DUAL CAPACITY FOR NUTRIENT UPTAKE IN TETRAHYMENA IMPORTANCE OF THE ORAL UPTAKE SYSTEM FOR FE AND CU UPTAKE

by

LEIF RASMUSSEN

EDUARDO ORIAS*)

The Biological Institute of the Carlsberg Foundation
16 Tagensvej, DK-2200 Copenhagen N

*) on sabbatical leave of absence from Department of Biological Sciences, University of California, Santa Barbara, Calif. 93106, U.S.A.

Key words: Tetrahymena pyriformis. ciliate - Food-vacuole-less mutant - Uptake of Fe and Cu

We have reported that a mutant of *Tetrahymena pyriformis* with heat-sensitive development of the oral apparatus can be grown indefinitely without food vacuoles if the medium is supplemented with folinic acid and a mixture of trace metal salts. We report here that the trace metal mixture can be replaced completely and specifically by salts of iron and copper. Fe(II) and Fe(III) are interchangeable. Addition of citrate has proven useful to reduce precipitate formation and improve the reproducibility of growth of the mutant cell. Thus it appears to serve as an Fe buffer.

From the increased concentrations of Fe and Cu required to permit good growth of the mutant strain at 37°C, we conclude that the oral uptake system plays a much more important role in the case of these two metals than the surface uptake system. The oral uptake system may facilitate Fe uptake in at least two ways: a) by a mechanical concentration of precipitates affected by the ciliary membranelles surrounding the oral cavity and, b) by lowering of the pH of the food vacuole and thereby releasing Fe from precipitates and from complexes which cannot be transported across the membrane as such. The second factor may also be important in Cu uptake.

A specific effect of Mg and Fe uptake or retention in the mutant strain growing without food vacuoles has been detected. The significance of this effect remains unclear.

Practical implications of the findings are discussed.

1. INTRODUCTION

Relative roles of *oral* and *surface* uptake in the nutrition of *Tetrahymena* have posed important questions. The availability of a *Tetrahymena* mutant with a heat-sensitive capacity to form food vacuoles (12) permitted a new attack on this problem. The strain can be indefinitely propagated in growth media supplemented with vitamins and trace metals at high concentrations (15). This paper shows that the trace metals can be replaced with Fe and Cu salts; possible roles of the uptake system for the uptake of Fe and Cu are discussed.

2. MATERIALS AND METHODS

2.1 Strains

The wild type parental inbred strain D of *Tetrahymena pyriformis,* mating type III of syngen 1, and the mutant strain NPl have been used. The phenotype of strain NPl has been described previously (12). It forms no food vacuoles at 37°C.

2.2 Media

The basal medium which supports optimal growth of the *wild type* strain consists of 2% proteose peptone broth, supplemented with 36 μM Fe salts, supplied either as Fe(II) or Fe(III). This medium was routinely supplemented with 2 mM tri-sodium citrate, dihydrate, to avoid formation of precipitates, and is designated medium I or MI. In some early experiments this medium was supplemented with the vitamins of the chemically defined medium (14), either in the standard, low concentrations (vit-MI) or in the high concentrations of 1 mg/l (Vit-MI).

The medium which supports growth of the mutant cell, NPl, at the restrictive temperature, 37°C, consists of 2% proteose peptone broth supplemented with 2 mM tri-sodium citrate, dihydrate, 0.9 mM ferrous ammonium sulphate, 50 μM copper sulphate and calcium folinate (1 mg/l) and is designated medium II, or MII. In early experiments this medium was supplemented with the trace metal salts of the chemically defined medium (14), either in the standard, low concentrations (tm-MII) or in 25-fold higher concentrations (TM-MII), see Table 1. This medium could be supplemented with the standard concentration of the vitamin complement from the chemically defined medium (vit-TM-MII) or with all the vitamins (14) supplied at 1 mg/l (Vit-TM-MII).

