Applied Organometallic Chemistry (1989) 3 515–521

0268–2605/89/03607515/$03.50

The effect of arsenicals on cell suspension cultures of the Madagascar periwinkle (*Catharanthus roseus*)

William R Cullen, Deepthi Hettipathirana and John Reglinski*
Chemistry Department, University of British Columbia, Vancouver, BC, Canada V6T 1Y6

Received 31 May 1989 *Accepted 26 August 1989*

***Catharanthus roseus* cells were grown in the presence of arsenite, arsenate, methylarsonate and dimethylarsinate. Cell growth and arsenical uptake were monitored. Reduction of arsenate, methylation of arsenic and demethylation of methylarsenic species are described. Alkaloid production by the cells is dramatically influenced by the presence of arsenicals. ^{1}H NMR studies of methylarsonate uptake by whole cells of *C. roseus* are reported.**

Keywords: *Catharanthus roseus*, NMR spectra, arsenic compounds, alkaloid metabolites, methylarsenicals, uptake, growth, methylation

INTRODUCTION

Only a limited amount of information is available about the arsenic species present in terrestrial plants, even though it is well documented[1] that arsenic uptake by plants sometimes results in very high localized concentrations of the element. In particular, Benson and Nissen[2,3] report that following uptake of [^{74}As]arsenate (AsO_4^{3-}), via the roots, by plants such as corn, pea and melon, arsenite can be extracted from the plant. When some plants are grown in nitrate- and/or phosphate-deficient conditions prior to exposure to arsenate, methylation of arsenic to simple compounds, presumably methylarsenic(V) acids, is found, together with the formation of more complex and unidentified arsenicals.

We have initiated studies on the interaction of terrestrial plants with arsenicals as part of our continuing investigation of the biogeochemistry of arsenic. However, rather than work with whole plants we have chosen to investigate plant-tissue cultures, since, in principle, this offers a more convenient system. This paper describes some of our initial results from work with cell suspension cultures of *Catharanthus roseus*, the Madagascar periwinkle. This plant produces alkaloids such as vindoline that can be used as precursors for the synthesis of commercially important anticancer agents[4–6] and there is considerable interest in modifying growth conditions of cell suspension cultures of *C. roseus* in order to influence specific alkaloid production. Considerable effect can be expected because of the similar response of living systems to arsenite (AsO_3^{3-}) exposure and heat-shock.[7,8]

EXPERIMENTAL

Cell suspension cultures of *Catharanthus roseus* were usually grown in 1-B5 medium[9,10] containing a known weight of the arsenical compound (as sodium salt) under investigation. The medium (100 cm^3) was autoclaved and inoculated with a suspension of *C. roseus* cells (15 cm^3) taken from a culture that had already reached stationary phase after 10 or 11 days incubation. The ratio 15 cm^3 of cell suspension per 100 cm^3 of culture was maintained in all experiments, including controls, desribed in this work. The cultures were incubated in a gyratory shaker at 130 rpm. The growth was monitored by weighing the cells from control cultures every few days. Other parameters used to monitor the growth were refractive index and the pH of the residual medium, as well as physical appearance and colour of the cells and the cell aggregates.[9] Cells were isolated after the appropriate

*Present address: Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow G1 1XL, UK.

period of growth by filtering the suspension through a Miracloth filter under vacuum. The isolated cells were washed with distilled water. Wet weights and the dry weights after freeze-drying were recorded. The residual media were analysed for arsenic by hydride generation as well as by graphite furnace atomic absorption spectrometry.[11] Alkaloid metabolites were extracted from sonicated cells grown in alkaloid producing medium following the procedure of Kutney *et al.*[12] Solutions in ethyl acetate were chromatographed (HPLC, C_{18}-reverse phase) by using as eluent CH_3CH/H_2O (40/60) made 1% in Et_3N.

NMR studies

Cells of *C. roseus* were harvested at stationary phase, washed three times with deuterium oxide to remove excess medium, and then packed into a 5 mm NMR tube. A Bruker WH-400 MHz spectrometer was used to record all spectra. The spin-echo NMR spectra were recorded by using the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence[13–16] with a delay time (t) of 30 ms. A small pre-saturation pulse was applied to the water resonance prior to accumulation. Typical data for the spin-echo NMR spectra include an acquisition time of 0.426 s and a spectral width of 5000 Hz. The 90° pulse was generated using a 13.0 μs pulse width. The free induction decay was collected in 4K of data-points zero-filled to 32K. A 0.1 Hz line-broadening function was applied during Fourier transformation. Data were accumulated for 15 min per spectrum.

