields and on the roadsides as a noxious weed."

Helminthosporium euchlaenae Zimmermann was described by Zimmermann [1904, 1, 2] as occurring on the leaves of *Euchlaena mexicana* Schrad (= *E. luxurians* Dur. and Ashers. = *Reana luxurians* Dur.). This host plant is an annual grass with a habit like maize, growing to a height of 5-10 ft. or more, and is occasionally cultivated in the States on the Gulf of Mexico as a forage cereal, where it is known by the name of "Teosinte."

The strain of *Helminthosporium cynodontis* used in the work about to be described was purchased in May 1932 from the Centraalbureau voor Schimmelcultures, Baarn, Holland, and was received by them in January 1929 from Dr Charles Drechsler of the Bureau of Plant Industry, United States Department of Agriculture, Washington. It is very probably the strain described by Drechsler [1923] in his monograph, and isolated by him from diseased Bermuda grass.

The strain of *Helminthosporium euchlaenae* used by us was purchased in May 1932 from Baarn, where it was isolated in May 1929 by Dr van Beyma thoe Kingma as a contamination of a culture of *Pythium* received by the Centraalbureau voor Schimmelcultures from Sideris, Hawaii, in April 1929.

When these two fungi were grown on a purely synthetic medium containing glucose as the sole source of carbon, good growth was obtained in each case, the chief obvious characteristic of which is the chocolate-brown colour of the reverse of the mycelium. The dried mycelium on extraction with chloroform gave in the case of *H. cynodontis* a considerable quantity, and in the case of *H. euchlaenae* a smaller quantity, of a substance crystallising in bronze-coloured plates and having the appearance of bronze powder. This substance, which we propose to term *cynodontin*, has the empirical formula $C_{15}H_{10}O_6$ and has not previously been described. From its origin and general properties it was considered to be a near relative of helminthosporin (I) which Charles, Raistrick, Robinson and Todd [1933] described as a metabolic product of the plant pathogen *Helminthosporium gramineum* Rabenhorst, and the constitution of which has been settled by synthesis [Raistrick, Robinson and Todd, 1933].

Actually it was found possible to convert helminthosporin ($C_{15}H_{10}O_5$, 4:5:8-trihydroxy-2-methylanthraquinone) into cynodontin ($C_{15}H_{10}O_6$) by means of manganese dioxide and sulphuric acid. The substance is not a powerful mordant dyestuff and hence the entering hydroxyl should be in the 1-position. Cynodontin is therefore, in all probability, 1:4:5:8-tetrahydroxy-2-methylanthraquinone (II).

The extracted mycelium from *H. cynodontis* afforded in addition to cynodontin, but in small relative amount, a colouring matter having the colour reactions of helminthosporin; this was not completely purified but its presence was demonstrated by conversion into triacetylhelminthosporic acid (III). The product from *H. euchlaenae* consisted of practically pure cynodontin.

EXPERIMENTAL.

Preparation of crude pigment. 35 litres of Czapek-Dox medium of the following composition were made up: glucose, 50.0 g.; $NaNO_3$, 2.0 g.; KH_2PO_4 , 1.0 g.; KCl, 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $FeSO_4 \cdot 7H_2O$, 0.01 g.; distilled water, 1000 cc. 350 cc. quantities of this medium were distributed in each of 100 1-litre conical flasks and sterilised. A measured quantity, previously determined, of sterile *N* sodium hydroxide solution was then added to each flask to bring the p_H of the medium to 8.0. Each flask was sown with a suspension, in sterile Czapek-Dox medium, of *Helminthosporium cynodontis* Marignoni (Catalogue No. Ag. 120).

The cultures used for inoculation had been grown for about 3 weeks on Czapek-Dox agar at 30°. After inoculation the flasks were incubated at 30° for 7 weeks. At the end of this time the mycelium, which was greyish on the surface with a chocolate-brown reverse, was filtered off, washed carefully with cold water, pressed and dried in a vacuum-oven. The dried mycelium in two experiments weighed 230 g. in each case. It was ground to a fine powder in a coffee-mill and exhaustively extracted with chloroform in a large Soxhlet apparatus, until the chloroform extract which, in the early stages was of a beautiful eosin-red colour, had become almost colourless. On cooling there separated from the chloroform solution (about 1½ litres) in a typical experiment 7.5 g. of bronze-coloured plates having a melting-point of 258–260°. The chloroform mother-liquors were evaporated to about 250 cc. and from this there separated a second fraction weighing 1.25 g. and melting at 250–255°. The filtrate from this fraction was evaporated to dryness, dried *in vacuo*, and extracted thoroughly with light petroleum (B.P. 40–50°) giving a third fraction which weighed 0.55 g. and melted at 208–218°. The light petroleum solution on evaporation yielded 47.6 g. of residue consisting mainly of fat together with very small amounts of pigmented material. The total yields of crude pigments in the two experiments referred to above were 9.30 and 9.74 g., while the total yields of crude fat were 47.6 and 49.6 g.