Table I

Trace metal salts of the chemically defined medium in standard (tm) and in 25-fold higher concentrations (TM).

	tm		TM	
	μg/100 ml	μM	μg/100 ml	μM
$Fe(NH_4)_2(SO_4)_2.6H_2O$	1400	36	35000	900
$ZnSO_4.7H_2O$	450	15	11000	375
$MnSO_4.4H_2O$	160	7.1	4000	180
$CuSO_4.5H_2O$	30	1.2	750	30
$Co(NO_3)_2.6H_2O$	50	1.7	1250	43
$(NH_4)_6Mo_7O_{24}.4H_2O$	10	0.08	250	2

2.3 Experimental procedures

Cultures were grown at 37°C in either 1 ml of medium in a 15 mm x 150 mm test tube, or in 10 ml in screw-capped conical flasks of 125 ml capacity. The cultures were not agitated. Cultures were routinely tested for the presence of vacuole-forming revertants of strain NPl.

3. RESULTS

3.1 Growth stimulation of NPl by Fe and Cu ions

Cells grown in complete medium were inoculated into culture flasks containing complete medium *without* the trace metal mixture, but individually supplemented with each of the trace metal salts at the same concentration as in the complete medium (TM). Only the Fe(II)-supplemented culture grew to an extent comparable to the culture fully supplemented with all six metal salts (Fig. 1, Panel B).

In order to determine if Fe(II) was a sufficient supplement, cells from the Fe(II)-supplemented culture were used to inoculate separate cultures of the same medium, but supplemented with each of the remaining trace metal ions at the concentration shown in Table I, column TM. These results are shown in Fig. 1, Panel C. As seen, only the culture supplemented with both Fe(II) and Cu(II) grew

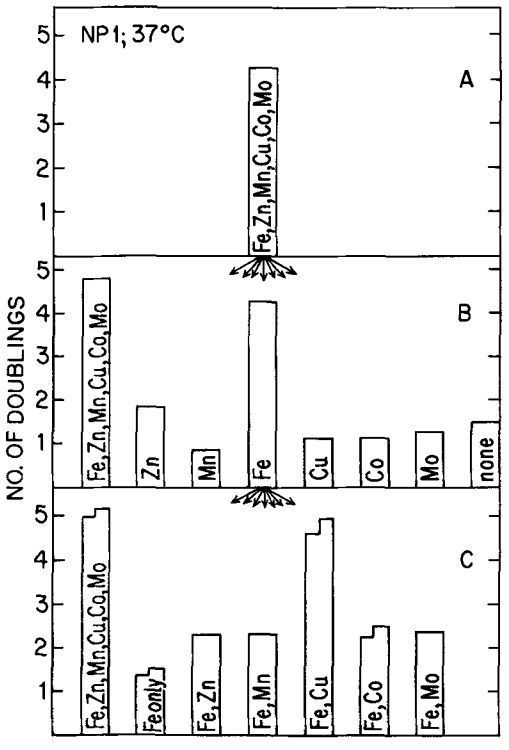

Figure 1. Identification of Fe and Cu ions as the important components of the trace metal mixture for growth of NPl at 37°C. *Panel A:* Growth of cells in Vit-TM-M(II) medium, containing all the trace metal salts in the concentration shown in the right column of Table I. *Panel B:* Growth of cells inoculated from the culture in Panel A into separate culture flasks. They contain all Vit-tm-M(I) medium and are individually supplemented with the metal salts indicated, supplied in 25-fold the standard concentrations, TM. *Panel C:* Growth of cells inoculated from the Fe supplemented culture in Panel B into flasks containing Vit-M(I) plus 1 mM Fe(II), individually supplemented with the trace metal salts indicated in TM concentrations (Table I). The column to the left represents growth in a fully supplemented medium and the column next to it, growth in a culture supplemented with Fe only. Initial cell concentration in all cultures: 5000 cells per ml; growth period: 16 hr.

as well as the fully-supplemented parallel culture. Serial subcultures of NPl in the Fe, Cu-supplemented medium continued to grow at the same rate as the fully supplemented cultures. Thus Fe and Cu salts at concentrations of 1 mM and 50 μM, respectively, replaced the TM mixture in satisfying the trace metal deficiency experienced by NPl growing without food vacuole formation.

3.2 The Fe requirement

3.2.1 Are Fe(II) and Fe(III) equivalent?

Dose response curves to these Fe-compounds are shown in Fig. 2. Fe(II) and Fe(III) satisfied the iron requirement equally well. The optimum response is obtained at 1-2 mM. Since the two forms of iron are rather easily interconvertible, the relative proportion of the two forms in the medium is unknown.