An extensive description of the pulse sequence and its effects on the NMR spectra of whole cells can be found elsewhere.[13–16]

RESULTS AND DISCUSSION

The growth cycle of a *C. roseus* cell suspension culture is usually composed of a short lag phase when there is no apparent cell division, then an exponential growth phase when the growth rate is a maximum, followed by a short stationary phase when the biomass yield is a maximum and constant. During the stationary phase, which is usually reached when a nutrient in the medium is depleted, there is no net increase in biomass. After stationary phase there is a drop in biomass due to cell lysis, as in Fig. 1. In 1-B5 medium the limiting nutrient is sucrose, the carbon source, and *C. roseus* cell

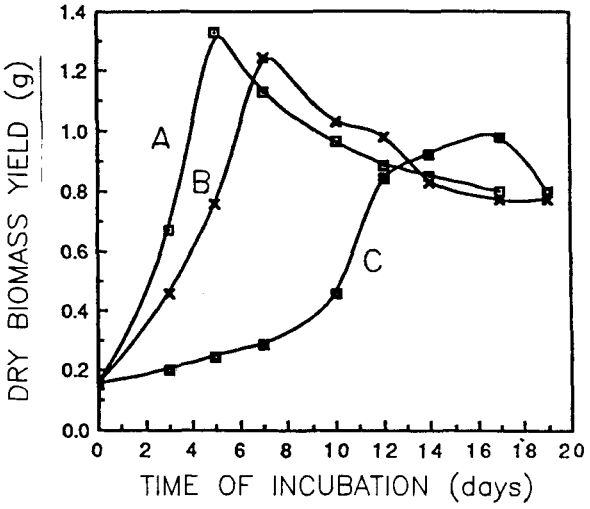

Figure 1 The effect of arsenate on the growth cycle of *C. roseus*: A, control; B, 2 μg cm^{-3} (ppm) arsenic as arsenate; C, 4 μg cm^{-3} arsenic as arsenate.

suspension cultures usually reach stationary phase after about five to ten days of incubation. This time can vary slightly, depending on the size of the culture (it is faster in smaller volumes of medium) and on whether wet or dry weight is used as the indicator.

The effect of arsenate (AsO_4^{3-}) on the growth cycle of *C. roseus* cultures is also shown in Fig. 1. At concentrations that do not inhibit growth completely there is a markedly longer lag phase, which depends on the concentration of arsenate, before the exponential growth phase starts. This phenomenon indicates an initial inhibition of growth by arsenate until the cells adapt to the arsenic-containing environment. Arsenite (AsO_3^{3-}) also causes a delay in growth, but it is not as pronounced as for arsenate. This effect is not observed for either methylarsonate or dimethylarsinate.

At low concentrations of arsenicals, cultures of *C. roseus* appear healthy and the biomass yields are comparable with those of control cultures grown in the absence of arsenic; however, at higher concentrations of arsenic compounds there is considerable aggregation and discoloration of cells, the extent of discoloration depending on the concentration. Inhibition of growth is apparent at higher concentrations of arsenic compounds.

Minimum inhibitory concentration (MIC) values for arsenicals can be estimated from plots of the dry biomass yield of the cultures, harvested after a specific time (usually 12–15 days) to allow stationary state to be achieved, relative to the initial concentration of the arsenical.

Figure 2 shows such a plot for arsenate. The MIC of arsenate is low, ($\sim 5\ \mu g\ cm^{-3}$ as arsenic) making it the most toxic of the four arsenic species studied toward the growth of *C. roseus*. Above this level there is a sharp fall in the cell yield and the cultures appear to be under much stress.

The arsenate uptake from medium initially containing 2 and $4\ \mu g\ cm^{-3}$ (ppm) arsenic (near the MIC value) was studied as a function of time (Fig. 3).

