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Induction of the Macrostomal Form of *Tetrahymena vorax* by a Synthetic Ferrous Iron Chelate of Hypoxanthine and Uracil

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ABSTRACT

Tetrahymena vorax is a polymorphic ciliate capable of forming two cell types that depend on environmental cues. One type, called the microstomal form, feeds on bacteria and other small particulate matter. The other, the macrostomal cell, is a carnivore and feeds on small ciliates or flagellates. Large numbers of synchronously differentiating macrostomal cells can be induced by suspending microstomal cells in stomatin, an exudate produced by a potential ciliate prey, *Tetrahymena pyriformis*. The active principle in stomatin has been identified as a ferrous iron chelate of hypoxanthine and uracil (Smith-Somerville et al., 2000). They produced a synthetically derived chelate with strong biological activity equivalent to that of stomatin. The purpose of our study is to use *our* chemicals and *our* strain of *Tetrahymena vorax*, and following the methods of Acevedo-Chavez et al., 1996 as modified by Smith-Somerville et al., 2000 to synthesize an active chelate. Our results show that while lower levels of chelate (1, 5, and 10 ug/ml) had little or no effect on levels of differentiation, chelate concentrations in the range of 100 ug/ml induced macrostomal differentiation equivalent to 78 % of the stomatin control value. Our results confirm findings published by Smith-Somerville et al. 2000.

Key words: autophagic vesicle, differentiation, iron chelate, macrostome, microstome, stomatin.

INTRODUCTION

Tetrahymena vorax is a fresh water polymorphic ciliated protist. One form, termed the microstome (Fig. 1), feeds by accumulating bacteria and small particulate matter in phagosomes that form successively at the base of the oral apparatus. Under the right circumstances, the microstomal form differentiates into large carnivorous cells called macrostomes (Fig. 2) that is capable of feeding on small ciliates and flagellates (Buhse, 1966). In addition to the larger oral apparatus, the macrostomal form is equipped with a

large pouch into which prey is engulfed and processed by digestion. Differentiation to the macrostomal form is triggered by stomatin (Buhse, 1967), a collection of catabolic products released by a potential prey, *Tetrahymena pyriformis* (Buhse, 1965). Stomatin is heat stable, is most effective at acidic pH values, and exhibits a pale yellow color in solution (Buhse, 1966). A bioassay has been developed to test the inductive qualities of stomatin preparations. In this assay 70-90 % of microstomal cells are induced to differentiate synchronously into the macrostomal phenotype 6 to 8 hr following addition of 3 mg/ml stomatin (final concentration) (Buhse, 1967).

Recently, the active principle that initiates microstomal to macrostomal differentiation has been identified as an ferrous iron chelate of hypoxanthine (6 hydroxy-purine) and uracil (2,4-dioxyprymidine) (Smith-Somerville et al., 2000). Earlier studies by LeBoy, et al., 1967, showed that nucleotide catabolic products including hypoxanthine and uracil were excreted by *Tetrahymena pyriformis*. As pointed out by Ryals et al., 2002, it is not known whether the chelate is assembled before or after excretion or what role, if any, the complex has in iron transport or metabolism. The chelate, a paracrine released by a potential prey, is postulated to act as a ligand binding to the surface of the undifferentiated microstomal form and activating a signal transduction system leading to macrostomal differentiation (Ryals et al., 1999). A biologically active iron chelate has been synthesized using the methods of Acevedo-Chavez et al., 1996 as modified by Smith-Somerville et al., 2000. Addition of 50 ug/ml of chelate to microstomal cells induced macrostomal differentiation at levels equivalent to the stomatin control (Smith-Somerville et al., 2000). The synthetic chelate exhibits many of the same characteristics as stomatin: it is heat stable, a property of chelates (Ueno et al., 1992), active at acid pH values, and is a pale yellow color in solution. A review of *Tetrahymena vorax*, including the macrostomal induction system, has been published (Ryals et al., 2002)

The purpose of this study is to synthesize the iron chelate using *our* chemicals and test its activity against *our* strain of *Tetrahymena vorax* using the stomatin bioassay system for macrostomal induction. Positive results from these experiments will serve as a prelude to further studies aimed at delineating the stoichiometrics of the active complex. It is postulated that only certain configurations of the chelate induce macrostomal differentiation (Ryals et al., 2002).

