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Studies on ferroverdin, a green iron-containing pigment produced by a *Streptomyces* Wak. species (**)

The submerged culture production of ferroverdin, an iron-containing green pigment of a novel type, by a new species of *Streptomyces* Wak. is reported.

The morphological properties of this micro-organism are described, and the optimum culture conditions for pigmentation in shake flasks and in stirred fermenters are determined.

The preparation and identification of reductive and alkali degradation products of ferroverdin are reported ; on the basis of their structures it is suggested that ferroverdin is the ferrous complex of the *p*-vinylphenylester of 3-nitroso-4-hydroxybenzoic acid.

Introduction

In a previous communication from this laboratory the purification and preliminary characterization of ferroverdin, a green iron-containing pigment produced by a new *Streptomyces* species, were reported (Chain, Tonolo & Carilli 1955). Magnetic measurements showed that the iron atom was divalent (Ehrenberg 1956).

The present paper reports the morphological features of the new micro-organism, gives details of the methods of production in submerged culture and purification of ferroverdin and the experimental evidence which has allowed the assignment of structures to ferroverdin and its degradation products. A preliminary note on the structure of ferroverdin has been published (Ballio, Bertholdt, Chain & Di Vittorio 1962).

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A. Methods and materials

I. *Strain*

The strain (No A-305 of this collection) was an unidentified species of *Streptomyces* Wak., isolated in this laboratory from a sample of soil collected at Leopoldville (Congo) in 1952.

II. *Microscopic observations*

These were carried out on fresh unstained or gram-stained mycelium according to Conn, Bartholomew & Jennison (1954).

III. *Culture media*

(1) *Solid media*

The culture was maintained on a glucose-yeast-agar medium with the following composition: glucose 1 g, yeast extract 0.1 g, agar 1.5 g, tap-water 100 ml., pH 6.8 to 7. Every month the strains were transferred on to a new slant. They were maintained at room temperature, after growing for a week at 24 °C.

(2) *Media for submerged fermentation.*

Unless otherwise stated a medium with the following composition was used for the production of ferroverdin, both in shake flasks and in stirred fermenters: Glucose 1 % ; yeast extract 0·5 % ; $FeCl_3 . 6H_2O$ 0·002 % ; tap-water ; pH 6·8 to 7. The culture media were sterilized for 20 min at 100 °C, followed by a further period of 20 min at 120 °C. The same medium was used for preparing the seed cultures.

IV. *Fermentation methods*

(1) *Shake flasks.*

Cotton-wool plugged 500 ml. Erlenmeyer flasks containing 100 ml. of culture medium were used on a rotary shaker at 24 °C (Paladino 1954); under the conditions of agitation (220 rev/min, eccentric throw 6 cm) the aeration rate in the absence of mycelium was about 28 ml. O_2 per 100 ml. of sulphite solution per hour. The shake flasks for the seed cultures were inoculated with a mixture of spores and vegetative mycelium obtained by growing a culture on an agar slant for 7 to 10 days. The spores and mycelial mat were scraped off the slant with a spatula and transferred into the liquid medium in the shake flasks. After 4 days' submerged growth in the seed flasks 10 ml. of mycelial suspension were used to inoculate the production shake flasks containing 100 ml. of fresh medium.

(2) *Stirred fermenters.*

Jacketed stainless-steel fermenters of 90 l. total capacity (Paladino, Ugolini & Chain 1954) were used with 50 l. of culture medium. Aeration was effected through a ring sparger with an air flow varying from 40 to 50 l./min at an over-pressure of 1 atm.

The fermenter was provided with a top-driven stirrer rotating at 220 rev/min and fitted with an eight-bladed turbine propeller (ratio diameter of fermenter to diameter of propeller 3.5 : 1). The 90 l. fermenters were inoculated with 5 l. of a mycelial suspension grown in another 90 l. fermenter for 4 days, which in turn was inoculated with 1 l. of vegetative mycelium grown in a 3 l. shake flask for 4 days. The 3 l. shake flasks were inoculated with 100 ml. of mycelial suspension obtained in the production shake flasks as described in the preceding paragraph.

V. *Analytical methods*

(1) *Ferroverdin.*

A routine assay for ferroverdin contained in the mycelium was carried out as follows. A duplicate sample of 100 ml. of culture was filtered, the mycelium thoroughly washed with distilled water, dried by suction as much as possible and stirred into 10 ml. hot methanol. The methanolic suspension was filtered and the extraction with methanol repeated until the extract was colourless. The methanolic filtrates were pooled, the volume determined and a sample used for colorimetric measurements (EEL colorimeter, orange filter no. 607). A calibration curve was prepared with a crystalline sample of ferroverdin as standard ; the curve was linear up to 70 µg/ml. Results were reported in mg ferroverdin per 100 ml. culture.

(2) *Glucose.*

Glucose was estimated in the culture filtrates by the method of SOMOGYI (1952).

(3) *Nitrogen.*

The total nitrogen was determined by a modified Kjeldahl method.

(4) *Dry weight.*

100 ml. of the mycelial suspension was filtered through paper on a Büchner funnel, washed thoroughly with three volumes of water, and dried at 85 °C for 24 hr.

(5) *Iron.*

Total iron in the culture filtrates was estimated by a colorimetric method with o-phenanthroline (SNELL & SNELL 1949), after digestion with perchloric and sulphuric acid, followed by dithionite treatment to destroy excess perchloric acid.

(6) *Spectrometry.*

The u.v. and visible spectra were recorded on a Cary spectrophotometer Model 11, the i.r. spectra on a Perkin-Elmer 21 spectrophotometer, the n.m.r. spectra on a Varian Model U-4300 B high resolution spectrometer operated at 60 Mc/s (resonance line positions reported in τ-values, relative to tetramethyl silane as internal standard). A Rudolph Model 200 S spectropolarimeter was used for optical rotation measurements at 537 nm of ethanolic solutions of ferroverdin.

(7) *Chromatography.*

(*a*) *Paper chromatography.* For the characterization of the coloured breakdown products of ferroverdin descending paper chromatography at 25 °C on Whatman no. 1 paper (washed with 2N acetic acid and followed by water) was used, with the following solvent systems: no. 1, *n*-propanol-ammonia (*d* 0.88)-water (60 : 30 : 10); no. 2, *iso*butyric acid 1 N-ammonia (100 : 60); no. 3, *n*-butanol-pyridine-water (60 : 40 : 30); no. 4, ethanol-1 N ammonium acetate pH 7.5 (75 : 30)

(*b*) *Column chromatography.* Purification of ferroverdin and the ferrous complex of 3-nitroso-4-hydroxybenzoic acid methylester was accomplished on alumina (Merck, standardized according to Brockman). The ferrous complex of 3-nitroso-4-hydroxybenzoic acid was irreversibly bound to alumina; therefore, partition chromatography on a column of Whatman's cellulose powder (ashless standard grade for chromatography) prewashed with *n*-propanol-water (9 : 1) was used.

(*c*) *Gas chromatography.* This was used for the purification of *p*-ethylphenol. A Fractovap Model B (C. Erba) was used, with a column 2 m long and 6 mm wide of Celite 22-Apiezon L run at 200 °C with hydrogen as mobile phase (at rate of 6 l. h).

(*d*) *Paper electrophoresis.* An apparatus very similar to that described by Markham & Smith (1952) and a pH 4 buffer (0.1 M sodium citrate 560 ml., 0.1 N hydrochloric acid 440 ml.) were used. A potential gradient of 25 V/cm was applied to 40 cm-long strips of Whatman No. 1 paper.

(8) *Microanalyses.*

Microanalyses were by Professor Margherita Marzadro at the Istituto Superiore di Sanità and by Dr. F. Pascher, Bonn.

VI. *Chemicals*

Chemicals used for shake flasks fermentations were of pure grade; glucose for fermentations in stirred fermenters was a technical grade product containing more than 90% glucose. The yeast extract was an autolysate of brewer's yeast purchased from A. Costantino and Co.; it contained 0.15 mg iron/g. The other nitrogen sources, namely pepton, acid and enzymically hydrolyzed casein, corn-steep, soya and pea-nut meals, were commercial products.

Chemicals used for quantitative assays and for chemical studies were of analytical grade. All solvents were redistilled before use.

The ferroverdin used in the present chemical investigations was in part the pure product prepared during previously described studies (Chain *et al.* 1955), and in part a partially purified material obtained according to a method outlined in the experimental section.

With the exception of *p*-ethylphenol, which was purchased from Fluka, Switzerland, authentic reference compounds were prepared by simple procedures described in the literature, i.e. 3-amino-4-hydroxybenzoic acid by reduction of 3-nitro-4-

hydroxybenzoic acid, 3-acetylamino-4-hydroxybenzoic acid by mild acetylation of 3-amino-4-hydroxybenzoic acid, methyl-3-acetylamino-4-methoxybenzoate by diazomethane treatment of 3-acetylamino-4-hydroxybenzoic acid, 3-acetylamino-4-methoxybenzoic acid by alkaline hydrolysis of the corresponding methylester, *p*-ethylanisol by methylation of *p*-ethylphenol. With the exception of methyl-3-acetylamino-4-methoxybenzoate, all the above compounds were known products and their physical properties were in excellent agreement with those reported in the literature. Methyl-3-acetylamino-4-methoxybenzoate crystallized from ether-light petroleum in prisms (m.p. 128-130 °C) and corresponded in all its properties with an analyzed product described below (p. 21).

B. Morphological observations

By A. Carilli and A. Tonolo

(1) *Morphological appearance of A-305 on agar.*

The colonies grown on glucose-yeast extract agar reached a diameter of 3 to 5 mm after 10 to 15 days' growth. They were round, pulvinate, with an even border and a smooth surface (Fig. 1 *a*, Tav. I). Aerial mycelium at first white became, on sporulation, grey-white to grey. Sporophores were straight, short, or curved, not forming any spiral (Fig. 2, Tav. I). Spores were globose to oval, 1.5 µm long and 0.8 µm wide; under the electron microscope they looked hairy (Fig. 3, Tav. I).

The vegetative mycelium had a deep green colour (Fig. 1 *b*); no pigment diffused into the agar. Completely green colonies without aerial mycelium were also observed.

The strain was Gram +, not acid-fast, aerobic, saprophytic with typical earth odour; optimum of growth 24 to 37 °C, no growth at 60 °C. It showed no antagonistic properties against bacteria or fungi.

(2) *Morphological appearance of* A-305 *in submerged culture.*

In submerged culture the mycelium was organized in pellets of 0.2 to 1.5 mm diameter (Fig. 4, Tav. I). In stirred fermenters filamentous mycelium was occasionally observed; in this case pigment formation was consistently absent.