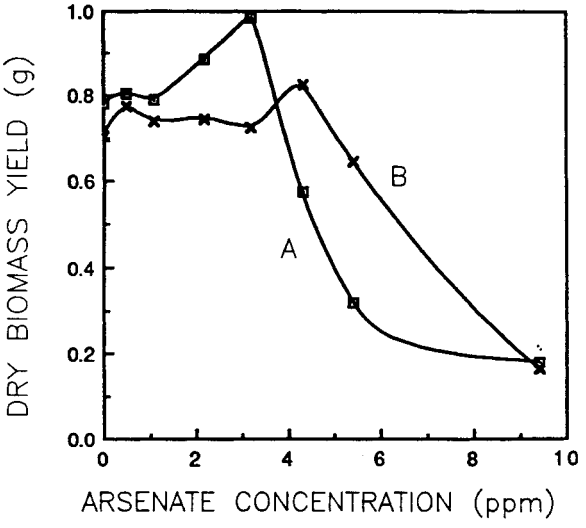

Figure 2 The effect of arsenate on the biomass yield of *C. roseus*. Cells were harvested after 13 days' growth (A), or 17 days' growth (B), in media containing the indicated initial concentration of arsenic as arsenate.

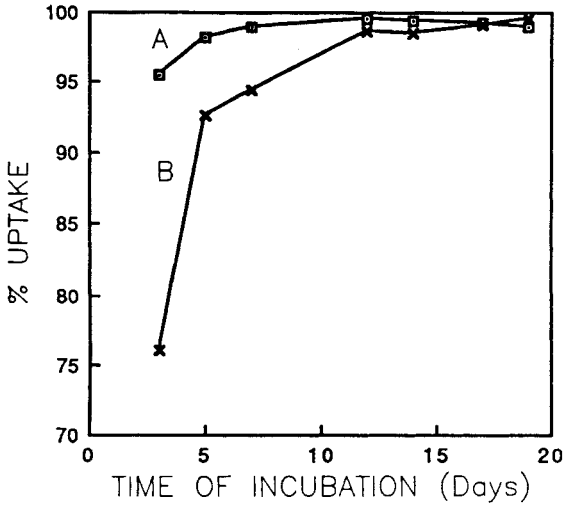

Figure 3 The uptake of arsenate with time by *C. roseus* from media containing (A), 2 $\mu g\ cm^{-3}$ (ppm) arsenic as arsenate; (B), 4 $\mu g\ cm^{-3}$ arsenic as arsenate.

Almost 95% of the arsenate is taken up by the third day of incubation from a medium initially containing 2 $\mu g\ cm^{-3}$ of arsenic as arsenate. Approximately 75% of the arsenic was taken up from a medium containing 4 $\mu g\ cm^{-3}$ of arsenate in the same time. Uptake reaches 99% or higher in about 10 days in both media. A slight increase in arsenic level in the media is observed after about 12 days. This increase can probably be attributed to cell lysis with ageing, which releases the incorporated arsenic back into the media.

Almost 95% of the arsenic left in the media is in the form of *arsenite*. A control study on autoclaved media containing arsenate but no plant cells failed to find any reduction. Thus, the reduction of arsenate is attributed to the living plant cells and may be a part of a detoxification system, since arsenite seems to be less toxic to *C. roseus*. As mentioned in the Introduction, whole plants reduce arsenate to arsenite;[2,3] some micro-organisms also carry out this transformation.[1]

The MIC value for arsenite is $\sim 9\ \mu g\ cm^{-3}$, as shown in Fig. 4. This Figure also shows that after 13 days the percentage of uptake of arsenite from the medium is effectively 100%, if the initial concentration is below the MIC value. The high uptake of both arsenate and arsenite by healthy cells indicates that active processes are involved.

The effect of methylarsonate, a widely used herbicide, on biomass yield is seen in Fig. 5. Unlike

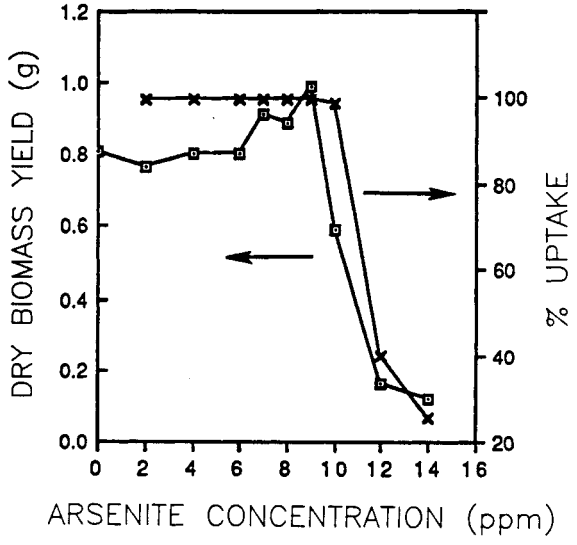

Figure 4 The effect of arsenite on the biomass yield of *C. roseus*. Cells were harvested after 13 days' growth in media containing the indicated initial concentration of arsenic as arsenite. The uptake of arsenite from the medium after 13 days' growth is also shown.

