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# Application of Whole Cell NMR Techniques to Study the Interaction of Arsenic Compounds with *Catharanthus roseus* Cell Suspension Cultures

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**$^1$H spin–echo NMR spectroscopy of intact cells of *C. roseus* facilitates monitoring changes inside the cells on treatment with arsenicals. This *in situ* detection method is non-invasive and non-destructive in comparison to other available biochemical methods. Short term uptake of the arsinicals, methylarsinate MMA and dimethylarsenate DMA, by *C. roseus* cells that have reached stationary phase in 1-B5 medium, is followed by using NMR spectroscopy, and in particular, the Carr–Purcell–Meiboom–Gill pulse sequence. An increase in the peak height of the methylarsenic resonance over a period of 11 h is indicative of uptake of each arsenical. However, there is no evidence of any biotransformation products in the $^1$H NMR spectra. The accumulation site of DMA is probably the vacuole as is seen from the change in the chemical shift of DMA as it moves into a compartment of lower pH. Biochemical changes associated with the presence of arsenicals are evident in the $^1$H NMR spectra of *C. roseus* cells isolated at different stages in the growth cycle. Although uptake has been demonstrated by other analytical techniques, the resonances corresponding to both MMA and DMA are not observed in the $^1$H NMR spectra of cells growing in media containing each arsenical. The association of these arsenicals with large biomolecules in the cell may account for these absences. In this event, the spin–spin relaxation time of the arsenic species would shorten and the signals would not be seen in the spin–echo NMR spectrum. In cells growing in the presence of MMA, a new resonance is observed at a chemical shift position 2.2 ppm after 15 days of growth. The shift in position of the resonance, from 1.75 ppm expected at physiological pH, may indicate an altered environment around the arsenic species such as high intracellular acidity.**

**Keywords: Arsenic, organic arsenic, *C. roseus*, cells, NMR, biomolecules**

## INTRODUCTION

NMR spectroscopy is an important technique for the study of biological fluids and intact cells because of its non-destructive and non-invasive nature[1] and the use of this technique, to study the effect of an added chemical species on plant cell metabolism *in vivo*, has considerable potential. *In vivo* NMR spectroscopy offers the capability to follow metabolic changes continuously on a single sample so is free from uncertainties associated with biochemical methods involving analysis of cell extracts. It also yields information on compartmentation in plant cells; the site at which a chemical species is accumulated being important in the evaluation of its effect on metabolism.[2] Membrane transport of any molecule that gives an observable NMR signal can also be detected directly.[3]

In principle, $^1$H NMR spectroscopy has an advantage in that the sensitivity of the proton is greater than any other nucleus. However, the abundance of hydrogen in biological material results in complicated $^1$H NMR spectra, making their study more difficult. Moreover, the intense water resonance can obscure a large portion of the spectrum and also create a dynamic range problem during data acquisition.

Various approaches are employed to simplify the proton NMR spectra of biological systems. Presaturation of the water resonance is one commonly used water suppression method.[1] The broad envelope of signals arising from membrane and plasma proteins can be eliminated by using specific pulse sequences which make use of differences in relaxation times between large protein molecules and smaller solute molecules in the cells.[1] The Hahn spin–echo pulse sequence, one such approach, establishes a delay in acquisition in order to selectively eliminate signals from macromolecules, and application of spin–echo NMR spectroscopy to intact red blood cells was first

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reported in 1977.[4] Such studies of intact blood cells and plasma are of great clinical interest as they can provide information on drug metabolism,[5,6] the binding of several heavy metal species including Hg(II) and $CH_3Hg^+$ in erythrocytes[7] and the pH inside the human erythrocyte.[8]

$^1$H Spin–echo NMR studies have been used to monitor the biochemistry of arsenicals in human erythrocytes where dramatic changes observed in the spectra on exposure to dimethylarsinate (DMA) indicate oxidative stress.[9] The decrease in intensity of the DMA signal with time is attributed to the reduction of the arsenical to a $Me_2As$—S— species bound to a transmembrane protein.[9] Related studies indicate adduct formation of another arsenical, phenyldichloroarsine, with sulphydryl containing compounds in guinea pig red bloods cells.[10]

The Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence, $(90°_x-(t-180°_y-t)_n$-acquisition), is a modified version of the Hahn spin–echo sequence that makes use of multiple 180° refocusing pulses during the spin–spin relaxation period. The CPMG pulse sequence has been used in combination with a preaturation pulse that suppresses the water resonance, in order to obtain NMR spectra of human erythrocytes and plasma.[2,11] Recently, Schripsema *et al.*[13] used a $^1$H NMR technique employing the CPMG pulse sequence to analyze the cell extracts and the medium from a *Tabernaemontana divaricata* plant cell suspension culture. They were unsuccessful in their attempt to apply this technique to intact plant cells.[13]