(3) *Surface growth on different carbon sources.*

The technique of Pridham & Gottlieb (1948) was used for testing the efficiency of different carbon sources to support growth of A-305 on agar slants. Results are reported in Table 1.

Table 1. — Effect of different carbon sources on surface growth of *Streptomyces* Sp., strain A-305 *

carbon sources	growth † (after 15 days at 24 °C)	carbon sources	growth † (after 15 days at 24 °C)
D-glucose	+ +	lactose	+ +
D.mannose	+ +	cellobiose	+ +
D-galactose	+ +	maltose	+ +
D-fructose	+	raffinose	±
L-xylose	+	dextrin	+ +
L-arabinose	+ +	inulin	—
D-sorbitol	—	starch	+ +
D-mannitol	±	glycerol	+ +
dulcitol	—	Na succinate	±
meso-inositol	+ +	Na citrate	±
sucrose	+	none	—

* Two experiments, each in triplicate.

† + + + = excellent growth ; the whole inoculated area was fully developed after 2 to 3 days incubation. + + = good growth ; the inoculated area showed a cluster of colonies after 4 to 5 days incubation. ± = poor growth ; the inoculated area showed very few punctiform colonies. — = no growth.

C. Fermentations

By E. B. Chain, F.R.S., A. Tonolo and Lidia Vero-Barcellona

(1) *Effect of added iron on the production of ferroverdin.*

The influence of different concentrations of iron on ferroverdin production and growth is shown in Table 2.

Table 2. — Effect of added iron on ferroverdin production and growth *

iron, added as $FeCl_3 . 6 H_2O$ to the nutrient medium (µg/ml.)	ferroverdin (mg/100 ml.)	mycelial dry weight (g/100 ml.)	ferroverdin (mg/100 mg of dry mycelium)
0	0·802 ± 0·11	0·296 ± 0.04	0·27
0·8	1·745 ± 0·14	0·318 ± 0.02	0·54
2	2·643 ± 0·31	0·332 ± 0.03	0·79
4	3·246 ± 0·39	0·365 ± 0.04	0·89
8	3·420 ± 0·51	0·390 ± 0.02	0·87
16	0·480 ± 0·15	0·295 ± 0.05	0.16
32	0·220 ± 0·18	0·200 ± 0.03	0·11
64	0·000 ± 0·00	0·206 ± 0.05	0·00
128	0·000 ± 0·00	0·000 ± 0.00	0·00

* Three experiments, each in duplicate. Readings after 4 days growth.

Table 3. — Effects of ferrocyanide and ferricyanide on ferroverdin production *

Fe compounds added	Fe added (µg/ml.)	ferroverdin (mg/100 ml.)	mycelial dry weight (g/100 ml.)	ferroverdin (mg/100 mg of dry mycelium)
none	0	0.675 ± 0.09	0.321 ± 0.02	0.21
$FeCl_3 . 6 H_2O$	4	4.005 ± 0.11	0.340 ± 0.06	1.17
$K_4Fe(CN)_6 . 3 H_2O$	2	2.225 ± 0.10	0.344 ± 0.07	0.64
	4	1.850 ± 0.09	0.310 ± 0.02	0.59
	6	1.050 ± 0.12	0.337 ± 0.04	0.31
$K_3Fe(CN)_6$	2	1.843 ± 0.08	0.297 ± 0.03	0.62
	4	1.973 ± 0.13	0.298 ± 0.04	0.66
	6	2.185 ± 0.15	0.339 ± 0.05	0.64

* Three experiments, each in duplicate. Readings after 4 days growth.

As can be seen from Table 2, there is an optimum concentration of iron for the production of ferroverdin. The amount of iron (0.75 µg/ml.) contained in the standard nutrient medium was below the optimum which was reached at about 4 to 8 µg/ml. of added Fe. At higher iron concentrations there was a sharp drop in both growth and ferroverdin production. Ferroverdin was also produced when iron was added in form of the ferro and ferricyanide complexes, though less than in presence of ferric chloride (Table 3).

(2) *Effect of ferroverdin production and growth of different carbon sources.*

Table 4 shows that glucose could be substituted by other carbon sources without impairment of the ferroverdin yields. Among the products tested, fructose and xylose gave poor ferroverdin production at both iron levels used, whereas sucrose was a poor carbon source only at lower iron concentration.

(3) *Effect of substituting yeast extract by other organic nitrogen sources.*

Yeast extract could be substituted by pepton, acid hydrolyzed and enzymically hydrolyzed casein, the latter giving somewhat higher yields of ferroverdin. Soya and pea-nut meals, while giving good growth when supplemented with sodium sulphate, failed to give rise to ferroverdin production ; corn-steep liquor gave only low yields of ferroverdin (Table 5). It was at first thought that the low production of ferroverdin in the presence of corn-steep liquor was due to the high iron content of the latter. This, however, cannot be the explanation because the iron content of enzymically hydrolyzed casein, which gives very good ferroverdin yields, is almost as high as that of corn-steep liquor.

(4) *Course of fermentation.*

The course of a typical ferroverdin fermentation in shake flasks in the standard medium is shown in Figure 1, and in a stirred fermenter of 90 l. capacity in Figure 2.

Table 4. — Effect of different carbon sources on ferroverdin production and growth *

carbon sources (all at 1% concn.)	Fe added (µg/ml.) 0·8 ferroverdin (mg/100 ml.)	0·8 mycelial dry weight (g/100 ml.)	0·8 ferroverdin (mg/100 mg of dry mycelium)	4 ferroverdin (mg/100 ml.)	4 mycelial dry weight (g/100 ml.)	4 ferroverdin (mg/100 mg of dry mycelium)
glucose	1·390 ± 0.19	0·299 ± 0.02	0·46	2·760 ± 0·11	0·342 ± 0·02	0·80
fructose	0·350 ± 0.02	0·265 ± 0.01	0·13	0·585 ± 0·03	0·290 ± 0·03	0·20
xylose	0·157 ± 0.02	0·194 ± 0.01	0·08	0·507 ± 0·09	0·300 ± 0·02	0·16
mannitol	1·208 ± 0.12	0·249 ± 0.02	0·48	2·300 ± 0·30	0·374 ± 0·04	0·76
sucrose	0·162 ± 0.05	0·169 ± 0.01	0·09	3·625 ± 0·15	0·371 ± 0·03	0·97
lactose	0·900 ± 0.09	0·207 ± 0.03	0·43	3·875 ± 0·29	0·374 ± 0·04	1·03
starch	1·550 ± 0.17	0·294 ± 0.02	0·52	3·920 ± 0·20	0·330 ± 0·01	1·18
glycerol	1·030 ± 0.10	0·241 ± 0.04	0·42	2·340 ± 0·18	0·268 ± 0·03	0·87

* Two experiments each in duplicate. Readings after 4 days growth.

Table 5. — Effect of the different nitrogen sources on the ferroverdin production and growth *

N sources †	Fe added (µg/ml.) 0 ferroverdin (mg/100 ml.)	0 mycelial dry weight (g/100 ml.)	0·8 ferroverdin (mg/100 ml.)	0·8 mycelial dry weight (g/100 ml.)	4 ferroverdin (mg/100 ml.)	4 mycelial dry weight (g/100 ml.)
yeast extract	0·568 ± 0.16	0·266 ± 0.02	1·156 ± 0·15	0·319 ± 0.05	3·745 ± 0.65	0·350 ± 0.05
pepton	0·545 ± 0.18	0·109 ± 0.04	1·490 ± 0·19	0·122 ± 0.02	2·255 ± 0.80	0·120 ± 0.04
casein (enzymically hydrolyzed)	2·092 ± 0.03	0·192 ± 0.04	2·056 ± 0·28	0·190 ± 0.03	4·275 ± 0.71	0·258 ± 0.02
casein (acid hydrolyzed)	0·511 ± 0.14	0·236 ± 0.05	1·042 ± 0·12	0·196 ± 0.08	3·125 ± 0.57	0·264 ± 0.03
corn-steep	0·202 ± 0.11	0·309 ± 0.03	0·146 ± 0·09	0·277 ± 0.05	0·000 ± 0.00	0·267 ± 0.02
soya meal ‡	0·000 ± 0.00	0·230 ± 0.01	0·000 ± 0·00	0·265 ± 0.04	0·000 ± 0.00	0·300 ± 0.05
pea-nut meal (‡)	0·000 ± 0.00	0·111 ± 0.02	0·000 ± 0·00	0·110 ± 0.01	0·000 ± 0.00	0·115 ± 0.02

* Three experiments, each in duplicate. Readings after 4 days growth.

† The nutrient medium had the following composition: glucose 1%, tap water, pH 7.0 and nitrogen sources (g/100 ml.) in the following quantities: yeast extract 0.5, pepton 0.33, casein enzymically hydrolyzed 0.30, casein acid hydrolyzed 0.50, corn-steep 0.90; soya meal 1, pea-nut meal 1. With these amounts added the total nitrogen content of the culture media was in each case 57.5 mg/100 ml. and the iron content (µg/ml.) 0.75 for yeast extract, 0.55 for pepton, 2.35 for enzymically hydrolyzed casein, 0.7 for acid hydrolysed casein, 2.65 for corn-steep liquor, 1.2 for soya meal, 1.8 for pea-nut meal.

‡ Supplemented with 0.05% of Na_2SO_4.

