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# THE STRUCTURE OF MELANINS AND MELANOGENESIS—V*

## USTILAGOMELANIN[1,2]

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**Abstract**—The black pigment from *Ustilago maydis* spores has been studied. Analytical data and degradation experiments show it to be related to catecholmelanin.

BROWN or black pigmentation is often noted in plants during normal growth or when tissues are injured and exposed to air. Although these pigments are widespread, very little is known of their structure and biological function.

These dark pigments are so difficult to isolate that research had tended to centre on the identification of possible precursors (tyrosine, dopa etc.) and of enzyme systems capable of causing oxidative polymerization (tyrosinase, laccase etc.). For example Bertrand[3] found both tyrosine and tyrosinase to be present in dahlia tubers.[4]

Since in a single tissue numerous substances may occur which are susceptible of enzymic oxidative polymerization,[5] the identification of tyrosinase and substrates in dark plant tissues has not led to reliable conclusions as to the structure of the pigments responsible for the dark colouration.

One of the first chemical reports on plant dark pigments was that of Griffoen,[6] who isolated a black product from a *Dyospyros* heartwood, which by alkali fusion yielded catechol, protocatechuic acid and phloroglucinol. Wedekind[7] extracted a black amorphous substance from West-African ebony, whose elemental analysis suggested the molecular formula $(C_3H_2O)_n$. No identifiable product was obtained upon degradation. De Vries[8] isolated from *Tagetes patula* fruits a black substance with an atomic ratio only slightly differing from $C_x(H_2O)_y$. More details are available on the structure of aspergillin, an alkali-soluble, black metabolite of *Aspergillus niger*

* Part IV. R. A. Nicolaus, M. Piattelli and E. Fattorusso, *Tetrahedron* **20**, 1163 (1964).

[1] Part of this work appeared as a short communication on *Tetrahedron Letters* No. 15, 997 (1963).

[2] This investigation was supported in part by the National Cancer Institute, research grants CA-05520-04 and -05, Public Health Service, U.S.A.

[3] G. Bertrand, *C.R. Acad. Sci. (Paris)* **122**, 1215 (1896).

[4] The fact that many tissues containing both precursor and enzyme do not present pigmentation was ascribed to the presence of inhibitors.

[5] For example, tyrosine, dopa, tyramine, hydroxytyramine and epinine were found in *Sarot hamnus scoparius* (P. Correale and I. Cortese, *Naturwiss.* **40**, 57 (1953)).

[6] K. Griffoen, *Konink. Akad. Wetenschap. Amsterdam* **36**, 897 (1933).

[7] E. Wedekind, *Ber. Dtsch. Chem. Ges.* **68**, 2363 (1935).

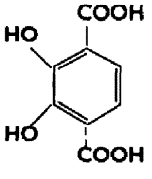
[8] M. A. De Vries, *Over de vorming van Phytomelaan bij Tagetes patula L. en enige andere, Composieten* Buurman, Leiden (1948).

van Tiegh. Quilico[9] showed that this pigment of high mol. wt. contains C, H and O in a ratio of 5:4:2, as well as a small percentage of N. In the aspergillin molecule there are carboxyl groups and phenolic hydroxyl groups. The obtaining of mellitic acid by oxidation of aspergillin indicates the presence of condensed ring systems. Quilico also pointed out the close similarity between aspergillin and humic acids, dark amorphous substances from soil and peat, which, like aspergillin, yield mellitic acid by oxidative degradation. More recently, three black plant pigments were isolated[10]; a preliminary degradation study revealed that they were not of "indole" type, different from the animal melanins studied thus far.

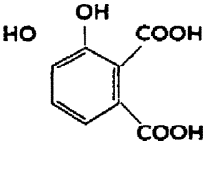
Considering the level of present knowledge, more detailed studies on dark plant pigments appear to be desirable.

Due to their insolubility, these pigments, like animal melanins, must be isolated by removing all the other tissue components by means of solvents and hydrolysing agents. Since lignin is the most difficult tissue component to remove, we chose the lignin-free *Ustilago maydis* DC. (corn smut, corn ergot) spores as starting material in order to isolate, purify and analyse a dark plant pigment.