MATERIALS AND METHODS

Cell maintenance

Tetrahymena vorax microstomal cells and *T. pyriformis* cells were grown in 50 ml of Loefer's medium [15 g proteose-peptone, 5 g bacto-casitone, 5 g glucose, 2 g NaCl, 1 g NaH_2PO_4, 1 g K_2HPO_4, 0.5 g yeast extract] (Loefer et al., 1935) contained in 500 ml Erlenmeyer flasks. Cells were grown at 20°C and sub-cultured every two days (10 mls of culture to fresh medium) following methods of Buhse, 1967.

Prepartion of stomatin

Stomatin preparation was carried out following Buhse, 1967. Fifty mls of a two-day old culture of *T. pyriformis* cells was transferred to 1 liter of Loefer's medium contained in 2.8-liter low form flasks. The inoculated cells were grown for two days (48 hr) at room temperature with moderate shaking. At the end of this time, the cells were removed by

centrifugation at 1500 RPM in a Sorvall centrifuge (model RC-5B) equipped with GSA rotor capable of holding six 250 ml plastic bottles. The concentrated cells were washed 3 times by centrifugation, suspended in a final rinse (100 ml) of double distilled water, and placed in a Roux flask over night at 20°C. The next day, the cells were removed by centrifugation (1500 RPM) in 50 ml conical centrifuge tubes using a IEC HN-2 table top centrifuge (rotor radius 10 cm). The resultant "conditioned media" was centrifuged twice more in a Sorvall RC-5B at 5000 RPM using the SS34 rotor. The cell-free liquid was concentrated by roto-evaporation to dryness, weighed, re-suspended in double distilled water, filtered using a Nalgene 0.5 um filter attached to a 10 ml syringe, and diluted to yield a final concentration of 6 mg/ml stomatin. The stomatin preparation was dispensed into 1-ml volumes (6 mg/ml) and stored frozen at minus 20°C .

Scanning Electron Microscopy

Tetrahymena cells were washed twice in 2.49×10^{-4}M sodium phosphate and 7.04×10^{-4}M magnesium sulfate buffer at pH 7.0. They were then pelleted and resuspended in 600 ul of wash buffer, 5 ul IGSS gelatin (Amersham Biosciences), 40 ug BSA (Sigma), 1.25 ul Tween 20 (Sigma), and 50 ul Sodium Azide (Sigma) and brought to volume of 5 mls. They were fixed by adding 200 ul of 2.5 % gluteraldhyde and 200 ul of a 1% osmium tetraoxide each of which was suspended in the sodium/magnesium buffer at pH 7.0. The cells and fixing solutions were mixed thoroughly, placed in a tray of ice, and microwaved (Pelco Biowave) for 30 seconds at power level 1(100 % power). The microwaved cells were left on ice for 25 min, then washed twice for 5 min each in a buffer. They were then suspended in a graded ethanol series of 10, 25, 40, 50, 65, 75, 85, and 95% followed by three washes in 100 % ethanol for 5 minutes each. A drop of the dehydrated cells was placed on a poly-L-lysine coated coverslip and critical-point dried (Balzers CPD030 critical point dryer). The dried samples were then mounted on an aluminum specimen stub with double sticky tape, coated with 15nm gold (Anatech Hummer Jr. sputter coater), and viewed using a JEOL 5600LV scanning electron microscope.

Preparation of the iron chelate

The iron chelate was prepared by the methods of Acevedo-Chavez et al., 1966, as modified by Smith-Somerville et al, 2000. Briefly, 1 mM hypoxanthine, 1 mM uracil, and 10 ml of 2 mM ferrous sulfate solution was added to 200 ml of a glycine-HCl buffer (0.084 g glycine, 0.66 g NaCl and 1.87 g HCl made up in 500 ml). The chelate solution (pH 1.5) was air dried by stirring slowly for two weeks at room temperature. The resultant residue was washed twice in cold (4°C) double distilled water and oven dried at 50°C for 2 days. Seven mg of dried chelate was dissolved in 7 ml of inorganic medium, IM (1 g Na_2HPO_4, 1 g KH_2PO_4, and 0.3 g $MgSO_4$ per liter) and the pH was adjusted to 5.7 by addition of dried Na_2HPO4. This preparation was then divided into seven 1ml samples and frozen at minus 20°C.

