Applied Organometallic Chemistry (1990) 4 119–125

0268-2605/90/020119-07 $05.00

The uptake of arsenicals by *Candida humicola*

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Received 14 November 1989

The uptake of arsenate by *Candida humicola* requires an active transport system and may operate through low- and high-affinity sites. Arsenite, methylarsonate and dimethylarsinate are deduced to enter the cell by slow passive diffusion.

Keywords: Arsenate uptake, *Candida humicola*, active transport, arsenicals, diffusion, two-site model

INTRODUCTION

The production of volatile arsines from arsenate and other arsenic compounds by micro-organisms is well documented.[1–3] Studies with the yeast *Candida humicola* have shown that trimethylarsine is produced when the organism is cultured in the presence of arsenate, arsenite, methylarsonate, dimethylarsinate and trimethylarsine oxide.[4–7] Furthermore, arsenite, methylarsonate and dimethylarsinate are produced when broken *C. humicola* cells are incubated with arsenate.[8] Thus the stepwise mechanism of Scheme 1 for the conversion of arsenate to trimethylarsine, first suggested by Challenger,[1,2] is feasible, although by no means established in every detail.[3]

In general, the individual reactions in this pathway are difficult to study, although it has been possible to look at the final step (i.e. the reduction of trimethylarsine oxide to trimethylarsine) in some detail due to the formation of a volatile, easily detectable product.[7]

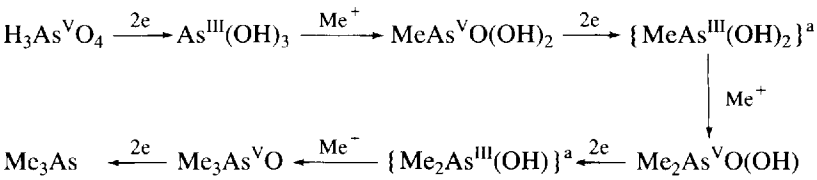
The initial step in the biological transformation of arsenate to trimethylarsine is presumed to be the entry of arsenate into the cell. Arsenate uptake has been monitored for several organisms, usually with the objective of studying its effect on phosphate uptake.[9–21] A common transport system for phosphate and arsenate is proposed for the bacteria *Micrococcus lysodeikticus*[9] and *Streptococcus faecalis*,[19,20] and also the yeasts *Saccharomyces cerevisiae*[10,16] and *Rhodotorula rubra*.[13,15] Two systems for the uptake or arsenate and phosphate are present in *Escherichia coli*.[18]

In this communication we have characterized the uptake of arsenate by *Candida humicola*, and compare it with three other metabolizable arsenicals.

EXPERIMENTAL

Organism

The organism used in all experiments was *Candida humicola*, a fungus isolated from domestic sewage by Cox and Alexander.[4,5] It was maintained in a minimal salts–glucose medium consisting of $(NH_4)_2SO_4$ (2.0 g), KH_2PO_4 (0.1 g), $MgSO_4.7H_2O$ (0.05 g), $FeSO_4.7H_2O$ (0.0018 g), thiamine hydrochloride (0.01 g), and glucose (10.0 g) per litre of 0.05 mol dm^{-3}, pH 5, potassium succinate/succinic acid buffer.[4]



Scheme 1

[a]These species are unknown as monomers

Arsenicals

Non-radioactive arsenic compounds were obtained as follows: sodium arsenate ($Na_2HAsO_4.7H_2O$) and sodium arsenite ($NaAsO_2$) from Baker Chemical Co.; sodium methylarsonate ($CH_3AsO(ONa)_2.6H_2O$) from Alfa Inorganics; dimethylarsinic acid [$(CH_3)_2AsO_2H$] from Fisher Scientific Co.; ^{14}C-labelled methylarsonic acid (specific activity 10 mCi mmol^{-1}), dimethylarsinic acid (specific activity 11.2 mCi mmol^{-1}), from ICN; and sodium [^{74}As]arsenate (specific activity 1 mCi mmol^{-1}) from Amersham Chemical Corp.

