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Effects of Copper on Phagocytosis in *Tetrahymena*

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Summary

Addition of copper, corresponding to 100 ppm, to the normal 2% proteose peptone medium is tolerated by *Tetrahymena*. This concentration of copper stimulates phagocytosis to a maximum value which is reached gradually during the first 1 hour exposure, and which is maintained during continuous exposures. Cell proliferation is resumed after a lag period, although at a decreased rate. Cells exposed to copper contain small refractile granules, previously proposed to represent an ion-regulating system; the number of granules remains constant in proliferating cells. Higher concentrations of copper also resulted in an elevated rate of phagocytosis but at the same time cell mortality was observed; this lack of transition between inhibited phagocytosis and cell mortality may be ascribed to the physiological role of copper. The high amount of organic matter in the growth medium protects against the toxic effects of copper, thus in the absence of organic matter *Tetrahymena* tolerated only a 100-fold lower concentration of copper than that tolerated in the growth medium. However, cells which had initiated granule formation (for example for regulation of calcium) prior to starvation and exposure to copper, were more resistant to copper than cells which had not yet activated this mechanism, perhaps because of the low capacity of starved cells for protein synthesis.

Keywords: Cell proliferation; Copper; Detoxication; Phagocytosis; *Tetrahymena pyriformis*.

1. Introduction

Copper is essential for living organisms. However, since only trace amounts are required for metabolic functions (FOX 1972), cells must have a mechanism for eliminating excess copper to prevent toxic effects. Malfunction of copper metabolism is seen in Wilson's disease, a congenital disorder in man, and as a result copper accumulates in the tissues, apparently due to an impairment of the normal excretion of the metal (WALSHE 1966, EVANS 1973, HILL 1977). On the other hand, some organisms may adapt to high external concentrations of copper (BRYAN 1976); such an adaptation, apparently also genetically

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determined, must be correlated with an efficient regulation of the intracellular concentration of the metal and of excretion of accumulated copper.

Copper has primarily a biological role in association with specific proteins of which the metal forms an integral part (WILLIAMS 1966, EVANS 1973, ÖSTERBERG 1974, HILL 1977, O'DELL and CAMPBELL 1971). In mammals, the copper proteins are involved in functions such as electron transfer reactions, transport of oxygen molecules, and in the transport and storage of copper ions; in the latter functions metallothioneins may play an important role (KOJIMA and KÄGI 1978). At the cellular level an accumulation of copper occurs in lysosomes (KOENIG 1963, MCNARY 1963, BARKA, SCHEUER, SCHAFFNER, and POPPER 1964, GOLDFISCHER 1965, LINDQUIST 1968, GEDIGK 1969, BRUN and BRUNK 1970, EVANS 1973, SCHEINBERG and STERNLIEB 1976, GASBARRINI, CORAZZA, and BONVICINI 1979).

In the present study, *Tetrahymena,* a common freshwater ciliate, has been exposed to excess copper. A study was made on the effects of the metal on the capacity of the cells to form food vacuoles and to proliferate; the former function usually ceases under toxic conditions (NILSSON 1979). The purpose has been to compare the effects of copper with those of lead, a non-essential heavy metal to which *Tetrahymena* shows a high tolerance (NILSSON 1978, 1979).

2. Material and Methods

Tetrahymena pyriformis GL-8 was grown axenically at 28 °C in a 2% proteose peptone (PP) medium enriched with 0.1% liver extract and salts (PLESNER, RASMUSSEN, and ZEUTHEN 1964); the medium contains 1.9 ppm copper. Some experiments were carried out in 0.5% and 1% PP media. The cell density of the populations was determined in triplicates using a Coulter Counter model ZB. Cells were used in the exponentially multiplying growth phase (4–10 $\times$ 10^4 cells/ml) and, in some instances, at the early stationary phase of growth. Starved cells were obtained by subjecting cells to 3 washings in an inorganic salt (IM) medium (HAMBURGER and ZEUTHEN 1957) in which they were left for 1 hour prior to use; this medium contains no copper. The pH of the PP and IM media was 6.5 and 7.1, respectively.