The spores, extracted with light petroleum and subsequently with ethanol, were kept in hydrochloric acid at room temp for 14 days. After washing with water and then with ethanol, the crude product was continuously extracted first with ethanol and then with tetrahydrofuran. It was further purified by prolonged treatment with hot hydrochloric acid. The resulting black pigment proved to consist of C, H and O as well as small amounts of N and inorganic matter. It was amorphous and completely insoluble in all solvents. It contains acid functions which are esterified by methanol, and reacts with diazomethane to yield a red-brown product. When heated at 200°, it does not evolve carbon dioxide, and is not apparently affected by hot hydriodic acid. Oxidation with hydrogen peroxide did not give any identifiable product. Pyrolysis gave small amounts of a single phenol, identified by chromatography and electrophoresis as catechol. Among the degradation products of alkali fusion catechol, protocatechuic acid and salicylic acid were identified. Since other phenolic acids were detected by paper chromatography, the presence of the *o*-dihydroxybenzenedicarboxylic acids I–IV was suspected. Acid IV, not reported in the literature, was prepared by



I



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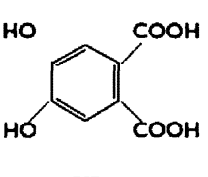
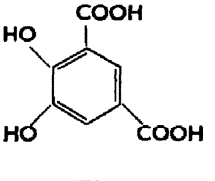


III



IV

oxidation of 5-formylvanillic acid to 4-hydroxy-5-methoxyisophtalic acid and subsequent demethylation. None of these acids, which proved to be easily identifiable by paper chromatography, was present among the degradation products of ustilagomelanin.

[9] A. Quilico, *I pigmenti neri animali e vegetali.* Tip. Fusi, Pavia (1937).
[10] Part IV of this series.

The catalytic reduction of ustilagomelanin at 200° in the presence of Pd–C yielded, besides catechol, smaller quantities of 3,3',4,4'-tetrahydroxybiphenyl. Reduction with sodium in pyridine afforded small amounts of catechol.

It is to be noted that these degradation products were obtained in very low yields. This can in part be justified by the fact that much of the pigment was recovered at the end of each degradation. On the other hand, the adoption of more drastic experimental conditions to degrade a greater percentage of starting material did not give better results because of complete breakdown of ustilagomelanin into fragments of no structural significance.

The results obtained from the degradation experiments suggest that the structure of ustilagomelanin is closely related to that of catecholmelanin. This dark polymer,

TABLE 1

| | C | H | N | $OCH_3$% |
|---|---|---|---|---|
| Ustilagomelanin | 62·7 | 3·4 | 1·0 | — |
| Catecholmelanin | 60·5 | 3·2 | 1·4 | — |
| Ustilagomelanin ($CH_3OH$) | 63·9 | 4·6 | 1·0 | 3·3 |
| Catecholmelanin ($CH_3OH$) | 61·2 | 3·4 | 1·3 | 3·2 |
| Ustilagomelanin ($CH_2N_2$) | 64·9 | 5·0 | 1·7 | 12·5 |
| Catecholmelanin ($CH_2N_2$) | 64·8 | 4·7 | 1·3 | 23·0 |

which can be prepared from catechol by oxidation in the presence or not of the enzyme, is considered to be built up of catechol and *o*-quinone units, but it is unknown at what positions and by means of what bonds these units are linked together.

Titration with alkali showed catecholmelanin to contain strongly acid functions with dissociation constants in the region $pK = 3$–7. The presence of quinonoid hydroxyl groups and/or carboxyl groups deriving from oxidative ring splitting can account for these strongly acid dissociations. Titration curves showed also the presence of phenolic hydroxyl groups ($pK = 8$–11·5). If one assumes that a single phenolic hydroxyl group per unit was titrated in the high pH region, about 54% of the polymer would consist of reduced phenolic units. On the other hand, if two phenolic hydroxyl groups per unit were titrated, the polymer would contain about 24% of reduced units.[11]

In order to compare ustilagomelanin with catecholmelanin, the latter was prepared by enzymic oxidation of catechol at pH 6·8. Like ustilagomelanin, it was esterified with methanol and reacted with diazomethane. The analytical data on ustilagomelanin, catecholmelanin and their derivatives are summarized in Table 1.

A striking difference appears in the values of the methoxyl content of the pigments after reaction with diazomethane. A higher ratio between the number of phenol and quinone units in the synthetic melanin can account for this difference.

