that they interact with a site in the receptor or a complementary receptor binding site on G/F. The latter seems more likely since INA uncouples not only the hCG/LH receptor but also the response to follicle-stimulating hormone (FSH) (data not shown). It is also interesting to note that the addition of low INA concentrations followed by irradiation might lead to the generation of functionally uncoupled membranes or cells, which may be helpful in future analysis of the membrane-bound receptor system.

Acknowledgments

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Registry No. 5-Iodonaphthyl 1-azide, 63785-44-4; 5-diazonionaphthyl 1-azide, 88000-66-2; adenylate cyclase, 9012-42-4.

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Formaldehyde Metabolism by *Escherichia coli*. In Vivo Carbon, Deuterium, and Two-Dimensional NMR Observations of Multiple Detoxifying Pathways†

Brian K. Hunter, Kathryn M. Nicholls, and Jeremy K. M. Sanders*

ABSTRACT: ^{13}C NMR has been used to demonstrate the metabolism of dilute solutions of labeled formaldehyde by *Escherichia coli* to methanol, formate, carbon dioxide, and several other unidentified metabolites which contain labeled CH_2 groups. Aeration of bacterial suspensions within the spectrometer dramatically increased the rate of oxidation to formate and carbon dioxide. Deoxygenation with nitrogen gas virtually abolished all metabolism, as did the exposure of bacteria to very high formaldehyde concentrations. Deuterium NMR of whole cells in deuterium-depleted water further demonstrated the conversion of formaldehyde-d_2 to methanol-d_2, ruling out a formaldehyde dismutase as an important species. Two-dimensional proton–carbon chemical shift correlation was used to reveal the chemical shifts of the protons attached to ^{13}C labels in metabolites. The results indicate that formaldehyde is efficiently detoxified by the bacterial cell through a route or routes which do not appear to involve tetrahydrofolate. This detoxification may be in competition with the lethal antibacterial processes associated with formaldehyde.

Formaldehyde occurs in low concentrations in cells as the adduct N^5,N^{10}-methylenetetrahydrofolate, which is an important intermediate in the reactions of the C_1 pool. When tetrahydrofolate adducts are used as carriers, one-carbon units at the formate, formaldehyde, and methanol oxidation level are transferred to other molecules during biosynthesis (Benkovic, 1980). The major source of methylenetetrahydrofolate is the reaction between tetrahydrofolate and serine, catalyzed by serine hydroxymethyltransferase:

serine + tetrahydrofolate ⇌
glycine + methylenetetrahydrofolate

Since this reaction is reversible, it can also generate serine if methylenetetrahydrofolate is provided by another route such as the oxidative degradation of glycine. An alternative route to the adduct is direct chemical reaction of formaldehyde with tetrahydrofolate itself (Benkovic, 1978). Serine may subsequently be converted to pyruvate and, after decarboxylation and derivatization, to acetyl-CoA. In this way carbon atoms

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which originate from formaldehyde could enter the normal metabolic pathways of the cell. At the same time formaldehyde is widely used, either in native or in masked form, as an antibacterial agent in clinical and industrial settings (Browne et al., 1978; Gidley & Sanders, 1983; Taylor et al., 1969; Walker, 1964).

Prompted by our earlier work on mechanisms of antibacterial formaldehyde delivery (Gidley & Sanders, 1983) we have been using NMR spectroscopy of intact *E. coli*[1] to investigate the biological fate of formaldehyde. We report here not only conventional ^{13}C NMR observations but also (i) the use of deuterium NMR as an effective probe of the fate of hydrogen atoms during metabolism and (ii) a two-dimensional proton–carbon correlation method for following the fate of C–H bond pairs. As a result of these experiments we also report the discovery of some aerobic metabolic pathways which appear to act as effective detoxifying mechanisms in *E. coli*.