3.2.2 Does citrate induce the Fe requirement?

To be sure that citrate was not enhancing the Fe requirement, the dose response experiment above was repeated, but citrate was omitted from the medium. The results obtained with Fe(III) are shown in Fig. 3. Comparison with Panel A of Fig. 2 shows that no significant difference was introduced by adding citrate. Thus citrate, even though it chelates Fe, still allows enough Fe to be taken up.

The results obtained with Fe(II) in the absence of citrate are not shown. Although qualitatively similar results were obtained, the cultures without citrate showed much variation between duplicate flasks. We assume that the amount and nature of the Fe precipitates and their availability to the cells varied from culture to culture. These results also clearly show the utility of adding citrate to the medium to keep Fe uniformly available.

3.2.3 Specificity of the Fe requirement

Is Fe specifically the element which limits the NPl cells when they are unable to form food vacuoles at 37°C? It is not sufficient to show that Fe restores a good growth rate. A different and active metal ion could be a contaminant in the commercial Fe preparation, or could be complexed to a component of the medium, but be displaced by Fe, and thus made available to the NPl cells.

We therefore determined whether Fe could be replaced with other salts required by living systems. The results shown in Fig. 1, Panel B, already suggested that the active metal ion was not likely to be Zn, Mn, Cu, Co and Mo. In addition, V, B, Cr and Ni were tested together. The results gave no evidence that any of these metal ions can replace Fe, even at con-

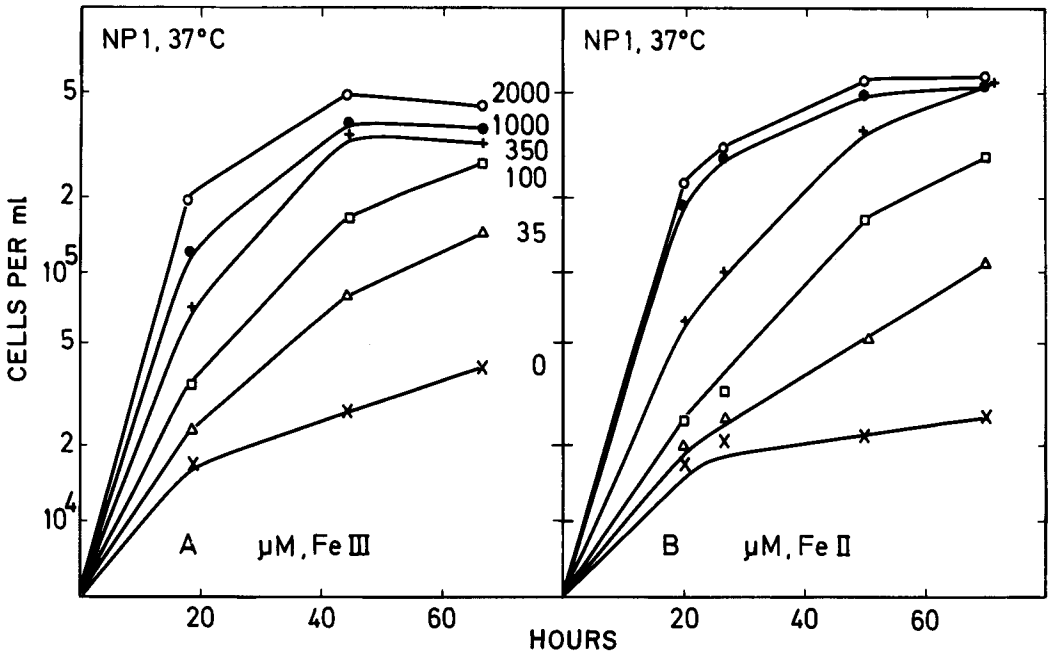

Figure 2. Response of strain NPl to ferric chloride (Panel A) and ferrous ammonium sulphate (Panel B) at 37°C. The numbers associated with each of the curves represent the Fe salt concentration (μM). Composition of the medium: MII (see Table I) minus Fe plus varying concentrations of Fe. Source of inoculum: NPl cells depleted for Fe.

centrations higher than those they might have as contaminants of the Fe preparations (Table II).