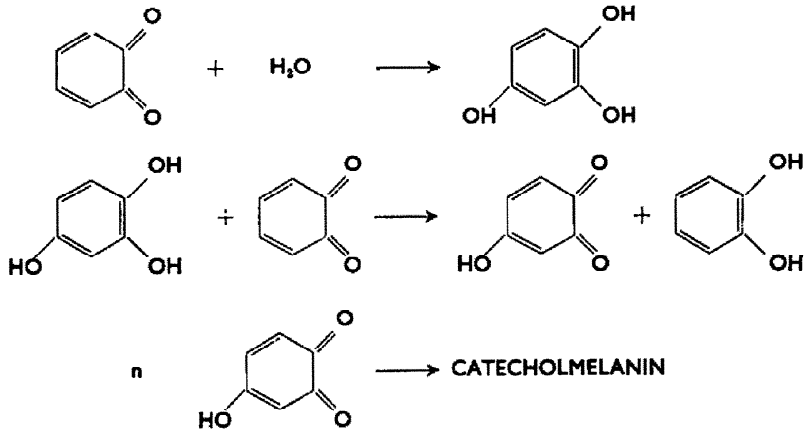
Pyrolysis, alkali fusion, reduction with sodium in pyridine and catalytic reduction of catecholmelanin gave the same products as in the case of ustilagomelanin.

[11] H. S. Mason in M. Gordon, *Pigment Cell Growth*. A.P. New York (1953).

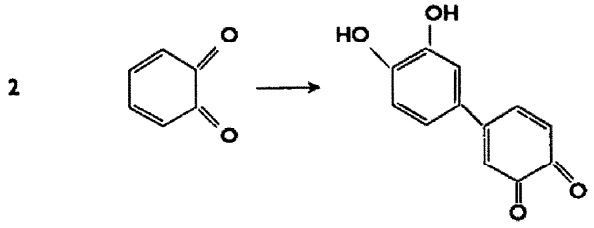
These results indicate that ustilagomelanin is a "catechol polymer." This hypothesis was supported by the examination of the nonacidic fraction of the ethanolic extract of *Ustilago maydis* spores, which revealed the presence of a single phenol, *viz.* catechol.

ESR spectrum of ustilagomelanin, which shows a well resolved triplet structure, is consistent with the hypothesis that the monomer is catechol. Furthermore, the catechol radical which gives rise to this triplet is probably bifunctional in the polymer, since it must retain 2 ring hydrogen atoms to show this hyperfine structure.[12]

As a consequence of the similarity between the natural pigment and the synthetic one, the data obtained from the biogenetic study of catecholmelanin can be utilized in the study of ustilagomelanin. Previous research[13] done in this field had demonstrated that the first step of melanogenesis from catechol is the formation of *o*-benzoquinone. It is not yet known how the reaction proceeds from there. According to Nelson *et al.*,[14] *o*-benzoquinone gives rise to hydroxyhydroquinone; by reaction of hydroxyhydroquinone with *o*-benzoquinone, catechol and hydroxyquinone are formed. Polymerization of the latter compound leads to catecholmelanin.

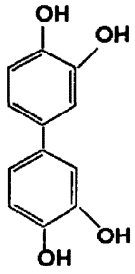



According to Mason,[15] the key reaction in melanogenesis from catechol is the condensation of *o*-benzoquinone according to the following scheme:




[12] M. S. Blois, private communication.
[13] C. E. M. Pugh and H. S. Raper, *Biochem. J.* **21**, 1370 (1927).
[14] H. Wagreich and J. M. Nelson, *J. Biol. Chem.* **115**, 459 (1936);
H. Wagreich and J. M. Nelson, *J. Amer. Chem. Soc.* **60**, 1545 (1938);
J. M. Nelson and C. R. Dawson, *Advances in Enzymology* **4**, 99 (1944).
[15] H. S. Mason in M. Gordon, *Pigment Cell Biology*. A.P. New York (1959).