There are several difficulties associated with the spin–echo technique. The intensities of the resonances depend on the spin–spin relaxation time of each solute species and thus do not reflect the absolute concentration.[11] Depending on the length of the spin–spin relaxation period, the spin–spin coupling constant, and the nature of the multiplet pattern, strongly coupled resonances can be greatly reduced in intensity. The spectra have phase modulated signals and as a result, peak integration is not possible.[14] However, the peak heights of signals do reflect the relative ratio of the solute species. Thus, the relative change in concentration of a solute species can be determined if a suitable reference compound is introduced into the sample or if it is established that there is an invariant species present inside the cell.[14]

The growth and secondary metabolism of *C. roseus* cell suspension cultures have been extensively studied, much of the interest being focused on the biosynthesis of indole alkaloids, especially vinblastine and vincristine, which are valuable anticancer agents.[15] The effect of arsenicals on the growth and alkaloid production in *C. roseus* cell suspension cultures has been reported recently[16] and a preliminary account of the novel application of $^1$H spin–echo NMR spectroscopy on *C. roseus* has been published.[17]

Application of $^1$H spin–echo NMR spectroscopy to study the interaction of arsenicals with *C. roseus* cells is described. Two approaches have been employed. In the first approach, $^1$H spin–echo NMR spectroscopy is used to follow the rapid biochemical changes, continuously, in a cell sample, on exposure to high concentrations of arsenic compounds. Transport of the arsenic compounds, methylarsonate (MMA) and DMA, across the cell membrane and accumulation inside the cells are also observed. Preliminary results from this study have been previously published.[17] The second approach involves recording the temporal variation in the NMR spectra of *C. roseus* cells growing in the presence of arsenicals.

## EXPERIMENTAL

### Materials and Methods

#### NMR parameters

*C. roseus* cells were harvested at the stationary phase, washed three times with deuterium oxide ($D_2O$, MSD Isotopes, Canada) to remove excess medium and then packed into a 5 mm NMR tube (Norell 507-HP). $^1$H NMR spectra were recorded on a Bruker WH 400 spectrometer by using a standard 5 mm probe. The Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence $(90°(t-180°-t)_n$, with $n=2$, was used to obtain the spin–echo NMR spectra and the delay time ($t$) was typically 30 ms, a value found to be optimal for the present application. A schematic representation of this pulse sequence is given in Fig. 1. The decoupler was on for water suppression during a period of 1 s before the start of the CPMG pulse sequence and during the pulse sequence. An acquisition time of 0.426 s was employed and the spectral width was 5000 Hz. Typically 90° and 180° pulse widths were 11 and 22 ms, respectively. The free induction decay was collected in 4 K of data points zero filled to 32 K.

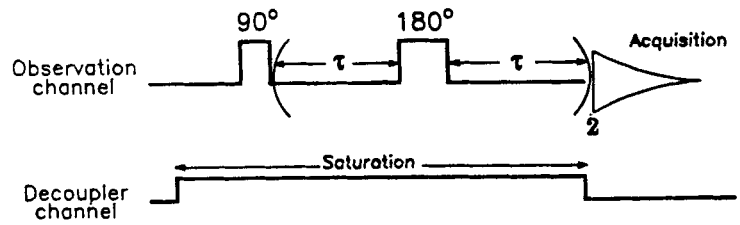


**Figure 1** A schematic representation of the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence.

A 0.1 Hz line broadening function was applied during Fourier transformation. All samples were maintained at 27 ± 2 °C and spun at 20 Hz during data collection. Generally, 200 transients were collected for each spectrum.

In short term uptake studies, a cell sample was prepared by packing the NMR tube with *C. roseus* cells (0.5 mL, packed cell volume) from a culture grown in 1-B5 medium[18] that had reached stationary phase. A specific amount of the arsenic compound was dissolved in 0.5 ml of $D_2O$ and then added to the NMR tube. The *C. roseus* cell line, AC-3, was initiated from a mature leaf explant and is usually maintained in 1-B5 medium.

In continuous monitoring studies, *C. roseus* cell suspension cultures were grown in 250 ml flasks containing 100 ml of alkaloid production medium[19] (APM) each. The control cultures did not contain any added arsenic. Others contained either 4 ppm of methylarsonate, 15 ppm of dimethylarsinate, 3 ppm of arsenate or 4 ppm of arsenite. Cells were removed aseptically at different times of growth and prepared for the NMR experiment.