^{13}C NMR is a well-established, although not yet widely applied, technique for monitoring carbon metabolism in whole cells [see, for example, Burton et al. (1980), den Hollander et al. (1981), Foxall et al. (1983), Mackenzie et al. (1982), and Ogino et al. (1982)]. It generally relies on the detection of ^{13}C-labeled substrates and products vs. a relatively low background of natural abundance signals. The detection of proton metabolism by NMR is more difficult. The substrate and metabolities of interest are likely to be masked by the resonances of other cell components, both large and small molecules, and by the water resonance which corresponds to 110 M protons. Suspending the cells in deuterium oxide is clearly helpful, but 100% replacement of protons is neither practical nor desirable from the point of view of many living cells. Residual water can be suppressed by many methods including selective saturation (Haasnoot, 1983; Wider et al., 1983), selective excitation (Stoesz et al., 1978), and spin echoes (Agris & Campbell, 1982; Nicholson et al., 1983; Rabenstein & Nakashima, 1979), but each of these methods has its drawbacks, and suppression ratios of better than 10^3 are difficult to achieve reliably. Macromolecule resonances can be suppressed through use of spin-echo methods (Agris & Campbell, 1982; Nicholson et al., 1983; Rabenstein & Nakashima, 1979) or by exploiting the spin diffusion which follows saturation of part of the macromolecule spectrum (Akasaka, 1979; Rabenstein et al., 1979). Thus, it has been possible by using one or more of these techniques to use proton NMR to observe metabolism within simple cells such as erythrocytes (Simpson et al., 1982), to characterize changes in cell constituents during differentiation (Agris & Campbell, 1982), and to compare blood and plasma constituents from different mammalian species (Nicholson et al., 1983). However, there seems little hope that metabolism of specified substrates at the millimolar level will be easily monitored in complex cells by this approach. Recent advances in heteronuclear spin-echo and inverse polarization transfer experiments appear to give attractive routes to the "selective" detection of protons bound to ^{13}C (Bendall et al., 1981; Foxall et al., 1983; Freeman et al., 1981). In practice the selective detection is generally achieved by alternate addition and subtraction of spectra which differ only in the phase of those carbon-bound protons; problems of the efficiency of suppression of the remaining signals therefore remain.

Virtually all of these problems should disappear if deuterium NMR is used to follow the metabolism of low molecular weight deuterium-labeled substrates. The natural abundance of deuterium is only 0.015% so background signals are intrinsically weak and 6600-fold enrichment is possible (as compared with 90-fold for ^{13}C). Deuterium line widths are 20 times more sensitive to slow tumbling than are those of protons (Bernstein et al., 1979; Zens et al., 1976), and this further suppresses the macromolecular background. The signal due to solvent HOD is normally equivalent to 16 mM deuterium, but this can be reduced almost 100-fold by brief suspension of the cells in deuterium-depleted water, which is commercially available. Monitoring of millimolar biochemistry should therefore be accessible. Furthermore, as we show in this paper, the ability to follow the appearance or disappearance of geminal H–D coupling provides a powerful tool for in vivo studies of enzyme mechanism.

An alternative approach is to detect proton resonances indirectly through two-dimensional ^1H–^{13}C chemical shift correlation (Bax & Morris, 1981). As this is an experiment which detects only ^{13}C magnetization, only those protons which are attached to carbon can be detected, and no suppression of water is needed. This approach has the further feature that the carbon spectrum is spread into an extra dimension, thereby reducing the chance of overlap.

In this paper we demonstrate the complementary application of both of these new approaches to proton metabolic studies, together with the conventional ^{13}C method, to formaldehyde metabolism in *E. coli*. Our results indicate that this metabolism is in competition with the lethal antibacterial processes associated with formaldehyde.

Experimental Procedures

Paraformaldehyde (91% ^{13}C) was obtained from Prochem and Merck Sharp & Dohme and sodium formate (91.7% ^{13}C) from Prochem. Paraformaldehyde (98% D) was obtained from Merck Sharp & Dohme. Deuterium-depleted water was obtained from Sigma Chemical Co. and BDH. All other chemicals were AR grade. *E. coli*, strain MU 352 from the Manchester University collection of bacteria, was a gift from Dr. M. C. Allwood, Addenbrooke's Hospital, Cambridge. Bacteria were grown at 37 °C with forced aeration to stationary phase in 2-L cultures by using Spizizen's (1958) salt medium containing 0.1% ammonium chloride in place of 0.2% ammonium sulfate. After 9-h growth (OD at 680 or 500 nm ca. 1.0) the cells had reached early stationary phase and were harvested. They were washed twice with chilled distilled water and finally pelleted by centrifugation at 4 °C. The resulting pellet could then be treated in various ways, depending on the spectroscopic or microbiological experiment.

For whole cell ^{13}C NMR the pellet was suspended in water (6 mL, containing 20% D_2O to provide a lock signal) to give a total sample volume of about 10 mL. This suspension was stored, usually for no more than 1–2 h, at 4 °C until required. In these relatively thick suspensions the cell volume accounts for approximately 20% of the total, and cell density is ca. 10^{12} mL^{-1}. Cells treated in this way show no significant loss of viability on resuspension in nutrient medium, even after many hours in the NMR spectrometer. For some metabolic runs the bacterial suspension was diluted 10–100-fold with 20% D_2O.

For deuterium NMR half the pellet was suspended in deuterium-depleted water (3 mL) and left to stand at room temperature for 20 min. The cells were then collected by centrifugation and resuspended in fresh deuterium-depleted water (3 mL).

The antibacterial effectiveness of formaldehyde under the conditions used for NMR experiments was tested by exposing

[1] Abbreviations: d, doublet; *E. coli*, *Escherichia coli*; FID, free induction decay; NMR, nuclear magnetic resonance; q, quartet; s, singlet; THF, tetrahydrofolate.

bacterial suspensions at the standard concentration (10^{12} mL^{-1}) to solutions containing 0, 1, 10, and 50 mM formaldehyde for times of 5–180 min. The resulting mixtures were then added to nutrient salt medium, and growth was monitored for up to 10 h.