An examination of the products of low mol. wt. formed during the enzymic oxidation of catechol led to the isolation of the following compounds[16]: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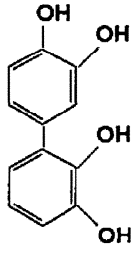
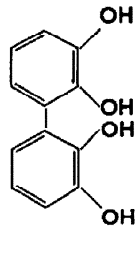
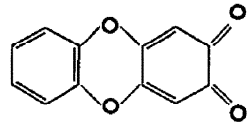
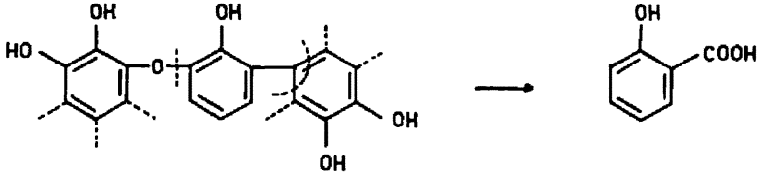

VI

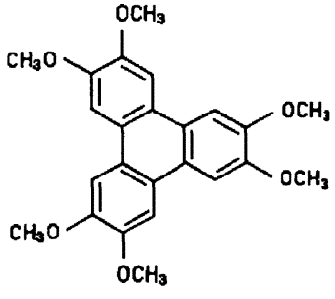

VII

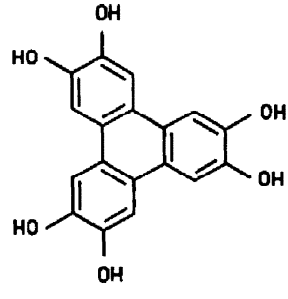

VIII

The formation of V, VI and VII shows that any one of the four unsubstituted positions in catechol can be involved in oxidative coupling; the formation of VIII indicates that not only C—C but also C—O links are formed during melanogenesis from catechol. The results obtained from degradation of ustilagomelanin and catecholmelanin are in agreement with the presence of both C—C and C—O linkages. The formation of protocatechuic acid by alkali fusion and of 3,3′,4,4′-tetrahydroxybiphenyl by catalytic hydrogenation concords with the presence of C—C linked units. On the other hand, ether linkages may provide an explanation of the formation of catechol from the reduction on both pigments with sodium in pyridine. A plausible interpretation of the formation of salicylic acid from partial structures including ether linkages is illustrated by the following scheme:



Further insight into the structure of these pigments was afforded by studying a simple catechol polymer of known structure formed by units bound through linkages of a single type, *viz.* C—C. Oxidation of veratrole with ferric chloride yielded, along


IX


X

[16] W. G. C. Forsyth and V. C. Quesnel, *Biochim. Biophys. Acta* **25**, 155 (1957); W. G. C. Forsyth, V. C. Quesnel and J. B. Roberts, *Ibid.* **37**, 322 (1960).

with other products, compound IX, m.p. 297–298°, whose highly symmetrical structure is apparent from the NMR spectrum, consisting in only two bands at 5·89τ and 2·24τ (3:1 ratio; $OCH_3$ and aromatic H respectively). Demethylation of IX with hydrobromic acid gave 2,3,6,7,10,11-hexahydroxytriphenylene (X). When this compound was subjected to alkali fusion, a number of phenolic substances were obtained, among which protocatechuic acid was identified, whereas acid III was not found. Therefore, the fact that no *o*-dihydroxybenzenedicarboxylic acid was obtained in the alkali fusion of ustilagomelanin and catecholmelanin does not rule out the presence in these pigments of catechol units linked by C—C bonds.

## EXPERIMENTAL

All m.ps are uncorrected. IR spectra were taken on a Perkin–Elmer Infracord instrument in KBr pellets and UV spectra on a Beckman DB spectrophotometer. NMR spectrum of IX was recorded in $CDCl_3$ solution (3% w/v) with tetramethylsilane as internal reference, on a Varian Associates A-60 spectrometer. Chromatograms were carried out on Whatman no. 1 paper (descending technique) using the following solvent systems: n-butanol–acetic acid–water (60:15:25; BAW); 0·005 N HCl (HCl); $CHCl_3$–MeOH–formic acid–water (100:10:0·4:9·6 organic phase; CMFW); EtOH–33% ammonia–water (80:4:16; EAW). Electrophoresis on Whatman no. 1 paper was carried out for 1 hr at 16 V/cm in the following electrolytes: 0·05 M pyridine formate (PF); 0·2 M borate buffer pH 8·7 (BOR). As spraying reagents, the following were used: 3% ethanolic $FeCl_3$; 2% phosphomolybdic acid.

Tentative identification of compounds was always substantiated by co-chromatography and co-electrophoresis with authentic specimens. Analyses were carried out by E. Thommen, Dept. Org. Chem., University of Basel, Switzerland.