Stock solutions of 5,000 ppm and 50 ppm of copper (cupric chloride or sulphate) were used for experiments with cells in PP and IM media, respectively. Addition of copper salts alters the pH of the media, hence the pH was adjusted with NaOH.

The capacity of the cells to form food vacuoles was tested by a 10 minutes exposure to carmine particles, as described previously (NILSSON 1972). The number of carmine-containing vacuoles was counted in 100 cells and the means of the samples were expressed as percentage of those of control cells (100%). In tests on reversibility, the cells were washed in fresh PP medium after a 1 hour exposure to copper. In growth experiments the cell density was determined at ½ hour intervals over a 5–6 hours period; in each experiment, the test cultures and the control culture all originated from the same population.

The cellular content of ATP was determined using the bioluminescent firefly luciferase assay for ATP (MYHRMAN, LUNDIN, and THORE 1968) and a LKB-Wallace "Luminometer" 1250 to measure the output. The handling of the cells has been described recently (NILSSON 1980) and the initial reading was taken 1 minute after withdrawal of the cell sample.

ATP standard curves were prepared in the presence of PP medium and PP medium with added copper; incidentally, the presence of 100 ppm copper increases the light output by about 15%.

The cells were observed *in vivo* using a Reichert Zetopan light microscope. Cell motility was determined on micrographs of a 1 second exposure using a chartometer to measure the paths of the cells (300 cells/sample); the results have been expressed as the percentage of the mean speed of control cells. The diameter of food vacuoles was measured on micrographs of cells after a 10 minutes uptake of carmine particles.

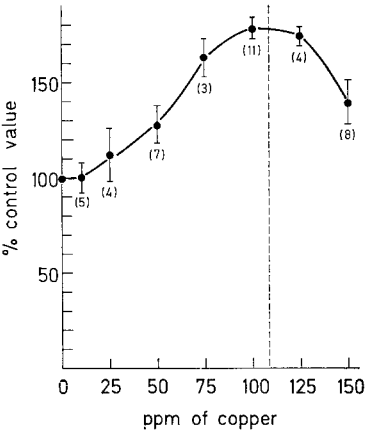

Fig. 1. The effects of a 1 hour exposure to copper on the rate of food vacuole formation in cells in normal (2% PP) growth medium. Cell mortality occurs in concentrations above 100 ppm copper (indicated by the broken line), see text for further explanation. The number of experiments is indicated in parenthesis and the standard deviation of the mean is indicated by vertical bars

3. Results

The capacity of *Tetrahymena* to form food vacuoles after a 1-hour exposure to different concentrations of cupric chloride is depicted in Fig. 1. A dose-dependent, stimulatory effect of copper is seen on the rate of phagocytosis, in concentrations higher than 10 ppm copper. Maximal stimulation occurs after addition of cupric chloride, corresponding to 100 ppm copper, the concentration above which a dose-dependent effect on cell mortality is seen, this transition is indicated by the broken line in the figure. Although cell deaths occur in the high concentrations, the remaining cells in the population form food vacuoles in the normal manner (Nilsson 1976) and at an elevated rate up to 175 ppm copper where the rate is 115% of that of control cells, whereas in 200 ppm copper the rate of food vacuole formation decreases to 35% of that of control cells (5 experiments). The mean percentage of dead cells is 7, 25, 37, 55, and 95% after a 1-hour exposure to 150, 175, 200, 225, and 250 ppm copper, respectively.

