Formaldehyde Metabolism by *Escherichia coli*. Detection by in Vivo ^{13}C NMR Spectroscopy of *S*-(Hydroxymethyl)glutathione as a Transient Intracellular Intermediate†

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ABSTRACT: In vivo ^{13}C NMR has been used to detect the transient formation of *S*-(hydroxymethyl)glutathione ($GSCH_2OH$) from glutathione and [^{13}C]formaldehyde in *Escherichia coli*. Two-dimensional ^1H–^{13}C shift correlation was used to locate the chemical shift of the formaldehyde-derived protons of the adduct. The adduct $GSCH_2OH$ is formed by chemical reaction in the first few minutes after cells are challenged with formaldehyde and remains within the cell until consumed by metabolism.

Formaldehyde is generated as an intermediate breakdown product in the metabolic detoxification of many foreign compounds in mammalian systems (Ahmed & Anders, 1976; Kreiter et al., 1985; Steenkamp, 1982); it is further metabolized to formate by an appropriate dehydrogenase. Glutathione has been suggested as a cofactor for many dehydrogenases, acting via formation of *S*-(hydroxymethyl)glutathione, the hemithioacetal adduct ($GSCH_2OH$) (eq 1), although direct

$$GSH + CH_2(OH)_2 \rightleftharpoons GSCH_2OH + H_2O \quad (1)$$

evidence for hemithioacetal formation in vivo has so far been lacking (Koivusalo et al., 1982; Kreiter et al., 1985; Uotila & Koivusalo, 1974; Weiner et al., 1982). We have previously shown that formaldehyde detoxification by *Escherichia coli* can be followed by in vivo NMR[1] spectroscopy (Doddrell et al., 1984; Hunter et al., 1984) and have determined the structures of the major metabolites that ultimately appear in the medium (Hunter et al., 1985). We now report that in vivo NMR spectroscopy allows the direct observation of the generation and consumption of *S*-(hydroxymethyl)glutathione ($GSCH_2OH$) within *E. coli* cells that are actively detoxifying formaldehyde. Adduct formation occurs before the accumulation of significant amounts of end products and is presumably an intermediate step in the biosynthesis of one or more of the end products.

Experimental Procedures

E. coli K12 were obtained from Dr. J. Wood (Guelph, Ontario, Canada) and *E. coli* NCIB 8797 from the Colworth culture collection (Unilever Research Ltd). They were maintained at 4 °C on slopes of tryptone soy agar and subcultured every 3 months. A growth inoculum was formed by aseptically transferring a scraping from a slope to 10 mL of tryptone soy broth. This was grown at 37 °C to an optical density of about 1.2 in 24 h. Five milliliters of this culture was transferred to 0.5 L of sterile minimal medium. This medium was based closely on that of Spizizen (1958): 3 g of KH_2PO_4, 7 g of K_2HPO_4, 0.5 g of $(NH_4)_2SO_4$, and 1.5 g of sodium acetate in 500 mL of distilled water, giving pH 7.0. A 1 M solution of $MgSO_4$ was autoclaved separately; 200 μL was added to the medium prior to inoculation.

The bacteria were grown with aeration at 37 °C to a variety of optical densities, usually OD_{540nm} = 1.2–1.4 in 12–16 h. Bacteria were harvested by centrifugation for 15 min at 4 °C. The pellet was resuspended in a 0.85% saline solution and again centrifuged as before. The bacterial pellet was stored on ice until required (up to 8 h) and then resuspended in 1% phosphate buffer (pH 7) to a volume of 9 mL. NMR samples (3.5 mL) were made from 3 mL of this suspension, plus 0.5 mL of D_2O (99.8%, Aldrich) to provide a lock signal. This gave a cell density of about 10^{11} cells/mL. A 600 μM stock solution of [^{13}C]formaldehyde was prepared by autoclaving [^{13}C]paraformaldehyde (91% ^{13}C, Prochem) in distilled water with a drop of concentrated hydrochloric acid overnight at 135 °C. This solution was assayed according to the Nash test (Nash, 1953). Fifty-eight microliters of the [^{13}C]formaldehyde solution was added to the bacterial suspension, giving a final concentration of 10 mM, and NMR observation was started within 1 min.