the response to inorganic arsenicals, there is a gradual decrease in biomass yield with increasing concentrations of the arsenical. It is difficult to define an MIC value from this graph; even 4 $\mu g\ cm^{-3}$ has an inhibitory effect. The biomass yield is reduced to 50% at an arsenical concentration of ~8 $\mu g\ cm^{-3}$. The uptake of methylarsonate decreases gradually with increasing initial arsenic concentration (Fig. 5). Here again, the uptake curve parallels the biomass curve indicating that uptake of methylarsonate is related to the number of living cells in the culture.

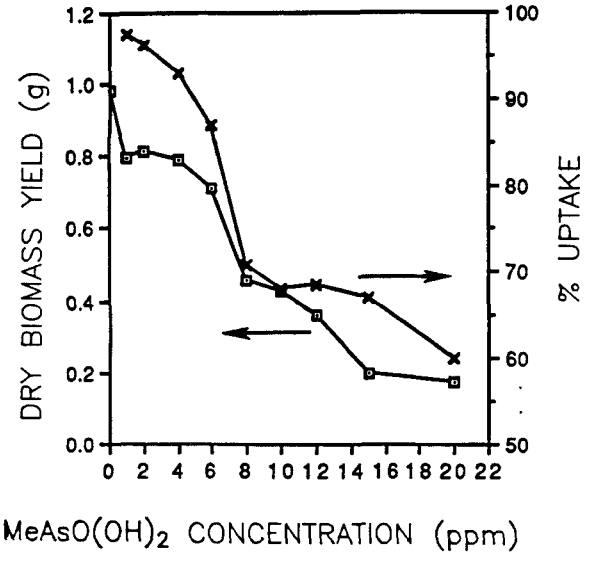

Figure 5 The effect of methylarsonate on the biomass yield of *C. roseus*. Cells were harvested after 12 days' growth in media containing the indicated concentration of arsenic as methylarsonate. The uptake of arsenic from the medium after 12 days' growth is also shown.

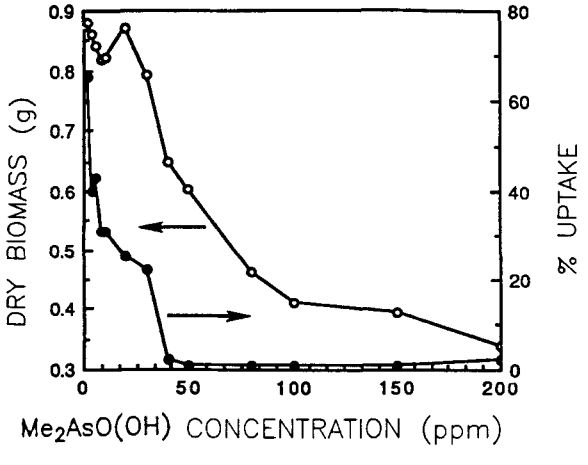

Figure 6 The effect of dimethylarsinate on the biomass yield of *C. roseus*. Cells were harvested after 12 days' growth in media containing the indicated concentration of arsenic as dimethylarsinate. The uptake of arsenic from the medium after 12 days' growth is also shown.

Dimethylarsinate ($(CH_3)_2AsOO^-$) is the least toxic to *C. roseus*. The data of Fig. 6 show that even at 20 $\mu g\ cm^3$ of arsenic the biomass yield after 12 days' growth is little changed from the control. The yield drops to 50% at an arsenic concentration of ~50 $\mu g\ cm^3$. The percentage uptake curve for dimethylarsinate is also somewhat different, in that the extent of uptake is much smaller even at low concentrations where there is good growth (Fig. 6). The highest uptake is around 70% at 2 $\mu g\ cm^{-3}$ of arsenic. This percentage rapidly levels off to ~20%. At initial concentrations above 50 $\mu g\ cm^{-3}$ the uptake is almost zero.

