

## Effects of various toxicants on uptake of amino acids and their incorporation into proteins by *Tetrahymena pyriformis*

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### Summary

We have investigated the effect of various inorganic and organic toxicants on uptake of amino acids and their incorporation into proteins by *Tetrahymena pyriformis*. We have studied some heavy metals - Cd, Cu, Zn and Cr (dichromate) and organic compounds (2,4-dinitrophenol, sodium pentachlorophenate, 4-chlorophenol and the pesticide Dicamba™). All these toxicants inhibited both activities. Furthermore, the inhibition patterns of amino acid uptake and protein synthesis were similar. This resemblance was observed with variously labeled amino acids: [<sup>35</sup>S]-methionine, a mixture of [<sup>35</sup>S]-methionine and [<sup>35</sup>S]-cysteine or a mixture of [<sup>14</sup>C]-amino acids. The ratios of inhibition of amino acid uptake/incorporation were quite close to one in most cases. Most probably, the toxicants tested inhibited protein synthesis and amino acid uptake indirectly, by affecting some other cell process influencing both uptake and incorporation. These results urge to exercise caution when interpreting data on the effect of toxicants on protein synthesis.

**Key words:** amino acid uptake, amino acid incorporation, heavy metals, organic toxicants, *Tetrahymena pyriformis*

### Introduction

The ciliate *Tetrahymena* is an organism of choice to study the effect of toxic chemicals because, among other factors, of its sensitivity and variety of its cellular and biochemical responses (Sauvant et al., 1999). T.W. Schultz and his collaborators have set up the Tetratox database (Seward et al., 2001) listing more than 2000

organic compounds toxic to *Tetrahymena*. However, few studies have dealt with the influence of various toxicants on amino acid uptake and incorporation into proteins by this ciliate. We have found no data on the effect of heavy metals on the overall protein synthesis in *Tetrahymena*, although there are a few studies on specific proteins that could be used as bio-markers (Sauvant et al., 1999). Investigations involving organic

toxicants and pharmaceutical drugs are also scarce and their conclusions are inconsistent. Dimethyl sulfoxide (Nilsson, 1980), acetaminophen and aspirin (Okano et al., 1984), lindane (Al-Chalabi and Al-Khayat, 1989) and dichloroisoproterenol (Ryals and Yang, 1997) have been reported to have, or not to have, an effect on *Tetrahymena* amino acid uptake or protein synthesis.

Amino acid incorporation is widely used to study the influence of a given condition on the nature and amount of synthesized proteins. It has been extensively exploited to assess the effect of various toxicants on general protein synthesis as well as on the synthesis of specific proteins (Sanders, 1993). However, simple as this approach might appear, it should be standardized to the conditions under study (Appel et al., 1992). Besides, it is often simply assumed that a certain experimental condition directly affects the protein synthesis process as such, though, as a matter of fact, many other factors may be involved: amount of mRNA and protein translation machinery components (Campbell and Weissbach, 1980), production of cell energy (Findly et al., 1983), amino acid transport and availability (Fink and Zeuthen, 1980), etc. The latter can influence protein synthesis in many ways. For example, amino acids are used by *Tetrahymena* even more for energy production and storage than for incorporation into proteins (Raugi et al., 1975, Stein and Blum, 1980). Less than 10% of the arginine uptake, and sometimes as low as 1%, finds its way into proteins when cells are grown under standard conditions when the access to amino acids is not restricted (Eichler, 1989).

Studies in other systems have shown that amino acid uptake is (Nilsson and Selstam, 1975) or is not (Brostrom et al., 1987; Sayegh et al., 1992) an important factor in inhibition or stimulation of protein synthesis. In toxicity and pharmacology investigations, such a correlation between amino acid uptake and incorporation into protein was very seldom investigated, let alone taken into account to interpret the data. Various compounds seem to act differently. For instance, aclacinomycin inhibition of protein synthesis is caused by a reduced amino acid uptake (Schaefer et al., 1988), while that brought about by rhein is not (Castiglione et al., 1990). Moreover, the effect of toxicants on a particular cell process might be caused by an overall inhibition of many other cell activities rather than by a highly specific impact upon the process itself. Such an indirect effect of dichloroisoproterenol on amino acids uptake and protein synthesis through an impairment of vacuole formation has been shown in *Tetrahymena vorax* (Ryals and Yang, 1997).