Experimental design

For these experiments, *T. vorax* microstomal cells were washed three times in inorganic medium and suspended in one half ml of inorganic medium contained in 15 x 1 cm Kimax culture tubes. The cells were exposed to several chemical compounds that also were dissolved in one half-ml aliquots. **(1)** Inorganic medium at pH 5.7 functioned as the negative control. **(2)** Three mg/ml stomatin final concentration functioned as the positive control (Buhse, 1967). **(3)** Various concentrations of the synthetically derived chelate (1,

5, 10, 50 and 100 ug/m final concentration) were added to the remaining culture tubes containing the cells and incubated at 20°C for 8 hr before being killed. The percentage of macrostomal differentiation in each tube was determined microscopically (Buhse,1967).

RESULTS

We determined the morphological effects of the chelate on treated populations and compared them to the negative (IM) and positive (stomatin) controls. Differences between the microstomal and macrostomal cells are easily seen in the photomicrographs (Figs. 1 & 2). The microstomal cells are elongate and small, while the macrostomal cells are larger and contain the prey receptacle which occupies over 80 % of the cell volume. Fig. 3 shows results from experiments placing microstomal cells in inorganic medium. There are very few macrostomal cells (arrows). The similar results were obtained for cells suspended in 1 ug /ml chelate, shown in Fig. 4. Three mg /ml stomatin (Fig. 5) and 100 ug/ml the chelate (Fig. 6) induce comparably higher levels of macrostomal cells. However, the levels of macrostomal induction were consistently lower than the stomatin control and values reported by Smith-Somerville et al. 2000 for comparable levels of chelate.

Next, we quantified our data by determining the percentage of macrostomal cells induced by each treatment. The data depicted in Table I show two experiments using different concentrations of the synthetic iron chelate of hypoxanthine and uracil. For trial 1, we dissolved 1000 ug of chelate in 1 ml then diluted this stock solution to 200 ug/ml. This stock was further diluted to give a final concentration of 100, 50, 10, 5, and 1 ug/ml after mixing 1:1 with washed microstomal cells. While determining the percentage of macrostomal cells, we noticed some of the chelate particles hadn't dissolved which may help to explain why we attained lower percentages of macrostomal induction than Smith-Somerville et al., 2000. In the dry state, the chelate appeared as a greenish powder, which dissolved completely prior to freezing. It is possible that upon freezing at this concentration some of the chelate precipitated. When we conducted the second experiment (shown as trial two), we dissolved 500 ug of chelate in 1 ml and diluted this stock to give the five chelate concentrations listed in Table I. Using this method, the chelate was dissolved completely and no particles were seen following thawing of the chelate. In each case, the percentage of macrostomal differentiation induced by stomatin was slightly more than 80 % while the negative control values hovered around 5 to 6 %. Table I shows (from left to right) the average results from trial 1 and 2 separately, and the combined average for the two experiments. The treatments were replicated three to five times (N) within each sample set and the experiment was repeated twice using the same batch of chelate. The standard error of the mean ($sigma_x$) was calculated for each chelate concentration tested. Finally, each treatment was normalized against the stomatin control that was arbitrarily set at 100 % differentiation. High chelate levels (50 or 100 ug/ml) of synthetically derived iron chelate promote macrostomal differentiation whereas lower levels (1 or 10 ug/ml) did not promote much macrostomal differentiation. While there is a fair amount of variability between experiments as evidenced by the relatively high standard error, it is clear that as the chelate concentration is increased progressively higher levels of differentiation are obtained culminating in 57.7 and 78.4 % of the stomatin control for 50 and 100 ug/ml, respectively.