The aqueous metabolism solution separated by filtration from the mycelium and still containing about 0.4 % of glucose was evaporated *in vacuo* to a total volume of about 1 litre, giving a very dark-coloured turbid liquid. This was filtered and acidified slightly with concentrated hydrochloric acid, and four volumes of absolute alcohol were added to it with constant shaking. There thus separated a considerable quantity of gummy material, examination of which has shown it to be a carbohydrate. Further work on this material is in progress.

In experiments using *Helminthosporium euchlaenae* Zimmermann (Catalogue No. Ag. 116), the cultural conditions followed, *i.e.* medium used, incubation temperature and period of incubation, were the same as those previously described for *H. cynodontis*. The method of isolating the pigment was also the same, but the yields obtained were somewhat different. The dried mycelium in two experiments weighed 247 and 264 g. respectively, and total yields of crude pigment of 2.36 and 5.06 g. were obtained in the form of bronze-coloured plates melting between 256 and 259°. The total yields of crude fat in the two experiments were 33.9 and 37.4 g. A considerable amount of gummy material which has proved to be a carbohydrate was also isolated from the evaporated aqueous metabolism solution by a method similar to that employed with *H. cynodontis*.

Products from Helminthosporium cynodontis.

Cynodontin. Fraction I of the crude coloured material (M.P. 258–260°) from *H. cynodontis* furnished, after one recrystallisation from pyridine, pure cynodontin, M.P. 260°, unchanged by further recrystallisation. It crystallises in brownish leaflets with a magnificent bronze lustre. Like helminthosporin it is sparingly soluble in most organic solvents to give solutions which are slightly bluer in tone than those of the former substance. It is insoluble in cold dilute sodium carbonate solution but dissolves in aqueous sodium hydroxide to a deep blue-violet solution. Solutions in concentrated sulphuric acid are blue and have a fine red fluorescence. Cynodontin has practically no dyeing properties. (Found: C, 63.1; H, 3.4. $C_{15}H_{10}O_6$ requires C, 62.9; H, 3.5 %.) Fraction II of the crude material had M.P. 250–255° and consisted essentially of cynodontin.

Cynodontin was found to be identical in every respect with a specimen of 1:4:5:8-tetrahydroxy-2-methylantraquinone prepared by the oxidation of helminthosporin (see below).

Tetra-acetylcynodontin. Cynodontin (0.5 g.) was acetylated by heating to boiling for a few minutes with acetic anhydride containing a trace of sulphuric acid. After pouring into water the precipitated acetate was recrystallised several times from glacial acetic acid, when it was obtained as a mass of bright yellow needles, m.p. 224–225°. (Found: C, 60.9; H, 4.1. $C_{23}H_{18}O_{10}$ requires C, 60.8; H, 4.0 %.)

1:4:5:8-Tetrahydroxy-2-methylantraquinone from helminthosporin.

Helminthosporin (2 g.) was dissolved in concentrated sulphuric acid (15 cc.), and finely powdered manganese dioxide (1.5 g.) added gradually with constant stirring, the temperature being maintained between 50 and 60°. When all had been added the mixture was kept at 50–60° for a further 15 minutes and then poured into water; the brownish-violet precipitate produced in this way became reddish-brown (hydrolysis) on boiling for a short time with water. The product after recrystallisation from pyridine still contained unchanged starting material. Accordingly it was acetylated with acetic anhydride containing a trace of sulphuric acid in the usual manner and the acetate purified. After four recrystallisations from glacial acetic acid the melting-point of the acetate became constant at 224–225° (mixed m.p. with helminthosporin acetate, of m.p. 223–224°, *ca.* 194°). The purified acetate (0.6 g.) was warmed on the water-bath with 15 % sodium hydroxide solution (30 cc.) during 2 hours when it dissolved slowly to a deep blue-violet solution. After acidifying with hydrochloric acid and boiling for a few minutes to coagulate the precipitate, the latter was collected and recrystallised from pyridine. It separated as brownish leaflets with a fine bronze lustre, m.p. 260°. (Found: C, 63.1; H, 3.5. $C_{15}H_{10}O_6$ requires C, 62.9; H, 3.5 %.) This material showed all the properties of cynodontin and a mixed m.p. with the latter substance showed no depression.

That this product is indeed 1:4:5:8-tetrahydroxy-2-methylantraquinone is clear from the evidence already mentioned and also for the following reasons. The new hydroxyl group cannot occupy a β -position in the anthraquinone nucleus, as the oxidation product is insoluble in cold sodium carbonate solution and is not a derivative of alizarin, being an exceedingly feeble dyestuff.