The aim of this work was to determine the extent, to which the exposure of *Tetrahymena pyriformis* to various heavy metals or organic toxicants could influence

both the uptake of amino acids and their incorporation into proteins. We used [<sup>35</sup>S]-methionine, a mixture of [<sup>35</sup>S]-methionine and [<sup>35</sup>S]-cysteine or a mixture of the [<sup>14</sup>C]-amino acids to monitor these two processes. We observed that various heavy metals (Cd<sup>2+</sup>, Cr(VI), Cu<sup>2+</sup>, Zn<sup>2+</sup>) or organic toxicants (2,4-dinitrophenol, pentachlorophenolate, 4-chlorophenol, 3,6-dichloro-2-methoxybenzoic acid (Dicamba™) and phenol) inhibited both uptake and incorporation of amino acids. We noted that the inhibition patterns generated by organic toxicants were more complex than those brought about by heavy metals. Moreover, in most, but not all, cases, the level of incorporation inhibition was closely correlated to the uptake inhibition. Again, the relationship was clearer in the case of heavy metals but more blurred in the case of some organic compounds.

## Material and Methods

**Cell culture and incubation.** *Tetrahymena pyriformis* (strain GL) was maintained in a standard culture medium (0.5% proteose-peptone, 0.5% tryptone, 0.02% K<sub>2</sub>HPO<sub>4</sub>) at 18°C. Prior to an experiment, cells were conditioned to grow at 25°C in a growth medium (1% proteose-peptone, 0.25% yeast extract and 0.2% glucose) for 48 hours. A culture was then grown for 48 hours after inoculating 1 mL of the conditioned culture into 100 mL of growth medium at 25°C.

Cells were concentrated and washed once in 10 mM Tris (pH 7.5) using the device already described (Lang and Gauthier, 1993). Cell concentration was brought to 2 × 10<sup>5</sup> cells/mL with 10 mM Tris (pH 7.5). Labeled amino acids were added to the mixture (0.5 μCi/mL), which was then distributed in tubes containing the required amount of the toxicant under study. Incubation was at 25°C for the required duration. We used either [<sup>35</sup>S]-methionine, a commercial equimolar mixture of [<sup>35</sup>S]-methionine and [<sup>35</sup>S]-cysteine or a commercial equimolar mixture of the 20 common [<sup>14</sup>C]-amino acids.

**Measurement of amino acid uptake.** At the required times, aliquots of 500 μL were withdrawn from the incubation mixture and transferred into a microtube containing 900 μL of cold 10 mM Tris. After a 10 min centrifugation at 14200 ×g, the cell pellet was washed with 1.5 mL of cold 10 mM Tris, and then solubilized with 500 μL of 10% sodium dodecylsulfate (SDS). The resulting suspension was transferred to a scintillation vial. The microtubes were rinsed twice with 250 μL of 10% SDS which were also added to the vial. Radioactivity was then measured after addition of 6 mL of scintillation cocktail.

**Measurement of incorporation into proteins.** At the required times, aliquots were withdrawn from the incubation mixture and transferred into a microtube containing the same volume of 10% SDS. After thorough

mixing, aliquots of 20  $\mu\text{L}$  were spotted on GF/A glass fiber filters. The filters were then put in a beaker containing at least 10 mL of cold 10% trichloroacetic acid (TCA) per filter. The TCA was brought to a boil for 10 min and ice was then added to cool down the solution until the filters sank in the bottom of the beaker. Filters were then rinsed twice with cold 5% TCA followed by two additional rinses with cold acetone (at least 5 mL of liquid per filter). Each filter was then transferred into a scintillation vial to which 500  $\mu\text{L}$  of 1 M NaOH was added and heating at 50°C was applied for 2 hours. After addition of 200  $\mu\text{L}$  of 6 M HCl, 6 mL of scintillation fluid was added and the radioactivity was measured. Under these conditions, severely limiting the amount of amino acids in the medium, most (> 90%) of the uptaken labeled amino acids were incorporated into protein in controls (no toxicant added).