^{13}C-Labeled formaldehyde solutions were prepared by heating the appropriate amount of paraformaldehyde in water at 115 °C in an autoclave for 30 min. The resulting solutions, which were generally about 50 mM in formaldehyde, were assayed by the sodium sulfite method (Walker, 1964). They were stable almost indefinitely. A 200 mM stock solution of formaldehyde-d_2 was prepared by heating the labeled paraformaldehyde with deuterium-depleted water at 120 °C in a sealed tube. The solution was assayed as above.

^{13}C spectra at 100.6 MHz were obtained in Cambridge on a Bruker WH 400 spectrometer by using 2-mL samples in 10-mm diameter tubes. ^{13}C spectra at 50.3 MHz were obtained at Queen's on a Bruker CXP 200 spectrometer by using 10-mL samples in 20-mm diameter tubes. Experiments involving aeration or nitrogen bubbling within the spectrometer were carried out at Queen's. Gas was introduced through a glass tube which had been drawn to a fine capillary at one end. This tube was held centrally within the NMR sample tube by two Teflon baffles which also served to protect the probe from any frothing due to over-vigorous bubbling. A second, narrower, glass tube sat in an eccentric hole in the baffles and served to hold the end of the capillary off the bottom of the sample tube. The whole arrangement was secured to the top of the sample tube by masking tape. Compressed air or nitrogen gas were blown in to the top of the bubbler tube through a drying tube which protected the sample from oil and other foreign matter. Provided that the capillary diameter was sufficiently small, there was only a very slight deterioration in spectral resolution during bubbling. Bacterial samples for metabolic study were prepared by mixing suspensions of bacteria and solutions of substrate to give a final concentration of 10 mM substrate. Pulse widths of 45° were used on both spectrometers. Relaxation delays of 1 or 2 s were generally inserted between pulses, and transients were collected into 4K or 8K data points. Sweep widths were 31 250 Hz (100 MHz) or 15 000 Hz (50 MHz). Most spectra were recorded with continuous proton broad band decoupling; cold nitrogen gas was blown through the probe continuously to keep the temperature close to 25 °C. In preliminary experiments external pyrazine was used as chemical shift standard at δ 145.8. Under these conditions the hydrated formaldehyde signal appeared at δ 83.2; in subsequent work formaldehyde itself was the standard. In metabolic experiments either 500 transients were collected every 2.1 s or 1000 transients every 1.06 s, corresponding in both cases to just under 20 min per spectrum. Sample spinning was not used. In general exponential broadening of 15 Hz was used to improve signal/noise. Spin–lattice relaxation times (T_1) were estimated by saturation recovery; absolute precision is unlikely to be better than ±20%, but the relative values are reasonably accurate.

Deuterium NMR spectra were acquired at 61.4 MHz on the WH 400 instrument by using a spectral width of 1000 Hz, 4K data points, 2.05-s acquisition time, no relaxation delay, and 65° pulse width. In some experiments the samples were contained in an 8-mm diameter tube; this tube was placed in a 10-mm tube containing hexafluorobenzene as fluorine lock. On other occasions, samples were placed directly in 10-mm tubes, and spectra were acquired unlocked. In metabolic experiments blocks of 512 transients were acquired, corresponding to 20 min per spectrum. The HOD resonance was assigned a chemical shift of δ 4.80, and other signals were referenced to this.

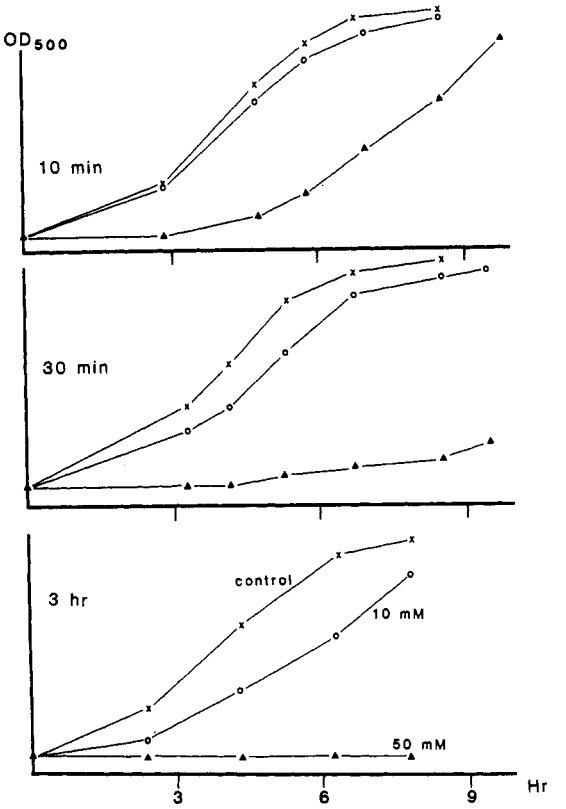

FIGURE 1: Growth curves for *E. coli* in salt medium following challenge of aqueous suspensions by different formaldehyde concentrations (0, 10, and 50 mM) for various times (10, 30, and 180 min). Growth curves for the suspension which had been exposed to 1 mM formaldehyde were indistinguishable from the controls and are not shown.