[^{74}As]Arsenite was prepared by the reduction of [^{74}As]arsenate.[22] Freshly prepared reducing solution (0.1 cm^3) (0.28 g sodium metabisulphite, 15 cm^3 water, 2 cm^3 1% sodium thiosulphate, and 0.25 cm^3 7.5 mol dm^{-3} sulphuric acid) was added to 0.1 cm^3 [^{74}As]arsenate and allowed to stand for 40 min. The reaction mixture was applied to a 3 cm × 0.7 cm anion-exchange resin column (Dowex AC1-X8-acetate, 100–200 mesh, supplied by Bio-Rad Laboratories). [^{74}As]Arsenite was eluted with water, whilst unreacted [^{74}As]arsenate was retained by the column.

Assay for the uptake of arsenate by *C. humicola*

The assay was based on that reported by Jung and Rothstein.[10] A 16–24 h culture of *C. humicola* (25 cm^3) was centrifuged and washed twice with 0.05 mol dm^{-3} pH 5 potassium succinate–succinic acid buffer. Cells were resuspended in this buffer at a concentration of 25 mg wet weight cm^{-3} (18 mg wet weight is equivalent to 1 mg dry weight). The cell suspension (1 cm^3) was mixed with 0.475 cm^3 buffer and incubated with stirring at 18 °C for 5 min. The [$^{74,75}As$]arsenate solution (25 μL) was added; the final arsenate concentration in the reaction mixture was 0.1 mmol dm^{-3}, and the total radioactivity of [^{74}As]arsenate was between 2 × 10^4 and 4 × 10^4 cpm. At various known times after the addition of arsenate, 100 μL of the reaction mixture was removed and placed on a polycarbonate filter disc of pore size 0.8 μm and diameter 25 mm (Nucleopore Corporation). The buffer was removed by suction, and the cells retained by the filter were washed free of extraneous arsenate with a continuous stream of 5 cm^3 of ice-cold buffer. The cells were counted after placing the filter in a vial with 10 μL of Bray's scintillation fluid.

Treatment of data

From the activity of [^{74}As]arsenate in the 0.1 mmol dm^{-3} arsenate reaction and that of the cells on the discs, the amount of arsenate taken up by the cells was calculated. The graph of arsenate accumulated against time was plotted, and the initial rate of arsenate uptake was taken to be the initial slope of this graph. A background run, in which cells were omitted from the reaction, showed that about 50 cpm or 0.5 nmol of arsenate was adsorbed by the filter. This amount was subtracted from all readings.

RESULTS

The accumulation of arsenate by *Candida humicola* under the standard assay conditions (cell concentration 1.7 mg wet wt. cm^{-3}; arsenate concentration 0.1 mmol dm^{-3}; pH 5; temperature 18 °C) had the characteristics seen in Fig. 1. The accumulation increased linearly with time for the first 60 s. Subsequently the uptake increased more slowly to a maximum value of 3.7 mmol (g wet wt cells)$^{-1}$. The initial rate of uptake, the slope of the linear section of the graph, is equivalent to 42 mmol arsenate s^{-1} (g wet wt)$^{-1}$. This rate can be compared with values found in other systems. For example, the corresponding values for *Saccharomyces cerevisiae* (25 °C,

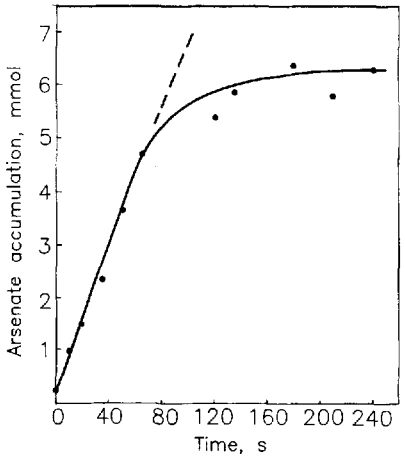


Figure 1 Arsenate accumulated by *Candida humicola* as a function of time. Cells of 1.7 mg wet weight were exposed to a solution of 0.1 mmol dm^{-3} arsenate, pH 5, 0.05 mol dm^{-3} succinic acid–potassium succinate, at 18 °C.

pH 4.5, arsenate concentration 1 mmol dm^{-3}) is 53 mmol s^{-1} g^{-1}, for Bakers yeast (25 °C, pH 5, arsenate concentration 2 mmol dm^{-3}) is 3 mmol s^{-1} g^{-1}, and for *Micrococcus lysodeikticus* (pH 7.8, arsenate concentration, 0.1 mmol dm^{-3}) is 15 mmol s^{-1} g^{-1}.[9,10]

Variations made in the parameters of the standard assay effected the following changes.