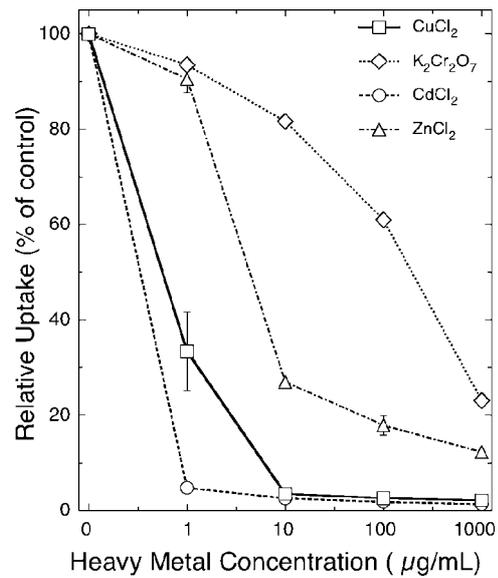
### Results

We first investigated the effects of various heavy metals ( $\text{Cd}^{2+}$  as  $\text{CdCl}_2$ ,  $\text{Cu}^{2+}$  as  $\text{CuCl}_2$ , Cr(VI) as  $\text{K}_2\text{Cr}_2\text{O}_7$  (dichromate), and  $\text{Zn}^{2+}$  as  $\text{ZnCl}_2$ ) on the uptake of [ $^{35}\text{S}$ ]-methionine by *Tetrahymena* cells (Fig. 1). These four heavy metals decreased the uptake of the amino acid compared to a control (without heavy metal). However, their patterns were different. Dichromate brought about a more gradual decline than the three other substances, reaching about 25% of the control at 1 mg/L. On the other hand,  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  precipitously decreased uptake at lower concentrations, bringing it below 5% of the control at 1 and 10  $\mu\text{g}/\text{mL}$ , respectively.  $\text{Zn}^{2+}$  had an intermediary effect, similar to that of dichromate at low concentration but more like  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  at higher concentrations. So the divalent cations had a much stronger effect compared to the hexavalent anion in the form of dichromate.

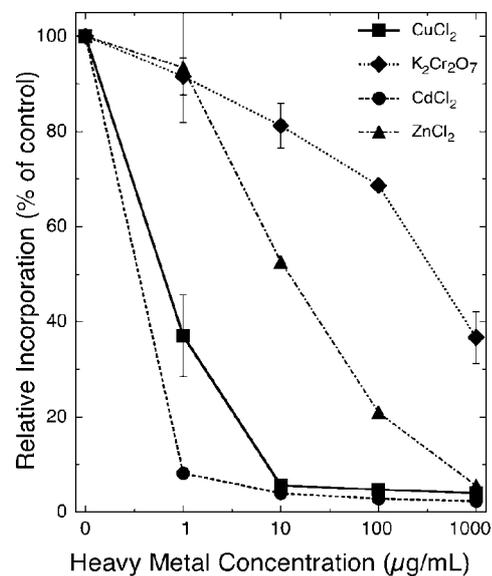
The effect of heavy metals on incorporation of [ $^{35}\text{S}$ ]-methionine into proteins (Fig. 2) followed the same pattern as observed in the above uptake experiments: a rapid and pronounced drop in presence of  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$ , a slower decrease in presence of dichromate, and an intermediary effect with  $\text{Zn}^{2+}$ .

We noticed the similarity in the patterns of inhibition of amino acid uptake and their incorporation into proteins. So we studied the correlation between these two processes by plotting one as a function of the other. The ratios of uptake and incorporation were strongly correlated with a linear relationship close to 1 (Fig. 3) for  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$ . For Cr(VI), the relation between uptake and incorporation was closer to 0.75. In all cases, the coefficient of determination ( $r^2$ ) was larger than 0.99.

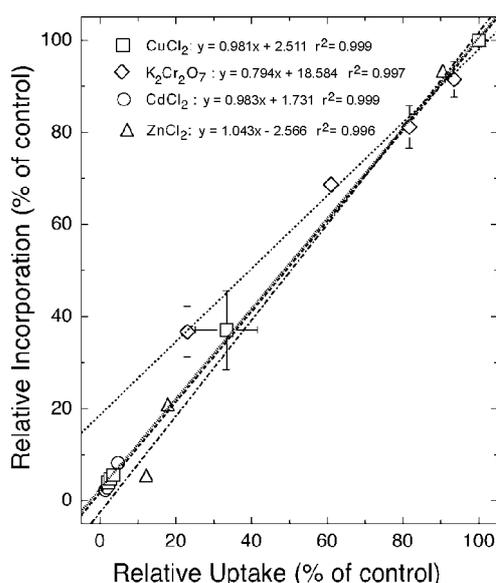
Organic toxicants also reduced methionine uptake, although their patterns were a bit more complex (Fig.