Metal ions which have higher affinities for organic ligands (Cu(II), Zn(II), Ni(II), Co(II)) were then tried at high concentrations, up to 3 mM, Table III. If Fe was not the principal active ion, then some of these ions might displace it from a complex not available to the cell, and thus be able to replace Fe. Even though the other ions tested allowed some residual multiplication of the cells, apparently none of them can replace Fe.

From the inability to replace Fe with other trace metals, we conclude that it is very likely that the NPl cells are actually starved for Fe under conditions in which they cannot form food vacuoles.

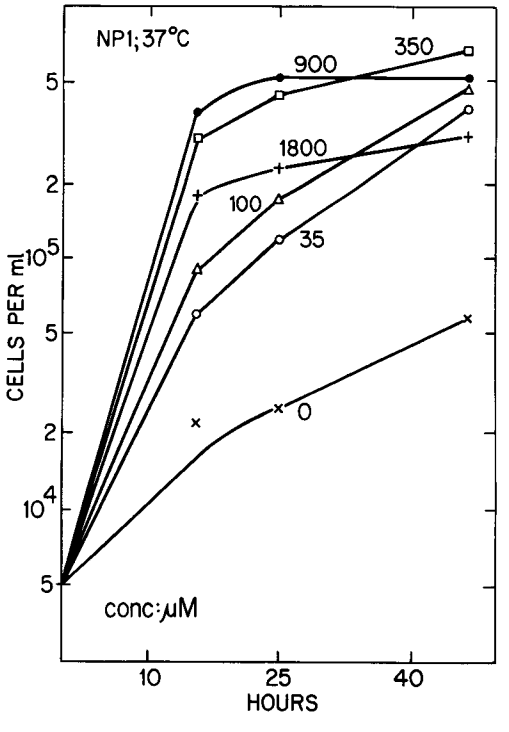

Figure 3. Response of strain NPl to ferric chloride in the absence of sodium citrate. Composition of the medium: MII minus citrate and Fe plus varying concentrations of Fe. Source of inoculum: NPl cells depleted for Fe.

3.3 Cu requirement

3.3.1 Growth rates of NPl as function of Cu concentration

The optimum Cu concentration for the growth

Table II

Inability of V, B, Ni, and Cr to replace Fe in stimulating growth of NPl cells at 37°C.

Medium	10^3 cells per ml after 0 h	20 h	42 h
M II minus Fe	2	8	11
+ V, B, Ni, Cr-mix	2	8	11
+ Fe (II)	2	91	333
+ Fe (II) + V, B, Ni, Cr-mix	2	102	257

M II medium: 2% proteose peptone broth supplemented with 2mM tri-sodium citrate, 1 mg calcium folinate/l, 1mM ferrous ammonium sulphate and 50 µM copper sulphate.
Source of inoculum: early stationary NPl cells grown in M II medium. Fe (II): ferrous ammonium sulphate. The V, B, Ni, Cr-mix provided (mg/l): NH_4VO_3: 4.6; H_3BO_3: 5.7; $NiSO_4.6H_2O$: 4.5; $CrK(SO_4)_2.12H_2O$: 9.6. These concentrations are 10 times higher than recommended by Hutner (7).

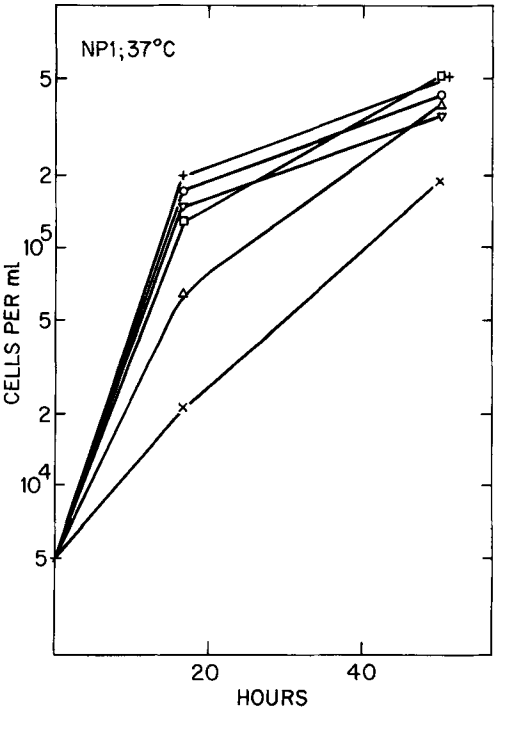

Figure 4. Response of strain NPl to copper sulphate at 37°C. Composition of the medium: Vit-TM-MII minus Cu plus varying concentrations of Cu. The curves from below represent growth responses to 0, 1.25, 4, 12.5, 40, 125 µM, Cu respectively. Source of inoculum: NPl cells grown for two transfers without addition of Cu.

