12. THE COLOURING MATTERS OF *PENICILLIUM CARMINO-VIOLACEUM* BIOURGE, WITH A NOTE ON THE PRODUCTION OF ERGOSTEROL BY THIS MOULD

By H. G. HIND

University College, Nottingham, and the Lister Institute, London

(Received 27 November 1939)

IN recent years great advances have been made in the chemistry of moulds, and many compounds new to organic chemistry have been isolated. Much attention has been devoted to colouring matters; many of these have been obtained chemically pure, and in some instances the constitutions of the compounds have been determined, particularly by H. Raistrick and co-workers.

Cultures of *P. carmino-violaceum* Biourge grown on carbohydrate media are characterized by a colouring of the medium, yellow when acid and claret red when alkaline. This pigment was observed by Krause & Ellis [1937] who rightly assumed that the colour changes in the medium with change of pH were due to indicator properties of the pigment. It was further pointed out that the pigment was precipitated by acid from alkaline solution; however, it was not isolated in a state of chemical purity. The mycological characteristics of *P. carmino-violaceum* are described by Thom [1930].

The present investigation has revealed that *P. carmino-violaceum* Biourge produces two pigments of molecular formulae $C_{20}H_{16}O_7$ and $C_{16}H_{12}O_6$ respectively. These compounds are indistinguishable by their colour reactions, both probably being complex hydroxyanthraquinones. The name carviolin is suggested for the compound $C_{16}H_{12}O_6$ and carviolacin for the compound $C_{20}H_{16}O_7$.

Carviolacin is a monomethyl ether, and, on account of its high carbon content, must possess a cyclic structure. Reduction with zinc dust of an alkaline solution of the pigment caused the colour to change from red to orange, and addition of H_2O_2 or shaking with air reversed the colour change; alizarin shows similar reactions with the above reagents, but in this case the colour change is purple to orange. Although the structure of carviolacin has not yet been determined, the following points have been established:

(1) Treatment with dimethyl sulphate and alkali gives a compound $C_{23}H_{22}O_7$ which is a trimethoxy derivative of the pigment.

(2) Zinc dust distillation yields 2-methylantracene.

Carviolin is also a monomethyl ether. Its general reactions indicate quinonoid properties. The tendency of the compound and its derivatives to retain solvent of crystallization has rendered investigation difficult. After many analyses and rigorous purification the formula $C_{16}H_{12}O_6$ was assigned to the compound.

Its structure has not been determined but certain of its reactions have been studied.

(1) Acetylation with acetic anhydride and pyridine yields a triacetate having invariably one molecule of water of crystallization.

(2) Methylation with dimethyl sulphate and alkali gives a compound $C_{19}H_{18}O_6$ which is a trimethoxy derivative of $C_{16}H_{12}O_6$.

(3) On simultaneous reduction and acetylation a penta-acetate of the leuco-compound was obtained which had a very strong blue fluorescence in acetic acid solution.

EXPERIMENTAL

Cultures

The mould culture used in the investigation was isolated and kindly presented by Miss M. Ellis of University College, Nottingham. A culture of the mould was identified as *P. carmino-violaceum* Biourge by Prof. Thom of the U.S. Department of Agriculture.

A culture of *P. carmino-violaceum* Dierckx, supplied by the Centraalbureau voor Schimmelcultures, Baarn, did not produce any anthraquinone-like pigments under the same experimental conditions as those used in the following experiments.

Cultural conditions

Since the pigments were more readily obtainable from the mycelium than from the metabolic solutions, it was desirable that the pigments should be retained in the mycelium as long as possible. Early experiments had demonstrated that the pigments are soluble in alkali and therefore the medium had to remain acid during the period of growth. A study of the effect of the source of carbon on the rate of change of *pH* during growth was made. The mineral salts used were the same throughout but different sugars were employed in the various tests. The cultures were set up in 300 ml. conical flasks and each flask contained 100 ml. of solution of the following composition: carbohydrate 5 g.; KNO_3 1 g.; KH_2PO_4 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25 g.; H_2O 100 ml. The cultures were grown at room temperature. The *pH* was determined from time to time by means of a B.D.H. capillator. In most cases the experiments were carried out in duplicate. The compounds tested as source of carbon were maltose, fructose, glucose, sucrose, lactose and glycerol.