The stimulating effect of copper on phagocytosis reaches a maximal value gradually during the first 1-hour exposure to 100 ppm copper, as shown in

Fig. 2 *A* (3 experiments). The elevated rate of vacuole formation remains constant when tested at intervals during the following hour and whenever tested during a 24-hour exposure (6 experiments). An increased cell motility of copper-treated cells could possibly correlate with their increased rate of phagocytosis; however, the average speed of cells in 100 ppm copper was found to be only 12% faster than that of control cells. Moreover, the ATP content of cells exposed to 100 ppm copper for 1/2, 1, and 3 hours was 115, 101, and 88%, respectively, of the control value (5 experiments), thus at

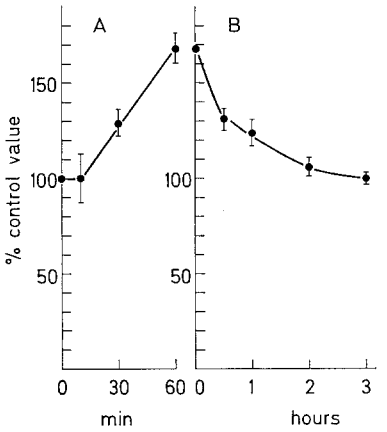

Fig. 2. The effect of a 1 hour exposure to 100 ppm on cells in normal (2% PP) medium. *A* time-dependent effect on the rate of food vacuole formation (3 experiments). *B* recovery of the rate of food vacuole formation after a 1 hour exposure (5 experiments). The standard deviation of the mean is indicated by vertical bars. Note the different time scale in the figures

the time of resumed cell proliferation the ATP content is low. The stimulatory effect of copper on food vacuole formation is reversible, thus after a 1-hour exposure to 100 ppm copper the normal rate is restored about 3 hours after removal of external copper, as shown in Fig. 2 *B* (5 experiments).

The rate of cell proliferation was determined in 100 and 150 ppm copper. After a variable lag period, about 3 hours, the cells proliferated but at a decreased rate; the generation time was prolonged by a factor of 1.4 and 1.7 in 100 and 150 ppm, respectively, above the rate of control cells (6 experiments). *In vivo* observations of copper-treated cells revealed the presence of small refractile granules, typical of slowly growing cells (NILSSON 1976, 1978); however, once cell proliferation was resumed the number of these granules remained almost constant in the cells. The size of newly-formed food vacuoles, as observed after labelling with carmine particles, was smaller in copper-treated than in control cells. The average diameter of the vacuole in copper-treated cells was only 80% of that of vacuoles in control cells

(90 determinations), indicating that during formation of one vacuole, copper-treated cells only take up half of the volume taken up by control cells. In contrast, during prolonged exposure to copper cells contained 6–8 large digestive vacuoles, whereas control cells contained 1–2 large vacuoles; this finding indicates that copper induces vacuole fusion and/or interferes with the normal reduction in vacuolar size during digestion. The final cell density of cells exposed to 100 ppm copper corresponds to that observed in control populations; however, in the stationary growth phase where control cells also

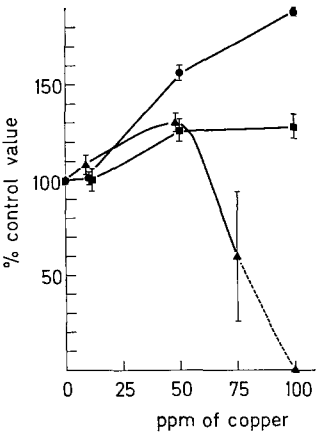

Fig. 3. The effect of a 1 hour exposure to copper on the rate of food vacuole formation in cells in media of different concentrations of proteose peptone (PP): (●) 2% PP (normal growth medium), (■) 1% PP, and (▲) 0.5% PP. The standard deviation of the mean (3 experiments) is indicated by vertical bars. The broken part of the 0.5% PP curve symbolizes a high cell mortality, see text for further explanation

form the small refractile granules, the copper-treated cells contain an increasing number of these granules and cell mortality was observed earlier in these cultures than in control cultures.