The assignment of NMR resonances of cell metabolites was carried out by comparing spin–echo NMR spectra of cell extracts with those of whole cells. The spectra of cell extracts were recorded as a function of pH, before and after the addition of the expected compounds. If the additional signal coincides with the resonance of interest in the extract, the assignment is correct. Some resonances were assigned using this approach.

A limitation in this approach is the absence of many resonances observed in whole cell spectra, in the spectra of cell extracts. During the extraction, many chemical associations present in the cell may be destroyed. The metabolites may also come into contact with cellular components, such as enzymes and membrane proteins, that are present in separate compartments within a living cell. These differences in the chemical environment surrounding cell metabolites present in cell extracts, hinder the assignment of resonances.

2-D spin–echo correlated (COSY) NMR experiments were carried out on whole cells as well as on cell extracts. The spectra of whole cells were noisy, probably as a result of changes occurring in the cells during the time taken for acquiring the COSY spectrum. Hence these spectra were not useful in the peak assignment. Nevertheless the spectra of cell extracts provided useful information in some cases.

## RESULTS AND DISCUSSION

### General features of $^1$H NMR spectra of *C. roseus* cells

A typical $^1$H NMR spectrum of *C. roseus* cells obtained after suppression of the water resonance is depicted in Fig. 2(a). The cells were isolated from a *C. roseus* cell suspension culture grown in 1-B5 medium that had reached stationary phase

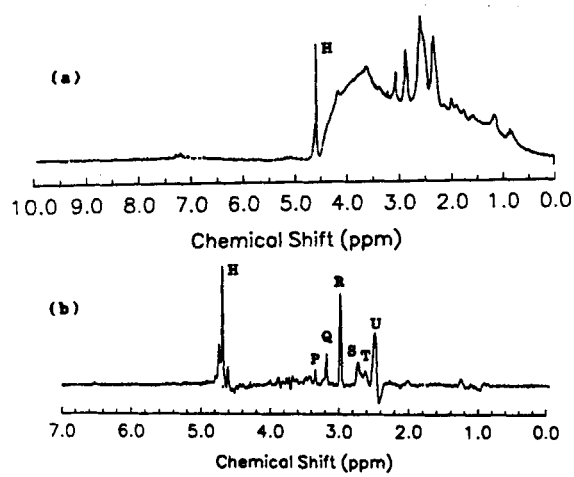


**Figure 2** 400 MHz $^1$H NMR spectra of a cell suspension of *C. roseus* (Cells were grown in 1-B5 medium for 10 days and then suspended in $D_2O$). (a) The spectrum measured with suppression of water signal by the application of a pre-saturation pulse. (b) The spin–echo spectrum measured using the CPMG pulse sequence, $t = 30$ ms, number of scans = 200.

after 10 days of growth. This spectrum is charactrized by a water resonance (peak H at 4.7 ppm) and a broad envelope of overlapping signals arising primarily from the membrane and plasma proteins.

To eliminate this broad envelope of resonances, a modified version of the spin–echo pulse sequence, the CPMG pulse sequence (Fig. 1) was used.[1] This pulse sequence creates a time delay ($4t = 120$ ms) between signal generation and accumulation. The selective elimination of the broad, poorly resolved signals from large protein molecules is achieved on the basis of their short relaxation times. Only the signals from the small molecules in the cytoplasm are observed in the resulting spectrum depicted in Fig. 2(b), where resonances P, Q and R probably arise from small, motile molecules in the cells. Peaks S, T and U are substantially broader than the other resonances and may arise from slowly tumbling species in the storage vacuoles.[1] These resonances are generally found in spin–echo NMR spectra of *C. roseus* cells that have reached stationary phase in 1-B5 medium, even though some variation in the relative intensities can be observed.

The $^1$H NMR spectra of *C. roseus* cells, obtained at other times in the growth cycle, show variation depending on the growth stage and nutrient composition in the growth medium. Effort was put into the identification of these signals only if they were found to be affected by arsenical treatment.

## Short term effects and uptake of methylarsenicals by *C. roseus* cells

The short term uptake of the methylarsenicals, MMA and DMA, by *C. roseus* cells was monitored continuously over a period of 11 h by exposing cells packed into an NMR tube to the appropriate arsenical. Mature *C. roseus* cells that had reached stationary phase in standard 1-B5 medium were used in the study. Cells that are packed into a NMR tube, usually in deuterated water, are under stress and expected to undergo changes in their biochemical content with time. Consequently, a control was obtained by monitoring *C. roseus* cells packed into a NMR tube in the absence of arsenic as a function of time.