The maltose medium apparently gave a good yield of the pigment but it was unsuitable because the *pH* rose too quickly, with the result that the pigment diffused into the medium. In the lactose medium the mould grew somewhat less vigorously and pigment production was apparently much retarded. Media containing carbohydrates other than lactose became yellow after a few days, while in the case of the lactose medium no such coloration was observed and it was only after about 20 days in this medium that an orange pink tinge appeared. For the purpose of pigment production and maintenance of acidity which facilitated isolation the glycerol medium was the most suitable and it was therefore employed.

500 ml. quantities of the culture medium were placed in 2.5 l. culture bottles which were sterilized in steam for 1.5 hr. on each of three successive days, and then kept for a further 7 days to ensure that sterilization was complete. They were then inoculated with spore suspensions in sterile water, the spores being obtained from stock cultures on solid media. The bottles were kept at room temperature.

Extraction of the pigments

The mycelium was removed from the culture bottles when the *pH* had risen to about 6.5 (usually 10–12 weeks). The mycelium was then undulating and white to pale bluish-green with reddish droplets on the surface, exuded from the mycelium. The culture medium was orange-yellow in colour, and the reverse of the mat was deeply coloured a rich golden brown. The mycelium was filtered

from the medium, washed with water and further treated by one of the following methods.

Method 1. The washed mycelium was pressed dry and extracted in a Soxhlet extractor with acetone. The residue from the acetone extraction was a greyish-white material containing no colouring matter. The acetone and water were removed *in vacuo* from the extract and the resulting dark brown pasty mass was extracted twice with a large amount of light petroleum (B.P. 40–60°). The residue was then extracted with hot absolute alcohol, 100 ml. per g. of solid, and filtered. The extract contained the anthraquinone-like pigments, and left undissolved some dark brown material which constituted about half the weight of the petroleum-insoluble fraction. To the alcoholic solution, 2 vol. of light petroleum (B.P. 40–60°) were added and a further quantity of dark brown material was precipitated. This was discarded. On removal of the solvents a brown solid remained which was still greasy to the touch and contained the anthraquinone-like pigments. This constituted about 3% of the dry weight of the mycelium. Further purification was effected by extraction with light petroleum.

Method 2. The mycelium was dried over CaCl_2 in a desiccator, and the dried material was powdered by grinding in a small hand mill. The powdered mycelium was then extracted with light petroleum (B.P. 40–60°) in a Soxhlet extractor. This removed the greater part of the fat and facilitated later work. The pigment from the mycelium was then extracted continuously for 15 hr. with acetone. The acetone was removed and the acetone residue in turn was continuously extracted with ether for 15 hr. when the anthraquinone-like pigments passed into the ether. This method was less satisfactory than method 1 as the mycelium darkened during drying and less effective extraction of the pigment was obtained.

The preparation properties and derivatives of carviolin

Isolation as acetate. Raistrick *et al.* [1934] described the purification of anthraquinones from the extracts of the mycelia of species of *Helminthosporium* by acetylating the crude material and fractionating the acetates. The same method has been particularly applicable for separating the pigments of *P. carmino-violaceum* Biourge.

The pigment concentrates obtained as described above were acetylated for 4 hr. on the water bath at 100° with acetic anhydride (10 ml. per g.) and pyridine (2 ml. per g.). The cooled reaction mixture was poured into 20 vol. of cold water and filtered. On crystallizing from acetic acid, a compound separated which crystallized in pale cream-green needles, M.P. (after four recrystallizations from acetic acid) 209°. It was insoluble in cold dilute NaOH, but readily dissolved on warming to form a blood-red solution. It was soluble in conc. H_2SO_4 to give a red solution. (Found (on specimens from different batches of pigment, dried at 110° *in vacuo*): C, 59.4, 59.7; H, 4.7, 4.5; MeO, 7.5, 7.2, 7.0; acetyl, 28.7%. $\text{C}_{16}\text{H}_9\text{O}_3(\text{O}.\text{OC}.\text{CH}_3)_3 \cdot \text{H}_2\text{O}$ requires C, 59.5; H, 4.5; MeO, 7.0; acetyl, 28.3%.)