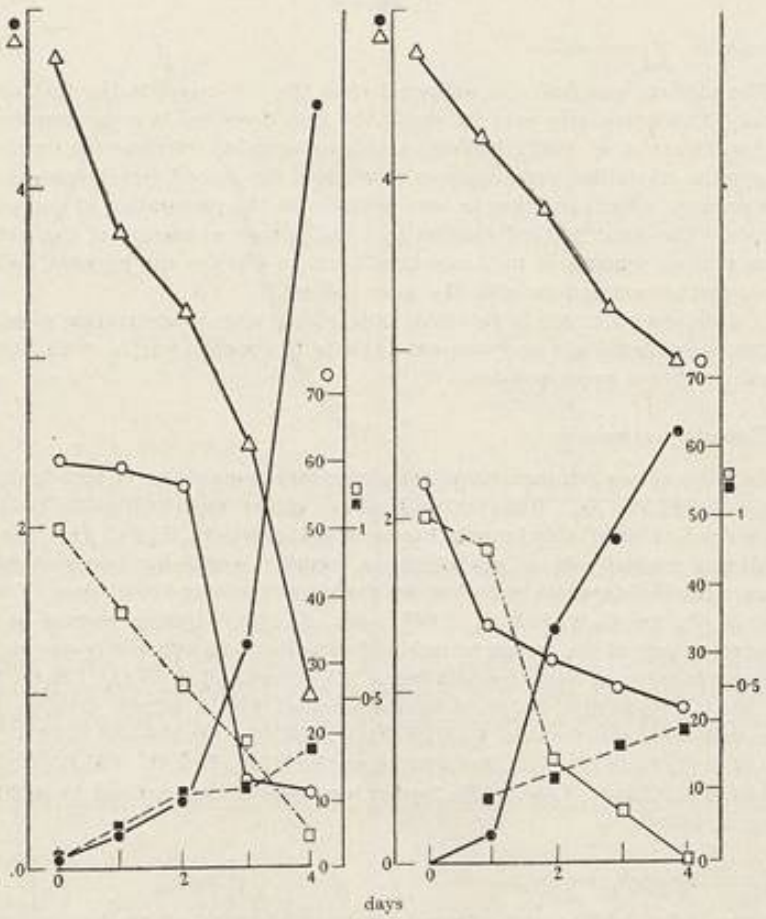


FIGURE 1

FIGURE 2

FIGURE 1. Course of a typical ferroverdin fermentation by *Streptomyces* sp., strain A-305, in shake flasks in standard medium. □ glucose (g/100 ml.); ○ total N in culture medium (mg/100 ml.); ● ferroverdin (mg/100 ml.); △ Fe^{3+} (µg/ml.); ■ dry weight (g/100 ml.).

FIGURE 2. Course of a typical ferroverdin fermentation by *Streptomyces* sp., strain A-305, in a 90 l. stirred fermenter in standard medium. Same symbols as in Figure 1.

D. Chemical studies

By A. Ballio, H. Bertholdt, E. B. Chain, F.R.S., and V. Di Vittorio

I. *General*

(1) *Isolation of ferroverdin.*

The pigment can easily be extracted from the wet mycelium by methanol or ethanol. Two procedures were followed: the first, described in a previous communication (Chain *et al.* 1955), includes a chromatographic fractionation on alumina and gives a crystalline pure compound, whereas the second (see below) yields a crude product, which, however, is very suitable for the preparation of degradation products. The latter method consists in a preliminary extraction of the wet mycelium with an amount of methanol insufficient to dissolve the pigment, followed by two further extractions with the same solvent.

A deep green solution is obtained, from which, after concentration at reduced pressure, a semisolid oil separates out; this is thoroughly washed with light petroleum to give a green powder.

(2) *Elementary composition.*

In the previous communication the elementary composition of ferroverdin was given as $C_{30}H_{24}FeN_2O_8$. However, on the basis of new analytical values on a pure sample dried at 100 °C this formula has to be changed to $C_{30}H_{20}FeN_2O_8$. The compound was originally in all probability a complex containing two molecules of carbon tetrachloride which were lost by drying *in vacuo* at 100 °C (loss of weight found 30.0% for $C_{30}H_{20}FeN_2O_8 . 2\,CCl_4$, calc. 34.4%). During storage at room temperature part of the carbon tetrachloride was lost, and apparently one molecule of water was taken up (loss of weight found 17.6%, for $C_{30}H_{20}FeN_2O_8 . 1\,H_2O . 1\,CCl_4$ calc. 20.8%). The new analytical figures obtained with a sample dried at 100 °C agree well with the formula $C_{30}H_{20}FeN_2O_8 . 1\,H_2O$. (Found—C: 59.07, 59.16, H: 3.52, 3.71, Fe: 9.07, N: 4.26, 4.62%. Calc. for $C_{30}H_{20}FeN_2O_8 . 1\,H_2O$; C: 59.03, H: 3.63, Fe: 9.15, N: 4.59%). No further loss in weight was noticed by additional drying at 160 °C.

(3) *Properties of ferroverdin.*

Ultra-violet, visible, and infra-red spectra made with a pure ferroverdin preparation are shown in Figures 3 and 4. The pigment is optically inactive (optical rotation measured at 537 nm). It does not melt at temperatures up to 300 °C.

Ferroverdin is fairly soluble in methanol, ethanol, acetone, ethyl acetate, acetic acid, dimethyl sulphoxide, nitromethane, nearly insoluble in ethyl ether and practically insoluble in water, aqueous acids and alkali, benzene, light petroleum, chloroform, carbon tetrachloride, carbon disulphide. It is quite stable when dissolved in organic solvents, as well as in neutral or acidified aqueous alcoholic solution;

on alkalinization, however, of an aqueous ethanolic solution the u.v. spectrum undergoes a very rapid change (see Figure 3) which is irreversible. The iron atom is very firmly bound in ferroverdin ; it cannot be displaced by any of the common

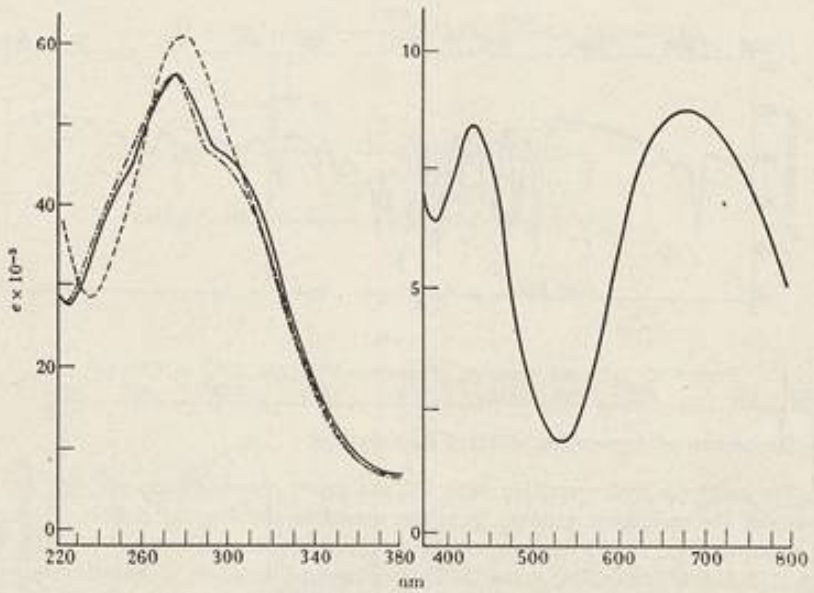


FIGURE 3. Ultraviolet (left) and visible (right) spectra of ferroverdin. ——— in methanol, – – – – in alkaline methanol, —.—.— in acid methanol.

chelating agents, such as ethylenediaminetetra-acetic acid, α : α-dipyridyl, *o*-phenanthroline, 1-nitroso-2-naphthol-3,6-disulphonic acid. Oxidizing agents, such as cold hydrogen peroxide or potassium permanganate, do not affect the colour of the

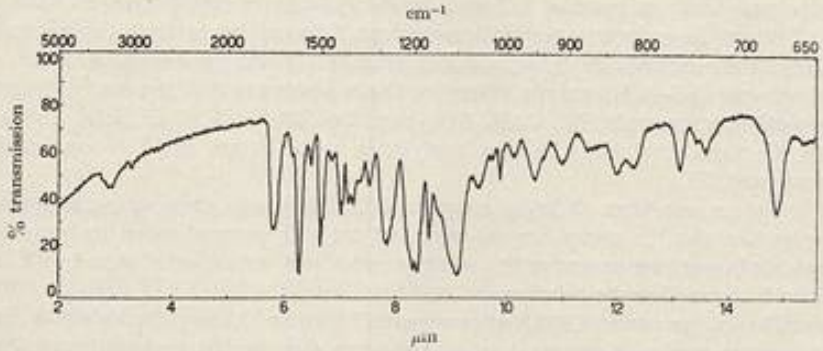


FIGURE 4. Infra-red spectrum of ferroverdin in KBr pellet.

pigment, whereas sodium dithionite decolorizes very rapidly an aqueous methanolic solution of ferroverdin. Decolorization was also observed on catalytic hydrogenation and on reduction with zinc and acetic acid.

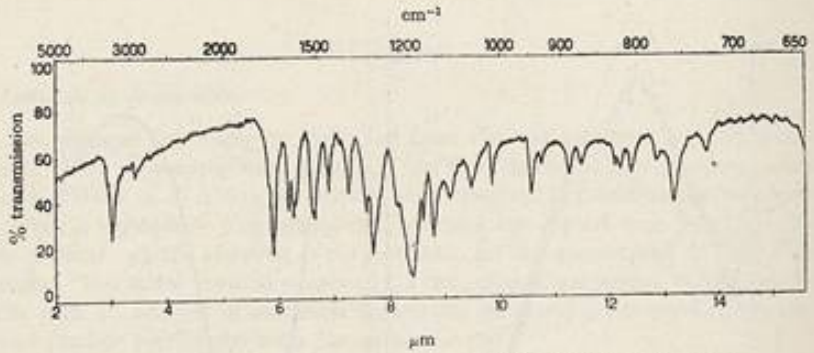


FIGURE 5. Infra-red spectrum of compound I ($C_{13}H_{13}NO_3$) in KBr pellet.

(4) *Derivatives of ferroverdin obtained by reduction.*

On catalytic hydrogenation with Pd the green colour disappears, but the solution of the reduction product is rather unstable, tending to darken; however, on concentration in a hydrogen atmosphere a crystalline compound (I, $C_{13}H_{13}NO_3$, m.p. 172-174 °C) could be isolated. This compound contains a diazotizable amino group and gives a weak brown-greenish colour with ferric chloride, indicating the presence of aromatic amino and hydroxy groups. The presence of these groups was corroborated by the i.r. spectrum (KBr) (Figure 5) which shows also the presence of an ester group (1695 cm^{-1}).

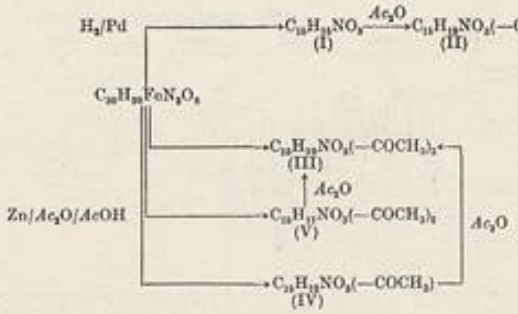
On acetylation this compound gives a crystalline product (II, $C_{21}H_{21}NO_6$, m.p. 125-127 °C) which contains 4 C—CH_3 groups and no active hydrogens. The same compound could also be obtained from ferroverdin by reductive acetylation (zinc and acetic anhydride) followed by catalytic hydrogenation.