DISCUSSION

Our findings show that the synthetically derived chelate induces levels of macrostomal differentiation within the range of the stomatin controls. The level of differentiation is positively correlated with the concentration of the chelate. Higher quantities (50 and 100 ug/ml) induce about 60 % and 80 % of stomatin controls, respectively, whereas lower quantities of chelate elicit levels of differentiation equivalent to that of cells suspended in inorganic medium. Our results are comparable with previous results (Smith-Somerville et al., 2000) that showed higher levels of chelate induced higher levels of macrostomal differentiation. In their case, 50 ug/ml induced 90 % of stomatin controls. For our experiments, 50 ug of chelate induced slightly less than 60 % of control values. These differences can be explained by the following or a combination of the following arguments as they are not mutually exclusive and may in fact be acting synergistically. Smith-Somerville et al., 2000 grew *T. vorax* microstomal cells on an iron containing medium prior to exposing them to the synthetic chelate, whereas we grew cells on Loefer's medium. They carried out their experiments at pH 5.0 while our experiments were run at pH 5.7. It has been shown that the iron chelate is most active at lower pH values than higher pH levels (Ryals et al., 2002). Thus, it is possible that at the higher pH value some of the active ferrous iron chelate was dissociated by oxidization to the ferric form resulting in a decrease of specific activity of our preparation. This conclusion may be directly tested by buffering our chelate to pH 5.0 to determine if there is a significant increase in activity. Another difference between our studies and those of Smith-Somerville et al, 2000 is that in their study the chelate was stored dry and dissolved just prior to use whereas ours was frozen immediately then thawed prior to use. One possibility is that freezing and thawing may have caused particles to precipitate (see Fig. 6) thus lowering the absolute concentration of the chelate. Finally, it is also possible that chelate preparations differ with respect to their ability to induce macrostomal differentiation. The stoichiometry of the iron chelate is not known and several arrangements could be formed. Thus, it is possible that some of these support formation of the macrostomal forms, while others support less differentiation (Ryals et al., 2002). The overall specific activity of the preparation would depend on this mixture. Therefore, and because of this, the level of activity of each batch of chelate may differ. The fact that doubling the concentration of chelate from 50 to 100 ug/ml leads to a significant increase in differentiation from 52 % to 70 % of the stomatin control supports this conclusion.

We plan further studies using the synthetic chelate approach. Using X-Ray diffraction it should be possible to determine the stoichiometry of various elements of the chelate. In anticipation of these studies, we present two possible structures of the chelate (Fig. 7). This model is consistent with coordination potential between hypoxanthine and uracil, however, several other structures are possible.

The question of when and where the chelate is formed remains to be resolved. One possibility is that the chelate forms as a by-product of autophagic vesicle activity. Autophagic vesicles are responsible for degradation of organelles and their formation is triggered by a variety of stresses including starvation (Reggiori & Klionsky, 2000; Mizushima 2007). Once produced, the digested end-products of autophagy including amino acids, proteins, fatty acids, and nucleic acids catabolic products are further hydrolyzed in the mitochondria to produce energy (Levine and Yaun, 2005).

Tetrahymena pyriformis cells form vesicles when they cease growing in organic medium as they enter stationary phase, a stage of growth. Cells enter stationary phase growth because of starvation, and/or oxygen depravation, and/or build up of toxic compounds due to metabolism of large numbers of cells maintained in batch culture within a confined space (Geise, 1962). Evidence that these vesicles are autophagic vesicles include the following observations: they are bounded by a double membrane that contains partially digested organelles and they bind a specific autophagic membrane antibody (data not shown).

We propose that these vesicles facilitate chelate formation because of their extreme internal conditions including acidic pH levels that are achieved following their fusion with lysosomes. The conditions within these vesicle are similar to those described for the production of the synthetic chelate (Smith-Somerville et al, 2000) that also requires reducing conditions and low pH values. We postulate that the small molecules formed and released from the vesicles including the chelate may easily diffuse out of the cell into the suspending medium. Once it is released from the cells, the chelate functions as a paracrine ligand that induces microstomal to macrostomal differentiation in *Tetrahymena vorax*. If this hypothesis is true, it should be possible to prevent stomatin production by blocking autophagic vesicle formation by use of inhibitors such as wortmannin or by knocking out genes that control autophagy specifically.

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Table 1. The percentage of macrostomal differentiation induced by synthetically derived stomatin and compared to stomatin control.

Chelate Conc. (μg/ml)	Trial 1 % Differentiation	N	σ_x	Trial 2 % Differentiation	N	σ_x	Trials 1 & 2 % Differentiation	σ_x	Control (Stomatin) % Differentiation
1	11.8	5	7.9	4.4	5	0.8	8.2	6.8	9.8
5	7.5	5	5.4	5.4	5	1.2	6.4	4.1	7.7
10	6.1	5	1.7	5.1	5	1.2	5.6	1.6	6.7
50	51.1	5	7.4	45.2	5	7.1	48.1	7.8	57.7
		4							
100	68.7	3	9.5	58.6	5	6.4	65.3	8.9	78.4
Stomatin*	83.5	5	2.3	83.3	4	4.8	83.4	3.9	100
I.M.	6.9	5	6.9	5.9	5	2.1	6.4	2.1	7.6

*Stomatin 3mg/ml

Fig. 1. SEM of the oral apparatus of a microstomal cell showing one of the membranelles (M) and the undulating membrane (UM). The oral apparatus functions by filter feeding on bacteria and/or small particles. A current of water containing the bacteria and produced by the membranelles is forced through the undulating membrane which acts as a baleen trapping bacteria and particles which then fall into the cytostomal region and induce food vacuole formation.