The recrystallized acetate was hydrolysed by refluxing with 2*N* H_2SO_4 containing 40% alcohol for 4 hr., during which time the acetate slowly dissolved, giving finally a clear deep yellow solution, from which crystals of carviolin separated on cooling. The pigment was recrystallized 3 times from alcohol, once from pyridine and finally 3 times from acetic acid, from which solvent it separated as chrome yellow needles, M.P. 286°. (Found (for compound dried at 160° *in vacuo*): C, 63.7; H, 4.2; MeO, 10.7%. $\text{C}_{16}\text{H}_{12}\text{O}_6$ requires C, 64.0; H, 4.0; MeO, 10.3%.) The final crystallizations were performed to remove

alcohol of crystallization, which was retained most tenaciously. If the final crystallization was from alcohol, the methoxyl content as shown by Zeisel estimation was always high, even after vigorous drying.

Direct isolation of carviolin. The acetone extract (method 1), if filtered, invariably yielded a quantity of dark brown material which on drying turned yellowish in colour. 1 g. of this was extracted with dilute ammonium hydroxide which gave a blood-red solution and a dark insoluble residue. The solution was filtered and acidified with HCl, and a chrome yellow precipitate formed. This was suspended in 100 ml. water, heated to 80° and treated with alcohol until the solution was clear. On cooling, a chrome yellow crystalline pigment separated (0.1 g.). This was recrystallized 4 times from 50 % alcohol and water and had m.p. 286°. (Found (on material dried at 110° *in vacuo*): C, 63.6; H, 4.3; MeO, 11.1 %. $C_{15}H_9O_5(OMe)$ requires C, 64.0; H, 4.0; MeO, 10.3 %.)

Carviolin is insoluble in water and HCl. It dissolves in dilute NH_4OH to give a clear blood-red solution, while in NaOH or KOH a bluer shade is obtained. Carviolin gives a red colour with conc. H_2SO_4 which changes to a reddish-purple on addition of boric acid.

The compound has acidic properties as shown by its solubility in alkali; carviolin cannot be extracted from NaOH by ether, but becomes extractable after acidification.

Carviolin dissolves in cold Na_2CO_3 to give a red solution, but is insoluble in bicarbonate. It is not a mordant dye. The pigment does not sublime in high vacuum at 200°.

Carviolin triacetate $C_{15}H_6O_2(O.CH_3)(O.COCH_3)_3, H_2O$. 40 mg. of carviolin were heated with 0.4 ml. of pyridine and 3 ml. of acetic anhydride for 3 hr. at 100°. The reaction mixture was cooled and 4 ml. of water were added when the product separated as minute cream-green needles (yield theoretical). These were recrystallized 3 times from acetic acid. m.p. 210° alone or mixed with a specimen isolated from the acetylated crude extract.

Carviolin trimethyl ether $C_{15}H_6O_4(OCH_3)_4$. 0.15 g. of carviolin was dissolved in 5 ml. acetone and 3 ml. dimethyl sulphate. Sufficient 2N NaOH was added from time to time to keep the reaction mixture alkaline. During the reaction carviolin trimethyl ether separated out crystalline. The acetone was evaporated from the alkaline solution and the product filtered (yield 0.12 g.). This was recrystallized from 96 % alcohol in gold-coloured rods, m.p. 186°. (Found (on material dried at 110° *in vacuo*): C, 67.0; H, 5.15; MeO, 34.2 %; mol. wt. (Rast), 348. $C_{15}H_6O_2(OCH_3)_4$ requires C, 66.7; H, 5.2; MeO, 36.2 %; mol. wt. 342.)