1 Temperature

The assay was carried out at various temperatures. The temperature dependence of arsenate uptake is shown in Fig. 2. There is no uptake at 4 °C, or at 60 °C. The maximum rate of accumulation, occurring at about 30 °C, is 76 mmol s^{-1} (g wet wt)$^{-1}$. These results are as expected for a mechanism of uptake dependent on viable cells. The rate of uptake at 30 °C is too high to be measured easily and accurately; hence the choice of a temperature of 18 °C for all other measurements.

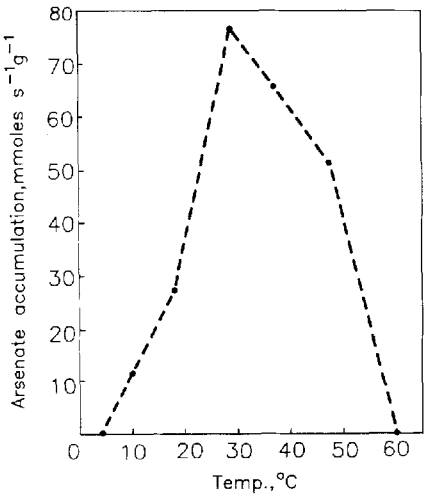


Figure 2 The effect of temperature on the rate of uptake of arsenate.

2 Cell concentration

Halving or doubling the cell concentration in the assay mixture had no effect on the rate of accumulation of arsenate per unit mass of cells.

3 pH

Harvested cells were washed twice with 0.85% sodium chloride solution and resuspended in this solution at a concentration of 50 mg wet weight cm^{-3}. The assay mixture contained: 0.5 cm^{-3} cell suspension; 0.74 cm^{-3} 0.1 mmol dm^{-3} buffer; 0.24 cm^{-3} H_2O; and 25 μL [^{74,75}As]arsenate solution. For pH 5, succinic acid–potassium succinate buffer was used; for pH 7, tris-HCl buffer was used. After filtering the cells, they were washed with cold 0.85% saline solution.

At pH 5, which is the pH of the culture media, the rate of uptake of arsenate was normal, whereas at pH 7 there was no uptake within the first 30 s after the addition of arsenate.

4 Autoclaved cells

Cells were autoclaved at 15 psi (72 Pa) for 15 min prior to assay for arsenate accumulation. Such cells showed no uptake of arsenate during the first 20 min following arsenate addition. Thus viable cells are required for arsenate uptake.

Dependence on external energy source

When glucose was added to the assay immediately before adding arsenate, so that the final concentration of the sugar was 1 mmol dm^{-3}, the rate of arsenate accumulation was not affected. When the non-metabolizable buffer, pH 5, 0.05 mol dm^{-3}, sodium phthalate–phthalic acid, replaced the usual succinate buffer, the rate of arsenate accumulation also was not affected. Thus, it can be assumed that the cells contain sufficient endogenous energy supplies for the uptake of arsenate. Shaking the cells for 12 h in distilled water prior to assaying for arsenate uptake did not remove their endogenous energy.