*Isolation of ustilagomelanin.* Spores of *Ustilago maydis* (40 g), mechanically isolated from corn smuts, were continuously extracted with hot light petroleum b.p. 60–80° for 12 hr and then with hot EtOH for 20 hr. After drying, the material (38 g) was suspended in conc HCl (300 ml) and kept at room temp for 4 days. After addition of water (300 ml) the suspension was centrifuged and the precipitate washed 3 times with 5% HCl. The crude dark pigment was again suspended in conc HCl and hydrolysis was allowed to proceed for 18 days; the HCl solution was removed after 6 and 12 days and replaced by fresh conc HCl. After washing with 5% HCl, water and finally EtOH, the crude pigment (8 g) was continuously extracted with hot EtOH for 48 hr and then with hot tetrahydrofuran for 48 hr. The material (4·5 g) was treated with boiling azeotropic HCl for 90 hr, washed with 5% HCl (10 times) and with water (20 times) and finally dried over $P_2O_5$ at 80° for 48 hr *in vacuo*, thus obtaining 4·1 g of a black amorphous powder, insoluble in any solvent, which on calcination left 1·3% ash. (Found: C, 62·7; H, 3·4; N, 1·0% calc. on ash-free basis.) The pigment was not apparently modified by treatment for 30 min with boiling 57% HI and did not evolve $CO_2$ when heated at 200° in vaseline oil.

*Alkali fusion of ustilagomelanin.* To NaOH (3 g), KOH (3 g) and $Na_2S_2O_4$ (50 mg), fused in a Pt-crucible, ustilagomelanin (2 g) was slowly added. The mixture was kept at 200° for 10 min and after cooling was dissolved in 2% $Na_2S_2O_4$ (150 ml). The solution was acidified with acetic acid (10 ml), clarified by centrifugation and extracted with ether (200 ml in 5 portions). From the combined ether extracts the solvent was removed *in vacuo*; the residue, dissolved in a sat. $NaHCO_3$ aq, was extracted with ether (50 ml in 4 portions). The combined ether extracts, after washing with water, were evaporated to dryness; in the residue, dissolved in water (0·5 ml), catechol was identified by paper chromatography in the solvents BAW and HCl and by paper electrophoresis in BOR (spray reagents: $FeCl_3$ and phosphomolybdic acid). The acidic degradation products were recovered from the $NaHCO_3$-solution by acidification with conc HCl and extraction with ether. Paper chromatography in the solvents BAW, HCl and CMFW and paper electrophoresis in the electrolytes PF and BOR (spray reagents: $FeCl_3$ and phosphomolybdic acid) showed the presence of protocatechuic acid and salicylic acid.

*Reaction of ustilagomelanin with methanol.* A suspension of ustilagomelanin (500 mg) in MeOH (30 ml) sat. with dry HCl was kept at room temp for 48 hr. The pigment, collected by centrifugation, was washed with MeOH (10 times) and dried at 80° over $P_2O_5$ *in vacuo* (490 mg). (Found C, 63·9; H, 4·6; N, 1·0; $OCH_3$, 3·3% calc. on ash-free basis.)

*Reaction of ustilagomelanin with diazomethane.* To ustilagomelanin (1 g), suspended in ether

(10 ml), diazomethane (1 g) was added. After 12 hr, an additional amount (1 g) of diazomethane was added and the suspension allowed to stand for 12 hr at room temp. The red–brown pigment was collected by centrifugation, washed with MeOH (3 times) and with ether (3 times) and dried at 80° over $P_2O_5$ *in vacuo* (1 g). (Found: C, 64·9; H, 5·0; N, 1·7; $OCH_3$, 12·50% calc. on ash-free basis.) The pigment gave a negative test for methylenedioxy groups.

*Pyrolysis of ustilagomelanin.* Ustilagomelanin (100 mg) in a Pyrex tube was heated to 450° in a slow stream of $N_2$. The material condensed in the cold zone of the tube was dissolved in EtOH (1 ml) and analysed by paper chromatography (solvents: BAW and HCl) and electrophoresis (electrolyte: BOR). The papergrams, sprayed with $FeCl_3$ and phosphomolybdic acid, revealed the presence of a single phenol which was identified as catechol.