Fig. 2. SEM showing the oral apparatus of a macrostomal cell. This carnivorous cell feeds by engulfing small ciliates through its large cytostomal opening (CY). Engulfed prey organisms become trapped in the cytopharyngeal pouch (PP) which eventually forms a food vacuole were digestion occurs. Membranelles 2 and 3 (M2 and M3, respectively) are indicated on the micrograph. Unlike the microstomal cell, the membranelles of the macrostomal form appear to have no role in prey capture. The 5 um bar shown in Fig. 1 applies to Fig. 2 as well.

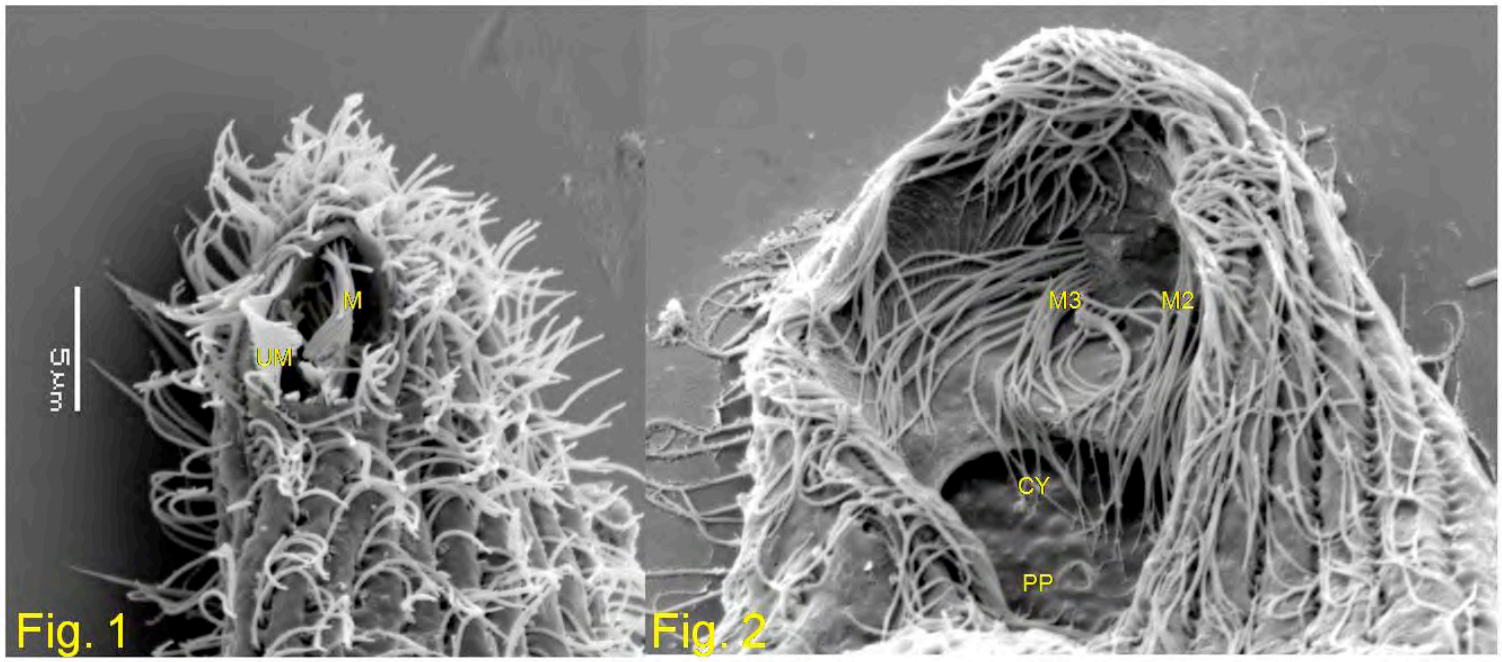


Fig. 3-6. *T. vorax* cells exposed to l ug/ ml chelate and 100 ug/ml synthetic chelate. The levels of macrostomal cells induced by inorganic medium (Fig. 3) were very low and equivalent to that induced by 1 ug/ml chelate (Fig. 4). The levels of differentiation initiated by 3 mg/ ml stomatin (Fig. 5) and 100 ug/ml chelate (Fig. 6) were similar, though the level of differentiation for chelate-treated cells were slightly lower (See Table I for a quantitative assessment). The brownish fiberous material visible in this photograph is most probably normal protein constituents of stomatin that are precipitated by Bouins fluid.