### Methylarsonate

Methylarsonate was introduced into *C. roseus* cells at two dosages. At the high dosage (3 mg 0.5 ml$^{-1}$ of the cell suspension), the methylarsenic

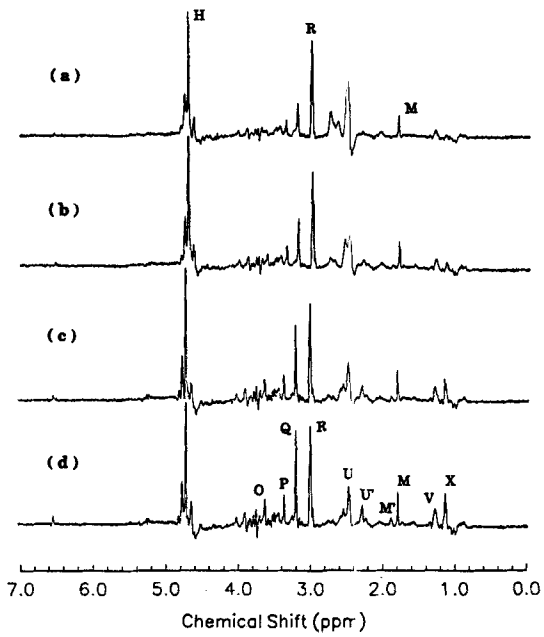


**Figure 3** $^1$H spin–echo spectra of a suspension of *C. roseus* cells treated with MMA (0.3 mg 0.5 ml$^{-1}$ of packed cells). Each spectrum was recorded: (a) 1 h, (b) 3 h, (c) 7 h, (d) 10 h after packing into the NMR tube and treatment with MMA.

NMR signal was found to swamp the signals from cell components. More meaningful results were obtained after treating the cells with a lower dosage of MMA (0.3 mg 0.5 ml$^{-1}$ of cell suspension) and the NMR spectra obtained 1, 3, 7 and 10 h after treatment are depicted in Fig. 3. At this dosage, the intensity of the methylarsenic signal (peak M) at 1.79 ppm increases with time and the chemical shift does not show a significant variation over time.

Peak R at 3.0 ppm, a prominent resonance in the spectrum, is characteristic of all cells at stationary phase when grown in 1-B5 medium. The intensity of the peak is not affected by the addition of arsenicals or time in the NMR tube. The relative signal intensity of the methylarsenic resonance as measured against the invariant signal R, increases with time, which is consistent with cellular uptake of MMA and is depicted in Fig. 4. The increase in peak intensity is associate with cell uptake of the substrate when a moiety moves from a NMR less sensitive (outside) to a more sensitive region (inside).[3,12] When the spin–echo pulse sequence is used there are differences in magnetic susceptibility inside and outside the cell. The increase in intensity of peak M is rapid at first and slows down after about 3 h. This indicates that the cytosolic capacity of the cell to accumu-

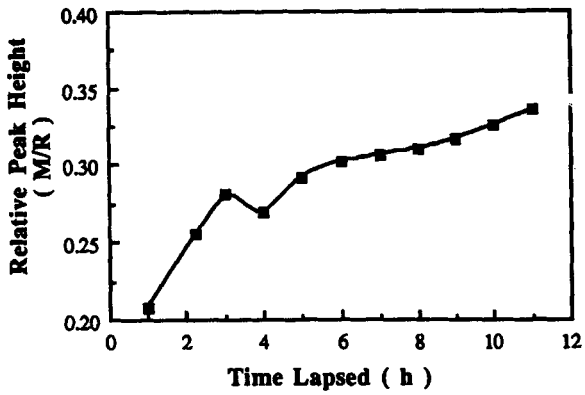


**Figure 4** The variation of the relative signal intensity of methylarsenic resonance (M/R) with time.

late MMA is larger than its immediate ability to transform it.

Eleven hours after adding MMA to the cells, very little MMA was found to be left in the supernatant, confirming that the bulk of the arsenical is inside the cell. This can be easily established by inverting the NMR tube and centrifuging the contents to remove the cells to the capped end, which allows the supernatant to be analyzed.