**Fig. 1.** Effect of some heavy metals on uptake of [ $^{35}\text{S}$ ]-methionine in *Tetrahymena pyriformis*. (Cells were incubated as described in Material and Methods in presence of various heavy metals for 2 hours.) Note the logarithmic scale on the concentration axis. Mean of two (Cu and Zn) or three (Cd and Cr) different experiments  $\pm$  standard deviation; if no error bar is shown, deviation is smaller than the symbol.



**Fig. 2.** Effect of some heavy metals on incorporation of [ $^{35}\text{S}$ ]-methionine into proteins in *Tetrahymena pyriformis*. (Cells were incubated as described in Material and Methods in presence of various heavy metals for 2 hours.) Note the logarithmic scale on the concentration axis. Mean of two (Cu and Zn) or three (Cd and Cr) different experiments  $\pm$  standard deviation; if no error bar is shown, deviation is smaller than the symbol.

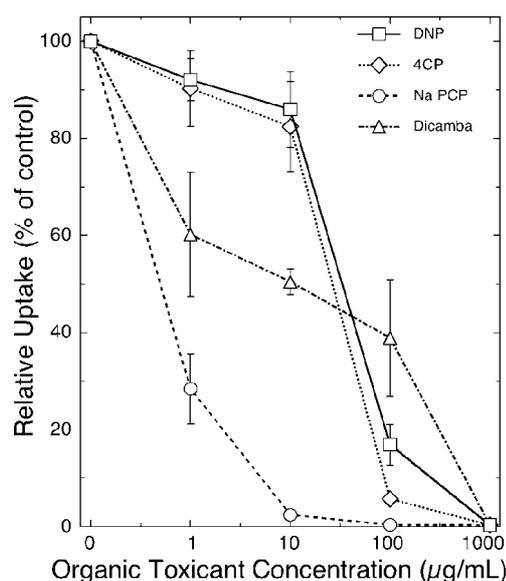


**Fig. 3.** Effect of some heavy metals on the ratio of amino acid uptake and incorporation into proteins. (Cells were incubated as described in presence of various heavy metals for two hours.) Mean of two (Cu and Zn) or three (Cd and Cr) different experiments  $\pm$  standard deviation; when no error bar is shown, deviation is smaller than the symbol.

4). For instance, 2,4-dinitrophenol and 4-chlorophenol had a biphasic effect: quite mild above 10  $\mu\text{g/mL}$ , but very marked after, which lowered the incorporation level below 20% of the control at 100  $\mu\text{g/mL}$ . The pesticide Dicamba<sup>TM</sup> (3,6-dichloro-2-methoxybenzoic acid) had an even more complicated pattern: rapid drops before 1  $\mu\text{g/mL}$  and after 100  $\mu\text{g/mL}$ , with only a slight diminution between 10 and 100  $\mu\text{g/mL}$ . On the other hand, sodium pentachlorophenate rapidly reached an almost complete inhibition at 10  $\mu\text{g/mL}$ .

Organic toxicants also inhibited methionine incorporation into proteins (Fig. 5) in a pattern rather similar to the uptake inhibition. Here again, we sought to assess the extent of this likeness. The ratio of uptake and incorporation was high, gradually decreasing from almost 1.0 to 0.8 in the row: sodium pentachlorophenate, Dicamba, 4-chlorophenol, 2,4-dichlorophenol (Fig. 6). There was a strong coefficient of determination ( $r^2 > 0.93$ ) for all organic toxicants.

We proceeded to ascertain if these observations on methionine could be generalized to other amino acids. So we compared the effect of inorganic and organic toxicants (1  $\mu\text{g/mL}$ ) using either [<sup>35</sup>S]-methionine, a mixture of [<sup>35</sup>S]-methionine and [<sup>35</sup>S]-cysteine or a mixture of [<sup>14</sup>C]-amino-acids (Table 1). The uptake/incorporation ratio was close to unity with the various labeled amino acids for most of the toxicants tested, indicating that amino acids uptake was the main limiting factor in their incorporation into proteins.