Carviolin tribenzoate $C_{15}H_6O_2(OCH_3)(O.COC_6H_5)_3$. 40 mg. of carviolin were heated with 2 ml. pyridine and 5 ml. benzoyl chloride for 3 hr. on the water bath (100°). The cooled reaction mixture was poured into excess of water and the oily drops which separated were collected with 10 ml. chloroform. 2N Na_2CO_3 was added and the two layers left in contact for 30 min. with occasional shaking in rotary manner. The chloroform layer was separated; to it were added 40 ml. alcohol and the chloroform was evaporated on the water bath till crystallization commenced. On cooling, the benzoate separated quantitatively. It was recrystallized from alcohol and chloroform in the same manner without loss, since it was apparently insoluble in cold alcohol (yield 80 mg.; theoretical yield for tribenzoate of $C_{16}H_{12}O_6$, 81.6 mg.), m.p. 240°. (Found (dried at 110° *in vacuo*): C, 72.0; H, 4.2; MeO, 5.3 %. $C_{37}H_{24}O_9$ requires C, 72.5; H, 3.9; MeO, 5.1 %.)

Leuco-carviolin penta-acetate. 0.1 g. carviolin was dissolved in 8 ml. of acetic anhydride; 0.5 ml. pyridine and 0.5 g. magnesium powder were added in small

quantities at intervals while the material was kept at 100°. The evolution of H₂ by this method was more copious and satisfactory than when zinc dust was used. The product was filtered, cooled and poured into cold water when an oil separated which hardened and crystallized on standing. After standing overnight it was crystallized from acetic acid in which solvent it had a blue fluorescence. The compound was finally recrystallized 3 times from 60% acetic acid. Cream coloured rods, M.P. 247°. (Found (on material dried at 110° *in vacuo*): C, 61.0; 60.8; H, 5.0, 4.9; MeO, 6.6%. C₁₅H₆(OMe)(O.COCH₃)₅ requires C, 60.9; H, 4.7; MeO, 6.1%.) A product (M.P. 261°) was obtained by the above method from anthraquinone for comparison with the penta-acetate of leuco-carviolin. This compound was evidently oxanthranol diacetate [Liebermann, 1888]. It had a blue fluorescence which strongly resembled that of leuco-carviolin penta-acetate.

Zinc dust distillation. A zinc dust distillation carried out using 100 mg. of carviolin yielded a small quantity of distillate, but no crystalline material was isolated.

Preparation, properties and derivatives of carviolacin

Isolation. The mother liquors, obtained by crystallizing the acetate of carviolin, in the isolation of that compound (*q.v.*) were invariably deep brown in colour. These were evaporated to dryness *in vacuo* and then treated with 2*N* NaOH on the water bath for 20 min. Filtration removed a quantity of dark material which was discarded, and on acidifying the wine-red solution a brown precipitate separated which crystallized from dilute alcohol and water in light brown needles. On heating they darkened at 220° and softened with decomposition at 243°. (Found (on material dried at 110° *in vacuo*): C, 65.3; H, 4.1; MeO, 8.4%; mol. wt. (Rast), 410. C₂₀H₁₆O₇ requires C, 65.2; H, 4.4; MeO, 8.4%; mol. wt. 368.) Carviolacin has also been obtained directly from the mycelium, but the yield was poor. There is no doubt that carviolin and carviolacin are unchanged by the acetylation and hydrolysis, and the acetylation method gives a satisfactory separation of the pigments.

Carviolacin is indistinguishable from carviolin by its colour reactions and the colour of its solutions, and is apparently closely related to that pigment. It has a tendency to dissolve in Na₂CO₃. It is insoluble in bicarbonate and it is not a mordant dye. It is more soluble in aqueous solvents than is carviolin, and unlike this compound it is soluble to some degree in water and dilute HCl. Efforts to sublime carviolacin have failed.