Speciation studies

When cells are harvested after growth in 2 $\mu g\ cm^{-3}$ (ppm) methylarsonate ($CH_3AsO_3^{2-}$) (12 days), freeze-dried, and extracted with 1 mol dm^{-3} sodium hydroxide (NaOH), the bulk of the borohydride ($NaBH_4$)-reducible arsenicals in the extract has retained one methyl group (95%); this is probably unchanged methylarsonate. Methylation to dimethylarsenic species takes place (4%), as well as demethylation to inorganic species (1%). Likewise, dimethylarsinate is largely recovered unchanged with ~12% demethylation to a monomethylarsenical; traces of inorganic and trimethylarsenicals are present.

Effect of arsenicals on alkaloid production

The effect of methylarsenicals on alkaloid production by *C. roseus* is shown in Fig. 7. The cells were grown to stationary phase in the presence of methylarsonate or dimethylarsinate and the alkaloids extracted by the Kutney *et al.* procedure.[12] Alkaloid production is dramatically suppressed by both arsenicals with notable changes in the fractions eluting at 7.87, 8.52, and 23.61 min. Of particular interest is the compound(s) of retention time 7.53 min produced by growth in the presence of the arsenicals. The biochemical effect of the methylarsenicals is clearly profound but little can be said until the particular metabolites are isolated and identified.

