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Isolation and Properties of Ferrichrome A

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A second iron-binding compound has been isolated in crystalline form from the smut fungus, *Ustilago sphaerogena*. When this organism is grown on a chemically defined medium in the absence of added iron, several iron-binding substances can be detected in the culture medium. The major component, as shown by paper chromatography, has been purified and crystallized. Since it is related to ferrichrome, the first such chelate isolated as a product of this organism's metabolism, it is proposed to name the new compound "ferrichrome A."

The concerted efforts of research groups both in this country and abroad have led to a general understanding of the route of porphyrin biosynthesis,[1] and recent work with cell-free systems will no doubt result in major advances in the mechanism of protein formation.[2] Little is known, however, of the pathway of metabolism of iron, the third and very important constituent, of iron porphyrin proteins.

The smut fungus, *Ustilago sphaerogena*, has been shown to form large quantities of a type of cytochrome-*c*.[3a,b] This property, combined with the general advantages of the use of microörganisms in metabolic studies, renders this organism a particularly valuable tool in the study of the role of iron in metabolism.

The isolation and general properties of ferrichrome, one of the iron chelates produced by *U. sphaerogena*, have been recently described.[4-8] Like coprogen[9,10] and the "terregens factor,"[11] it acts as a growth stimulant for *Pilobolus* and *Arthrobacter* species.

We have found that when *U. sphaerogena* is cultured in a medium without added iron, *i.e.*, sub-optimal amounts, several iron-binding substances are found in the cell free medium. The iron addition compounds of these chelates are quite highly pigmented and are therefore easily detected by paper chromatography. The major component appearing on these chromatograms has been crystallized from water in the form of regular plates. Since this substance contains iron, ornithine and glycine, it is obviously related to ferrichrome, and has therefore been designated "ferrichrome A."

However, the new compound differs from ferrichrome in the following respects: it contains serine in addition to those amino acids found in ferrichrome; it has less nitrogen and iron, is very soluble in methanol and sparingly soluble in water. Ferrichrome, on the other hand, is very soluble in water and is crystallized from methanol. Furthermore, the two substances are easily separable by paper chromatography. The R_f values for ferrichrome and ferrichrome A are 0.40 and 0.51 with butanol–acetic acid–water and 0.68 and 0.52 with 80% methanol as the developing solvent with the paper buffered at *p*H 7.0 with 0.1 *M* phosphate buffer. Another striking difference is the acidity of ferrichrome A in contrast to the neutrality and lack of buffering capacity of ferrichrome in the *p*H range of 2.5 to 9.5. The ultraviolet and infrared spectra also show distinct differences to those of ferrichrome.

The composition of coprogen,[9,10] the iron-containing compound of the Lederle group, differs from that of ferrichrome A. It contains more carbon and hydrogen, less nitrogen than either ferrichrome and its iron content is intermediate between that of the two ferrichromes. The distinctive solubility characteristics of coprogen are reflected in its behavior on paper chromatography. The R_f of coprogen with 80% methanol and buffered paper is 0.80, greater than that of either of the ferrichromes.

Terregens factor differs from all of the above substances in that it contains a large number of amino acids.[11]

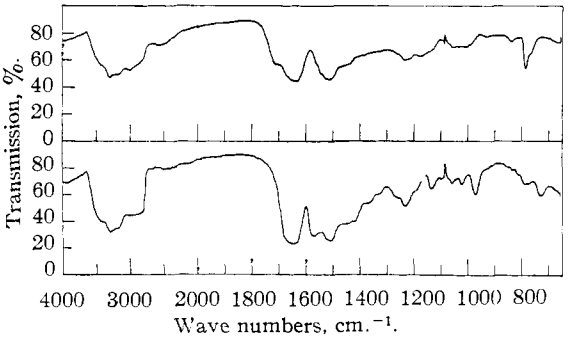

Fig. 1.—Infrared spectra of ferrichrome and ferrichrome A: top curve, ferrichrome A; bottom curve, ferrichrome.

Experimental

Production.—Ferrichrome A is produced when *U. sphaerogena* is grown on a modified Grimm–Allen medium[12] without added iron. The composition of the medium is as follows: potassium sulfate, 1.0 g.; dipotassium phosphate, 3.0 g.; ammonium acetate, 3.0 g.; sucrose, 20.0 g.; citric acid, 1.0 g.; thiamin, 2.0 mg.; trace elements to give in mg. per l. copper, 0.005; manganese, 0.035; zinc, 2.0; magnesium, 80.0. The *p*H is adjusted to 6.8 with concentrated ammonium hydroxide and the volume made to one liter with distilled water.