Carviolacin trimethyl ether C₁₉H₁₀O₃(OCH₃)₄. 0.1 g. of carviolacin was dissolved in 8 ml. of acetone and 7 ml. of dimethyl sulphate were added. 2*N* alkali was added from time to time to keep the reaction mixture alkaline, and finally the acetone was evaporated on the water bath when the derivative separated (0.08 g.). This was recrystallized from alcohol as yellow needles, M.P. 214–215°. (Found (on material dried at 110° *in vacuo*): C, 67.3; H, 5.1; MeO, 30.2, 30.4%. C₁₉H₁₀O₃(OMe)₄ requires C, 67.3; H, 5.4; 4MeO, 30.2%.)

Acetylation of carviolacin. An acetyl derivative was prepared to study its properties as an aid in the pigment separation. 20 mg. carviolacin, 1 ml. acetic anhydride and 1 drop of conc. H₂SO₄ were heated on the steam bath for 2 hr. The reaction mixture was cooled and poured into water, but partially dissolved. The solution was extracted with 20 ml. of CHCl₃ and the two layers were separated. 20 ml. alcohol were added to the CHCl₃ extract and the CHCl₃ removed on the water bath. On standing overnight the acetate separated in yellow needles. These were recrystallized 4 times from alcohol and water (M.P. 230°).

Zinc dust distillation. 250 mg. of carviolacin were mixed with 1.4 g. of zinc dust and a further 0.2 g. zinc dust was added to cover the mixture in the distillation apparatus. On heating, a colourless crystalline compound collected in the cooler parts of the tube, together with some yellow oily liquid. On cooling, the contents of the tube were extracted with ether and the extract was filtered. This gave a yellowish solution, which showed a green fluorescence. The ether was evaporated and the brown crystalline material was recrystallized twice from alcohol. It formed plates with greenish fluorescence (M.P. 203°) with a tendency to sublime above 185°, and had M.P. 204° when mixed with 2-methylantracene (M.P. 206°). The mixed M.P. with anthracene (M.P. 212°) was 189°. There seems little doubt that the compound obtained by zinc dust distillation of carviolacin is 2-methylantracene.

Ergosterol

In the present work it was found that on concentrating the petroleum extracts of the dried mycelium (method 2) colourless needles separated which crystallized readily from alcohol. These crystals were insoluble in dilute HCl and dilute NaOH, and had M.P. (after recrystallizing 4 times) 156°. The following positive reactions indicate that the substance was a sterol.

(1) Conc. H_2SO_4 added to an acetic anhydride solution gave a red colour which changed rapidly to green.

(2) A blue colour was given with CHCl_3 and CCl_3COOH [Rosenheim, 1929].

70 mg. of material were heated in a solution of 2 ml. benzoyl chloride and 0.5 ml. pyridine for 5 min. and the cooled solution was shaken with 25 ml. of $N \text{ Na}_2\text{CO}_3$ for 5 min. The product was collected (yield theoretical) and recrystallized from alcohol 5 times. It had M.P. 164°, the M.P. of ergosterol benzoate from ergosterol isolated from *P. puberulum* Bainier [Birkinshaw *et al.* 1931] being 163°.

SUMMARY

Investigation of the colouring matters of *P. carmino-violaceum* Biourge has led to the isolation of two complex anthraquinones which have been named carviolin ($\text{C}_{16}\text{H}_{12}\text{O}_6$) and carviolacin ($\text{C}_{20}\text{H}_{16}\text{O}_7$) respectively. The preparation of various crystalline derivatives of these pigments are described and throw light on their constitutions. Ergosterol was isolated from the mould.

I am deeply indebted to Prof. J. M. Gulland and Prof. H. Raistrick for much helpful advice.

REFERENCES

- Birkinshaw, Callow & Fischmann (1931). *Biochem. J.* **25**, 1977.
- Krause & Ellis (1937). *Ann. Bot., Lond.*, N.S. **1**, 499.
- Liebermann (1888). *Ber. dtsh. chem. Ges.* **21**, 1172.
- Raistrick, Robinson & Todd (1934). *Biochem. J.* **28**, 559.
- Rosenheim (1929). *Biochem. J.* **23**, 47.
- Thom (1930). *The Penicillia*. London.