When the reductive acetylation was not followed by catalytic hydrogenation a crystalline compound (III, $C_{21}H_{19}NO_6$, m.p. 155-156 °C) was isolated, which contained only 3 C—CH_3 groups. Varying the temperature and the acetylation time two other compounds (IV, $C_{17}H_{15}NO_4$, m.p. 227-228 °C; V, $C_{19}H_{17}NO_5$, m.p. 153-154 °C) were obtained, which by acetylation under reflux could be converted to compound III.

The i.r. spectrum (KBr) of compound IV (Figure 6) gives strong assignments for an acetylamino group, for an ester and for a hydrogen-bonded hydroxyl; the latter is in good agreement with a weak greenish colour reaction obtained with ferric chloride in methanolic solution.

The i.r. spectrum ($CHCl_3$) of compound V (Figure 7) shows bands which can be assigned to a phenolic acetate, an arylester, an acetylamino group, whereas in the i.r. spectrum ($CHCl_3$) of compound III (Figure 8) the NH band has disappeared

but two bands in the CO region are still present ; one of them has been assigned to the phenolic acetate, and the second broad band to an arylester and diacetylamino group. The i.r. spectrum ($CHCl_3$) of compound II shows bands nearly iden-



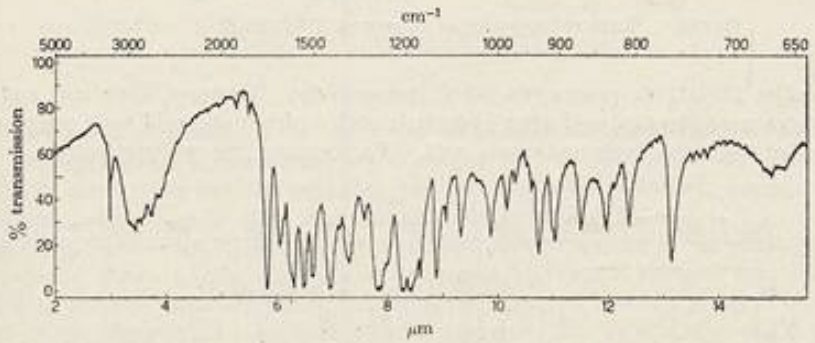


FIGURE 6. Infra-red spectrum of compound IV ($C_{17}H_{15}NO_4$) in KBr pellet.

tical with compound III. The n.m.r. spectra of compounds II and III show the presence of three acetyl groups and seven aromatic protons, in both compounds, of an ethyl group in compound II and a vinyl group in compound III.

Both compounds II and III were obtained in yields higher than 50% (62 and 86%, respectively), thus indicating that ferroverdin must be composed of two identical C_{15} units or of two closely related C_{15} units yielding identical reduction products.

(5) *Hydrolysis of the reduction derivatives of ferroverdin.*

The u.v. spectra of the products prepared by reductive acetylation of ferroverdin (II, III, IV, V) show that all of them undergo an irreversible change when kept at room temperature in methanolic aqueous sodium hydroxide. The chemical change taking place under these conditions was therefore investigated, and two known compounds were isolated from compound II, namely, *p*-ethylphenol and

3-acetylamino-4-hydroxybenzoic acid. Whereas the first coincided in all its properties with an authentic sample of *p*-ethylphenol, the second showed a melting point 10 °C higher than that of a several time recrystallized synthetic compound

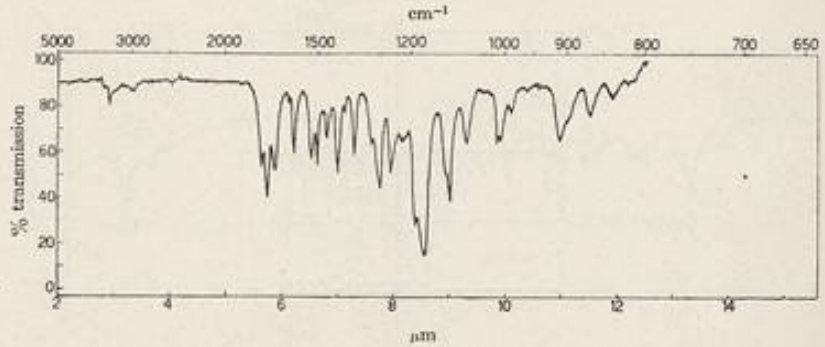


FIGURE 7. Infra-red spectrum of compound V ($C_{23}H_{17}NO_5$) in chloroform.

(needles 270-271 °C, prisms 260-261 °C, respectively). However, their u.v. and i.r. spectra were identical and after hydrolysis with hydrochloric acid both compounds yielded 3-amino-4-hydroxybenzoic acid. Furthermore the melting points, mixed

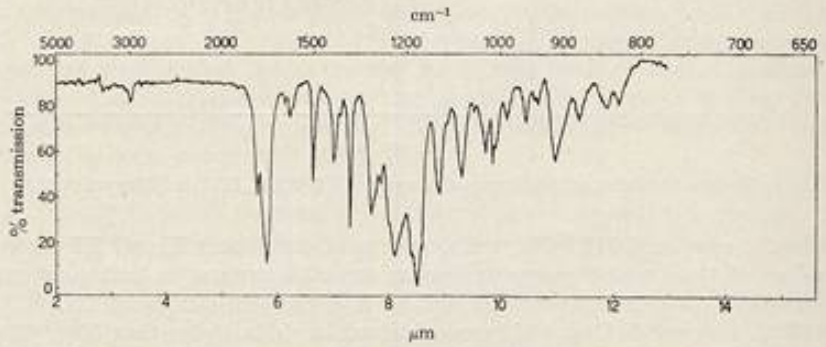


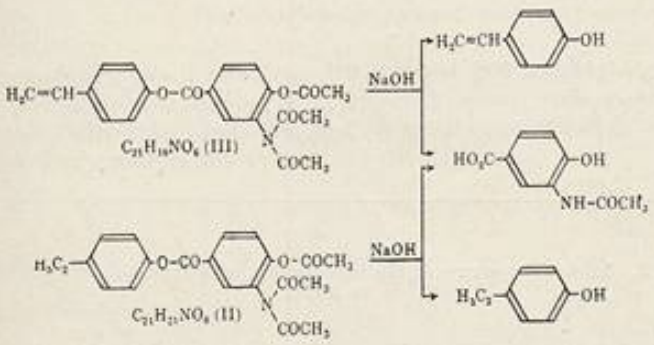
FIGURE 8. Infra-red spectrum of compound III ($C_{21}H_{15}NO_4$) in chloroform.

melting points, u.v. and i.r. spectra of their methyl ethers and methyl ether methyl esters were identical.

Compound III gave similar results, except that *p*-vinylphenol instead of *p*-ethylphenol was obtained. On hydrolysis with strong acids compound II yielded *p*-ethylphenol and 3-amino-4-hydroxybenzoic acid.

From these results and the previously discussed spectral properties of compounds II and III, reasonable structures could be assigned to these reduction derivatives of ferroverdin, namely those of the *p*-ethylphenylester and *p*-vinylphenyl-

ester respectively of 3-diacetyl-amino-4-acetoxybenzoic acid. In consequence compound I must be the *p*-ethylphenylester of 3-amino-4-hydroxybenzoic acid, compound IV and compound V the N-acetyl derivative and N, O-diacetyl derivative respectively of the corresponding *p*-vinylphenylester. These structures were confirmed by unambiguous synthesis of II.



With the identification of the above-mentioned compounds all the carbon and nitrogen atoms of ferroverdin are accounted for. As the elementary compositions of ferroverdin and the reduction product I differ only in iron, oxygen and hydrogen content, it is clear that the essential structural features of I must be present in ferroverdin. The latter must contain a vinyl instead of the ethyl group (as clearly shown by the structure of compound III) and a nitrogen function in a higher state of oxidation which, in conjunction with the hydroxy group in *o*-position to the nitrogen function, must also be responsible for the chelation of the iron atom.

(6) *Hydrolysis of ferroverdin.*

Further evidence for the correctness of this assumption was obtained by mild alkaline hydrolysis of ferroverdin which led to the formation of *p*-vinylphenol and a green iron-containing acid. The latter was shown to be identical with the iron complex of synthetic 3-nitroso-4-hydroxybenzoic acid by u.v., visible and i.r. spectra (Figure 9), paper chromatography and paper electrophoresis. Similarly, the methyl ester of the green degradation product of mild alkaline hydrolysis of ferroverdin was shown to be identical, by the same criteria, with the iron complex of the methyl ester of synthetic 3-nitroso-4-hydroxybenzoic acid. The possibility that the oxidation state of the nitrogenous function corresponded to that of a hydroxyl amino group, suggested by the elementary formula of ferroverdin reported in previous communication (CHAIN *et al.* 1955), was excluded by demonstrating that no trivalent iron was formed during the formation of the green complex from a model nitrosophenol (α-nitroso-β-naphthol) and ferrous ions. With

ferric ions no solvent, extractable, green complex was formed and the trivalent iron was recovered quantitatively in the aqueous phase.

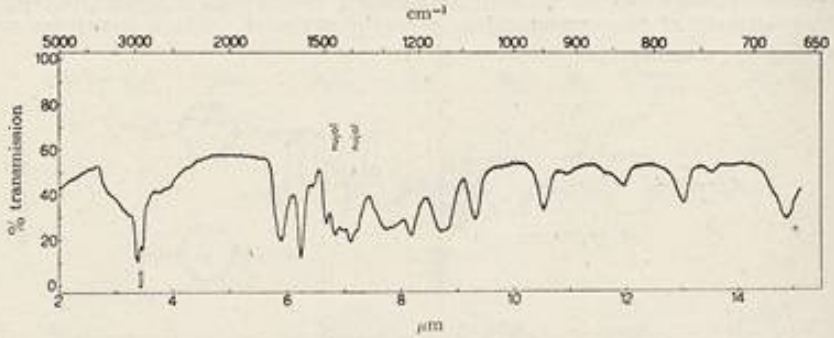
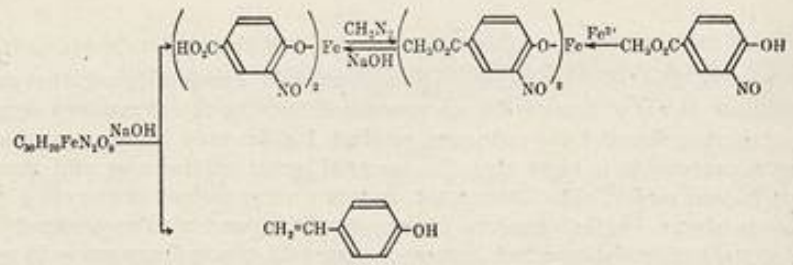
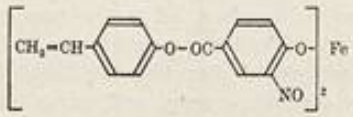


FIGURE 9. Infra-red spectrum of the iron complex of 3-nitroso-4-hydroxybenzoic acid in Nujol.