^{13}C NMR spectra were acquired at 100.6 MHz (Bruker WH400) or 62.6 MHz (Bruker WM250) in Cambridge. Generally 100–1000 transients were acquired by using 8K data points over 250 ppm. Gated proton decoupling was used to avoid excessive heating; 45° pulses were applied, with 1.5-s relaxation delay between pulses giving a repetition time of 2 s. During acquisition, the samples were held at 37 °C by a variable temperature unit. The samples were not aerated, so that the metabolism was effectively anaerobic. $^{13}CH_2(OH)_2$ was used as an internal standard (83.2 ppm); ^1H-coupled ^{13}C spectra and two-dimensional ^{13}C–^1H correlation spectra were obtained as described previously (Hunter et al., 1984).

In some experiments, successive spectra were acquired and stored to allow the time course of metabolism to be followed to completion. In others, the sample was placed on ice for 5–10 min as soon as the adduct peak at 66.6 ppm was observed. In one case, the sample was centrifuged and the pellet (resus-

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[1] Abbreviations: *E. coli*, *Escherichia coli*; $GSCH_2OH$, hemithioacetal of glutathione and formaldehyde; GSH, reduced form of glutathione; NMR, nuclear magnetic resonance; UV, ultraviolet.

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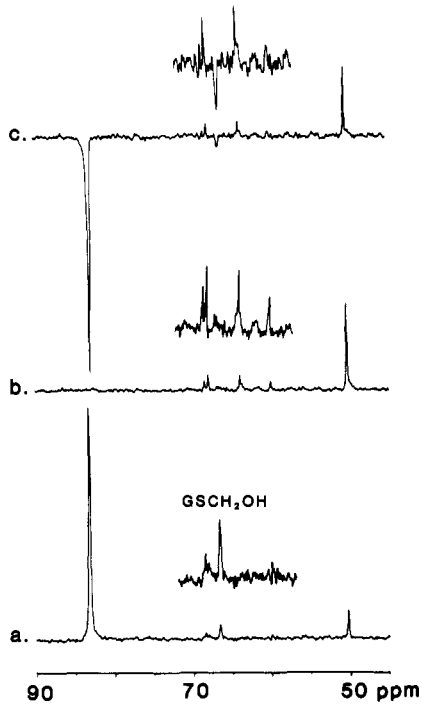

FIGURE 1: (a) Partial 62.6-MHz proton-decoupled ^{13}C NMR spectrum of a suspension of *E. coli* K12, showing the first 20 min (500 transients) of metabolism at 37 °C after the addition of 10 mM [^{13}C]formaldehyde. Formaldehyde (83.2 ppm) and methanol (50.2 ppm) are evident, together with $GSCH_2OH$ at 66.6 ppm. Exponential line broadening of 6 Hz was applied prior to Fourier transformation. (b) As in (a) but 1 h later. All formaldehyde has now been consumed. The peak at 66.6 ppm has disappeared, and new peaks have appeared at 68.4, 67.8 (propane-1,2-diol), 64 (glycerol), and 60 ppm (propane-1,3-diol). (c) Difference spectrum generated by subtracting (a) from (b). This clearly demonstrates the loss of formaldehyde and $GSCH_2OH$ with concomitant gain of the C_3 alcohols and methanol.

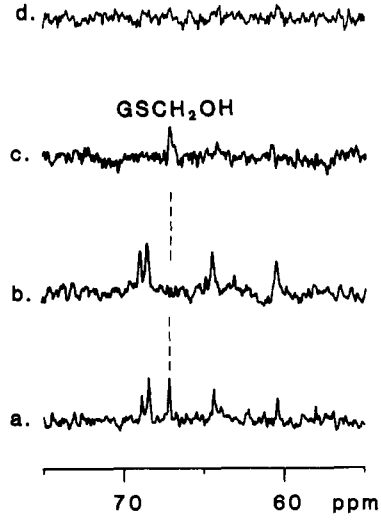

FIGURE 2: (a) As in Figure 1b but after the addition of a further 10 mM [^{13}C]formaldehyde. The reappearance of $GSCH_2OH$ is clearly seen. A total of 120 scans were acquired over 4 min. Line broadening of 6 Hz and zero filling to 16K data points were applied prior to Fourier transformation. (b) The supernatant from (a) obtained by centrifugation at 4 °C. This shows that the stable metabolites are extracellular. (c) The resuspended bacterial pellet from (a) after centrifugation. $GSCH_2OH$ is seen to be uniquely intracellular. (d) As the suspension of (c) was allowed to warm up to 37 °C, the peak due to $GSCH_2OH$ disappeared, indicating biochemical removal.