Two-dimensional correlation spectroscopy was carried out on the WH 400 by using the following pulse sequence:

$$\pi/2(^1\text{H})\text{–}\tau\text{–}\pi(\text{C})\text{–}\tau\text{–}D_1\text{–}\pi/2(^1\text{H}),\pi/2(\text{C})\text{–}D_2\text{–Acq } ^{13}\text{C, decouple H}$$

where τ is the incremented delay and D_1 and D_2 are fixed delays of 3.3 and 1.65 ms, corresponding to $(2J)^{-1}$ and $(4J)^{-1}$ for the C–H bonds concerned; 128 experiments were each recorded with 72 transients after two dummy scans in 2K data points, with spectral widths of 1200 and 5000 Hz in f_1 and f_2, respectively, and a relaxation delay of 3 s. FIDs were transferred by magnetic tape to an IBM 3081 computer in Cambridge. Data processing and plotting were carried out by using the CAM2D package (J. D. Mersh, unpublished results; Mersh & Sanders, 1982). By use of off-line data processing, it is convenient to explore a wide range of apodization combinations on the data. In this case the contour plot is based on a spectrum generated by using a 30°-shifted sine bell in each direction. However, this treatment produces distortions in the proton cross sections, as is apparent from inspection of the formaldehyde and methanol singlets (not shown). For the cross sections in Figure 8, sine-bell apodization was applied to the proton dimension, f_1, and a 90°-shifted sine bell in the carbon dimension, f_2.

Results

Viability Experiments. Figure 1 shows the growth curves for bacterial suspensions which had been exposed to various formaldehyde concentrations for different times before being incubated in the standard nutrient medium. It is clear that under the conditions used, 50 mM formaldehyde rapidly leads

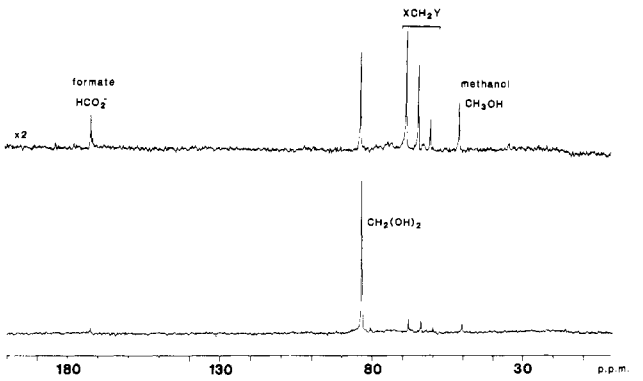

FIGURE 2: 100.6-MHz ^{13}C NMR spectra of a nonaerated *E. coli* suspension (10^{12} cells mL^{-1}) initially containing 10 mM ^{13}C-labeled formaldehyde. The lower spectrum was acquired during the first 20 min after formaldehyde addition; the upper spectrum was acquired 3 h later. A total of 500 transients was collected, with 2-s relaxation delay and 45° pulse width.

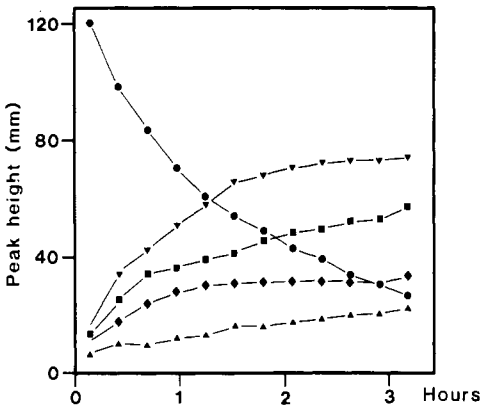

FIGURE 3: Formaldehyde consumption and metabolite production as a function of time for the experiment of Figure 2. Symbols: (●) formaldehyde (×1/2), (▼) 68 ppm product, (■) 64 ppm product, (♦) methanol, and (▲) formate.

to a complete loss of viability, and 1 mM formaldehyde has virtually no effect, even after a 3-h exposure. By contrast, exposure to 10 mM formaldehyde leads to a gradual effect on the growth rate over a period of hours. Most of the NMR work described below was carried out by using an initial formaldehyde concentration of 10 mM.