(7) *Structure of ferroverdin.*

It follows, therefore, that the structure of ferroverdin is represented by the formula :



The actual configuration of the iron and the ligand constituents is not yet defined, and several different structures can be envisaged around the ferrous ion. The final answer to this point will probably be given by electron spin resonance and X-ray crystallographic studies.

The structure of ferroverdin, like that of many microbial metabolites, has unusual features. As far as the authors are aware, it is the first nitrosophenol encountered in nature.

Synthetic green iron complexes of nitrosophenols (Baudisch & Karzeff 1912) have found industrial applications as dyes (Venkataraman 1952). Among other purposes they have been used in the manufacture of various salts from the waters of the Dead Sea as light-absorbing agents to accelerate the water evaporation. Vinylphenol has been found as a plant constituent, free in *Papaver somniferum* (Schmid & Karrer 1945), and as glucoside in *Viburnum furcatum* (Hattori & Imaseki 1959), but to the author's knowledge has not been encountered as a microbial metabolite.

The iron in ferroverdin is extremely firmly bound as a nitrosophenol complex. The capability of a natural compound of chelating iron in this manner is also unique and raises interesting biochemical and biological questions as to the function of this potent iron concentrating system and its biogenesis.

II. *Experimental*

(1) *Isolation of ferroverdin.*

The mycelium from a 50 l. fermentation tank (approximately 1.5 kg, of which the dry weight was about 10%) was centrifuged, washed on the centrifuge with 20 l. of water and mixed in a 5 l. Turmix blendor for 10 min with 1 l. of methanol/kg wet weight. The greenish brown filtrate was discarded, the mycelium then treated in the same way twice with methanol. The dark-green-coloured filtrates were combined and evaporated *in vacuo* (350 to 400 mm Hg) down to one-fifth of the original volume. On standing overnight at room temperature a semi-solid material separated. On shaking with light petroleum and filtration over a sintered plate a green powder finally resulted after thorough washing with light petroleum and air drying. The yield was on the average 800 mg.

This crude material differed from ferroverdin mainly by its much lower solubility in organic solvents, but after chromatography on alumina acquired the same properties. On degradation (reductive acetylation, hydrolysis) this crude material yielded the same products as did pure crystalline ferroverdin.

(2) *Reduction derivatives of ferroverdin.*

(*a*) *Catalytic hydrogenation of ferroverdin.* (Formation of the *p*-ethylphenyl-ester of 3-amino-4-hydroxybenzoic acid, compound I).

Ferroverdin (130 mg) was dissolved in methanol (150 ml.) and hydrogenated at 25 °C in the presence of a Pt catalyst (from 120 mg PtO_2) for 4 h. The light-brown solution was quickly filtered through cotton wool and the filtrate immediately concentrated *in vacuo* in a hydrogen atmosphere down to 50 ml.

Water (100 ml.) was added and distillation repeated until a volume of 20 ml. was reached. The turbid solution was kept overnight at 5 °C to allow crystallization, the crystals were separated by centrifugation and recrystallized first from water containing a small amount of ethanol and then from ether-light petroleum.

Light yellow prisms (61 mg ; 55% of theory), m.p. 172 to 174 °C *, were obtained. The product was soluble in methanol, ethanol, acetone, ether, chloroform, ethyl acetate and aqueous alkali, nearly insoluble in cold water and benzene, insoluble in light petroleum and carbon tetrachloride. It gave a greenish brown colour with ferric chloride, was diazotizable and coupled with diazotized sulphanilic acid. (Found—C : 70.16 ; H : 5.79 ; N : 5.36% ; calc. for $C_{15}H_{15}NO_3$—C : 70·02 ; H : 5.88 ; N : 5.44%).

Ultra-violet spectrum (ethanol-water)— $\lambda_{max.}$ (nm), at pH 5.5 : 233 ($\log \varepsilon = 4.44$), 271 ($\log \varepsilon = 4.08$), 312 ($\log \varepsilon = 3.80$) ; at pH 2.5 : 259 ($\log \varepsilon = 4.30$) ; at pH 12 : 246 ($\log \varepsilon = 4.24$), 300 (shoulder, $\log \varepsilon = 4.01$), 330 ($\log \varepsilon = 4.30$).

Infra-red spectrum (KBr) : see Figure 9.

(*b*) *Catalytic hydrogenation of ferroverdin, followed by acetylation of the hydrogenation product.* (Formation of the *p*-ethylphenylester of 3-diacetylamino-4-acetoxybenzoic acid, compound II).

Ferroverdin (14.5 mg) was dissolved in ethanol (10 ml.) and hydrogenated at room temperature in the presence of a Pd-$BaSO_4$ catalyst for 2 h. The catalyst was centrifuged off, the filtrate distilled *in vacuo* in a hydrogen atmosphere to dryness and the residue immediately refluxed with acetic anhydride (10 ml.) in the presence of anhydrous sodium acetate for 1 h. The solvent was evaporated *in vacuo*, the residue treated with water and shaken with ether (three times with 10 ml.). The combined ethereal layers were washed with water, dried and concentrated to a small volume. After addition of light petroleum until the appearance of slight turbidity and standing at — 20 °C, a crystalline compound (11.5 mg ; 61.7% of theory) separated, which could be recrystallized from ether by addition of light petroleum. In this way heavy prisms, m.p. 124 to 126 °C, were obtained. The product was insoluble in water, dilute mineral acid and alkali, very soluble in ether and chloroform. It gave no colour reaction with ferric chloride, was not diazotizable and did not couple with diazotized sulphanilic acid. The hydroxamic acid test was positive. The substance contained no O-methyl nor N-methyl groups. (Found—C : 65.95, 65.66 ; H : 5.86, 5.49 ; N : 3.69, 3.75 ; CH_3—(C) : 15.68, 15.75 ; CH_3CO— : 44.62, 44.30% ** ; mol. weight, by cryoscopy in benzene : 335, 327 ; calc. for $C_{21}H_{21}NO_6$—C : 65.78 ; H : 5.52 ; N : 3.65 ; CH_3—(C) : 15.68 ; CH_3CO— : 33.68% ; mol. weight : 383).

Ultra-violet spectrum (ethanol-water) : $\lambda_{max.}$ (nm), at pH 7 : 238 ($\log \varepsilon = 4.26$) ; at pH 2.4 : 238 ($\log \varepsilon = 4.27$) ; at pH 11.3 : 250 ($\log \varepsilon = 4.28$), 314 ($\log \varepsilon = 4.45$).

Infra-red spectrum ($CHCl_3$) : bands (C=O) at 1770, 1740 (shoulder), 1720 cm^{-1}.

Nuclear magnetic resonance spectrum ($CDCl_3$) : multiplet centred at 1.80τ (2 protons), multiplet centred at 2.70τ (5 protons), quartet centred at 7.31τ (2 protons), 7.65 and 7.69τ (only partially resolved peak, 9 protons), triplet centred at 8.74τ (3 protons).

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* All melting points (Kofler stage) are uncorrected.

** The high acetyl values (nearly one mole excess over theory) are due to the fact that under the experimental conditions of the acetyl determination method volatile *p*-ethylphenol is liberated and titrated together with the acetic acid.

(*c*) *Reductive acetylation of ferroverdin at about* 120 °C. (Formation of the *p*-vinylphenylester of 3-diacetylamino-4-acetoxybenzoic acid, compound III).

Ferroverdin (200 mg) was heated with zinc dust (3 g), acetic anhydride (75 ml.) and glacial acetic acid (7.5 ml.), in presence of anhydrous sodium acetate (1.5 g). After standing for some minutes at room temperature, the dark green solution became colourless and was then heated under reflux for 1 h. The solution was then concentrated *in vacuo* until salts precipitated, ether was added, the salts were filtered off and the filtrate distilled *in vacuo* to dryness. The residue was heated again under reflux in acetic anhydride (25 ml.) in the presence of anhydrous sodium acetate for 1 h, the solvent removed *in vacuo*, the residue treated with water (20 ml.) and shaken three times with ether (30 ml.). The ethereal solution was washed a few times with water, then dried with anhydrous sodium sulphate. The red-brown filtrate was treated with charcoal and the colourless solution then concentrated to a small volume. On standing at — 20 °C crystallization took place. The separated crystalline product was washed with a mixture of ether : light petroleum (1 : 1) and dried *in vacuo* ; yield 197 mg ; m.p. 153 to 154 °C. After concentration the filtrate gave further 24.5 mg of the same product. The total yield was 86.4 % of the theory. Recrystallization from the ether gave colourless rectangular plates (m.p. 155-156 °C), which showed birefringency in polarized light. This compound was insoluble in dilute mineral acid and alkali hydroxide, but readily soluble in ether and chloroform. In concentrated sulphuric acid it gave a colourless solution. The compound gave no colour reaction with ferric chloride, could not be diazotized, did not couple with diazotized sulphanilic acid, but gave a positive hydroxamic acid test. No active hydrogens, O-methyl, N-methyl groups were present. (Found—C : 65.94, 65.65 ; H : 4.94, 4.85 ; N : 3.59, 3.57 ; O : 25.51 ; CH_3—(C) : 11.24, 11.04 ; calc. for $C_{21}H_{19}NO_6$—C : 66.13 ; H : 5.02 ; N : 3.67 ; O : 25.17 ; CH_3—(C) : 11.82 %).

Ultra-violet spectrum (methanol-water)—$\lambda_{max.}$ (nm), at pH 7 : 247 ($\log \varepsilon = 4.47$) at pH 2.4 : 246 ($\log \varepsilon = 4.48$) ; at pH 11.3 : 250 ($\log \varepsilon$ 4.44), 314 ($\log \varepsilon = 4.42$).

Infra-red spectrum ($CHCl_3$) : see Figure 12.

Nuclear magnetic resonance spectrum ($CDCL_3$) : multiplet centred at 1.78τ (2 protons), multiplet centred at 2.70τ (5 protons), 3.02, 3.20, 3.33 and 3.52τ (1 proton), 4.18, 4.48 and 4.86τ (2 protons), 7.70 and 7.74τ (only partially resolved peak, 9 protons).