pended in D_2O) and supernatant were examined separately by NMR. Alternatively, the separated pellet and supernatant were each heated to 95 °C for 10 min before reexamination.

In a third experiment, a suspension containing $GSCH_2OH$ was boiled for 10 min prior to centrifugation (suspension A). In control experiments metabolism was observed at 2 °C, and metabolism was also investigated in a suspension that had been boiled prior to the addition of formaldehyde. In some experiments a second dose of formaldehyde was added once the original dose had been consumed.

To investigate the interaction of glutathione and formaldehyde in vitro, 10 mg of glutathione (reduced form, Sigma) was dissolved in 0.5 mL of D_2O to give a solution with pD 2.5. Under these acid conditions the $GSCH_2OH$, which forms almost instantaneously, is stable over a period of days. It was examined by a combination of ^{13}C and ^1H NMR spectroscopy, ^1H spectra being acquired at 400 MHz. To demonstrate the identity of the peak observed for $GSCH_2OH$ in vitro and in vivo, 10 mg of glutathione was dissolved in 0.5 mL of 0.2 M K_2HPO_4 and the pH was adjusted to pH 7. A total of 125 μL of 600 mM [^{13}C]formaldehyde was added; 75 μL of this solution was added to half the supernatant from a boiled bacterial suspension (suspension A, above) containing the metabolite at 66.6 ppm.

RESULTS

When an anaerobic suspension of late exponential phase *E. coli* K12 was challenged with 10 mM [^{13}C]formaldehyde, new peaks appeared in the ^{13}C NMR spectrum. A resonance at 66.6 ppm appeared almost instantly (Figure 1a) and decreased again over a period of 60 min; its consumption was more rapid than that of formaldehyde. Other metabolites, i.e., formate (172.3 ppm), methanol (50.2 ppm), propane-1,2-diol (67.8 ppm), glycerol (64 ppm), and propane-1,3-diol (60 ppm), appeared more slowly and remained [Figure 1b; cf. Hunter et al. (1984, 1985)].[2] Following complete consumption of the formaldehyde, a second challenge with 10 mM [^{13}C]formaldehyde led to the immediate reappearance of the 66.6-ppm signal and further metabolism.

When a formaldehyde-challenged bacterial suspension (Figure 2a) was cooled and centrifuged, the signal at 66.6 ppm was observed exclusively in the resuspended pellet (Figure 2c), showing that this compound is probably intracellular. In contrast, the end-product metabolites occurred predominantly in the supernatant (Figure 2b). When the resuspended pellet was warmed to 37 °C, the peak at 66.6 ppm disappeared rapidly (Figure 2d). If the resuspended bacterial pellet was boiled and cooled to 37 °C, then the peak at 66.6 ppm remained unchanged over several days. If, in similar experiments, the bacterial suspension was boiled and centrifuged, then all the products including the peak at 66.6 ppm occurred exclusively in the supernatant and remained over a period of hours.

If formaldehyde challenge is carried out and observed at 2 °C, the formation of the end products such as methanol is very much slower, as would be expected for enzymatically mediated reactions. However, the species giving the 66.6-ppm signal is still formed and accumulates to a much higher concentration. If bacteria are boiled, then this is the only product observed when [^{13}C]formaldehyde is added. This is true of bacteria harvested at any stage of growth.

A proton-coupled ^{13}C NMR spectrum showed the 66.6-ppm signal to be a triplet, J_{CH} = 159 Hz, indicating that it was due to XCH_2Y, where X and Y are electronegative; this coupling constant is too large for a CCH_2OH group. Two-dimensional ^{13}C–^1H correlation spectroscopy showed that the protons at-

[2] An additional, as yet unidentified, metabolite gives rise to a signal at 68.4 ppm in *E. coli* K12.