^{13}C NMR Spectroscopy. Aqueous solutions of 10 mM ^{13}C-labeled formaldehyde give a proton-decoupled spectrum containing essentially only a singlet at δ 83.2 due to formaldehyde hydrate $CH_2(OH)_2$. When suspensions of *E. coli* were challenged with formaldehyde and incubated in the spectrometer, new signals rapidly appeared. Adequate signal was obtained in about 20 min. Figure 2 shows spectra taken during the first 20 min of such an incubation and after 3 h, and Figure 3 plots the time course for the most prominent signals. After all the formaldehyde was exhausted, further quantities could be administered, and these were metabolized similarly. When bacteria were challenged with formaldehyde but kept at 4 °C for some hours, virtually no metabolism was observed to have occurred in the cold, but metabolism at the normal rate began as soon as incubation at 26 °C in the spectrometer began. No metabolism occurred in suspensions of heat-killed bacteria. Some of the new species were readily indentified by their chemical shifts and, in proton-coupled spectra, multiplicities and couplings. These are methanol (CH_3OH, δ 50.1, q, J = 141 Hz), formate (HCO_2^-, δ 172.2, d, J = 218 Hz), and, in several runs, carbon dioxide (δ 125.8, s). Additional, unidentified signals were also consistently seen at δ 59.8, 63.9, 68.0, and, on occasion, 183 (see below). The

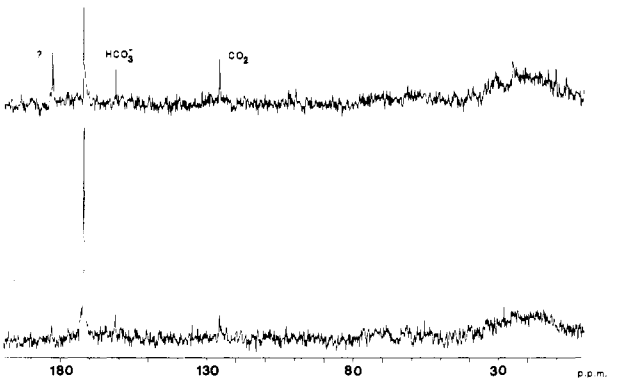

FIGURE 4: 100.6-MHz ^{13}C spectra of a nonaerated *E. coli* suspension (10^{12} cells mL^{-1}) initially containing 10 mM sodium [^{13}C]formate. Lower spectrum was acquired during the first 20 min and the upper spectrum some hours later.

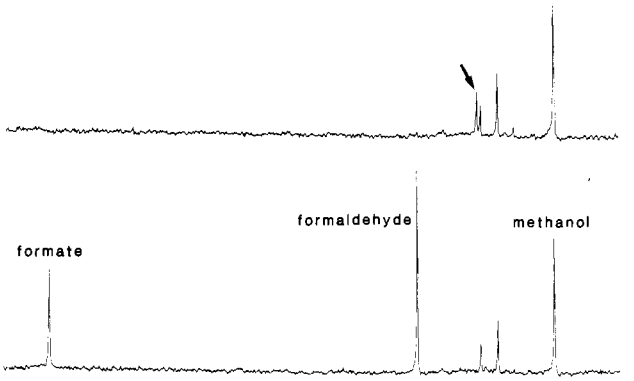

FIGURE 5: 50.3-MHz ^{13}C spectra demonstrating the effect of in situ aeration on formaldehyde metabolism. The lower spectrum is of a partially metabolized, nonaerated suspension (10^{12} cells mL^{-1}). The upper spectrum was acquired about 1 h after the beginning of aeration; the arrow indicates new metabolite at δ 68.9.

relative intensities of metabolite signals varied considerably from one experiment to another, with methanol and formate sometimes the most intense. Approximate relaxation times were formaldehyde hydrate (5 s), methanol (15 s), formate (7 s), δ 68.0 (3 s), δ 63.9 (2.3 s), and δ 59.8 (1.7 s). Thus, the present acquisition parameters significantly underestimate the proportion of metabolism to formate and methanol. Very little metabolism was seen in a suspension which had been incubated overnight with a 50 mM formaldehyde solution, and virtually none occurred after a 100 mM incubation.

Feeding a bacterial suspension with 10 mM ^{13}C-labeled sodium formate confirmed the assignment at δ 172.2 and led to the appearance of bicarbonate (δ 161.2), carbon dioxide, and the unknown at δ 183 (Figure 4). No formaldehyde, methanol, or other products were seen.

These experiments, carried out in suspensions which were neither aerated nor deoxygenated, indicated the presence of a reductive pathway from formaldehyde to methanol and an oxidative pathway to bicarbonate and carbon dioxide via formate. Aeration of a partially metabolized suspension in the spectrometer using the assembly described in detail under Experimental Procedures led to almost complete disappearance of the formate resonance within the time taken to acquire the next spectrum (Figure 5). A new signal also appeared at δ 68.9 (Figure 5). When aeration was begun at the start of a metabolic run, no formate was seen at all, small quantities of the δ 50–70 metabolites (*including* that at δ 68.9) were seen, but formaldehyde clearly disappeared more rapidly. This is presumably a result of rapid oxidation of formate to carbon dioxide, catalyzed by formate dehydrogenase (Gale, 1939).