(*d*) *Catalytic hydrogenation of compound III.* (Formation of *p*-ethyl-phenylester of 3-diacetylamino-4-acetoxybenzoic acid, compound II, from *p*-vinylphenylester of 3-diacetylamino-4-acetoxybenzoic acid).

Compound III (310 mg) was dissolved in ether (150 ml.) by refluxing and then hydrogenated in presence of Pd-$BaSO_4$ as catalyst for a few hours. The catalyst was filtered off, the filtrate evaporated to a small volume. On standing at — 20 °C heavy prisms separated (m.p. 125-127 °C ; mixed m.p. with compound II showed no depression ; yield 286 mg, i.e. 91.8 % of theory) showing birefringency in polarized light.

(*e*) *Brief reductive acetylation of ferroverdin at* 20 °C. (Formation of *p*-vinylphenylester of 3-acetylamino-4-hydroxybenzoic acid, compound IV).

Ferroverdin (135 mg) and zinc dust (600 mg) were shaken at 20 °C with a mixture of acetic anhydride (10 ml.) and glacial acetic acid (0.2 ml.); the green colour disappeared (about 5 to 10 min). The remaining zinc dust was filtered off, the filtrate was poured on ice (50 to 60 g) and stirred vigorously for 1 h. The precipitated product was extracted with ethyl acetate (50 ml.), the organic layer washed with aqueous sodium bicarbonate and water, dried and evaporated to a small volume. Crystallization took place on standing at — 20 °C; yield 78.6 mg. On concentrating the filtrate gave a further 22.3 mg of the same product. The total yield was 75.2% of the theory.

Recrystallization from ethyl acetate-ether gave colourless needles (m.p. 227-228 °C), which in polarized light showed birefringency. This compound was insoluble in dilute mineral acid, sparingly soluble in ethanol, ether, chloroform, ethyl acetate, but soluble in dilute sodium hydroxide solution. The compound was soluble in concentrated sulphuric acid with intensive red colour and gave weak green colour reaction with ferric chloride. (Found—C : 68.37 ; 68.19 ; H : 5.19, 5.10 ; N : 4.70 ; 4.92% ; calc. for $C_{17}H_{15}NO_4$—C : 68.67 ; H : 5.08 ; N : 4.71%).

Ultra-violet spectrum (ethanol-water)— $\lambda_{max.}$ (nm), at pH 7 : 244 ($\log \varepsilon = 4.49$); at pH 2.4 : 244 ($\log \varepsilon = 4.50$) ; at pH 11.3 : 251 ($\log \varepsilon = 4.46$), 361 ($\log \varepsilon = 4.45$).

Infra-red spectrum (KBr) : see Figure 10.

Refluxing in acetic anhydride and anhydrous sodium acetate gave a compound (m.p. 156-157 °C), which in the mixed melting point test showed no depression with compound III.

(*f*) *Reductive acetylation of ferroverdin at* 20 °C. (Formation of *p*-vinylphenyl-ester of 3-acetylamino-4-acetoxybenzoic acid, compound V).

Ferroverdin (130 mg) and zinc dust (2 g) were shaken at 20 °C with acetic anhydride (50 ml.) for 1 h. The zinc dust was filtered off and the filtrate distilled *in vacuo* to dryness. The residue was treated with water (10 ml.) and extracted with ether (three times with 40 ml.). The combined ethereal solutions were evaporated to dryness. The residue (146 mg) could be crystallized from ether, but the product did not possess a sharp melting point (135-154 °C) and proved to be a mixture of two substances. On extracting with a small amount of hot cyclohexane a product remained, which after several recrystallizations from a large volume of cyclohexane finally gave needles with a sharp melting point (153-154 °C), which in polarized light showed no birefringency. The mixed melting point with compound III (m.p. 155-156 °C) gave a depression to 135 °C. (Found—C : 67.32, 67.02 ; H : 5.25, 5.57 ; N : 4.09%, calc. for $C_{19}H_{17}NO_5$—C : 67.25 ; H : 5.05 ; N : 4.12%).

Ultra-violet spectrum (ethanol-water)— $\lambda_{max.}$ (nm), at pH 7: 248 ($\log \varepsilon = 4.49$); at pH 2.4 : 248 ($\log \varepsilon = 4.50$) ; at pH 11.3 : 251 ($\log \varepsilon = 4.42$), 315 ($\log \varepsilon = 4.43$).

Infra-red spectrum ($CHCl_3$) : see Figure 11.

Acetylation with acetic anhydride-anhydrous sodium acetate by refluxing for 1 h, and crystallization from ether gave a product with melting point 154 to 155 °C ; the mixed melting point with compound III now gave no depression.

(*g*) *Alkaline hydrolysis of compound II.* (Formation of 3-acetylamino-4-hydroxybenzoic acid and *p*-ethylphenol).

(i) 3-*Acetylamino*-4-*hydroxybenzoic acid.*

Compound II (1 g) was dissolved in ethanol (500 ml.) by warming on the water bath and, after cooling to room temperature, 2N sodium hydroxide (500 ml.) was added. After standing at room temperature for 24 h the brownish yellow hydrolysate was brought to pH 4 to 5 by addition of dilute hydrochloric acid and concentrated in a hydrogen atmosphere *in vacuo* to a volume of 350 to 400 ml. The receiver flask contained 2N sodium hydroxide (9 ml.) in order to trap the volatile product (see paragraph *g*, v).

The pH value of the concentrated hydrolysate was again adjusted to between 4 and 5 by addition of 2N sodium hydroxide, then further concentrated to a volume of 300 ml. On standing at 5 °C overnight a crystalline product (322 mg; 63.2% of theory) separated, which could be crystallized from acetone-chloroform in fine needles (m.p. 270-271 °C). This compound showed no birefringency in polarized light; it was soluble in concentrated sulphuric acid giving a colourless solution, in aqueous sodium bicarbonate, acetone, methanol and ethanol, insoluble in water and dilute mineral acids. It coupled with diazotized sulphanilic acid to give a yellow compound which, on treatment with dilute potassium hydroxide, turned orange-yellow. The compound was not diazotizable; in methanolic solution it gave a very weak brown greenish colour with ferric chloride; the hydroxamic acid test was positive. (Found—C: 55.38, 55.44; H: 4.92, 5.00; N: 7.01, 6.80; CH_3—(C): 8.53, 8.07; CH_3—CO: 21.69, 21.62; active H: 1.284, 1.255%; calc. for $C_9H_9NO_4$—C: 55.38; H: 4.64, N: 7.17; CH_3—(C): 7.70; CH_3—CO: 22.05; active H: 1.033%).

Ultra-violet spectrum (ethanol-water)—$\lambda_{max.}$ (nm), at pH 7: 233 ($\log \varepsilon = 4.30$), 253 and 290 (shoulders); at pH 2.4: 235 ($\log \varepsilon = 4.34$), 259 ($\log \varepsilon = 4.11$), 290 (shoulder); at pH 11.3: 243 ($\log \varepsilon = 4.16$), 284 ($\log \varepsilon = 4.19$).

Infra-red spectrum (KBr): bands at 3405, *ca.* 3000 (very broad), 1728, 1669, 1600, 1548, 1500 cm^{-1}.

(ii) 3-*Amino*-4-*hydroxybenzoic acid.*

The above compound (100 mg) was heated for 2 h at 90 to 100 °C with concentrated hydrochloric acid (5 ml.). The hot solution was treated with charcoal, and the colourless filtrate on cooling gave rectangular plates (m.p. 260-265 °C, dec.: yield 23 mg, i.e. 23.8% of theory), which showed birefringency in polarized light. The mixed melting point with 3-amino-4-hydroxybenzoic acid hydrochloride gave no depression.

(iii) 3-*Acetylamino*-4-*methoxybenzoic acid methylester.*

3-Acetylamino-4-hydroxybenzoic acid (120 mg), obtained from compound II by mild alkaline hydrolysis, was dissolved in methanol (20 ml.) and after addition of an ethereal solution of diazomethane (30 ml.), the solution was allowed to stand for 20 h at 20 °C. After evaporation to dryness the residue crystallized from ether-light petroleum to give prisms (m.p. 128-130 °C; yield 108 mg, i.e. 78.8% of

theory) which showed birefringency in polarized light. No N-methyl groups were present. (Found—C : 59.01, 58.77 ; H : 6.27, 6.23 ; N : 6.45, 6.25 ; CH_3O— : 27.11, 26.85% ; calc. for $C_{11}H_{13}NO_4$—C : 59.19 ; H : 5.87 ; N : 6.27 ; CH_3O— : 27.80%). This compound proved to be identical in all its properties with a sample of 3-acetyl-amino-4-methoxybenzoic acid methylester prepared from authentic 3-acetyl-amino-4-hydroxybenzoic acid ; the mixed m.p. gave no depression.

(iv) 3-*Acetylamino-4-methoxybenzoic acid.*

The above methylation product (67 mg) was dissolved in ethanol (10 ml.) and after addition of 2N potassium hydroxide (10 ml.) the solution was allowed to stand for 15 h at 20 °C. It was then freed from ethanol by distillation *in vacuo*, shaken with ether (20 ml.) and the aqueous layer acidified with dilute sulphuric acid, when a crystalline product precipitated. This was separated, washed with water, dried *in vacuo* and on crystallization from acetone-ether gave crystals (m.p. 266-268 °C ; yield 44 mg, i.e. 70% of theory) which showed birefringency in polarized light. The product was soluble in sodium bicarbonate solution, sparingly soluble in acetone and in ether, insoluble in water and in dilute mineral acid. (Found—C : 57.47, H : 5.18 ; N : 6.62% ; calc. for $C_{10}H_{11}NO_4$—C : 57.41 ; H : 5.30 ; N : 6.70%).

This compound proved to be identical in all its properties with an authentic sample of 3-acetylamino-4-methoxybenzoic acid (Simonsen & Rau 1917) ; the mixed m.p. gave no depression.

(v) p-*Ethylphenol.*

The alkaline solution of the volatile product in the receiver flask (see paragraph *g*, i) was freed from ethanol by distillation *in vacuo*, acidified by addition of 2N hydrochloric acid (10 ml.) and shaken with ether (three times with 100 ml.). The ethereal solutions were combined, dried and concentrated to a volume of 5 ml. using a Widmer column. From this solution a crystalline compound could be isolated by gas chromatography.

The crystalline compound (m.p. 39-40 °C) was soluble in 1N sodium hydroxide and in all common organic solvents. It gave no colour reaction with ferric chloride * and coupled with diazotized sulphanilic acid in the presence of sodium hydroxide to give a violet-red colour. Its behaviour, the u.v. spectrum in different solvents and the i.r. spectrum were identical with those of *p*-ethylphenol. (Found—C : 78.62 ; H : 8.17% ; calc. for $C_8H_{10}O$—C : 78.65 ; H : 8.25%).