Scheme I

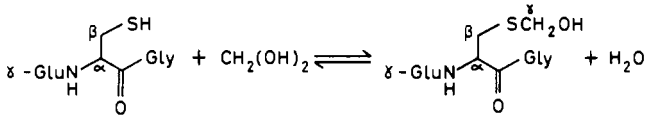

Table I: Selected ^{13}C and ^{1}H NMR Chemical Shifts and Coupling Constants in the Cysteine Residue of GSH and $GSCH_2OH$

	GSH	$GSCH_2OH$
	Chemical Shifts (ppm)	
C_α	56.9	54.8
C_β	26.2	33.6
C_γ		66.6
H_α	4.54	4.62
H_β	~2.92	3.13
$H_{\beta'}$	~2.92	3.01
H_γ		4.70
$H_{\gamma'}$		4.72
	Coupling Constants (Hz)	
$H_\alpha H_\beta$		5.1
$H_\alpha H_{\beta'}$		8.2
$H_\beta H_{\beta'}$		14.1
$C_\gamma H_\gamma$		159
$C_\gamma H_\beta$		4.3
$C_\gamma H_{\beta'}$		4.7
$H_\gamma H_{\gamma'}$		14.0

tached to this carbon resonate at 4.7 ppm.

Studies of the reaction of formaldehyde with model biomolecules (Tome & Naulet, 1981; R. P. Mason and J. K. M. Sanders, unpublished results) suggested structures similar to hydroxymethyl derivatives of cysteine (66 ppm), asparagine (68.2 ppm), arginine (65.2 ppm), glutamine (66.3 ppm), or ethanethiol (65.6 ppm), which all have J_{CH} around 160 Hz. However, none of these adducts gave peaks that precisely coincided with the peak at 66.6 ppm in formaldehyde-challenged *E. coli*.

As it was known that both glutathione and tetrahydrofolate are cofactors in formaldehyde oxidation (Koivusalo et al., 1982; Ku & Billings, 1983; Rose & Racker, 1966), mixtures of these with formaldehyde were examined. Tetrahydrofolate gave 66.8- and 75-ppm resonances and was dismissed. Glutathione produced a ^{13}C triplet at 66.6 ppm, J_{CH} = 159 Hz, and also proton signals at 4.7 ppm. The resonance at 66.6 ppm is shown unequivocally to be the hemithioacetal of glutathione (Scheme I) by the observation of a long-range coupling to the cysteine β-protons. Furthermore, the proton and carbon resonances of cysteine C_α and C_β shift on adduct formation. The data are summarized in Table I.

When a bacterial supernatant containing the peak at 66.6 ppm, produced by boiling formaldehyde-challenged cells, was mixed with an authentic mixture of GSH and formaldehyde containing $GSCH_2OH$, the 66.6-ppm signals were found to be coincident. If the bacterial supernatant was adjusted to pH 8, then the peak at 66.6 ppm rapidly disappeared. Shortly afterward, two new peaks, as yet unidentified,[3] grew at 61.3 and 61.9 ppm (J_{CH} = 156 Hz). Identical behavior is observed when authentic solutions of *S*-(hydroxymethyl)glutathione are made basic.[3] This observation firmly identifies the transient species as *S*-(hydroxymethyl)glutathione.

The growth period of the bacteria, prior to harvest, was found to be critical for observing the glutathione adduct. No adduct was observed for bacteria harvested in early exponential growth phase. Harvesting in late exponential growth phase or early stationary phase was found to be optimal. We have observed the glutathione adduct in *E. coli* grown on acetate, glucose, glycerol, or succinate minimal media and in both K12 and NCIB 8797 strains.

Discussion

These results demonstrate the transient formation in *E. coli* of *S*-(hydroxymethyl)glutathione following addition of formaldehyde. This adduct had been postulated as the true substrate for formaldehyde dehydrogenase on the basis of enzyme studies in vitro (Koivusalo et al., 1982; Strittmatter & Ball, 1965; Weiner et al., 1982), but its formation has not been previously observed in vivo.