The apparently high tolerance of *Tetrahymena* to copper added to the normal growth medium could be ascribed to the high content of organic matter in this medium, as previously observed on exposure to lead (Nilsson 1978). Copper was therefore added to cells in media of lower contents of proteose peptone (PP). The effect of different concentrations of copper on the rate of vacuole formation of cells in 0.5, 1, and 2% PP media is illustrated in Fig. 3 (3 experiments). The rate of phagocytosis is clearly affected in a dose-dependent manner by different amounts of organic matter in the corresponding concentration of copper. Moreover, cell mortality was seen in the weaker media; thus after a 1-hour exposure to 100 ppm copper in 1% PP medium the percentage of dead cells was 2%, whereas 5, 50, and 95% dead cells were found

in 0.5% PP medium with 50, 75, and 100 ppm copper, respectively. However, as observed in the normal growth medium, the surviving cells were still able to form vacuoles.

The effect of copper was also tested in the absence of organic matter. The cells were transferred to an inorganic salt (IM) medium in which they were starved for 1 hour before exposure to copper. These cells tolerated copper only in concentrations which were a factor of 100 lower than those tolerated by cells in the normal growth medium. However, the response of the starved cells exposed to 1 ppm copper varied greatly, either the majority of the cells

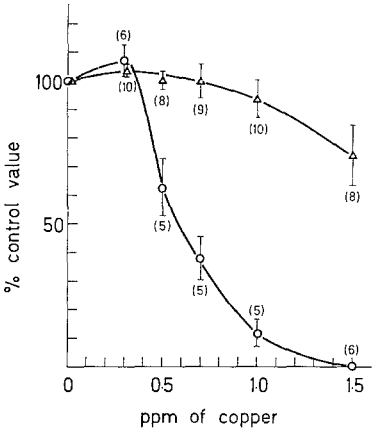

Fig. 4. The effect of a 1 hour exposure to copper on the rate of food vacuole formation in starved cells in inorganic salt medium (absence of organic matter). (○) cells from exponentially multiplying cultures prior to starvation. (△) cells from early stationary phase cultures prior to starvation. The number of experiments is indicated in parenthesis and the standard deviation of the mean is indicated by vertical bars

did not survive a 1-hour exposure or all cells survived and formed food vacuoles at the normal rate. This variation depended on the physiological state of the cells prior to starvation, *i.e.*, whether the cells were proliferating at a high rate (log. phase cells) or whether their rate of proliferation had decreased (stat. phase cells). The results of 16 experiments, grouped according to the physiological state of the cells prior to starvation, are shown in Fig. 4; incidentally, starved stat. phase cells form food vacuoles at a rate corresponding to 85% of that of starved log. phase cells. The two groups of cells show different response to copper with respect to food vacuole formation and cell mortality. Thus, starved log. phase cells showed some mortality in 0.7 ppm copper and in 1 ppm and 1.5 ppm copper the percentage of dead cells was 50 and 90%, respectively, whereas starved stat. phase cells showed a transition in 1.5 ppm copper with cell mortality varying from zero to 30%. However,

as observed for copper in general, although some cells died the surviving cells were capable of forming food vacuoles; moreover, all cells exposed to copper contained more refractile granules than seen in the starved control cells.

4. Discussion

Copper in excess amounts of that found in the growth medium has interesting effects on *Tetrahymena* as revealed by the present study. The rate of food vacuole formation shows intense stimulation and the usual transition between inhibited phagocytosis and cell mortality (NILSSON 1976) is lacking. Adaptation is indicated by resumed cell proliferation, although at a decreased rate, and these cells contain an almost constant number of small refractile granules, previously proposed to represent an ion-regulating system, which normally contain calcium and magnesium but, when added externally, will also accumulate strontium and lead (COLEMAN, WARNER, NILSSON, and BATT 1972, 1973, NILSSON and COLEMAN 1977, NILSSON 1979). The effects of copper are greatly influenced by the presence of organic matter.

