[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

A Crystalline Organo-iron Pigment from a Rust Fungus (*Ustilago sphaerogena*)[1]

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A crystalline organo-iron pigment has been isolated from the cells of the rust fungus *Ustilago sphaerogena*. The organism was cultured by large-scale fermentation and the pigment extracted from the cells with dilute alkali. Ammonium sulfate was used to deproteinize the extract, the pigment was taken into benzyl alcohol and crystallized from methanol. The recrystallized material was homogeneous on chromatography and specific extinction coefficient tests. The presence of iron was proven by emission spectrography and analyses. In aqueous solutions, *p*H 4 to 10, there is a broad absorption band with a maximum at 425 mμ. A negative hemochrome test, in addition to other evidence, shows the substance does not have the nature of a heme compound. It is proposed to name the substance "ferrichrome."

The work of Weisel and Allen[3] established that the rust fungus *Ustilago sphaerogena* could be propagated on several types of synthetic and natural culture media. These media produced cells that were white in color. However, they made the interesting observation that when cultured in a medium containing yeast extract, the organism became pink and the spectrum of cytochrome-*c* could be observed directly in a suspension of the cells. Finally, the presence in the pink cells of a type of cytochrome-*c*, which in some respects resembles and in other respects differs from the cow or horse heart product of Theorell,[4] was proven by the isolation of the hemoprotein.[5]

We found that high cytochrome production in the rust fungus was accompanied by the formation of a non-protein pigment. The following method was used for the isolation of this substance. An alkaline extract of the cells was deproteinized with ammonium sulfate and the pigment extracted with benzyl alcohol. A large volume of ether and a small volume of water were added to the benzyl alcohol in order to obtain the substance in aqueous solution. The latter was evaporated to dryness and the substance then crystallized from methanol. It is yellow in dilute and orange-red in concentrated solution. Emission spectrography and iron analysis showed it to be an organo-iron compound. All the available evidence indicates it is not directly related to the porphyrin series. Undoubtedly the main interest in this material, at least from the biochemical point of view, rests on the fact that it contains iron. Therefore, it is proposed to name the substance "ferrichrome."[6]

Experimental

Fermentation.[7]—The organism was cultured in a large-scale fermentation apparatus[8] equipped with attachments for agitation, aeration and temperature control. The medium used was 200 l. of tap water containing 1.0% glucose and 1.8% Difco yeast extract.[9] The tank and medium were autoclaved for one hour at 15 lb. pressure. One liter of a 24-hour culture of the organism in the same medium was used as inoculum. Aeration was maintained at the maximum possible rate that would not cause foaming. After incubation for 36 hours at 25°, the broth was pumped out into a holding tank. Dry weight (100°) determinations on small aliquots showed this broth to contain 2.0–2.4 kg. of dry cells.

Isolation.—The broth was thoroughly mixed with 7 kg. of Filter-cel and pumped through a filter press. Water was added to the cake to give a fluid suspension, the final volume of which was about 40 l. *N* NaOH was added to bring the *p*H to 10 and the mixture stirred mechanically for 4 hours, NaOH being added from time to time to maintain the *p*H in the region of 10. The mixture was again pumped through the filter press and this time the clear, brownish extract was saved.

The *p*H of the extract was reduced to 7 with sirupy phosphoric acid and 480 g. of technical ammonium sulfate added per l. of solution. The preparation was left at 5° for 3–4 hours, after which a white precipitate could be removed by filtration through fluted papers. The filtrate was completely saturated with ammonium sulfate and allowed to stand at 5° overnight. Next morning a further protein precipitate was removed by filtration to give about 20 l. of a clear orange-colored solution. This solution was extracted with two one-liter portions of benzyl alcohol, the benzyl alcohol layers were combined and washed with two 200-ml. portions of water. Six liters of diethyl ether and enough water to give a volume of about 300 ml. in the aqueous phase were then added to the washed benzyl alcohol solution. After vigorous shaking the aqueous layer was drawn off and replaced with fresh water. A total of three extractions were made so that a combined volume of about 1 l. of aqueous solution, containing the pigment, was obtained. The combined aqueous extract was shaken with 300 ml. of diethyl ether and the ether phase discarded. The aqueous solution was then concentrated to dryness under reduced pressure at 25°.

Crystallization.—The dry powder was briefly refluxed in 300 ml. of anhydrous methanol, filtered and the filtrate concentrated slightly on the steam-bath. The ferrichrome began to crystallize at once in long, yellow needles (Fig. 1). A second crop of crystals was obtained by concentration of the mother liquor. The combined yield was 0.8–1.0 g. After three recrystallizations from anhydrous methanol the material was dried at 110° under reduced pressure. The yield on each recrystallization was 85–90%.