It appears that the equilibrium shown in eq 1 has only been studied by UV spectroscopy to date (Uotila & Koivusalo, 1974). Even in vitro this relies upon a small change in the end absorption of the thiol group; in vivo there are so many chromophores within the cell that it is virtually impossible to assign changes to specific groups or processes. By contrast, ^{13}C NMR spectroscopy allows us to follow the fate of labeled formaldehyde molecules within the cell without interference from any other species. The complexity of the GSH–formaldehyde equilibrium, which is highly sensitive to pH, age of solution, and relative concentrations of glutathione and formaldehyde,[3] means that analysis of model systems or extracts may not provide a reliable representation of the processes occurring within the cell.

The observation of $GSCH_2OH$ upon addition of formaldehyde to heat-killed cells indicates that adduct formation is a chemical process; i.e., the equilibrium in eq 1 is not enzyme mediated. However, the results shown in Figure 2 suggest that consumption of the adduct is a biochemical process. Furthermore, the results of challenge experiments at 2 °C, where adduct formation occurs readily but consumption is much slower, are consistent with the two processes being chemical and biochemical, respectively. As yet, we have no *direct* evidence as to whether $GSCH_2OH$ is involved in the biosynthesis of any of the observed metabolites, although its involvement as the true substrate in formate production is very likely. GSH is clearly present in substantial quantities in *E. coli* cells, irrespective of the stage of harvesting.[4] However, it appears that the enzyme that consumes $GSCH_2OH$ is less active in cells harvested later, allowing significant buildup.

Recently, there has been a great deal of concern regarding the potential carcinogenicity of formaldehyde (Yodaiken, 1981). Cells may be exposed to formaldehyde externally in sterilizing solutions, e.g., noxythiolin (Gidley & Sanders, 1983), or in the atmosphere, e.g., released from urea–formaldehyde resins (Georghiou et al., 1983). Formaldehyde is also released intracellularly in the detoxification of a number of methylamines (Krieter et al., 1985; Steenkamp, 1982) and methanol (Goodman & Tephly, 1971). If the formaldehyde is not removed rapidly, it may cross-link proteins (Tome et al., 1985) or DNA (Chaw et al., 1980). The formation of $GSCH_2OH$ may act as a sink for formaldehyde, reducing the effective concentration available for cross-linking. It is possible, therefore, that the protective role of glutathione in cells may extend beyond the familiar scavenging of free radicals (Torchinsky, 1981). The results presented here indicate that it should be possible to study the role of glutathione in detoxification of formaldehyde and foreign compounds in other cell systems.

[3] Above pH 6, the glutathione–formaldehyde interaction is very complicated. The initially formed $GSCH_2OH$ rearranges slowly to a variety of products (R. P. Mason and J. K. M. Sanders, unpublished results).

[4] GSH itself can be detected in cells by ^{1}H spin-echo techniques (Brown et al., 1977; Nakashima & Rabenstein, 1986).

Our results may be viewed in another way: not only do they throw new light on formaldehyde metabolism but they also provide a crude assay for glutathione. They show that a naturally occurring species may be visualized and quantified within tissue by formation of a specific adduct with an infiltrated labeled reagent. The labeled product ($GSCH_2OH$ in this case) has characteristic properties that can report on the concentration of the species of interest. This concept is widely used in radioimmunoassay and has recently been applied to assay by NMR of Ca^{2+} and other metal ions in animal tissue (Smith et al., 1983) and asparagine in potato tissue (Mason et al., 1986). The concentration of GSH in the *E. coli* cells may be estimated as being in the millimolar range from our results. This conclusion is based on the following assumptions: a typical cell has a volume of 10^{-15} L, so that the total intracellular volume of 10^{11} cells is 10^{-4} L. Thus by comparing the signal intensity of $GSCH_2OH$ in the bacterial suspension before and after the addition of the GSH–formaldehyde mixture and knowing the concentration of GSH and formaldehyde in that mixture, we can estimate the original concentration. Our range is consistent with previously quoted values of 0.4–12 (Torchinsky, 1981) and 6.6–6.8 mM in *E. coli* K12 cells (Apontomel & Berends, 1975).

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Registry No. GSH, 70-18-8; $GSCH_2OH$, 32260-87-0; HCHO, 50-00-0.

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