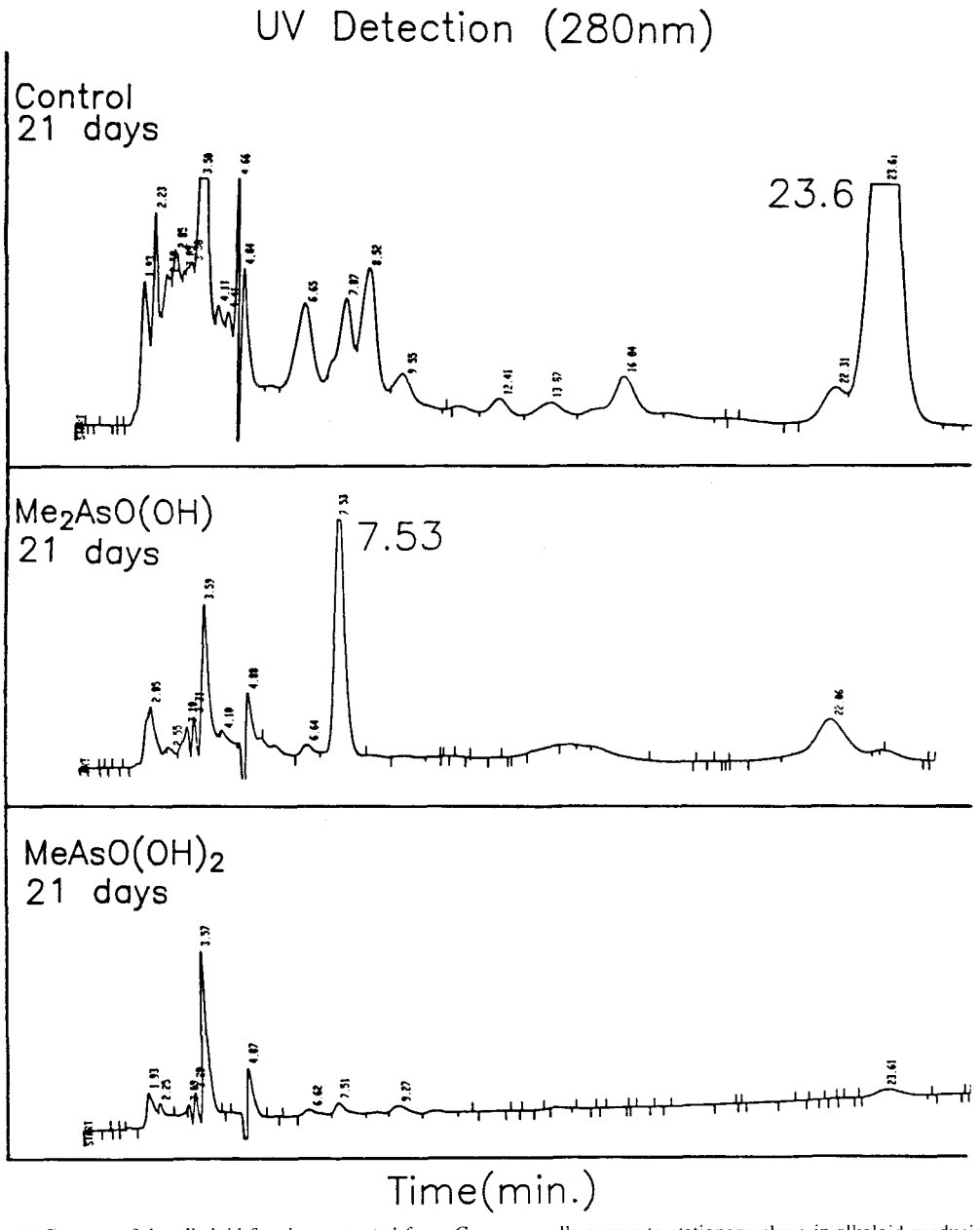

Figure 7 HPLC traces of the alkaloid fraction extracted from *C. roseus* cells grown to stationary phase in alkaloid-producing medium amended with dimethylarsinate (40 μg cm^{-3}) or methylarsonate (8 μg cm^{-3}).

NMR studies

The application of ^{1}H spin-echo NMR spectroscopy to plant cell cultures is novel. The initial spectra of cells at stationary phase, Fig. 8, still require full assignment and, in contrast to the erythrocyte[13–18] plant cell spectra, show considerable variation, implying that the biochemical contents of the cells are not identical from growth to growth. In particular the intensity of the resonance at 3.4 ppm varies widely.

The CPMG pulse sequence and the spin-echo spectrum are shown in Fig. 8.

Examples from a time-course study are shown in Fig. 9. Methylarsonic acid was introduced into the NMR tube containing the *C. roseus* cultures (0.3 mg per 0.5 cm^{-3} of cell suspension) at time zero. The