The spectra (Fig. 3) illustrate some other changes that take place in the cells with time. The spectra of a control culture of *C. roseus*, no arsenical added, show a similar variation with time. The broad, phase modulated peak U, centered around 2.5 ppm, changes its shape rapidly, accompanied by a decrease in intensity. After 10 h, the peak is replaced by a positive, broad peak. Both peaks S (2.76 ppm) and T (2.66 ppm) decrease in intensity with time and probably arise from the same molecule. These chemical shifts may be indicative of citrate which is known to be present in *C. roseus* cells at significant concentrations.[20] After 10 h, a small, broad peak remains centered at 2.7 ppm. The disappearance of peaks S and T could be a result of either the molecule being used up in the metabolic processes in the cell or hydrogen/deuterium exchange.

Other changes in the spectra include the increase in intensity of peaks O, P, U′, V and X, also observed in spectra of cells not treated with any arsenical. These changes may be attributed to the accumulation of by-products of cell metabolism, some processes being accelerated due to the rigid conditions in the NMR tube. For example, peaks O and X can be assigned to ethanol: the methyl resonance of ethanol falls at 1.17 ppm (peak X) at physiological pH and the signal arising from the $CH_2$ group of ethanol has a chemical shift position of 3.6 ppm (peak O).

A new peak at 1.88 ppm (M′) is first observed about 5 h after adding MMA an slowly increases in intensity. This peak can be assigned either to acetate or to a dimethylarsenic species, on the basis of its chemical shift. Attempts to confirm assignments by using cell extracts were not informative because of changes in environment. Methylation of MMA by *C. roseus* cells was observed in previous speciation studies,[17] but whether or not it takes place during the time frame of the present experiment (a few hours) is yet to be confirmed. A peak at 1.9 ppm, assigned to acetate, is observed in the NMR spectrum of a control cell sample 5 days after time zero. These cells were under considerable stress and acetate is a likely product of cell lysis. If acetate is present, M′ in Fig. 3, it could indicate that MMA exerts considerable stress on the cells.

**Dimethylarsinate**

Uptake of dimethylarsinate by cells that had reached stationary phase was monitored in a similar way, after treating the cells with DMA (0.15 mg 0.5 $ml^{-1}$ of cell suspension). (A higher dose swamps the signals from the cell components.) The spectra obtained at various time intervals after treatment are shown in Fig. 5.

The dimethylarsenic resonance, labelled D, is intense compared with the resonances from other cell metabolites. One hour after treating the cells with the arsenical, the dimethylarsenic resonance is seen at 1.75 ppm and it gradually shifts to 1.83 ppm 4 h after treatment. Thereafter, the position remains constant. This lower field shift of the dimethylarsenic resonance may be associated with the movement of DMA into the vacuoles provided rapid exchange between the vacuoles and the cytosol is possible. At pH $5.5 \pm 0.2$, the vacuolar pH of *C. roseus* cells,[21,22] the dimethylarsenic resonance appears at 1.83 ppm, whereas at pH 7.3 and higher (the pH of cytoplasm and outside medium), a chemical shift of 1.7 is expected. Thus, the major accumulation site of DMA appears to be the vacuoles in *C. roseus* cells.

An increase in the intensity of the dimethylarsenic resonance (peak D) with time is seen in Fig. 5. Fig. 6 shows the variation with time of the relative intensity of signal D measured against the invariant signal R. The increase in signal intensity demonstrates the transport of the substrate DMA from the NMR less sensitive region outside the

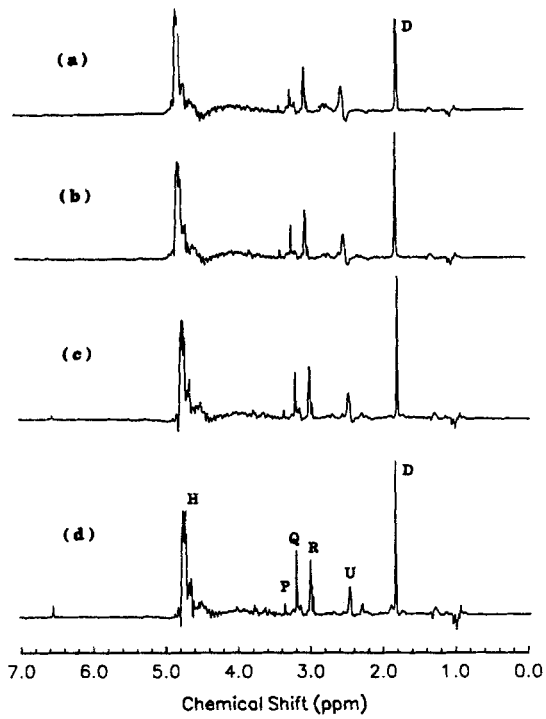


**Figure 5** $^1$H spin–echo spectra of a suspension of *C. roseus* cells treated with DMA (0.15 mg 0.5 ml$^{-1}$ of packed cells). Each spectrum was recorded: (a) 1 h, (b) 3 h, (c) 5 h, (d) 8 h after packing into the NMR tube and treatment with DMA.

cell to the more sensitive inside. The uptake appears to be rapid for the first 3 h and a slower increase is observed up to 11 h.