Table III

Inability of Cu, Zn, Ni and Co-ions at high concentrations to replace Fe in stimulating growth of NPl cells at 37°C.

Medium	10^3 cells per ml after 0 h	17 h	41 h
M II minus Fe	1	4	7
M II minus Fe + Fe (III)			
0.63 mM	1	26	260
1.3 -	1	30	300
2.5 -	1	70	440
3.0 -	1	x	x
M II minus Fe + Cu			
0.5 mM	1	3	9
1.0 -	1	3	6
2.0 -	1	3	3
3.0 -	1	2	1
M II minus Fe + Zn			
0.5 mM	1	4	10
1.5 -	1	4	7
2.0 -	1	4	7
3.0 -	1	4	8
M II minus Fe + Ni			
0.5 mM	1	4	17
1.0 -	1	4	22
2.0 -	1	3	7
3.0 -	1	7	x
M II minus Fe + Co			
0.5 mM	1	4	8
1.0 -	1	5	6
2.0 -	1	3	1
3.0 -	1	x	x
M II	1	80	260

M II: see legend for Table II. Fe (III): ferric chloride. Salts provided as sulphates, except for cobalt nitrate. Source of inoculum: 100-fold dilution of an early stationary phase culture grown in M II medium. x means that the cells died.

of strain NPl at 37°C was determined in Fig. 4. The optimum growth rate is obtained at 12.5 µM. At higher concentrations (up to 125 µM) there appears to be a slight inhibition. Even though the growth rate is low, the maximum yield of cells is eventually obtained even when Cu is not added to the medium.

3.3.2 Specificity of the Cu requirement

The results in Fig. 1, Panel C, already indicated

Table IV

Inability of various metal ions to replace Cu in stimulating growth of NPl cells at 37°C.

Medium	10^3 cells per ml after 0 h	19 h	43 h
Experiment 1			
M II minus Cu	1	11	87
+ V, B, Ni, Cr	1	11	86
M II	1	72	340
+ V, B, Ni, Cr	1	84	340
Experiment 2	0 h	15 h	42 h
M II minus Cu	1	29	340
+ 5 mM Ca	1	31	340
+ 5 mM Mg	1	35	340
+ 50 µM Cu	1	122	420

M II: see legend of Table II. Source of inoculum: early stationary NPl cells, grown in same medium for one transfer (about 7 fissions) in expt.1, and for two transfers (about 15 fissions) in expt. 2. Metals were supplies as follows: Ca as calcium chloride, dihydrate; Mg as magnesium sulphate, heptahydrate. V, B, Ni, Cr: as in Table II. The metal salts solutions were sterilized by filtration and added aseptically to the culture medium.

Table V

Lack of Cu requirement induced by high concentration of Fe in the wild type strain at 37°C.

Cells kept in	10^3 cells per ml after 18 h		
	1st transfer	2nd transfer	3rd transfer
M I minus Fe+1 mM Fe (II)	383	340	370
M I minus Fe+1 mM Fe (II) + 50 µM Cu	352	350	360

Cells were subcultured in the appropiate medium every day, at an initial concentration of 1000 cells per ml.
M I: see Methods section. Fe (II): ferrous ammonium sulphate.

that Zn, Mn, Co and Mo did not replace Cu, even at concentrations higher than those they might have as contaminants of commercial Cu salts. Four other metals (V, B, Cr and Ni) were also tested in this respect (Table IV). The results gave no evidence that any of these metals could replace Cu. Thus the NPl cells growing without food vacuoles appear to be starved for Cu as well as for Fe.