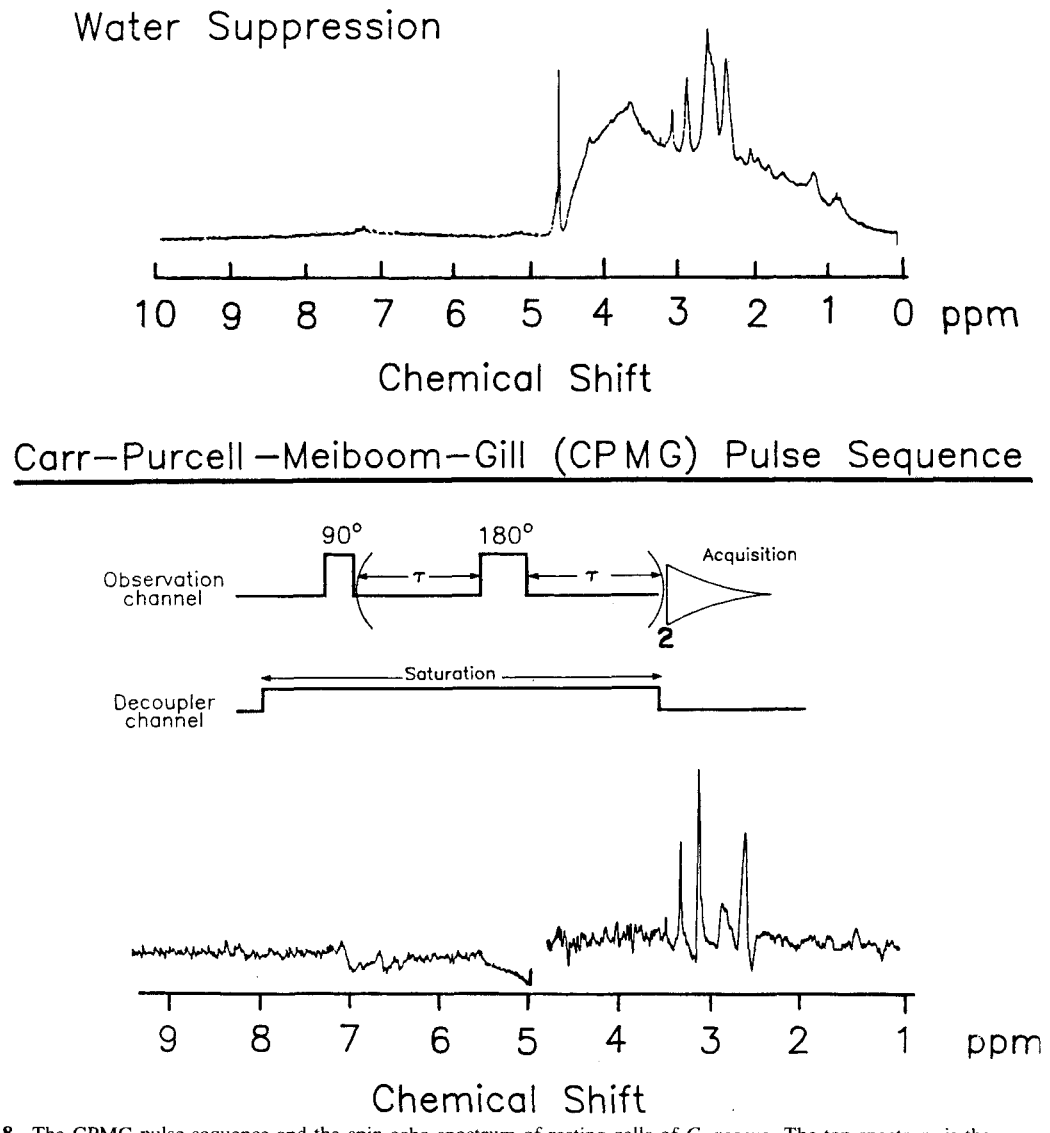

Figure 8 The CPMG pulse sequence and the spin-echo spectrum of resting cells of *C. roseus*. The top spectrum is the normal, water-suppressed, spectrum of the same sample.

arsenic–methyl resonance is clearly seen at 1.79 ppm in the first spectrum that was recorded after 60 min and its intensity increases further with time. This increase is consistent with cellular uptake of substrate, where a moiety moves from an NMR-insensitive (outside) to a more sensitive region (inside).[18] Inverting the NMR tube and centrifuging the contents removes the cells to the capped end and allows a simple method of quickly analysing the contents of the medium. This was found to have little methylarsonate present, confirming that the bulk of the arsenical is inside the cell.

It seems that in the case of *C. roseus* the cytosolic capacity of the cell to accumulate methylarsonate is larger than the cell's ability to transform it immediately. Other changes in the spectra with time are evident in Fig. 9, but again little can be said in the absence of specific assignments. The new resonance which appears at 1.87 ppm may be due to the production of dimethylarsinate.

Acknowledgements We thank the Natural Sciences and Engineering Research Council of Canada and the British Council (J R) for financial support, and Deborah Reimer for technical assistance.