Most of the biochemical changes in the cells with time, observed in other cell samples, are reflected in the spectra shown in Fig. 5. However, there are several peculiarities. Significant accumulation of ethanol is not observed in these cells because peaks at 1.15 (X) and 3.65 ppm (O) are absent. The intensity of an unidentified out-of-phase peak at 1.05 ppm (peak X′) increases with time. Peak V at 1.29 ppm can be assigned to lactate on the basis of its chemical shift at physiological pH and is also observed in a spectrum of a control cell sample. This peak increases as a function of time, indicating the accumulation of lactate.

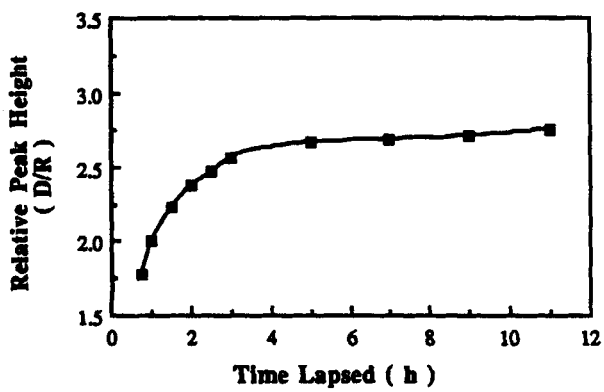


**Figure 6** The variation of the relative signal intensity of dimethylarsenic resonance (D/R) with time.

A resonance at 1.9 ppm is first observed in this cell sample after 7 h Figure 5(d), and increases in intensity with time; this peak is also present in MMA treated cells and is assigned either to acetate or a dimethylarsenic species. Another unassigned resonance at 1.78 ppm is also detected after 7 h of incubation and slowly increases with time. Either of these two resonances may arise from a metabolic product of the substrate, DMA.

## Long term effects of arsenicals on *C. roseus* cells

The longer term effects of arsenicals on the metabolism of *C. roseus* cells during its growth cycle can be monitored by $^1$H spin–echo NMR spectroscopy. Comparison of the spectra with those from a control culture aids in identifying any changes in the cellular contents associated with the presence of arsenicals.

### Methylarsonate

The cells growing in APM containing 4 mg dm$^{-3}$ of methylarsonate (MMA) were monitored by $^1$H spin–echo NMR spectroscopy. Six hours after transferring to the medium containing MMA, the NMR spectrum of the cells is as shown in Figure 7(a). The broad resonance H centered at 4.77 ppm arises from water and has been suppressed using a pre-saturation pulse. The intense resonances between 3.4 and 4.4 ppm and the small resonance S′ at 5.41 ppm are assigned to sucrose on the basis of their chemical shift positions. Thus, the rapid uptake of sucrose into the cells from the growth medium, APM, which contains 5% sucrose, is evident. The spectrum also contains resonances from other components present in 1-B5 grown cells that had reached stationary phase. These peaks at 3.0, 2.76, 2.68, 2.54 and 2.45 ppm, are observed in Figs 2–4.

The spectra of the cells show a close similarity to that of the control up to 5 days of growth. After 8 days growth in APM containing MMA [Figure 7(b)], signals arising from sugars still dominate, both sucrose (S′) and glucose being present. Glucose is indicated by the peak S″ at 5.2 ppm