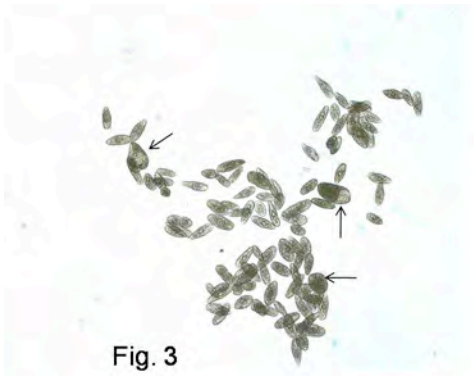


Fig. 3

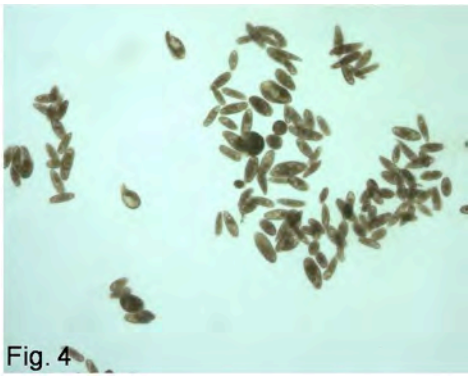


Fig. 4

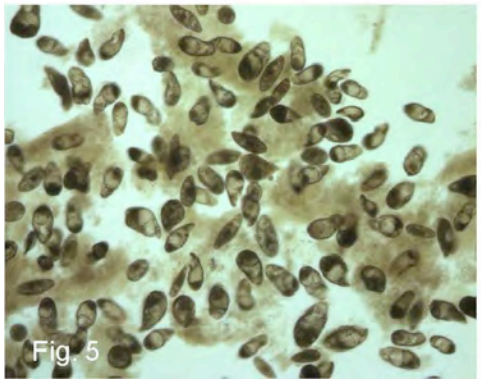


Fig. 5

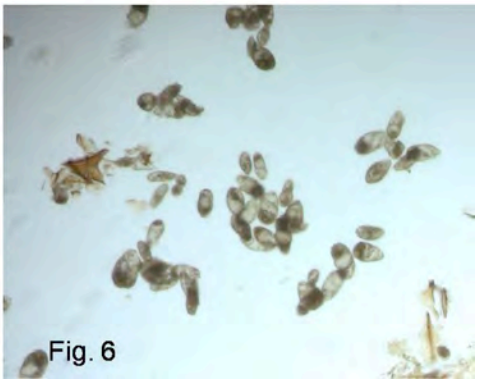
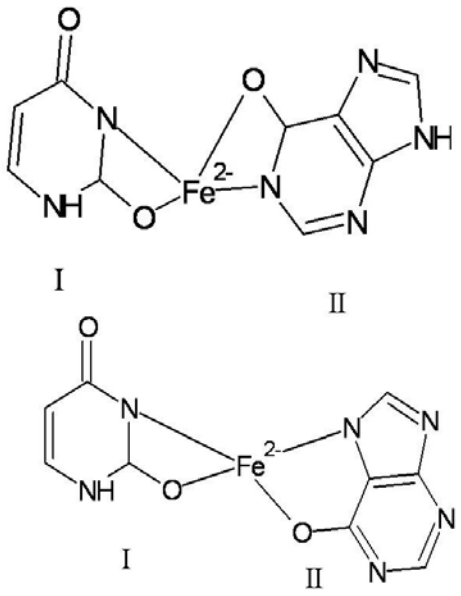


Fig. 6

Fig. 7. Possible structures of the ferrous iron chelate based on coordination potential between iron hypoxanthine and uracil. However, many other structures are possible. Please see the text for more details.



I = Uracil

II = Hypoxanthine