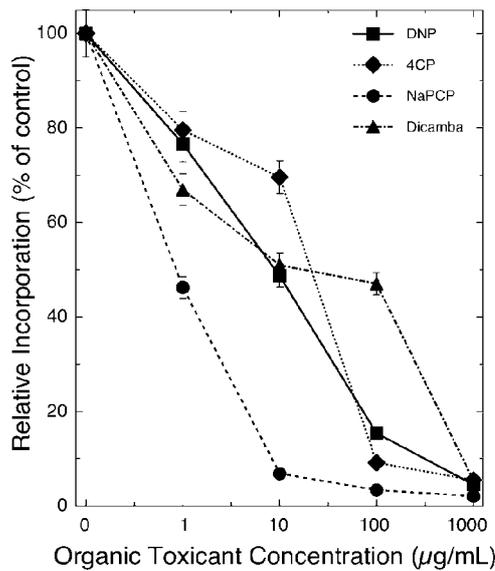


**Fig. 4.** Effect of some organic toxicants on uptake of [<sup>35</sup>S]-methionine in *Tetrahymena pyriformis*. (Cells were incubated as described in Material and Methods in presence of various organic toxicants for 2 hours.) Note the logarithmic scale on the concentration axis. Mean of two (Dicamba<sup>TM</sup> and DNP) or 3 (PCP and 4CP) different experiments  $\pm$  standard deviation; if no error bar is shown, deviation is smaller than the symbol. *Abbreviations:* NaPCP = sodium pentachlorophenate, DNP = 2,4-dinitrophenol and 4CP = 4-chlorophenol.

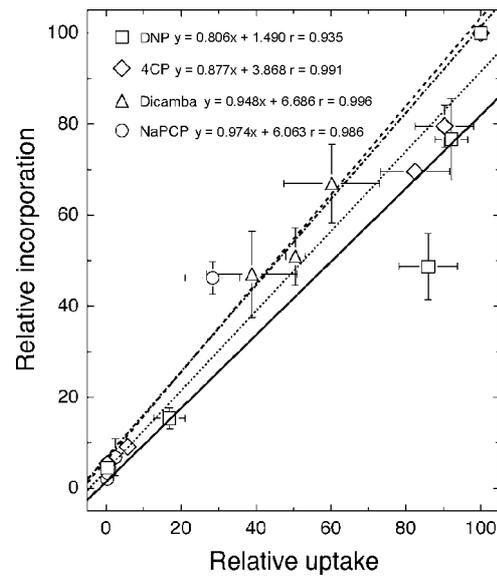
## Discussion

Our results show that, in *Tetrahymena*, all the toxicants tested inhibited both amino acid uptake and incorporation. This phenomenon seems to be independent of the type of labeled amino acids (or mixture thereof). Uptake/incorporation ratios were close to 1 in all heavy metals but Cr (Fig. 3). We cannot explain this difference, but it should be noted that Cr was in an anionic form (dichromate), while other heavy metals were added as divalent cations. Furthermore, the inhibition patterns seem to be simpler for divalent cationic heavy metals than for organic compounds, the latter having a more complex response (Fig. 1 and 2 compared to 4 and 5) and uptake/incorporation ratios less close to one (Fig. 3 vs 6). However, such a concomitant inhibition of both amino acid uptake and protein synthesis can be brought about by dimethyl sulfoxide in *Tetrahymena* (Nilsson, 1980) or by crystal violet in another protozoan, *Trypanosoma* (Hoffmann et al., 1995). In rat cells,  $\text{Cd}^{2+}$  inhibits both protein synthesis and amino acid uptake (Holt and Webb, 1986).

We have also shown that the inhibition of incorporation of amino acids into proteins by toxic compounds is strongly correlated with an inhibition of their uptake. This was the case for both inorganic and organic



**Fig. 5.** Effect of some organic toxicants on incorporation of [<sup>35</sup>S]-methionine into proteins in *Tetrahymena pyriformis*. (Cells were incubated as described in Material and Methods in presence of various organic toxicants for 2 hours.) Mean of two (Dicamba™ and DNP) or 3 (PCP and 4CP) different experiments ± standard deviation; if no error bar is shown, deviation was smaller than the symbol. Note the logarithmic scale on the concentration axis. Abbreviations as in Fig. 4.