3.3.3 Is the elevated Cu requirement induced by the high Fe concentration?

Since high concentrations of Fe are added to the medium, the Cu requirement might be only a secondary consequence of this addition. This could occur if, for example, there was competition for a common uptake system. Such interactions, involving other metal combinations, have been documented (1). We therefore determined whether the wild type parental strain, DIII, showed a higher Cu requirement when grown at high Fe concentrations. This was not the case (Table V). We interpret this to mean that a high concentration of Fe does not interfere with Cu uptake by the food vacuole route. We cannot rule out, however, an interference of Fe with the uptake of Cu through the secondary route that becomes the main source of Cu uptake for NPl in the absence of food vacuoles.

3.4 Effect of Mg at limiting Fe concentrations

As a part of the test of the specificity of Fe in stimulating NPl growth at 37°C, Ca and Mg were included (Fig. 5). Surprisingly, Mg – but not Ca – stimulated growth of NPl significantly under Fe-deficient conditions. The effective concentrations were high, approximately 5 mM.

We tested for the possibility that a similar effect of Mg would be observed for the wild type strain under conditions of Fe deficiency. The results showed no evidence of a similar effect of either Ca or Mg on strain DIII, Table VI. Thus the effect of Mg in Fe starved cells appears to be specific for NPl.

The results in Table IV, exp. 2, showed no effect of Mg on Cu starved cells; thus it appears that the effect of Mg is relatively specific for Fe.

4. DISCUSSION

4.1 Cu and Fe requirement of strain NPl at 37°C

Studies on growth requirements of a food vacuole-less mutant have forcefully called attention to the dual uptake capacity of *T.*

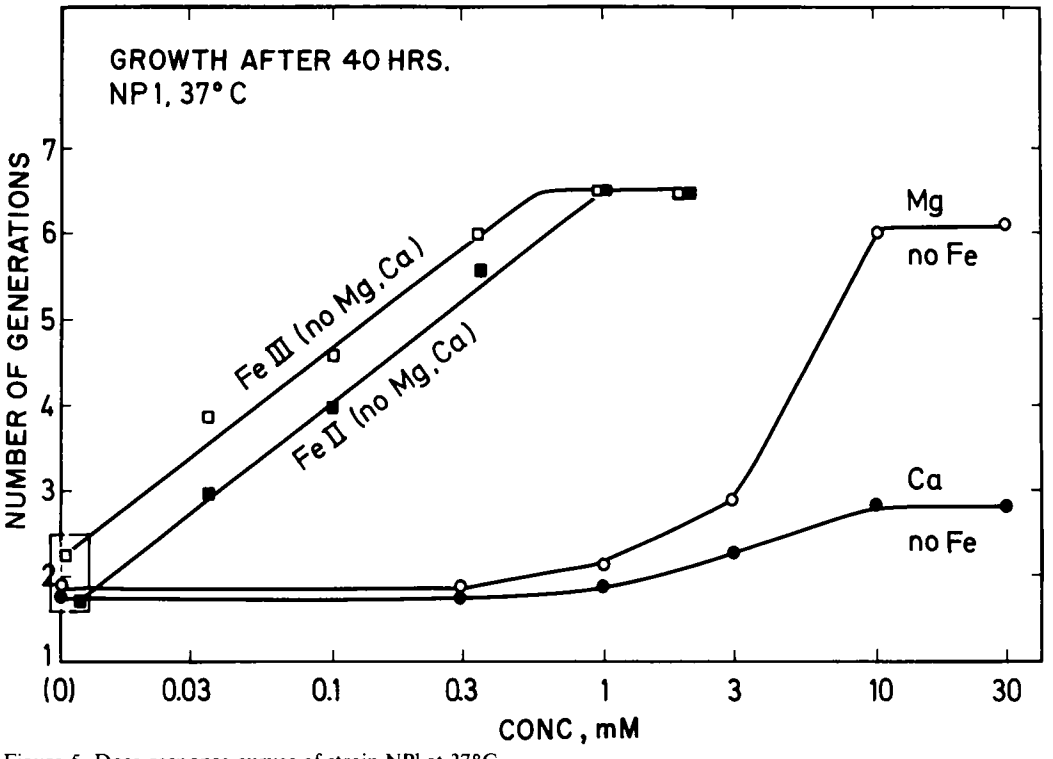

Figure 5. Dose response curves of strain NPl at 37°C to the addition of Ca and Mg ions under conditions of Fe-starvation. Nutrient substrate: MII minus Fe. Source of inoculum: NPl cells grown at 37°C in MII. Initial concentration 2000 cells per ml (Ca and Mg curves) and 5000 cells per ml (Fe curves). The dose response for addition of ferrous ammonium sulphate or ferric chloride is included for purposes of comparison. It has been obtained from the data plotted in Fig. 2. The composition of the medium for these Fe-addition experiments differs slightly, in that all the vitamins and all the trace metals (except Fe) were added. These differences should not affect the results.