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The medium is dispensed in 250-ml. aliquots in liter erlenmeyer flasks. After sterilization at 15 lb. steam pressure for 15 minutes, the medium is inoculated with 1 ml. of a one day culture grown on similar medium. The cultures are then incubated at 30° on a rotary shaker.

Appearance of the iron-binding compounds is followed by determining the increase in optical density at 425 mμ upon the addition of iron to the cell free medium. That is, the culture is centrifuged, and one ml. of the clear supernatant is added to 3 ml. of 0.1 *M* acetate buffer *p*H 4.6. To this is added 1 ml. of a solution of ferrous sulfate containining 1 mg. iron/ml. This is read at 425 mμ in a Beckman spectrophotometer using a solution of 4 ml. of the buffer and 1 ml. of the iron solution as a control. It should be noted that this method measures all iron compounds which absorb at 425 mμ.

Formation of the chelates as a function of time of incubation is shown in Table I.

TABLE I

FORMATION OF CHELATES AS A FUNCTION OF TIME OF INCUBATION

Time, hr.	48	72	96	120	144
Optical density, 425 mμ[a]	0.48	0.82	1.05	1.10	1.10

[a] Optical density per cm. Conditions for color development are given in the text.

The addition of 1.0 mg. of iron per l. of base medium prevents completely the appearance of any detectable iron binding compounds in the culture medium.

Ferrichrome A is isolated from the culture medium by a procedure similar to that utilized for ferrichrome. The culture is centrifuged to remove the cellular material. Iron in the form of ferrous sulfate is added to the supernatant until no further increase in optical density at 425 mμ is obtained. The identical compound is also isolated when ferric chloride is added. The solution is saturated with ammonium sulfate and is extracted with several aliquots of benzyl alcohol. The completeness of the extraction is followed by the disappearance of the characteristic color of ferrichrome A from the aqueous layer. The benzyl alcohol extracts are combined. Three volumes of diethyl ether and one-tenth volume of water are added. Ferrichrome A is found in the aqueous layer. The organic layer is extracted with small aliquots of water until it is free of ferrichrome A pigment. The extracts are combined, washed with diethyl ether to remove any benzyl alcohol present and are allowed to stand at room temperature where crystallization occurs.

Composition.[13]—The substance is hygroscopic and must be dried to constant weight at 100° under reduced pressure before analysis.

Anal. Found: C, 44.75; H, 5.80; N, 11.18; Fe, 5.3; ash, 7.4. Tests[14] for sulfur, phosphorus, and halogen were negative.

A sample was hydrolyzed in a sealed tube in 6 *N* HCl for 16 hours at 105°. The hydrolysate was evaporated to dryness, redissolved in water and subjected to two-dimensional paper chromatography using butanol–acetic acid and phenol–cresol systems.[15] The development with ninhydrin spray indicated the presence of glycine, serine and ornithine. Results obtained by chromatography of the 2,4-dinitrofluorobenzene derivatives substantiated these findings.[16]

Physical and Chemical Properties.—Ferrichrome A is very soluble in methanol, ethanol and propanol, to some extent in hot water, but is insoluble in acetone, petroleum ether, diethyl ether and chloroform.

Dilute aqueous solutions are acidic (*p*H about 3). Electrometric titration in distilled water at 25° in the automatic recording apparatus[17] shows a dissociation curve between *p*H 3 and 6. The neutral equivalent with *p*H 7.0 as the endpoint is 370. Buffering capacity is totally absent in the *p*H range 6 to 11.

Ferrichrome A is reduced with hydrosulfite with complete disappearance of the amber color and, like ferrichrome, is autoxidizable. Aqueous solutions are stable for at least several minutes at *p*H 11. However, in NaOH solutions more concentrated than 0.1 *N* the iron is precipitated as the hydroxide. We have not yet determined the stability constants or the metal-binding specificity of the ferrichromes. However, since ferrichrome was originally isolated from a *Ustilago* culture grown on a heterogeneous medium (yeast extract), the specificity for iron is presumably fairly high.

In 0.1 *M* phosphate buffer *p*H 7.0 ferrichrome A exhibits a broad band with a maximum at 440 mμ. Both the ultraviolet and the visible absorption are shifted to longer wave lengths as contrasted to ferrichrome. At a concentration of 0.05 mg./ml. the $E^{1\%}_{1\text{ cm.}}$ at 440 mμ in 0.1 *M* phosphate buffer *p*H 7.0 is 33.8. The infrared spectra of ferrichrome A and ferrichrome are given in Fig. 1. Both spectra were taken in a nujol mull.[18]

Biological Properties.—The *Pilobolus* strain which we have used for the microbiological tests exhibits considerable fluctuation in the level of ferrichromes required for optimum growth. However, good growth has been obtained at a level of 10 μg. of ferrichrome A per ml. in the Page medium.[19]

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