In its colour reactions the oxidation product closely resembles 1:4:5:8-tetrahydroxyanthraquinone as described by Fischer and Ziegler [1912].

2-Methyl-1:4:5:8-tetra-acetoxyantraquinone. The purified acetate obtained from the crude oxidation product of helminthosporin had m.p. 224–225° and crystallised in bright yellow needles. The same product was obtained by acetylating pure 2-methyl-1:4:5:8-tetrahydroxyanthraquinone with acetic anhydride and a trace of sulphuric acid. (Found: C, 60.7; H, 4.2. $C_{23}H_{18}O_{10}$ requires C, 60.8; H, 4.0 %.)

This acetate was identical in appearance with cynodontin acetate and the mixed m.p. with the latter substance showed no depression.

Examination of fraction III of crude material from H. cynodontis.

Fraction III (0.55 g.), which was obtained by evaporation of the chloroform mother-liquors after removal of fraction II, had an indefinite m.p. 208–218°. It appeared slightly lighter in colour than the earlier fractions and the colours of its solutions in concentrated sulphuric acid, sodium hydroxide and in organic

solvents were intermediate between those shown by cynodontin and helminthosporin. Indeed, it was possible to simulate the colours produced by fraction III by adding to solutions of cynodontin small amounts of helminthosporin.

Several attempts were made to isolate pure helminthosporin from fraction III by fractionation with various organic solvents, but with little success. Fractional crystallisation from acetic acid of the material obtained on acetylation was also tried and would doubtless have succeeded had larger quantities of material been available; thus the more soluble fractions yielded after repeated treatment a product whose reactions were more like those of helminthosporin acetate than cynodontin acetate. Its solution in sulphuric acid was reddish-violet in colour (still bluer than pure helminthosporin solutions) with the pronounced fluorescence characteristic of helminthosporin; the m.p. of this material was 210–215° but this was not depressed on mixing with a small quantity of helminthosporin acetate (m.p. 223–224°).

Confirmation of the view that fraction III contained helminthosporin was obtained in the following way. The original mixture (0.2 g.) was acetylated in the usual manner, the crude acetate dissolved in a mixture of acetic anhydride (4 cc.) and acetic acid (4 cc.) and oxidised at 90–100° by means of chromic anhydride (0.4 g.) in acetic acid (4 cc.). When the yellow precipitate obtained on diluting the reaction mixture with water was agitated with cold dilute sodium carbonate solution, a portion dissolved to a reddish-violet solution leaving behind unchanged cynodontin acetate (it had previously been ascertained that cynodontin acetate was practically unaffected by chromic anhydride under the conditions here employed). After filtering and acidifying the clear solution the reddish precipitate (30 mg.) was collected, re-acetylated by means of acetic anhydride containing a trace of sulphuric acid, and recrystallised several times from acetic acid. In this way a small quantity of material was obtained which showed all the properties of triacetylhelminthosporic acid (III). The m.p. of this product was low (230–232°) and owing to the very small quantity available further purification was impossible. The mixed m.p. with triacetylhelminthosporic acid (m.p. 235°) was 234°, leaving no room for doubt as to its identity.

From the evidence available it seems reasonable to conclude that helminthosporin is a constituent of the coloured products of *H. cynodontis*, although the amount present is very small indeed.

Products from Helminthosporium euchlaenae.

The coloured metabolic product proved to be almost homogeneous and yielded on recrystallisation pure cynodontin, m.p. 260°. (Found: C, 62.8; H, 3.8. $C_{15}H_{10}O_6$ requires C, 62.9; H, 3.5 %.) Its identity was confirmed by direct comparison with an authentic specimen of cynodontin; the appearance, colour reactions, m.p. and mixed m.p. of the specimens were identical as also were those of their acetates (m.p. and mixed m.p. 224–225°).

SUMMARY.

The mycelium of *Helminthosporium cynodontis* Marignoni and of *Helminthosporium euchlaenae* Zimmermann, when grown on a synthetic culture medium containing glucose as the sole source of carbon, has been shown to contain a hitherto undescribed, crystalline colouring matter, which has been called cynodontin. Cynodontin has been shown to be 1:4:5:8-tetrahydroxy-2-methyl-anthraquinone and is thus 1-hydroxyhelminthosporin. Helminthosporin, which

has recently been shown to be present in considerable amounts in the mycelium of the plant pathogen *Helminthosporium gramineum* Rabenhorst, is also present in very small amounts in the mycelium of *Helminthosporium cynodontis*, but is absent from *Helminthosporium euchlaenae*.

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