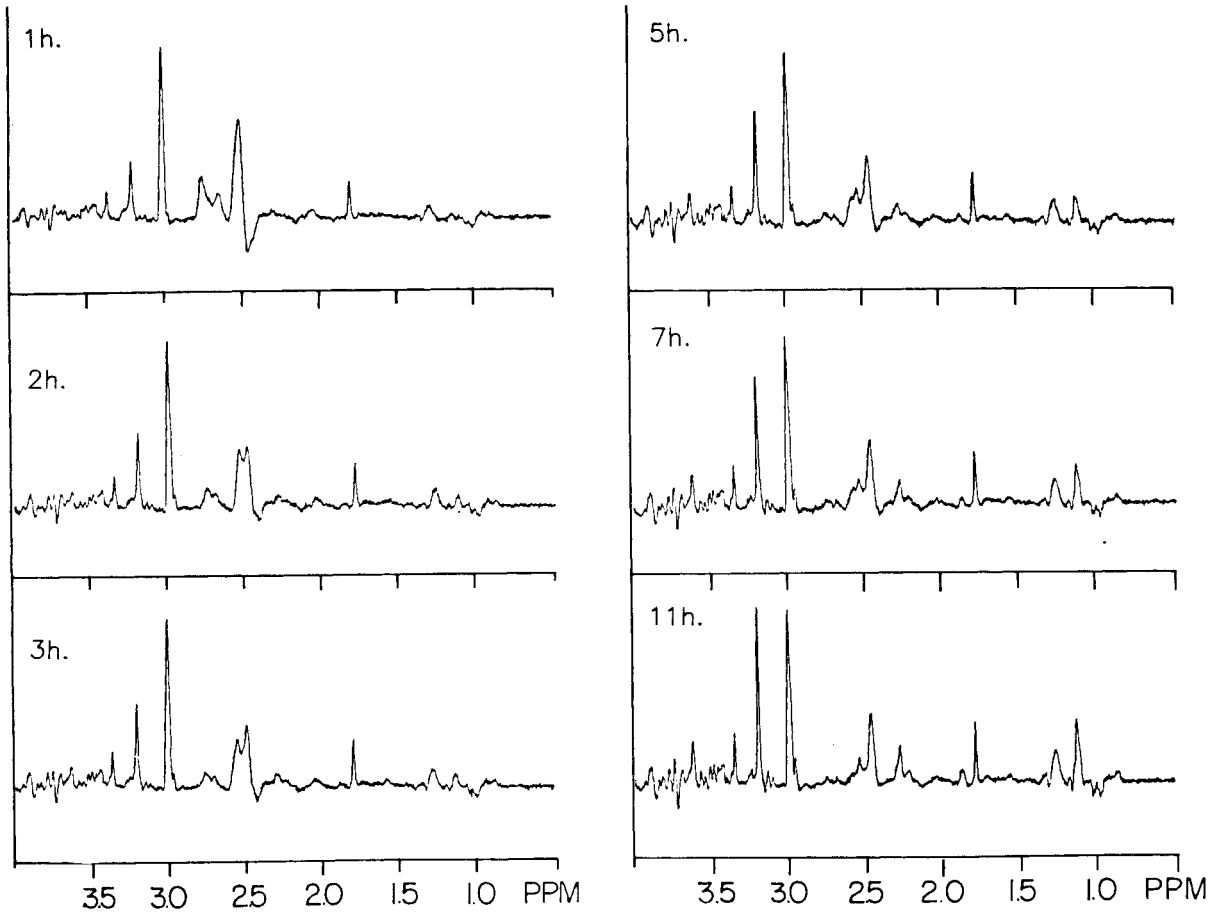

Figure 9 The variation of the ^{1}H NMR spectrum of *C. roseus* in the presence of methylarsonate.

REFERENCES

1. Cullen, W R and Reimer, K J *Chem. Rev.*, 1989, 89: 713
2. Nissen, P and Benson, A A *Physiol. Plantarum*, 1982, 54: 446
3. Benson, A A and Nissen, P In: *Biochemistry and Metabolism of Plant Lipids*, Wintermans, JF G M and Kuiper, P J C (eds), Elsevier, Amsterdam, 1982, p 121
4. DeLuca, V, Balsevich, J, Tyler, R T, Eilert, U, Danchuk, B D and Kurz, W G W *J. Plant Physiol.*, 1986, 125: 147
5. Kurz, W G W, Chatson, K B, Constabel, F,Kutney, J P, Choi, L P, Kolodziejczyk, P, Sleigh, S K, Stuart, K and Worth, B R *Phytochemistry*, 1980, 19: 2583
6. Smith, J I, Smark, M J, Kurz, W G W and Misawa, M *J. Exp. Botany*, 1987, 38: 1501
7. Key, J L, Lin, C Y and Chen, Y M *Proc. Natl. Acad. Sci. USA*, 1981, 78: 3526
8. Czarnecka, E, Edelman, L, Schöffl, F and Key, J L *Plant Mol. Biol.*, 1984, 3: 45
9. Gamborg, O L In: *Plant Tissue Culture Methods*, 2nd edn, Wetter, L R and Constable, F (eds), National Research Council of Canada (NRCC 19876), 1982
10. Gamborg, O L, Miller, R A and Ojima, K *Exp. Cell Res.*, 1968, 50: 151
11. Cullen, W R and Dodd, M *Appl. Organomet. Chem.*, 1988, 2: 1
12. Kutney, J P, Choi, L S L, Kilodziejczyk, P, Sleigh, S K, Stuart, K L, Worth, B B, Kurz, W G W, Chatson, K B and Constable, F *Phytochemistry*, 1980, 19: 2589
13. Brown, F F and Campbell, I D *Phil. Trans. R. Sco. London*, 1980, B289: 395
14. Rabenstein, D L, Millis, K K, Strauss, E J *Anal. Chem.*, 1988, 60: 1380
15. Rabenstein, D L and Nakashima, T T *Anal. Chem.*, 1979, 51: 1475A
16. Reglinski, J, Hoey, S, Smith, W E and Sturroch, R D *J Biol. Chem.* (in press)
17. Rabenstein, D L, Isab, A A and Reid, S R *Biochim. Biophys. Acta*, 1982, 720: 53
18. Brindle, K M, Brown, F F, Campbell, I D, Grathwhol, C and Kuchel, P W *Biochem. J.*, 1979, 180: 37