Purity.—The compound shrank and blackened at 240–242° (uncor.) without melting. In most preparations the first crystals gave weak tests for phosphorus but the latter could be removed by three recrystallizations. $E^{1\%}_{1\,cm.}$ at 425 mμ in methanol was 39.5, 39.4 and 39.8 on three successive recrystallizations from methanol. The recrystallized material was further examined for purity by chromatography on a 15 × 10 cm. column of activated Florosil. Exactly 10 mg. of ferrichrome was dissolved in 10 ml. of anhydrous methanol and the solution allowed to percolate

(1) Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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(3) P. Weisel and P. Allen, unpublished experiments.

(4) H. Theorell, THIS JOURNAL, **63**, 1804, 1812, 1818, 1820 (1941).

(5) J. B. Neilands, *J. Biol. Chem.*, in press.

(6) Ferrichrome may be related to the recently described "Coprogen" (C. W. Hesseltine, C. Pidacks, A. R. Whitehill, N. Bohonos, B. L. Hutchings and J. H. Williams, THIS JOURNAL, **74**, 1362 (1952)). These workers have also isolated another substance which has a similar infrared spectrum and a similar chemical composition to ferrichrome (personal communication). They found all three of these preparations to be highly active in promoting growth of the fungus *Pilobolus*. While our compound could be designated as another "coprogen," we prefer the name "ferrichrome" on the grounds that it describes the most interesting property of the new material, namely, the fact that it contains iron.

(7) G. Tener kindly assisted with the fermentation process.

(8) J. Stefaniak, F. B. Gailey, C. S. Brown and M. J. Johnson, *Ind. Eng. Chem.*, **38**, 666 (1946).

(9) Obtained from Difco Laboratories, Detroit, Michigan.

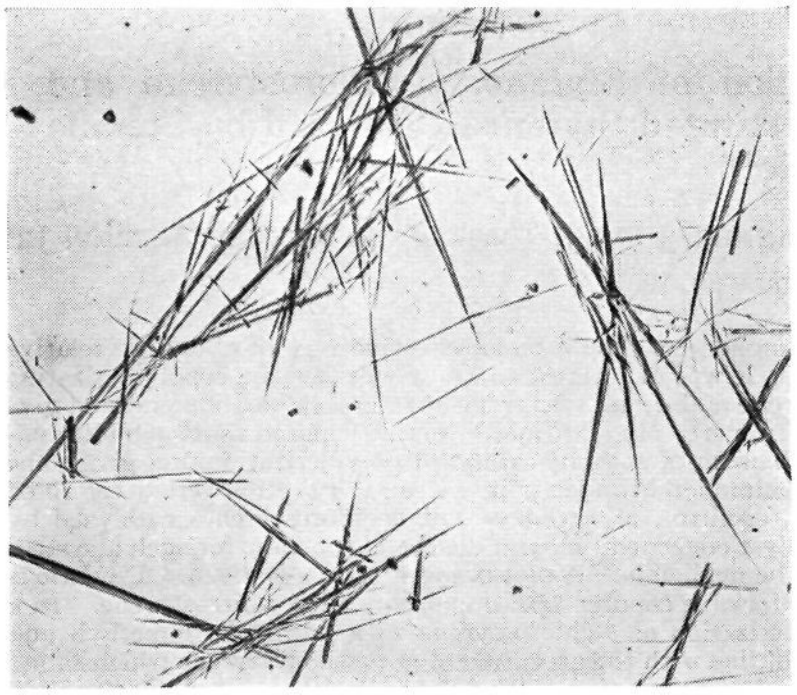

Fig. 1.—Crystalline ferrichrome from methanol.

into the column. On continued washing with anhydrous methanol the substance migrated slowly down the column. Only a single band was detected by both visual and ultraviolet examination. Ferrichrome is not fluorescent. The colored zone in the effluent was collected separately and concentrated slightly on the steam-bath to yield 9.4 mg. of crystals with $E^{1\%}_{1\text{ cm.}}$ at 425 mμ 39.4.

Composition.[10]—The substance was hygroscopic and had to be dried to constant weight at 110° under reduced pressure before analysis. One sample, after drying, was allowed to gain to constant weight and then redried. The loss in weight was 7.50%.

Anal. C, 44.02; H, 5.90; N, 16.55; Fe, 7.35; ash, 11.0; OMe, nil; mol. wt. by diffusion,[11] 700–800.

The elementary analyses suggest the formula $C_{27-29}H_{42-46}O_{12}N_9Fe$.

Physical and Chemical Properties.—Ferrichrome is freely soluble in water and hot methanol. It is sparingly soluble in ethanol, acetone, diethyl ether, chloroform and organic solvents in general. The isolated material has no acidic or basic properties both before and after crystallization from methanol. Electrometric titration[12] in distilled water failed to reveal a dissociation constant in the *p*H range 2.5–9.5.