*Reduction of ustilagomelanin with sodium in pyridine.* Ustilagomelanin (220 mg), anhydrous pyridine (1 ml) and Na (350 mg) were placed in a 3-necked flask, fitted with a mechanical stirrer, a thermometer and a reflux condenser. The mixture was heated to 140° for 4 hr. After cooling, the mass was diluted first with pyridine (6 ml), then with pyridine–water mixture 1:1 (10 ml) and eventually with water (30 ml). After acidification with conc HCl and centrifugation, the solution was extracted with ether (50 ml in 5 portions). The ether extracts were combined and dried over $Na_2SO_4$. The solvent was removed *in vacuo* and the residue taken up in water (0·3 ml). The solution, analyzed by paper chromatography (solvents: BAW and HCl) and electrophoresis (electrolyte: BOR), showed the presence of catechol.

*Catalytic hydrogenation of ustilagomelanin.* Ustilagomelanin (2 g), suspended in EtOH (100 ml), was hydrogenated at 200° and 200 atm for 36 hr in the presence of Pd–C (250 mg). Catalyst and unaffected melanin were removed by filtration; the filtrate was concentrated under red. press. to about 5 ml and analysed by 2-dimensional paper chromatography, using water and the top layer of a benzene–acetic acid–water mixture (2:4:1), in that order, as developing solvents. By spraying the chromatogram with $FeCl_3$, only two substances were detected, which were identified as catechol and 3,3′,4,4′-tetrahydroxybiphenyl by comparison with authentic specimens.

*Preparation and properties of catecholmelanin.* Catechol (1 g) was dissolved in 1300 ml 0·05 M phosphate buffer at pH 6·8. After addition of 22 mg of mushroom tyrosinase (500 U/mg), a rapid stream of $O_2$ was bubbled for 6 hr into the solution kept at 37°. After acidification with conc HCl, the dark precipitate was collected by centrifugation and washed with 1% HCl until catechol was completely removed. The catecholmelanin was then washed with water (3 times) and dried over $P_2O_5$ at 80° *in vacuo* for 48 hr (180 mg). (Found: C, 60·5; H, 3·2; N, 1·4% calc. on ash-free basis.) Permanganate oxidation, alkali fusion, catalytic hydrogenation, reduction with Na in pyridine and pyrolysis of catecholmelanin were performed in the experimental conditions described for ustilagomelanin and gave the same results. Analytical data of the products obtained from catecholmelanin by reaction with MeOH and diazomethane (experimental conditions used for ustilagomelanin) are reported in Table 1.

*Identification of catechol in* Ustilago maydis *spores. U. maydis* spores (5 g) were continuously extracted first with hot light petroleum b.p. 60–80° for 12 hr and then with hot EtOH for 12 hr more. The EtOH extract was taken to dryness and the residue dissolved in water (10 ml). The solution was extracted with ether (40 ml in 4 portions). The combined ether extracts, after washing first with a sat. $NaHCO_3$ aq and then with water, were evaporated to dryness. The residue was dissolved in water (0·2 ml) and the solution used for paper chromatography (solvents: BAW and HCl) and electrophoresis (electrolyte: BOR), using $FeCl_3$ and phosphomolybdic acid as spray reagents. The papergrams revealed the presence of a single phenolic substance, identified as catechol.

2,3-*Dihydroxyterephtalic acid* (I). Acid I was prepared according to Schmitt and Hahle[17]. $R_f$ in EAW 0·37 (blue–grey with $FeCl_3$).

3,4-*Dihydroxyphtalic acid* (II). It was prepared according to Freund and Horst,[18] $R_f$ in EAW 0·26 (blue–grey with $FeCl_3$).

4,5-*Dihydroxyphtalic acid* (III). Acid III was prepared according to Späth and Epstein,[19] $R_f$ in EAW 0·1 (deep green with $FeCl_3$).

4-*Hydroxy-5-methoxyisophtalic acid.* To a suspension of freshly prepared $Ag_2O$ (1·16 g) in water (20 ml), NaOH (2 g) was added. With continued stirring at 55–60°, 1·96 g 5-formylvanillic acid was

[17] R. Schmitt and H. Hahle, *J. Prakt. Chem.* **44**, 1 (1891).

[18] M. Freund and F. Horst, *Ber. Dtsch. Chem. Ges.* **27**, 332 (1894).