Arsenate concentration

The rate of uptake of arsenate as a function of arsenate concentration was investigated by changing the arsenate concentration from that used in the standard assay. Figure 3 shows that, when arsenate concentration is increased or decreased, there is a corresponding increase or decrease in the rate of arsenate uptake. At arsenate concentrations lower than 0.02 mmol dm^{-3} there seems to be a linear relationship between the rate of uptake and arsenate concentration. At concentrations greater than 0.02 mmol dm^{-3}, another linear relationship probably exists, but the rate of change in uptake rate is less marked. A greater concentration than 0.2 mmol dm^{-3} is required to saturate the cells,

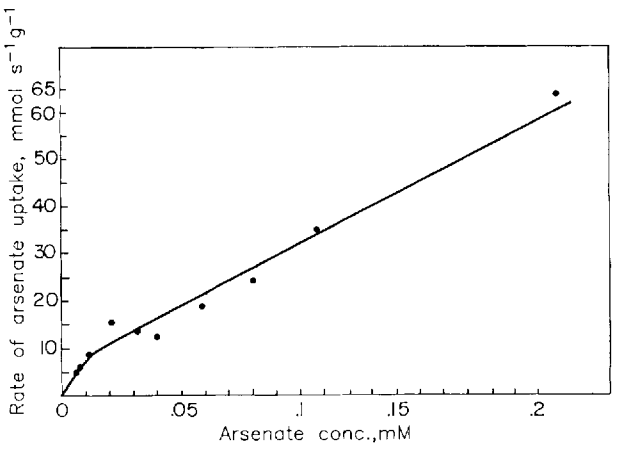


Figure 3 The rate of uptake of arsenate as a function of arsenate concentration.

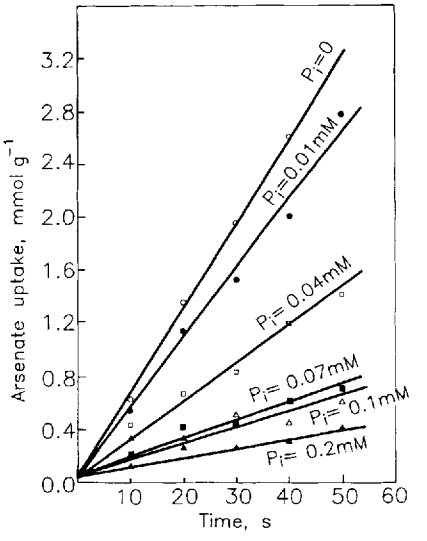


Figure 4 The effect of inorganic phosphate on arsenate uptake.

because a maximum rate is not reached at this concentration.

The effect of arsenate concentration in the assay on the final total arsenate accumulated by cells is shown in Table 1.

At the lowest-tested arsenate concentrations, all the arsenate is accumulated by the cells. At higher arsenate concentrations, the proportion of available arsenate accumulated decreases, although the actual amount accumulated increases. At all concentrations, the final cellular arsenate concentration (assuming a density of 1.0 g dm^{-3} for wet cells) is greater than the extracellular arsenate concentration.

Phosphate concentration

Phosphate was added at various concentrations to the assay. Figure 4 shows the effect of this on the accumulation of arsenate by *C. humicola* cells. A phosphate concentration as low as 0.01 mmol dm^{-3} (i.e. 10% of the arsenate concentration in the assay) had a noticeable effect on the rate of arsenate accumulation by the cells. The rates of arsenate accumulation as a function of phosphate concentration are plotted in Fig. 5. A phosphate concentration of 0.035 mmol dm^{-3} is sufficient to reduce by 50% the rate of arsenate accumulation. When the phosphate concentration is equimolar with arsenate (i.e. 0.1 mmol dm^{-3}), the rate of arsenate accumulation is 7 mmol s^{-1} g^{-1}, compared with 38 mmol s^{-1} g^{-1} in the absence of phosphate.

Inhibition by arsenate analogues and other arsenicals

The rate of arsenate uptake was assayed in the presence of sulphate, selenate, tellurate, arsenite, methylarsonate, dimethylarsinate and trimethylarsine oxide

Table 1 The effect of arsenate concentration on arsenate uptake by *C. humicola*

Arsenate concn mmol dm^{-3}	Total arsenate accumulated [mmol arsenate (g wet wt cells)$^{-1}$]	Available arsenate accumulated (%)
0.01	0.6	100
0.04	1.9	79
0.11	2.4	38
0.23	2.6	19
1.07	4.4	7
2.30	4.7	3.5