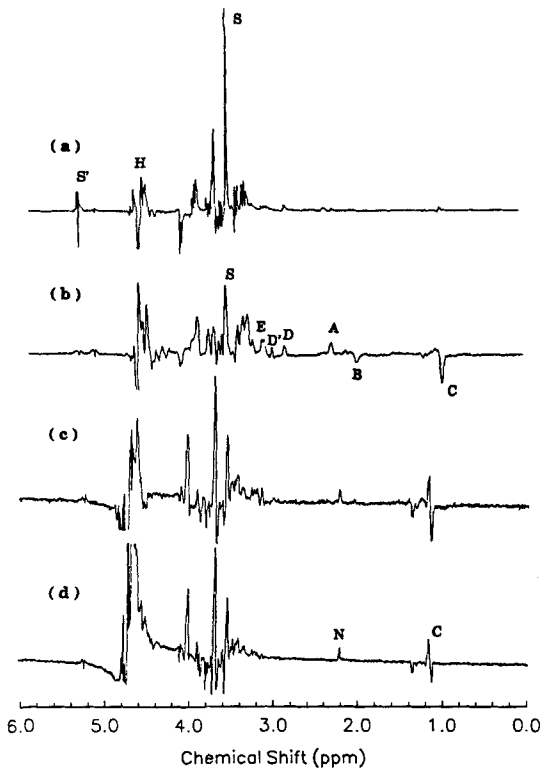


**Figure 7** Spin–echo NMR spectra of *C. roseus* cell suspensions grown in alkaloid production medium (APM) containing 4 ppm of methylarsonate (MMA): (a) 6 h after transferring to the new medium containing MMA, (b) 8 days after transfer, (c) 15 days after transfer, (d) 23 days after transfer.

(better seen in Figure 8(a)), which is assigned to the proton on the anomeric carbon of $\alpha$-glucase. A peak at 4.6 ppm should similarly indicate $\beta$-glucose. This peak is apparently buried under the resonances from other cellular components, and thus information about the anomeric equilibrium can not be obtained. The out-of-phase peak C at 1.17 ppm, assigned to ethanol, has a high intensity, similar to the spectra of other arsenical treated cells. The signal arising from the $CH_2$ group of ethanol, expected at a chemical shift position of 3.6 ppm, is probably buried under the more intense resonances from sucrose.

All NMR spectra recorded after 15 days of growth in APM containing MMA, show characteristic changes in the appearance of the spectra. Peaks are much narrower compared to the control (and DMA treated cells, see below). The signal to noise ratio is also poor. These phenomena are probably associated with some physical changes in the cells.

After 15 days of growth, cells still contain sugar, mostly glucose, as is seen in the NMR spectrum in Figure 7(c). A new resonance N at 2.21 ppm has appeared that is still present in Figure 7(c) (23 days of growth). In an attempt to establish its identity, spectra of extracts of cells grown in media containing MMA were obtained. Neither methanol nor aqueous cell extracts, resuspended in $D_2O$ (pH 3.75), contain a metabolite which gives rise to a signal at 2.21 ppm. The NMR spectrum of a suspension of the cell residue does not show this extra resonance either. Therefore, during the extraction process, the metabolite either undergoes decomposition or its molecular environment is altered such that it does not give rise to the expected NMR signal.

This extra resonance N in the NMR spectrum may be assigned to MMA itself, which is possibly sequestered in the vacuoles. *C. roseus* cell cultures growing in a medium containing MMA exhibit an unusually low pH. A 22 day old control culture has a pH of 5.8 whereas the pH of a MMA treated culture is 3.8. The lower pH (<3.8) in combination with a weak association with another molecule in the vacuole may cause the shift in chemical shift. (The chemical shift of MMA in $D_2O$ at pH 3.8 is 1.91 ppm.) The absence of a resonance assignable to MMA in the cell extracts may indicate that MMA is bound to a larger molecule with which it comes into contact during the extraction; the resultant short relaxation time may be responsible for its non-appearance in the spin–echo NMR spectrum. On spiking the cell extract with a relatively large dose of MMA (5 mg $1\ ml^{-1}$ of cell extract, the methyl resonance of MMA is observed at 1.91 ppm.

This lowering of the pH in these cultures suggests that acidic components accumulate in cells on treatment with MMA. Other workers have reported that malic acid accumulates in Johnson grass when treated with MMA. MMA acts as a specific herbicide against Johnson grass.[23] Malate accumulation has been observed in *C. roseus* cells when subjected to osmotic stress.[20,24] However, the presence of malic acid is not evident in the NMR spectra of *C. roseus* cells treated with MMA. At pH 3.9, malic acid is expected to give rise to two resonances at 2.8 and 2.7 ppm which converge to give a single broad peak at 2.9 ppm at more acidic conditions.