[19] E. Späth and H. Epstein, *Ber. Dtsch. Chem. Ges.* **61**, 334 (1928).

added. After 15 min, the mixture was filtered and the Ag washed with 30 ml hot water. The combined filtrates were acidified with HCl. The precipitate, collected by filtration, was suspended in a 10% $NaHSO_3$ solution. After 10 min, the precipitate was filtered off and washed with water. The crude product, crystallized from water, afforded 4-hydroxy-5-methoxyisophthalic acid (1·1 g) in colourless needles, m.p. 272–273°. (Found: C, 50·08; H, 4·17. $C_9H_8O_6$ requires: C, 50·95; H, 3·80%.)

4,5-*Dihydroxyisophtalic acid* (IV). 4-Hydroxy-5-methoxyisophthalic acid (500 mg) was refluxed for 5 hr with acetic acid (10 ml) and 48% HBr (10 ml). After cooling, water (100 ml) was added and the solution was extracted with ether (150 ml in 5 portions). The combined ether extracts were dried over $MgSO_4$, and the ether was removed by distillation under red. press., leaving the crude product. After

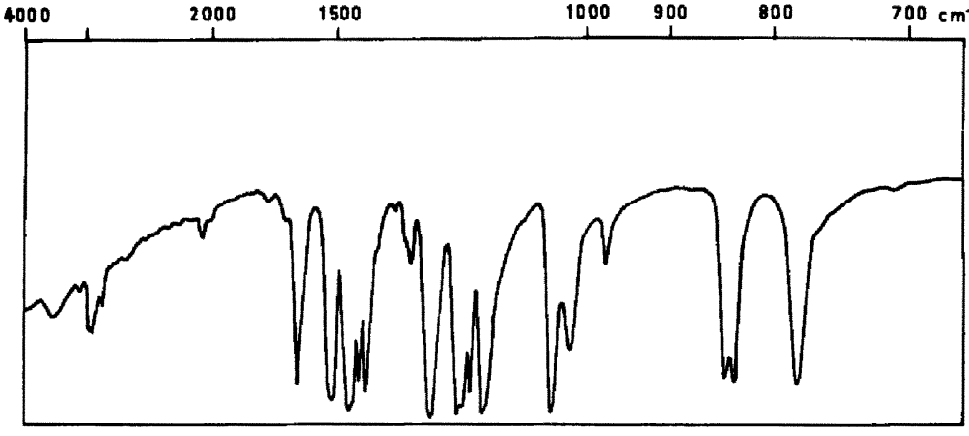



FIG. 1

crystallization from water, acid IV, m.p. 276° dec was obtained. (Found: C, 48·32; H, 3·18. $C_8H_6O_6$ requires C, 48·49; H, 3·05%.)

2,3,6,7,10,11-*Hexamethoxytriphenylene* (IX). Water (4·5 g) was added dropwise with stirring under $N_2$ at room temp to a mixture of veratrole (69 g) and $FeCl_3$ (40·6 g). The mixture was warmed at 75° for 20 min. After cooling, the precipitate was filtered off and washed with EtOH, 5N HCl and finally water. The crude product, dried at 120°, was purified by chromatography on a silicagel column (2 × 37 cm), using $CHCl_3$ as eluent. After crystallization from acetic acid and recrystallization from trifluoroacetic acid, IX (4·8 g) was obtained as colourless needles m.p. 297–298°. (Found: C, 70·18; H, 6·06; $OCH_3$, 44·9. $C_{24}H_{24}O_6$ requires: C, 70·57; H, 5·92; $OCH_3$, 45·6%; Mol. wt determined by ebullioscopic method in chloroform = 412; calc. for $C_{24}H_{24}O_6$ = 408·4.) NMR spectrum of IX consists in only two bands at 5·89$\tau$ and 2·24$\tau$ (3:1 ratio; $OCH_3$ and aromatic H respectively). IR spectrum of IX is reported in Fig. 1.

2,3,6,7,10,11-*Hexahydroxytriphenylene* (X). Compound IX (200 mg) was refluxed under $N_2$ for 15 hr with acetic acid (20 ml) and 48% HBr (20 ml). The solution was evaporated to dryness and the residue crystallized from water to give X (140 mg), as colourless m.p. > 310°. (Found: C, 66·52; H, 3·81. $C_{18}H_{12}O_6$ requires: C, 66·67; H, 3·73%.) By heating at 400° a mixture of X (50 mg) and Zn-dust (500 mg) a colourless sublimate was obtained. Its identity with triphenylene was proved by UV and IR spectra and mixed m.p.

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