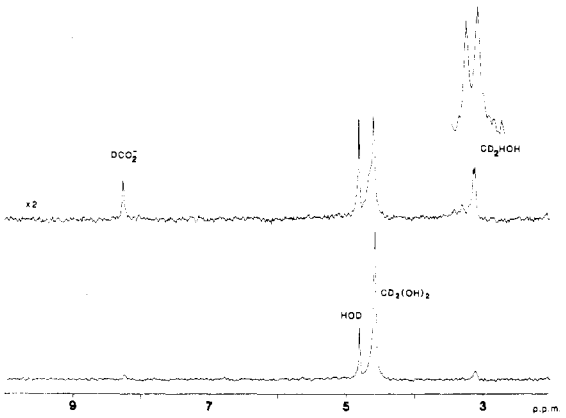

FIGURE 6: 61.4-MHz deuterium NMR spectra of a nonaerated *E. coli* suspension (10^{12} cells mL^{-1}) in deuterium-depleted water initially containing 10 mM formaldehyde-d_2. Lower spectrum was acquired during the first 20 min and the upper spectrum after some hours metabolism in the spectrometer. A total of 500 transients was acquired, with 65° pulses, 2-s acquisition time, and no relaxation delay.

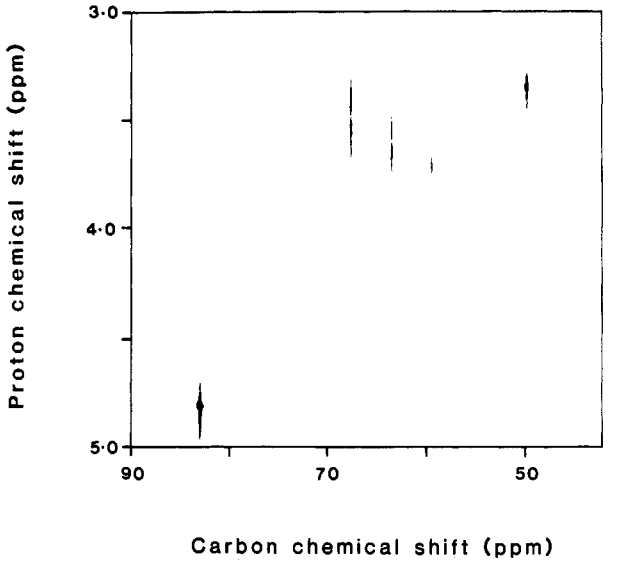

FIGURE 7: Contour plot of part of a two-dimensional chemical shift correlation spectrum. The sample was supernatant from a bacterial suspension which had extensively metabolized formaldehyde. See Experimental Procedures for details of sample preparation and spectroscopic parameters.

Bubbling of nitrogen gas through a bacterial suspension before 10 mM formaldehyde challenge led to an almost complete lack of metabolism even if aeration was subsequently applied. Nitrogen bubbling (30 min) followed by aeration for a similar time before formaldehyde challenge gave normal metabolism.

When formaldehyde-challenged bacteria were separated from the medium by centrifugation, the bulk of metabolite signal intensity appeared in the supernatant and very little in the resuspended cells. In control experiments, no reaction occurred when formaldehyde was added to the supernatant from either unchallenged or previously challenged bacteria.

Several as yet inconclusive experiments were carried out in an effort to identify the products giving rise to the signals between δ 59 and δ 70. The three peaks which are present in nonaerated suspensions all arise from CH_2 groups with proton couplings of ca. 140 Hz. Both shifts and couplings are consistent with structures such as XCH_2OH. Serine, as an obvious candidate if methylenetetrahydrofolate were to be formed, was added to the suspension and was shown not to be responsible for any of these three major signals. Lyophilization of supernatant followed by redissolution in water showed that all major metabolites except methanol were relatively involatile, although the δ 68.0 signal did decrease in intensity relative to the other signals. Sodium cyanoborohydride was added to a solution containing labeled unknowns, but no reaction was detectable by NMR. This observation rules out structures of the form NCH_2OH and NCH_2N which would be formed by simple chemical reaction of formaldehyde with amine groups and which would be reduced to *N*-methyl groups by cyanoborohydride (Benkovic, 1978; Gidley & Sanders, 1982). Efforts to identify these compounds continue.