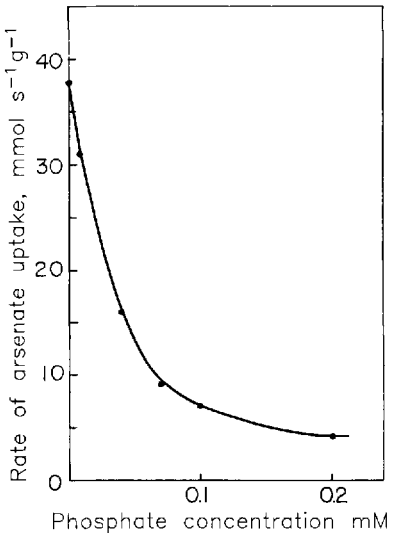


Figure 5 The effect of phosphate on the rate of arsenate uptake.

species. Each of these compounds was added simultaneously with arsenate. The concentration of the additive in the assay mixture was 0.1 mmol dm^{-3}. Table 2 shows the rate of arsenate uptake in the presence of each compound, expressed as a percentage of the rate of the control in which only arsenate is present.

Table 2 The effect of additives on the rate of arsenate uptake by *C. humicola*

Species[a]	Rate (% control)
Sulphate	66
Selenate	85
Tellurate	115
Arsenite	87
Methylarsonate	86
Dimethylarsinate	70
Trimethylarsine oxide	73

[a]The concentration of the additive was 0.1 mmol dm^{-3}

Sulphate, dimethylarsinate and trimethylarsine oxide significantly depressed arsenate accumulation by about 30% (cf. phosphate, which caused an 80% reduction in arsenate uptake).

In a second experiment of this type, cells were pre-incubated at 18 °C for 19 min with 0.1 mmol dm^{-3} arsenite, methylarsonate, dimethylarsinate or trimethylarsine oxide, before the assay was initiated by addition of arsenate. Pre-incubation with these arsenicals did not affect the subsequent rate of uptake of arsenate.

Electron-transport inhibitors

Table 3 shows the effect of pre-incubating cells for 10 min at 18 °C with various inhibitors of electron transport, and 2,4-dinitrophenol (2,4-DNP) (which uncouples oxidative phosphorylation). All inhibitors, with the exception of oligomycin, reduced the rate of arsenate uptake. The most effective was 2,4-dinitrophenol, which completely inhibited arsenate uptake.

Table 3 The effect of electron-transport inhibitors on the uptake of arsenate by *C. humicola*

Inhibitor (mmol dm^{-3})		Rate (% control)
Oligomycin	(0.1)	100
Urethane	(5.0)	58
HOQNO[a]	(0.5)	55
Cyanide	(10.0)	25
Azide	(10.0)	21
Rotenone	(0.5)	19
Antimycin A	(0.01)	15
CCCP[b]	(0.01)	0.4
2,4-DNP	(1.0)	0

[a]HOQNO, 2-*N*-heptyl-4-hydroxyquinoline-*N*-oxide; [b]CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

Uptake of arsenite, methylarsonate and dimethylarsinate

The uptake of each of arsenite, methylarsonate and dimethylarsinate added alone was assayed in the same way as arsenate uptake. There was no detectable uptake of arsenite, methylarsonate or dimethylarsinate within 30 min of adding these arsenicals to *C. humicola* cells.

In an attempt to induce uptake of arsenicals other than arsenate, *C. humicola* was cultured in the presence of 10 mmol dm^{-3} methylarsonate or dimethylarsinate, and the cells from such cultures were assayed in the usual way for uptake of methylarsonate or dimethylarsinate, respectively. As with untreated cells, no uptake was detected in the first 30 min of the assay.

It appears from this work that the first step in the metabolism of these arsencials to trimethylarsine must be a slow, passive diffusion of the compound into the cell not observed here. The slight inhibition of arsenate

uptake by dimethylarsinate is presumably of a non-competitive nature.