Table VI

Effect of high concentrations of Ca and Mg on Fe-starved wild type cells.

Medium	10^3 Cells per ml after 0 h	15 h	42 h
M I minus Fe	1	8	135
+ 5 mM Ca	1	8	120
+ 5 mM Mg	1	9	150
+ 0.36 mM Fe (III)	1	175	410

M I: see Methods section. Source of inoculum: DIII cells, propagated through two serial transfers (about 15 fissions) in the same basal medium.
Mg supplied as magnesium sulphate; Ca as calcium chloride; Fe (III): ferric chloride. Stock solutions were sterilized by filtration and added aseptically.

pyriformis: uptake mediated by the food vacuole (and accessory cell specializations), the *oral uptake system* (OUS) and uptake mediated by the cell membrane, the *surface uptake system* (SUS). We have argued that the mutant NPl has a genetically determined block of at least the food vacuole component of the OUS, and therefore we attribute nutritional differences between NPl and the wild type strains to the functioning of the OUS (12). We also assume (with some justification) that a blocked OUS is the only pertinent functional difference between NPl and the wild type strain at 37°C (unpubl. obs.). Given these assumptions, we interpret our results to mean that in the wild-type *Tetrahymena* the OUS system plays an important role in the uptake of Fe and Cu at the concentrations normally present in the standard growth media. Nevertheless the surface-uptake system can apparently mediate the uptake of adequate amounts of those compounds if provided in high enough concentrations.

4.2 Role of the OUS in the transport of Fe and Cu

These conclusions raise the question: In what

way can the OUS be so important for the uptake of these two metal ions? An important role for the hydrolytic enzymes of the food vacuole can be ruled out. However, as described below, the two compounds have properties that lead us to suggest that pH differences may be a major part of the explanation.

Fe(III) is probably never available at biologically meaningful concentrations as the simple hydrated complex $Fe(H_2O)_6^{3+}$. On the one hand, in the absence of other complexing agents, it will polymerize to give oxo- and hydroxo-derivatives whose solubility product is about 10^{-36} (11). One the other hand, Fe(III) can also form strong complexes to organic ligands – particularly to the elements O, N and S (11) – which also have low dissociation constants (17). Complexes with only a few highly specialized ligands have been shown to be capable of being transported into cells (3, 16). Thus it has been pointed out that Fe presents a challenging uptake problem to single-cell, water-dwelling organisms at nearly neutral pH (7, 9, 11, 16). This challenge has been met by some bacteria by secretion of ferric ironophores which can wrest the iron away from precipitates and other complexes and then be transported into the cell, see reviews by HUTNER (7), NEILANDS (11) and BYERS (3).

Tetrahymena could well make use of another biological invention, namely the collection of nutrients into an internal, membrane-bound food vacuole, whose pH can be manipulated. The pH of *Tetrahymena* food vacuoles does not appear to have been studied. However, extensive studies in the related ciliate *Paramecium* suggest that the pH undergoes a programmed change during the »life cycle« of a food vacuole, and that it reaches values as low as 1-3 (8, 10, 18). Since H^+ ions compete with the metal ion for the electron-donor groups of the ligand, a lowering of the pH increases the concentration of »free« Fe(III), and could thus make it available to Fe-chelating groups associated with transport sites on the food vacuole membrane.