Deuterium NMR Spectroscopy. Figure 6 (bottom) shows the 61.4-MHz deuterium NMR spectrum of 10 mM formaldehyde-d_2 acquired during the first 20 min after addition to a bacterial suspension in deuterium-depleted water. As expected, the solvent peak is considerably less intense than that of the labeled substrate. Sensitivity and time resolution were comparable to the ^{13}C experiments described above. Figure 6 (top) shows the result of several hours metabolism. No aeration was employed. Sharp signals from formate, methanol, and residual formaldehyde dominate the spectrum, but several smaller broad resonances are also visible near δ 3.5. When resolution enhancement and zero filling are applied, the methanol signal is seen to be a doublet, with a splitting of 1.7 (±0.3) Hz. Proton decoupling gave a singlet for the methanol, proving that the dominant species is CD_2HOH. The coupling constant is the expected size for this species. This observation rules out the presence of substantial amounts of formaldehyde dismutase ("Cannizzarase"?) activity catalyzing the reaction:

$$2CH_2(OH)_2 \rightarrow CH_3OH + HCO_2H + H_2O$$

Such an enzyme has been reported in a *Pseudomonad* (Kato et al., 1983) but would have been apparent in the deuterium experiment by the observation of a singlet corresponding to CD_3OH. The upfield line of the methanol doublet is consistently slightly more intense than the other line, and this may indicate the presence of small amounts of dismutase activity. However, other explanations are also possible (see Discussion). In a separate experiment, a bacterial suspension was challenged with methanol-d_3. As expected, neither formaldehyde formation nor any other change was observed.

The broad resonances near δ 3.5 were not readily identifiable but were presumed to correspond to the XCH_2Y metabolites appearing in the ^{13}C spectra. This correspondence was confirmed by two-dimensional NMR.

Two-Dimensional Spectroscopy. Several experiments were carried out, both on metabolizing bacterial suspensions and on postmetabolism supernatants. Figure 7 shows the contour plot and Figure 8 some cross sections from a ^{13}C–^1H two-dimensional correlation experiment which was carried out on a supernatant sample. A standard bacterial suspension had been fed 10 mM ^{13}C-labeled formaldehyde and allowed to metabolize until the formaldehyde was virtually exhausted. A further 5 mM formaldehyde was added, and metabolism was allowed to continue until approximately 12 mM formaldehyde had been consumed. The suspension was then centrifuged, and the supernatant was used for the 2D experiment. The contour plot shows, in addition to intense singlets for formaldehyde and methanol, complex multiplets in the proton dimension, f_1, for the carbon resonances at δ 68 and 64. These are more easily seen in the cross sections (Figure 8) which show the proton signals corresponding to a given ^{13}C chemical shift. It is clear that the proton chemical shifts do indeed correspond to the deuterium signals described above. It is also clear that the proton signals are more complex than those of formaldehyde or methanol; we ascribe this complexity to second-order character in the proton spectrum. We cannot be sure

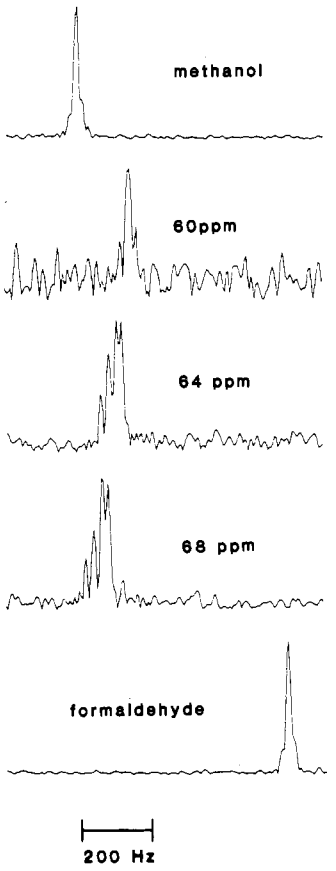

FIGURE 8: Proton cross sections through the same two-dimensional experiment as Figure 7.

at this stage whether this second-order character arises from nonequivalence of the two methylene protons attached to the labeled carbon or to strong coupling between those protons and protons attached to an adjoining, nonlabeled carbon, or both.

Projection of the 2D spectrum onto f_2 gives the normal ^{13}C spectrum, and projection onto f_1 gives the ^1H spectrum. Since this proton spectrum is a scaled version of the deuterium spectrum, it is clear that the protons remain attached to the carbon during metabolism.

Discussion

Spectroscopy. The pulse widths and acquisition rates used for the ^{13}C work represent a convenient compromise between two extremes. When rapid pulsing is employed, small molecule resonances are effectively saturated and disappear, but many signals due to polysaccharides and proteins are seen (K. M. Nicholls and J. K. M. Sanders, unpublished observations). On the other hand, the long delays between pulses which would be necessary to give accurate integration for small molecule metabolites would lead to unacceptably slow data acquisition and correspondingly poor time resolution in metabolic experiments. In deuterium NMR the long acquisition time and probable short relaxation times combine to give a more realistic estimate of relative concentrations.