A spectrometric determination of the volatile compound on a large sample (1 g) showed that it was formed in a yield of 91.7% of theory.

(vi) p-*Ethylanisol.*

The volatile product (47 mg) from mild hydrolysis of compound II was dissolved in a mixture of 1N sodium hydroxide (1.18 ml.) and water (3.5 ml.) and

* *Beilstein's Handbook* (Prager, Jacobson, Schmidt & Stern 1923) reports erroneously that *p*-ethylphenol gives a blue colour with ferric chloride.

after addition of purified methyl sulphate (0.036 ml.) the mixture was shaken for 30 min, then allowed to stand for 5 h at 20 °C. After heating the mixture in a closed flask for 1 h at 90 °C and cooling to room temperature, it was shaken three times with ether (20 ml.), the ethereal layers combined, dried, concentrated using a Widmer column and chromatographed on an alumina column (length 380 mm; diameter 11 mm). On elution with ether a sample of each 10 ml. fraction was taken, diluted with ethanol and examined for a spectral change of the maximum at 279 nm on addition of alkali (1 drop of 1N NaOH into the cuvette). All those fractions which did not show a change under these conditions were combined, evaporated using a Widmer column, and the oily residue was distilled *in vacuo* (b.p. 50-60 °C; 0.1 mm Hg). The colourless liquid was shown by u.v. and i.r. spectroscopy to be identical with *p*-ethylanisol.

(*h*) *Acid hydrolysis of compound II.* (Formation of 3-amino-4-hydroxy-benzoid acid and *p*-ethylphenol).

Compound II (80 mg) was dissolved in ethanol (30 ml.) and after addition of 10N sulphuric acid (30 ml.) refluxed for 10 h. For trapping of volatile products the condenser was connected through a tube with a 2N potassium hydroxide solution (10 ml.). After hydrolysis the solution was diluted with water (50 ml.) and concentrated *in vacuo*. The receiver flask contained the same solution of 2N potassium hydroxide used before for trapping the volatile products. On concentration of the hydrolysate a colourless clear solution was obtained, the u.v. spectrum of which at different pH values was similar to that of 3-amino-4-hydroxybenzoic acid. The content of the receiver flask was freed *in vacuo* from ethanol, acidified, shaken three times with ether (20 ml.) and the amount of *p*-ethylphenol in the combined and dried ethereal layers determined spectrophotometrically (yield 23.8 mg, i.e. 96.5% of the theory). The yield of 3-amino-4-hydroxybenzoic acid formed by acid hydrolysis of compound II was accurately determined by refluxing compound II (10 mg) with 6N hydrochloric acid (10 ml.); after dilution of the solution to 100 ml. it was concentrated *in vacuo* to remove completely the *p*-ethylphenol, and u.v. spectra at different pH values were determined. These were identical with the u.v. spectra of an authentic sample of 3-amino-4-hydroxybenzoic acid. From the spectrophotometric data a yield of 98% was calculated.

(*i*) *Alkaline hydrolysis of compound III.* (Formation of 3-acetylamino-4-hydroxybenzoic acid and *p*-vinylphenol).

Compound III (200 mg) was dissolved in ethanol (100 ml.). After addition of 2N sodium hydroxide (100 ml.) the solution was allowed to stand for 6 h at room temperature, then brought to pH 5 by addition of dilute hydrochloric acid and concentrated under hydrogen *in vacuo* to a volume of 70 ml. The receiver flask contained 0.5N potassium hydroxide (40 ml.) in order to trap the volatile product (see below). The concentrated hydrolysate, from which during distillation a small quantity of a solid product was precipitated, was then shaken twice with a mixture of chloroform and methanol (5 : 1, 60 ml.), twice with a mixture of ether and methanol (5 : 1, 60 ml.), and twice with ether (50 ml.). All organic layers were combined, dried, treated with charcoal and brought to dryness *in vacuo*. The re-

sidue was crystallized from ether (yield 50.3 mg, i.e. 49.1 % of theory). Recrystallization from acetone-chloroform or from dioxan gave needles with melting point 272 to 274 °C, which was not depressed on admixture with the nitrogen-containing hydrolysis product from compound II. The u.v. spectra at different pH values were also identical. The alkaline solution of the volatile product in the receiver flask (see above) was freed from ethanol by distillation *in vacuo*, then acidified with dilute hydrochloric acid and shaken three times with ether (50 ml.). The combined, dried ethereal solutions gave, on hydrogenation with Pd-$BaSo_4$ catalyst, a volatile compound, which by its behaviour and u.v. spectra in different solvents was shown to be identical with *p*-ethylphenol. Spectrophotometric measurements showed that before hydrogenation the above ethereal solution contained at least 35 mg *p*-vinylphenol, that is 55.5 % of theory. In another experiment the ethereal solution of the volatile, non-hydrogenated compound was evaporated under a Widmer column. The oily residue polymerized on distillation *in vacuo*. Only one drop of distillate was obtained which was used for u.v. and i.r. spectroscopy.

Ultra-violet spectrum (ether): $\lambda_{max.}$ (nm) 261, 295.

Ultra-violet spectrum (water): $\lambda_{max.}$ (nm), at pH < 2: 256, 290 (shoulder); at pH > 10: 282.

Infra-red spectrum (liquid): bands at 3300 (OH, broad) 1610, 1590, 1515, 838 (aromatic), 1630, 990, 902 (vinyl) cm^{-1}.

The compound gave, furthermore, the same colour reactions reported by Schmid & Karrer (1945) for *p*-vinylphenol.

(*j*) *Synthesis of compound II.* (*p*-ethylphenylester of 3-diacetylamino-4-acetoxybenzoic acid).

To 3-amino-4-hydroxybenzoic acid hydrochloride (994 mg) was added acetic anhydride (50 ml.) and anhydrous sodium acetate (1 g), and the mixture was heated under reflux for 1 h. The solvent was evaporated *in vacuo* to dryness and the residue treated twice with ethyl acetate (30 ml.). The suspension was filtered, the filtrate was evaporated *in vacuo*, the residue dissolved in thionyl chloride (10 ml.) and heated under reflux in water bath for 40 min. The excess of thionyl chloride then was evaporated and a solution of *p*-ethylphenol (640 mg) in pyridine (10 ml.) was added to the residue. After standing for 1 h at room temperature the reaction mixture was poured in small portions into ice-cold hydrochloric acid (30 ml.; 12.5 %) and extracted three times with ether (40 ml.). The combined ethereal layers were shaken with a saturated aqueous solution of sodium bicarbonate, dried, treated with charcoal and evaporated to dryness. Excess *p*-ethylphenol in the residue was removed by distillation *in vacuo* (0.1 mm Hg; 90 to 100 °C). The odourless residue was taken up in acetic anhydride (30 ml.), anhydrous sodium acetate (0.5 g) added and the mixture was heated under reflux for 1 h. The solvent was evaporated *in vacuo*, the residue treated with water (10 ml.) and shaken three times with ether (40 ml.). On concentration to a small volume, the combined ethereal layers, after drying and treatment with charcoal, gave a crystalline compound (yield 310 mg). Recrystallization from ether gave heavy prisms with melting point 124 to 126 °C, which on admixture with compound II

showed no depression. The u.v. spectrum of compound II was identical with that of the synthetic compound.

(3) *Behaviour of ferroverdin on alkali treatment.*

(1) *Formation of the iron complex of 3-nitroso-4-hydroxybenzoic acid and p-vinylphenol by alkaline hydrolysis of ferroverdin.*

(*a*) *Iron complex of 3-nitroso-4-hydroxybenzoic acid.* Ferroverdin (1.15 g) was dissolved in methanol (450 ml.) and treated under cooling and stirring with an aqueous 4.5N sodium hydroxide solution (130 ml.). After standing 3 h at room temperature the solution was neutralized with 6N aqueous sulphuric acid with cooling and kept overnight at 5 °C to allow separation of sodium sulphate. The mixture was filtered, the solids washed with methanol, and water (200 ml.) added to the pooled filtrate and washings ; after acidification to pH 5 with sulphuric acid the green solution was concentrated *in vacuo* below 50 °C. The receiver flask contained 2N sodium hydroxide (90 ml.) in order to trap the volatile product. After distillation of about 300 ml. of liquid, an equal volume of water was added to the distillation flask, and evaporation repeated ; this treatment was repeated once more. The concentrated dark solution was brought to pH 8 with sodium hydroxide and then shaken three times with an equal volume of ethyl acetate in order to remove a yellow substance. After acidification to pH 1.5 with sulphuric acid the green solution was repeatedly extracted with ethyl acetate (nearly 2 l.), until no more colour was extracted. The pooled organic layers were shaken with 0.1 M aqueous sodium bicarbonate (500 ml.), which removed all the colour from the ethyl acetate layer. The aqueous solution was separated, acidified and repeatedly extracted with ethyl acetate. The organic layers were washed twice with one-tenth volume of water (some green colour went into the aqueous phase during this operation), dried with sodium sulphate, filtered and evaporated *in vacuo* to dryness. The dark green solid was dried *in vacuo* at 110 °C over P_2O_5 (625 mg ; 86% of theory). This product gave a single green spot on paper chromatography with four different solvent systems and was suitable for preparing the methyl ester (see next paragraph). Further purification was achieved by chromatography on a cellulose column, as described below for the synthetic product. (Found—N : 6.93, 7·22% ; calc. for $C_{14}H_8FeN_2O_8$—N : 7·22%).

Ultraviolet and visible spectrum (methanol)—$\lambda_{max.}$ (nm), at pH 7 : 269 ($\log \varepsilon = 4.50$), 297 ($\log \varepsilon = 4.43$), 432 ($\log \varepsilon = 3.81$), 681 ($\log \varepsilon = 3.80$) ; at pH < 2 : 268 ($\log \varepsilon = 4.51$), 296 ($\log \varepsilon = 4.45$) ; at pH > 10 : 257 ($\log \varepsilon = 4.46$), 284 (shoulder, $\log \varepsilon = 4.39$), 327 (shoulder, $\log \varepsilon = 4.12$).

Ultra-violet and visible spectrum (water)—$\lambda_{max.}$ (nm) at pH 7: 265, 283 (shoulder), 445, 705, at pH < 2 : 268, 291 (shoulder), 445, 700 ; at pH > 10 : 259, 281 (shoulder), 450, 712.

Infra-red spectrum (Nujol) : see Figure 13.