**Dimethylarsinate**

$^1H$ spin-echo NMR spectra obtained from cells growing in APM containing $15\ mg\ dm^{-3}$ DMA are shown in Fig. 8. Six hours after transfer into

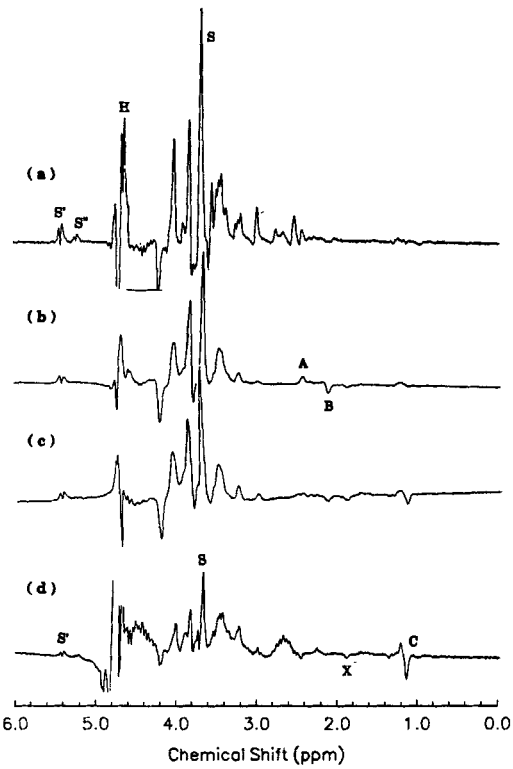


**Figure 8** Spin–echo NMR spectra of *C. roseus* cell suspensions grown in Alkaloid Production Medium (APM) containing 15 ppm of dimethylarsinate (DMA) (a) 6 h after transferring to the new medium containing DMA, (b) 8 days after transfer, (c) 17 days after transfer, (d) 28 days after transfer.

the new medium, the NMR spectrum of cells still resembles that of the control, Figure 8(a). Both sucrose and glucose are present as revealed by the peaks S, S′ and S″. The dimethylarsenic resonance is not observed which may indicate a relatively low concentration of DMA inside the cell. An alternative explanation is that any DMA taken up is invisible in the spin–echo spectrum because of its association with larger molecules in the cell.

The spectra of cells treated with DMA resemble those of the control through days 2–5 and no delay in growth can be detected. After 8 days of growth, the NMR spectrum of cells, Fig. 8(b), exhibits a few differences from that of the control. The major sugar is sucrose in DMA treated cells. The other difference is an extra resonance X at 1.92 ppm, a negative peak. The same trend is observed after 12 and 15 days.

Figure 8(c) shows the NMR spectrum of cells after 17 days of growth in the medium containing DMA. The major sugar present is sucrose (peak S′). Peak A at 2.43 ppm has almost disappeared but peak B at 2.15 ppm is still present. The new peak X at 1.9 ppm is more evident. The out-of-phase peak at 1.2 ppm may indicate the accumulation of ethanol.

The spectrum of cells after 28 days of growth is given in Fig. 8(d) which exhibits several new peaks associated with stationary phase such as the broad peaks at 2.67 ppm and 2.26 ppm as well as the peaks D (3.0 ppm) and E (3.2 ppm). The new peak X associated with DMA treatment is present as a negative peak at 1.9 ppm. This resonance could be assigned either to acetate or a dimethylarsenic species on the basis of chemical shift; however, both appear as positive peaks in a CPMG NMR spectrum. Association of the dimethylarsenic moiety with another small molecule in the cell may result in the shape and the phase change but there is no direct evidence. It is conceivable that any resonances from the rest of this molecule are buried under the large sugar resonances.

### Inorganic arsenicals

*C. roseus* cell suspension cultures growing in the presence of inorganic arsenicals were monitored over the duration of their growth cycle by using $^1$H spin–echo NMR spectroscopy. The signals arising from sugars dominate the spectra during the early part of the growth cycle. In cells growing in the presence of 3 ppm arsenate, it is evident that spectral changes are delayed in comparison to the control culture. This effect can be related to the delay in growth previously observed in *C. roseus* cell suspension cultures in the presence of arsenate.[17] A similar delay effect is not evident in cells treated with arsenite. A common feature in these spectra is the early appearance of peaks assigned to acetate, lactate and ethanol. The accumulation of these metabolites is an indication of the cell stress that accompanies treatment with inorganic arsenicals.

## CONCLUSION

Although $^1$H spin–echo NMR spectroscopy of intact plant cells retains many of the advantages of an *in situ* detection method, it has several limitations in monitoring the effect of arsenicals on plant cells. The complicated spectra preclude the complete assignment of resonances arising from many plant metabolites. The uptake of inorganic arsenic species cannot be monitored and

any association of methylarsenicals with intracellular proteins and large biomolecules can only be predicted from the disappearance of the methylarsenic resonance.

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