Carbon and deuterium NMR have proved to be powerful and complementary tools for monitoring the metabolic response of *E. coli* to formaldehyde challenge under a variety of experimental conditions. The former detected bicarbonate and carbon dioxide, while the latter elucidated the isotopic makeup of the products. Carbon clearly has the advantage of chemical shift dispersion, but deuterium is both easier and cheaper to incorporate synthetically into substrate molecules. On the basis of our work, the two approaches have similar sensitivities or, what is perhaps more significant, similar time resolution in a metabolic experiment. The quadrupole properties of deuterium which contribute to the lack of macromolecular background also place a limit of the size of substrate and metabolite which can be easily observed by direct observation. At molecular weights above 100 or 200 the increased line width will render geminal coupling to protons unobservable and could lead to severe problems of overlap, but the technique should be applicable to most types of "small" molecules provided that mobile sites such as side chains are labeled.

The two-dimensional experiment also appears to have potentially useful features. Perhaps the most remarkable feature illustrated in Figures 7 and 8 is the ability to record metabolite proton spectra at millimolar concentrations, or below, within a fraction of a ppm of the water resonance but with no interference from water or any other species. In addition, as Figure 8 makes clear, the experiment separates proton resonances which would be likely to overlap even at 400 MHz.

There are numerous NMR methods for detecting biosynthetic pathways using isotopically labeled materials including double labeling strategies for following the fate of bonds rather than individual nuclei (Garson & Staunton, 1979). These are usually used on isolated and purified products. There are also non-NMR isotope effect and incorporation methods for elucidating mechanisms of isolated and purified enzymes [see, for example, Albery & Knowles (1976a–c), Herlihy et al. (1976), Maister et al. (1976), Fletcher et al. (1976), Leadlay et al. (1976), and Fisher et al. (1976)]. The results presented here demonstrate that it should be possible in favorable cases to carry out both types of investigation by NMR in vivo in circumstances where the enzymes responsible have not been identified or isolated.

Biochemistry. It appears from our results that there is no significant incorporation of formaldehyde into the THF pool. Both formate and methanol are accessible through that route (Benkovic, 1980), but if it were the pathway employed, then we would also expect to see evidence for serine, methionine, and other methyl groups. There is in our experiments no evidence for such products.

The demonstration of an aerobic pathway from formaldehyde to formate and beyond is perhaps not surprising. Formate dehydrogenase has long been known in *E. coli* (Gale, 1939) as has the oxidation of acetaldehyde to acetate (Lees & Jago, 1977). Neither is the reduction to methanol in itself a striking observation apart from the fact that it is apparently aerobic and has not (to our knowledge) been previously described; the oxidation of methanol by methanol dehydrogenases is, by contrast, well-known (Dalton, 1981). Presumably our reduction to methanol requires NADH, or a similar coenzyme, and presumably the oxidation of formaldehyde generates NADH or its equivalent, so the observation in the deuterium experiments that CD_2HOH is the main product may be interpreted in one of two obvious ways, each additional to the conclusion that a dismutase is not important. We may conclude that either (i) the donor and acceptor coenzymes are not the same or (ii) the NADH pool is sufficiently large that the NADD generated in formate production is effectively diluted and little CD_3OH is formed. It may in fact be that the greater intensity of the high field line in methanol-d_2 is due to a small amount of methanol-d_3, shifted by an isotope effect.

It is clear that more work is required to characterize more fully both the nature and location of the formaldehyde metabolic processes occurring in *E. coli* and to establish the generality of these pathways in other organisms. These are

in hand. However, even at this stage it is possible to propose a model for the interaction of formaldehyde and bacteria. This is outlined in the following section.

Is Metabolism a Defense Mechanism? Given the rather effective antibacterial properties of formaldehyde, it is perhaps a surprise that its metabolism by *E. coli* is so diverse, vigorous, and apparently unexplored. Indeed we suspect that this metabolism has remained unexplored precisely because it was thought unlikely to occur. The bulk of our NMR work has been carried out at formaldehyde levels which have marginal antibacterial properties, and it has demonstrated that in the presence of air formaldehyde is effectively detoxified to apparently innocuous metabolites which are released into the medium. When, however, air is excluded during the early stages of formaldehyde challenge and is then readmitted, no such metabolism is observed. The bacteria appear to be metabolically inactive (dead?). Similarly when high (50 to 100 mM) concentrations are used, very little metabolism is seen. It would seem therefore that there is a competition between detoxifying metabolism and the lethal processes and that once the lethal processes have occurred, no formaldehyde biochemistry can take place. These high dose experiments also demonstrate that none of the metabolites we observe at lower doses cause the loss of viability when formaldehyde is fed to *E. coli*. We shall not speculate here on the nature of those lethal processes. Nor do we necessarily imply that there is a specific anti-formaldehyde response; the appropriate enzymes may be fortuitously present for quite different roles. Nevertheless, if this competition hypothesis is correct, then it opens the way to improving the antibacterial efficiency of formaldehyde (and possibly its derivatives) by suppression or inhibition of the "defense" mechanisms.

Registry No. Formaldehyde, 50-00-0; methanol, 67-56-1; formate, 71-47-6; carbon dioxide, 124-38-9.

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