Adaptation of *Tetrahymena* to excess copper is a gradual process. Maximal stimulation of phagocytosis is found after addition of cupric salts, corresponding to 100 ppm copper, to cells in normal growth medium. The stimulatory effect increases gradually during the first 1-hour exposure, indicating that an induced metabolic change, involving synthesis, is responsible for the change in phagocytic rate. Copper may enter *Tetrahymena* through the membranes of food vacuoles and of cilia, in a manner similar to that described for lead (NILSSON 1979). A balance in the intracellular concentration of copper is apparently established within the first 1-hour exposure, as indicated by the maintenance of an elevated rate of phagocytosis during continuous exposures and by the resumed cell proliferation; incidentally, in copper-tolerant algae equilibrium is established after a 30-minute exposure (MANDELLI 1969). In *Tetrahymena* adaptation seems to be correlated with accumulation of copper within the small refractile granules. Similar granules containing copper have been found in a variety of organisms exposed to the metal (WIESER 1968, WIESER and KLIMA 1969, BRYAN 1976, BROWN 1977, BALLAN-DUFRANCAIS, JEANETET, and QUINTANA 1979); they correspond to the mammalian lysosomes mentioned in the Introduction. The intracellular handling of copper will be discussed further below. Whether adaptation of *Tetrahymena* to excess amounts of copper is genetically determined, as is the case in other organisms (BRYAN 1976, FOSTER 1977), is not known but adaptation appears to be energy requiring.

Copper-treated cells apparently require excess nutrients, as indicated by their high rate of phagocytosis and their prolonged generation time. However, the volume of a newly-formed food vacuole in these cells corresponds only to

half the volume of a newly-formed vacuole in control cells; nevertheless, the total uptake of nutrients during a cell generation of copper-treated cells is 10–20% higher than that of control cells. Moreover although the ATP content of cells proliferating in the presence of copper is only 88% of that of control cells, the total amount of ATP per cell generation of copper-treated cells is 120% of that of control cells. Thus, the overall energy expenditure of cells exposed to 100 ppm copper is about 20% higher than that of control cells during a cell generation; this extra energy expenditure is likely to represent the cost of adaptation, although it amounts to less than that spontaneously inferred by the high rate of phagocytosis.

Copper interferes with the vacuolar membrane in *Tetrahymena.* To cover their nutrient requirements, copper-treated cells use twice as much membrane in the initial formation of food vacuoles as that used by control cells, thus copper interferes with the utilization of food vacuolar membrane. Furthermore, the unusually high number of large digestive vacuoles in copper-treated cells indicates that copper also interferes with the functioning of the food vacuolar membrane, either by induced fusion of food vacuoles or by inhibition of the normal reduction in vacuolar size during digestion (NILSSON 1976). That copper may alter the properties of membranes has been reported from other cell systems (ROTHSTEIN 1959, PETERS 1966, KOEFOED-JOHNSEN and USSING 1974, RIISGÅRD 1979, RIISGÅRD, NIELSEN, and SØGAARD-JENSEN 1980); in addition, an increased amount of cellular membranes was observed in copper-intoxicated rats (BARKA, SCHEUER, SCHAFFNER, and POPPER 1964). Moreover, the copper induced cell mortality may be ascribed to an effect on membranes; cell rupture would occur as a result of a forced high rate of phagocytosis and an insufficient supply of membrane to meet this demand. These rather unusual effects of copper on *Tetrahymena* may well be a reflection of the physiological role of the metal, not known in detail for this organism; excess copper may cause bolting effects on some metabolic reactions, somehow related to food vacuole formation, but not on related reactions. It is interesting that the stimulated rate of phagocytosis requires a 2- to 3-hour recovery period after removal of copper to return to the normal level; this finding could indicate a slow removal of all excess copper.