(*b*) p-*Vinylphenol.* The volatile product trapped in the alkaline solution was identified as *p*-vinylphenol by the same criteria used for the identification of *p*-vinylphenol formed during the alkaline hydrolysis of compound III.

(2) *Synthesis of the iron complex of 3-nitroso-4-hydroxybenzoic acid.*

The synthetic iron complex of the methyl ester of 3-nitroso-4-hydroxybenzoic acid (see next paragraph) was dissolved in methanol (30 ml.) and allowed to stand for 24 h at 20 °C after addition of 2 N sodium hydroxide solution (30 ml.). The alkaline hydrolysate was freed from methanol by distillation *in vacuo*, acidified with 1 N sulphuric acid and shaken with ethyl acetate (five times with 50 ml.). The dark green combined organic layers were shaken with a saturated solution of sodium bicarbonate, the aqueous layer was separated, acidified with 1 N sulphuric acid and shaken again with ethyl acetate (four times with 30 ml.). The organic layers were combined and dried with sodium sulphate; on distillation *in vacuo* to a small volume a black-green solid precipitated. It was separated, washed with ethyl acetate, dried and dissolved in *n*-propanol-water (10 ml.; 9 : 1, v/v). This solution was put on a cellulose powder column (length 600 mm; diameter 24 mm) and eluted with the same solvent. Only one green zone appeared. The fraction containing the iron pigment was freed from propanol by distillation *in vacuo*, acidified with 1 N sulphuric acid and shaken with ethyl acetate (five times with 50 ml.). The organic layers were combined, dried and on slow distillation *in vacuo* to a small volume a black-green product separated in small grains (yield 96 mg).

On drying *in vacuo* (2 h at 100 °C and 12 mm Hg) it lost 20.1% of weight (calc. for one mole ethyl acetate: 18.6%). The melting point was higher than 300 °C. (Found—N: 7.10, 7.04%; calc. for $C_{14}H_8FeN_2O_8$—N: 7.22%).

The acid obtained by alkaline degradation of ferroverdin and the synthetic product showed identical R_F value on paper chromatography with four different solvent systems (Table 6); they also had identical paper electrophoretic mobilities, u.v. spectra at different pH values, an i.r. spectra. This water-soluble compound showed neither growth promoting nor growth inhibiting activity on *Staphylococcus aureus*, *Sarcina lutea*, *Klebsiella pneumoniae*, *Escherichia coli*, *Mycobacterium smegmatis*, *Bacillus subtilis* at concentration of 0.5 mg/ml.

(3) *Methylation of the iron complex of 3-nitroso-4-hydroxybenzoic acid.* (Iron complex of methyl ester of 3-nitroso-4-hydroxybenzoic acid).

The partially purified iron complex of 3-nitroso-4-hydroxybenzoic acid (625 mg) prepared from ferroverdin was dissolved in a mixture of methanol (450 ml.) and ether (50 ml.). The green solution was cooled at 5 °C (methylation at room temperature caused degradation of the iron complex) and treated with a large excess of an ethereal diazomethane solution. After 2 hours' standing at 5 °C the solution was quickly evaporated *in vacuo* at room temperature. The black-green solid was dissolved in ethyl acetate, dried over sodium sulphate, and recrystallized after chromatography on alumina, as described below for the synthetic product. The yield was 420 mg (62.5% of theory). (Found—C: 45.90; H: 2.90; Fe: 13.63; N: 6.45%; calc. for $C_{16}H_{12}FeN_2O_8$—C: 46.18; H: 2.90; Fe: 13.42; N: 6.73%).

Ultra-violet and visible spectrum (methanol)— $\lambda_{max.}$ (nm), at pH 7: 270 ($\log \varepsilon = 4.53$), 298 ($\log \varepsilon = 4.47$), 434 ($\log \varepsilon = 3.84$), 677 ($\log \varepsilon = 3.85$); at pH < 2: 270 ($\log \varepsilon = 4.53$), 298 ($\log \varepsilon = 4.47$); at pH > 10: 270 ($\log \varepsilon = 4.53$), 298 ($\log \varepsilon = 4.47$).

Infra-red spectrum (KBr): band (C=O) at 1705 cm^{-1}.

(4) *Synthesis of the iron complex of the methyl ester of 3-nitroso-4-hydroxybenzoic acid.*

The nitrosation of methyl *p*-hydroxybenzoate was made following a method described by CRONHEIM (1947) for phenols. Finely powdered methyl *p*-hydroxybenzoate (15 g, 0.1 mole) was suspended in acetic acid (30 ml.) and water (50 ml.). By addition of sufficient sodium acetate the mixture was adjusted to pH 4.2. A solution of sodium nitrite (17.3 g, 0.25 mole) and crystalline cupric sulphate (12.5 g, 0.05 mole) in water (500 ml.) was prepared and added to the above mixture. After vigorous stirring the mixture for 4 days at room temperature a red-violet copper salt crystallized on top of the suspended white starting material. It was filtered off, washed with water, dried *in vacuo* (yield 16.6 g), suspended in 1 N sulphuric acid (100 ml.) and shaken 4 or 5 times with light petroleum (150 ml. each time) until the suspension of the red-violet copper salt became colourless. Filtration and drying of the insoluble part gave unchanged methyl *p*-hydroxybenzoate (11.5 g). The greenish layers of light petroleum were combined, washed twice with water (10 ml.) and shaken for some minutes after addition of water (20 ml.) and 1% ferrous sulphate solution (1 ml.). The aqueous layer became dark green and a product precipitated. The treatment with the ferrous sulphate solution was repeated until a sample of the organic layer did not show green colora-

Table 6. — PAPER-CHROMATOGRAPHIC AND PAPER-ELECTROPHORETIC MOBILITIES OF FERROVERDIN, AND OF THE FERROUS COMPLEXES OF 3-NITROSO-4-HYDROXYBENZOIC ACID AND OF THE METHYL ESTER OF 3-NITROSO-4-HYDROXYBENZOIC ACID

compound	chromatographic mobility (R_F)				electrophoretic mobility (cm/h travelled toward anode)
	solvent no. 1	solvent no. 2	solvent no. 3	solvent no. 4	
ferroverdin	0·93	0·95	0·91	0·94	0
ferrous complex of 3-nitroso-4-hydroxybenzoic acid, synthetic	0·40	0·62	0·65	0,60	7·7
same, from alkaline degradation of ferroverdin	0·40	0·62	0·66	0·60	7·7
ferrous complex of the methyl-ester of 3-nitroso-4-hydroxybenzoic acid, synthetic	0·93	0·90	0·86	0·94	0
same, from the acid obtained by alkaline degradation of ferroverdin	0·93	0·90	0·86	0·94	0

tion on further shaking with the ferrous sulphate solution. The aqueous layer was then filtered, the black-green residue washed with water and dried *in vacuo*. This product was dissolved in ethyl acetate (10 ml.) and adsorbed on alumina (column length 250 mm ; diameter 12 mm) and eluted with absolute ethanol. Only one green zone could be eluted.

This eluate was filtered and concentrated *in vacuo* to a volume of 10 ml.; after addition of benzene (50 ml.), the solution, on slow evaporation *in vacuo* almost to dryness, gave a crystalline compound with a melting point above 300 °C; yield 131 mg. (Found—N: 6.50, 6.32%; calc. for $C_{16}H_{12}FeN_2O_8$—N: 6.73%).

The methyl ester of the acid obtained by alkaline degradation of ferroverdin and the synthetic iron complex of the methyl ester of 3-nitroso-4-hydroxybenzoic acid showed identical R_f values on paper chromatography with four different solvent systems (Table 6); they also had identical paper electrophoretic mobilities, u.v. spectra at different pH values, and i.r. spectra.

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TABLE

TABLE I.

Fig. 1 *a*. Colony of *Streptomyces* sp., strain A-305 ; aerial mycelium. (Magn. × 10).

Fig. 1 *b*. Same colony as in figure 1 *a* ; green vegetative mycelium. (Magn. × 10).

Fig. 2. Sporophores and spores of *Streptomyces* sp., strain A-305. (Magn. × 2000).

Fig. 3. Spores of *Streptomyces* sp., strain A-305. (Electron micrograph, magn. × 20000).

Fig. 4. Pellets of 4 days old *Streptomyces* sp., strain A-305, grown in submerged culture in standard medium. (Magn. × 10).

Fig. 1 *a*

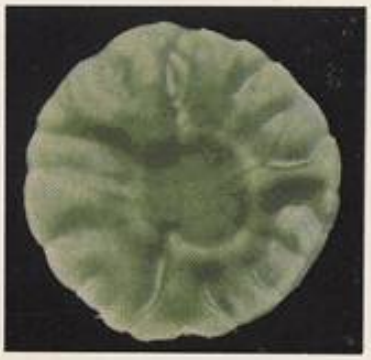

Fig. 1 *b*

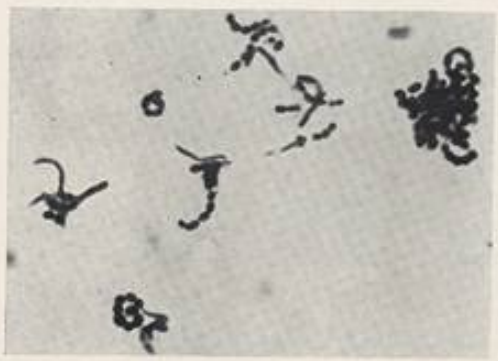

Fig. 2

Fig. 3

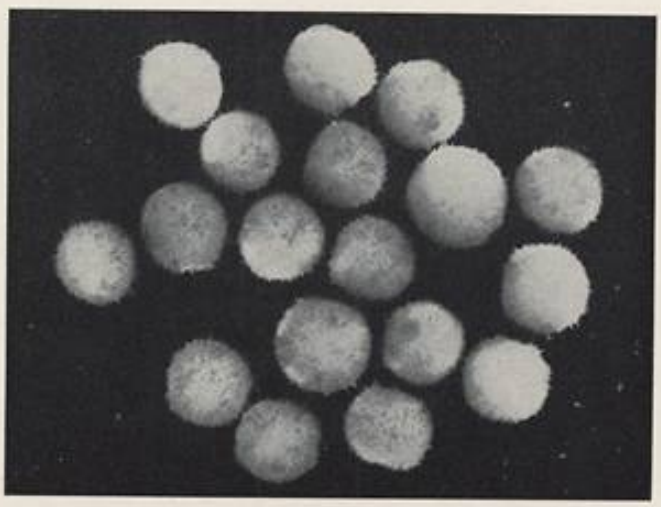

Fig. 4