The spectrum shown in Fig. 2 is that obtained in aqueous buffer solutions, *p*H 4–10, with the Beckman model DU spectrophotometer. In methanol the visible absorption is the same. The spectrum of aqueous solutions is stable indefinitely at room temperature and is not sensitive to light, $E^{1\%}_{1\text{ cm.}}$ (425 mμ) 39.6. The infrared spectrum[13] (mull) suggested the presence of amino or amide groups but did not allow the positive identification of any structural feature of the compound. Treatment with sodium hydrosulfite or oxalic acid completely decolorizes the compound.

Treatment with pyridine, alkali and sodium hydrosulfite fails to give a hemochrome test. Further evidence that ferrichrome is not closely related to the porphyrin series is found by inspection of Fig. 2. The Soret band of any heme compound is so intense that it is unmistakable, even when the heme is diluted out with some 20 times its weight of protein.

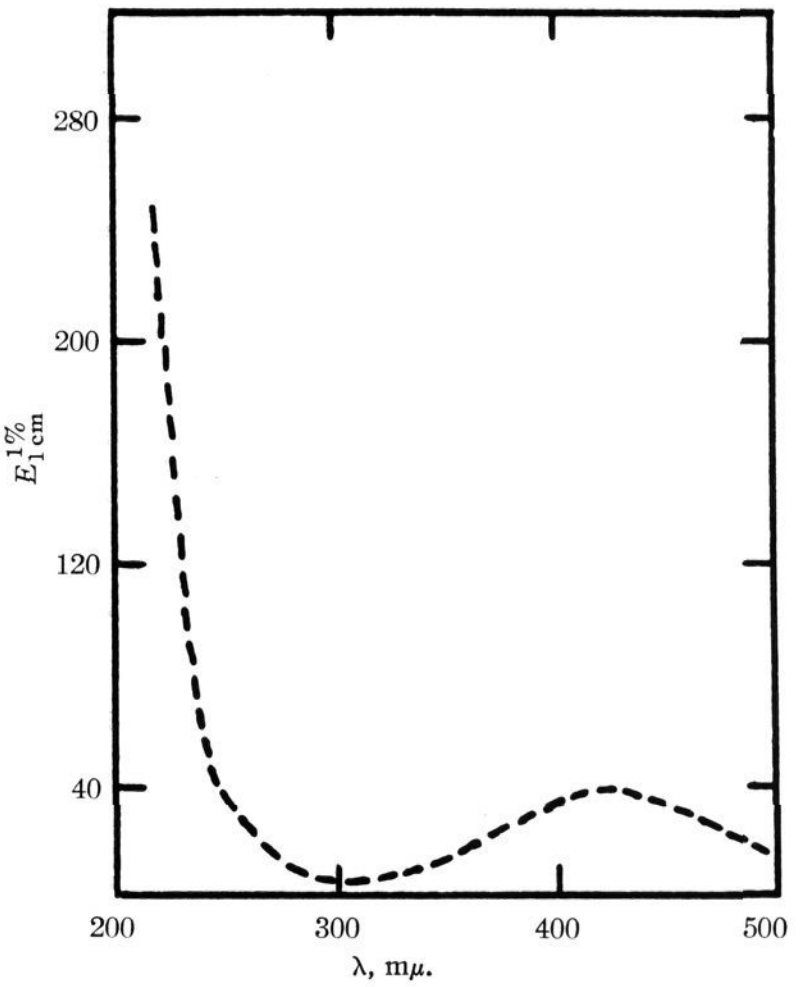

Fig. 2.—Absorption spectrum of ferrichrome in distilled water.

The structure of ferrichrome is under investigation.

Biological Properties.—Ferrichrome has no antibiotic activity against *S. aureus* or *B. subtilus*.[14] It was tested for a possible effect in the oxidation of glucose, succinate and ascorbic acid (cytochrome oxidase assay[15]). Both rat liver homogenates and cell homogenates of *U. sphaerogena*, the latter grown with and without iron in the medium, were used as enzyme sources. In these tests ferrichrome showed no activating or poisoning effect. The possibility exists, of course, that the substance represents a natural precursor or decomposition product of one of the hemoproteins.

It was mentioned above that the Lederle[6] group found ferrichrome to stimulate growth of the fungus *Pilobolus*. Since this organism has an absolute requirement for these organo-iron pigments, it might be easier to find their mechanism of action by using *Pilobolus*.

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(10) Microanalyses by Micro-Tech Laboratories and Clark Microanalytical Laboratories.

(11) J. H. Northrop and M. L. Anson, *J. Gen. Physiol.*, **12**, 543 (1929).

(12) Performed by R. M. Bock.

(13) Performed by D. Johnson.

(14) Performed by M. Larson.

(15) W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques," Burgess Publishing Co., Minneapolis, Minn., p. 139.