In unsupplemented 2% proteose peptone broth, all Fe may be complexed to soluble components, since filtration of the medium through *Millipore* membrane filters with 0.2 µm retention capacity does not change the concentration of the total Fe (about 0.2 µg/ml as measured by atomic absorption (Dr. S. CHRISTENSEN, pers. comm.)). Compounds known to complex Fe are present in the medium: amino acids (negative ions), peptides, nucleosides, vitamins etc., in addition to citrate. Another feature of the *Tetrahymena* OUS may come into play at higher concentrations of Fe, where oxo- and hydroxo-precipitates may occur. This is the ability of the ciliary membranelles to concentrate particulate matter in the size range up to 5 µm. It has been estimated that the concentration factor for India ink particles (approximately 0.2 – 0.5 µm in diameter) in the food vacuoles is around 500 (13). Thus the oral uptake system, through its remarkable capacity for mechanical concentration and for pH lowering, is well adapted to handle the geochemical and biological challenge posed by Fe. The hydrated form of Cu(II) does not present solubility problems nearly as serious as those of Fe(III). Cu does, however, form strong complexes with organic ligands. Indeed, among the remaining nutritionally most important transition elements (Zn, Mn, Cu, Co, Mo, V) Cu(II) generally forms the strongest complexes (reviewed by ANGELICI (2)). Thus the OUS system may also significantly facilitate the uptake of Cu, at least by lowering the pH and liberating the Cu from complexes which render it unavailable. Nothing is known about the ionophores involved in Cu transport across the cell membrane in *Tetrahymena*. Strain NPl may be a useful experimental system for the study of that mechanism.

4.3 Fe and Cu uptake through the surface uptake system

Fe uptake at 37°C by the SUS of strain NPl can be considered extremely poor at best. This suggests the absence of specialized chelators for Fe transport analogous to the catechols and hydroxamates produced by other unicellular organisms (3, 16). The dose response curves of NPl to Fe(III) are not significantly different in the presence or absence of citrate, and this suggests that the Fe(III)-citrate complex is unavailable to the SUS, and that a citrate-de-

pendent uptake system analogous to that in *Salmonella* does not play a nutritionally significant part in the SUS (18). The possibility remains that any of the above systems exists but is heat-sensitive in both strains, *i. e.*, inactive at 37°C. Heat-sensitive Fe uptake systems are known in certain wild type bacterial strains (5).

The only role we ascribe to citrate in the growth of NPl at 37°C – and our only reason for adding it to the medium – is to serve as an Fe buffer, *i. e.*, to chelate and thus solubilize the Fe precipitates which are otherwise formed. These precipitates introduce a source of variability from culture to culture, as they can interfere both with optical or electronic methods of cell counting, and with the fractionation and purification of cellular components. Whether Fe(III)-citrate complexes can be transported as such through the food-vacuole membrane is unknown.

4.4 Fe and Cu requirements of the wild type strain

We have confirmed for strain GL and inbred strain D of syngen 1 CONNER and CLINE's observation for strain W that 2% proteose peptone broth, even though it allows transplantable growth indefinitely, is nutritionally deficient for *Tetrahymena* (4). Fe corrects that deficiency. The addition of 36 μM Fe(II) or (III) yields a maximum growth rate for GL and strain DIII.

4.5 Mg stimulation of Fe-starved NPl cells

Mg can stimulate the growth of Fe-starved NPl, but not wild type cells. This effect of Mg may be related to the uptake (or possibly the retention) of Fe by the cell surface system and is somewhat specific for Fe. The finding that Mg is much more active than Ca tends to rule out the possibility that the Mg is simply displacing Fe (or some other metal) from a complex that sequesters that metal, since Ca in general has greater affinity than Mg for the organic ligands present in the growth medium (17). Mg may stimulate a membrane-located component of the Fe-transport system.

ACKNOWLEDGEMENTS

We thank Professor ERIK ZEUTHEN, head of the Biological Institute of the Carlsberg Foundation for valuable discussions at all stages of the present study. We also thank Drs. G. G. HOLZ, Jr., S. H. HUTNER and R. E. PEARLMAN for critical reading of the manuscript. Finally, we are indebted to Dr. S. CHRISTENSEN, Department of Physiology, University of Aarhus, for iron determinations of our nutrient media and Mrs. J. ORIAS and Mrs. B. DOHN for excellent technical assistance. We thank mag. scient. K. MAX MØLLER for helpful advice on problems of chelation. One of us, E. O., acknowledges gratefully support by NIH grant GM 21067 and by the Carlsberg Foundation, Copenhagen, Denmark.

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