Adaptation to excess copper requires control of the intracellular concentration of the metal, retention of absorbed copper, and elimination of accumulated copper, as mentioned in the Introduction. These mechanisms have been studied extensively in mammals, where exposure to copper occurs mainly through the digestive tract. As copper is liberated from ingested food it binds to free amino acids and to the mucosal metallothionein, a low molecular weight non-enzymic protein with a high binding capacity for metals (VALLEE and WILLIAMS 1966, KOJIMA and KÄGI 1978, CHERIAN and GOYER 1978). The copper-amino acid complexes are transported actively across the mucosa, whereas copper bound to metallothionein may become dissociated for transport

or may remain bound and return to the intestinal lumen (EVANS 1973); less than 30% of the ingested copper is absorbed (O'DELL and CAMPBELL 1971).

Metallothioneins may play a general role in metal detoxication. The proteins have been identified in the cytosolic fraction of some tissues from a wide range of organisms subjected experimentally to metals (KOJIMA and KÄGI 1978). Also in *Tetrahymena* a metallothionein-like protein has been found to accumulate on exposure to cadmium (YAMAGUCHI, WADA, ONO, and NAGAHASHI 1978); this protein might also play a role in the absorption of copper since both metals bind to metallothioneins (O'DELL and CAMPBELL 1971, EVANS 1973, CHERIAN and GOYER 1978). The intracellular site of the metallothionein-like protein in *Tetrahymena* is not known; however, in analogy with the mammalian systems it may be associated with membranes of digestive vacuoles and the small refractile granules, which are limited by a membrane resembling that of digestive vacuoles (NILSSON and COLEMAN 1977). Accumulation of copper within the small granules could be mediated by special ATPases, such as the calcium-activated ATPase isolated from *Tetrahymena* (CHUA, ELSON, and SHRAGO 1976); this ATPase shows increased activity at the stage of granule formation. Another point is that during continuous exposure to copper, with an assumed constant influx of copper, an increase would be expected in the number of refractile granules; however, this is not observed in the proliferating cells. One explanation is that the granules, which are of lysosomal type, could fuse with digestive vacuoles whereby accumulated copper could be eliminated from the cells with other debris through defecation, as previously suggested for accumulated lead (NILSSON 1979). Such a mechanism would be in accordance with the excretion of copper via feces in mammals.

The protective property of the organic growth medium towards toxic effects of heavy metals on *Tetrahymena* has previously been found on exposure to lead and nickel (NILSSON 1978, 1979, LARSEN and NILSSON 1980). The phenomenon is ascribed to the general binding, or chelation, of heavy metals to organic and inorganic compounds, thus leaving little in ionic form; a compositional change of the medium is also indicated by the lowering of the pH on addition of the metal salts. Moreover, such factors may in part explain the discrepancy in the concentration of copper claimed to be tolerated by *Tetrahymena* (RUTHVEN and CAIRNS 1973, BOVEE, BERGQUIST, WYTTENBACH, STERNHEIN, and TIPPITT 1977, present study). The finding of a 100-fold difference in the concentration of copper, tolerated by cells in the presence and absence of organic matter, is remarkable and indicates that ionic copper has a high toxicity. However, the toxicity of copper depends not only on the concentration of the metal, but also on the physiological state of the cells, as found in the present study. Cells, which are forming granules, are resistant to higher concentrations of copper than cells which have not yet activated this

mechanism prior to starvation and exposure to copper, probably because of the low capacity of starved cells for protein synthesis.

Although copper exerts unusual effects on *Tetrahymena*, undoubtedly related to the physiological role of the metal, the intracellular handling of copper appears to follow the same pathways as found for calcium and lead (NILSSON and COLEMAN 1977, NILSSON 1979). These observations confirm the general concept (BRYAN 1976) that organisms which have a mechanism for detoxication of one metal are likely to have an increased capacity for detoxication of other metals by co-accumulation within the same structure, which in the case of *Tetrahymena* is the small refractile granule. A modification of the concept would be a requirement for specific proteins for the binding of different metals, as may be the case (ALBERGONI, PICCINNI, and COPPELLOTTI 1980); tolerance to individual heavy metals would then be genetically determined, although a common mechanism for detoxication of metals already exists.

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