DISCUSSION

The temperature profile and the pH specificity of the uptake of arsenate by *C. humicola*, as well as the effects of electron-transport inhibitors and autoclaving cells, point towards an active transport mechanism for arsenate accumulation in the organism. The endogenous energy of the cells is sufficient for accumulation to occur. Starving cells of *S. faecalis*[19,20] did not take up arsenate unless glucose or galactose was added. Similarly starved cells of bakers' yeast, in which endogenous fermentation is low, showed no appreciable arsenate uptake.[10,16]

The change in kinetics of arsenate uptake with arsenate concentration, if real, is more complex than can be explained by a simple Michaelis–Menten model. It has been shown that for bakers' yeast there may be two sites of arsenate uptake, one with a low affinity for arsenate and another with a high affinity.[10] Although from the data here it is not possible to prove that a two-fold system is present in *C. humicola*, such a system could explain these observations. Thus for arsenate concentrations less than 0.01 mmol dm^{-3}, high-affinity sites are preferentially occupied and are responsible for arsenate uptake. At about 0.01 mmol dm^{-3} all high-affinity sites are saturated, and as the arsenate concentration is increased above 0.01 mmol dm^{-3}, low-affinity sites are used. An arsenate concentration greater than 0.2 mmol dm^{-3} is required to saturate the latter sites.

The reduction in arsenate uptake when phosphate is added is greater than can be accounted for by assuming equal affinities of the cells for phosphate and arsenate. For example, if arsenate and phosphate were indistinguishable, a mixture of 0.1 mmol dm^{-3} arsenate and 0.1 mmol dm^{-3} phosphate would be equivalent to 0.2 mmol dm^{-3} arsenate, and 0.1 mmol dm^{-3} phosphate would be equivalent to 0.1 mmol dm^{-3} arsenate. From Fig. 3, the uptake rate from 0.2 mmol dm^{-3} arsenate is 60/35 (=1.7) times faster than from 0.1 mmol dm^{-3} arsenate. Since in a mixture of arsenate and phosphate only the arsenate is labelled and detected, the expected arsenate-plus-phosphate uptake rate from 0.1 mmol dm^{-3} arsenate and 0.1 mmol dm^{-3} phosphate if the two ions were equivalent would be 0.85 times the uptake rate from 0.1 mmol dm^{-3} arsenate alone. In fact, the observed rate is only 0.18 times the rate from arsenate alone. This high degree of inhibition by equimolar (0.1 mmol dm^{-3}) phosphate can be compared with a 50% reduction in initial arsenate uptake by a marine yeast caused by phosphate when the arsenate concentration is 10^{-7} molar and the phosphate concentration is 10^{-6} molar. *Saccharomyces carlsbergensis* is claimed to be more permeable to arsenate than to phosphate, although in *Sac. cerevisiae* the rate of accumulation of phosphate is much faster than that of arsenate. With *C. humicola*, our results show that the inhibition of arsenate uptake increases as phosphate concentration increases, although the increase is less than directly proportional.

When the normal metabolism of *C. humicola* is inhibited either by electron-transport inhibitors such as azide and CCCP, or by the oxidative phosphorylation uncoupler 2,4-dinitrophenol (2,4-DNP), arsenate uptake is hindered. A wide range of inhibitors is effective, indicating that arsenate uptake is not linked to one specific step in the electron-transport chain. Cerbón[21] has shown that arsenate uptake from a very dilute arsenate solution (10^{-9} mol dm^{-3}) by *Sac. cerevisiae* is inhibited by about 75% by 10^{-4} mol dm^{-3} 2,4-DNP, by about 65% by $10-^{-3}$ mol dm^{-3} azide, and not at all by 10^{-3} mol dm^{-3} cyanide.

The rate of uptake of arsenate by *C. humicola* is unique among the arsenicals we have tested and the pK_a values of 2.25 and 6.67 are undoubtedly an important factor; however, those of methanearsonic acid [$MeAsO(OH)_2$] are 4.58 and 7.82, suggesting that simple ionization is not the controlling factor. This rapid uptake is in no way reflected in either a greater toxicity or a faster conversion to trimethylarsine under normal conditions of culture; thus the rate-limiting step is not transport into the cell.

Acknowledgements We thank the Natural Sciences and Engineering Research Council of Canada for financial assistance.

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