**Fig. 6.** Effect of some organic toxicants on the ratio of amino acid uptake and incorporation into proteins. (Cells were incubated as described in presence of various organic toxicants for two hours.) Mean of two (Dicamba™ and DNP) or 3 (PCP and 4CP) different experiments ± standard deviation; if no error bar is shown, deviation is smaller than the symbol. Abbreviations as in Fig. 4.

toxicants whatever the labeled amino acids used (Table 1). Of course, the simplest explanation for this correlation is to ascribe the reduced incorporation to an impairment of amino acid transport brought about by the toxicants. Thus the lowered entry of amino acids would deplete their cytoplasmic concentrations leading to a slowing down of protein synthesis. However, it should be pointed out that the amount of labeled amino acids put in the incubation medium is very small compared to the amount of free amino acids required and used by protein synthesis. In fact, in a system where proteins are metabolically labeled, protein synthesis is essentially driven by the internal pools of amino acids (Appel et al., 1992). So, a lessened amino acid uptake may not be an important factor in reducing protein synthesis, though it would impact the labeling level. However, we obtained basically the same results with three different amino acids or combinations (Table 1: methionine alone, methionine and cysteine, 20 amino acids). It would be surprising that all the toxicants tested would directly impair all amino acid transport systems, even roughly, in the same way. Both organic and inorganic toxicants inhibit phagocytosis in *Tetrahymena* (Nilsson, 1980; Nilsson, 1981), preventing amino acid from entering the cell via this route. In *Tetrahymena*, membrane-associated transporters take up amino acids, not phagocytosis

which captures proteins (Orias and Rasmussen, 1979; Leick, 1992). So, in our case, the former mechanism should predominate in transporting labeled amino acids into the cell. But here again, we cannot expect all the tested toxicants to inhibit all those transporters to a similar level. It should be also noted that the half-life of these transporters is so short that, rather paradoxically, protein synthesis inhibition could slow down amino acid transport (Blum, 1982; Eichler, 1989) even before the latter could inhibit the former.

These considerations lead us to think that both amino acid uptake and protein synthesis could be inhibited by a third activity. The likeliest possibility is energy production, since both amino acid transport and protein synthesis require large amounts of ATP. A large number of toxicants, both inorganic and organic, have been shown to alter mitochondrial structure and inhibit ATP production (Nilsson, 1980; Findly et al., 1983; Nilsson, 1995; Sparagano, 1995; Nicolau et al., 2004). This hypothesis could be tested by evaluating the effect of those toxicants on amino acid uptake or incorporation and on a cell activity highly dependant on energy production, such as swimming speed. However, our results did not rule out that some of the compounds tested could directly inhibit protein synthesis or amino acid transport or act at many levels.

**Table 1.** Effect of various toxicants on the amino acid uptake/incorporation ratio.

Toxicant	Amino acid uptake/incorporation ratio		
	Isotopic marker		
	[ <sup>35</sup> S]-methionine <sup>a</sup>	[ <sup>35</sup> S]-methionine and [ <sup>35</sup> S]-cysteine <sup>b</sup>	[ <sup>14</sup> C]-amino acids <sup>b</sup>
Heavy metals			
Cd <sup>2+</sup>	1.22 ± 0.21	1.13 ± 0.22	0.85 ± 0.11
Cu <sup>2+</sup>	1.18 ± 0.21	1.17 ± 0.20	0.95 ± 0.12
Cr (VI)	0.98 ± 0.05	1.21 ± 0.11	0.94 ± 0.15
Zn <sup>2+</sup>	1.03 ± 0.09	1.08 ± 0.20	0.92 ± 0.11
Organic toxicants			
2,4-Dinitrophenol	0.83 ± 0.18	1.18 ± 0.25	0.94 ± 0.02
4-Chlorophenol	0.88 ± 0.22	n.d.	n.d.
Pentachlorophenate	1.30 ± 0.56	1.17 ± 0.04	0.99 ± 0.19
Phenol	0.98 ± 0.11	1.12 ± 0.10	1.12 ± 0.27

**Note:** Cells were incubated for two hours in Tris 10 mM in presence of 1 µg/mL of a given toxicant. Amino acid uptake and incorporation were measured as described in "Material and Methods".

a - mean of two different experiments ± standard deviation; b - mean of four different experiments, two of which were performed in parallel at the same time with the same cell preparation; ± - standard deviation; n.d. - not determined.

On a more practical note, if one is to assess the influence of toxicants on protein synthesis, it would be well worthwhile to ascertain whether any of the effects observed could be related to amino acid uptake or, for that matter, to any cell process.

To conclude, we have shown that most of the slowing down of protein synthesis by several organic as well as inorganic toxicants is accompanied by a reduction of amino acid transport. Whether these two phenomena are linked by a causal effect or are themselves the consequence of a third event is unclear and has to be ascertained for each toxicant. More importantly, such a possible interplay between those cell processes should be taken into account in the design of investigations on the effect of toxicants and the interpretation of their results.

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