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In Vivo Enzymology: A Deuterium NMR Study of Formaldehyde Dismutase in *Pseudomonas putida* F61a and *Staphylococcus aureus*†

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ABSTRACT: High-resolution deuterium NMR spectroscopy has been used to follow the detoxifying metabolism of [D_2]formaldehyde in vivo in several bacterial species. Production of [D_2]methanol in *Escherichia coli* confirms that the oxidation and reduction pathways of metabolism are independent in this organism. Efficient production of equimolar quantities of [D]formate and [D_3]methanol in *Pseudomonas putida* F61a and *Staphylococcus aureus* implicates a formaldehyde dismutase, or "cannizzarase", activity. These observations imply that the unusual formaldehyde resistance in *P. putida* F61a is a direct result of efficient dismutation acting as a route for detoxification. Cross-dismutation experiments yield an enzymic kinetic isotope effect of ca. 4 for H vs D transfer and a similar spectrum of substrate specificity to the isolated enzyme. [D]benzyl alcohol produced by cross-dismutation of [D_2]formaldehyde and benzaldehyde in *P. putida* is demonstrated to have the *R* configuration by a novel deuterium NMR assay. Additionally, *S. aureus* produces methyl formate as a product of formaldehyde detoxification, apparently by oxidizing the methanol hemiacetal of formaldehyde.

Our understanding of the mechanisms of action of enzymes has largely come from studies of isolated material in well-defined, but artificial, media. However, enzymes actually operate in the context of the living cell, so it seems worthwhile to try to study enzyme kinetics and mechanism in this more "natural" environment. NMR[1] spectroscopy is potentially a powerful tool for this type of work, because it is both nonin-

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[1] Abbreviations: *B. catarrhlis*, *Branhamella catarrhlis*; *E. coli*, *Escherichia coli*; LADH, horse liver alcohol dehydrogenase; NAD, nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; *P. putida*, *Pseudomonas putida*; *S. aureus*, *Staphylococcus aureus*; *S. mutans*, *Streptococcus mutans*; TLC, thin-layer chromatography.

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vasive and highly selective, although it does suffer from an inherent lack of sensitivity. ^{1}H, ^{13}C, and ^{31}P NMR spectroscopies have all been used to study enzyme kinetics in cells, tissues, whole organs, and live animals, the emphasis generally being on the observation of turnover by a particular enzyme or of a flux along a known pathway [for reviews, see Brindle et al. (1987) and Elgavish (1987)]. We now report the use of deuterium (^{2}H) NMR to observe and investigate the operation of formaldehyde dismutases in live cultures of *Pseudomonas putida* F61a and *Staphylococcus aureus*. Some of these results have been discussed elsewhere in preliminary form (Mason et al., 1987a; Sanders, 1987).

Previous work in our laboratory (Hunter et al., 1984) showed that ^{13}C NMR could be used to monitor the detoxification of ^{13}C-labeled formaldehyde by *E. coli* and led to identification of all the major metabolites (Hunter et al., 1985). These experiments told us the fate of formaldehyde *carbon* rather than that of the *protons*, which could be quite different. Furthermore, we always found similar amounts of methanol and formate resulting from anaerobic formaldehyde metabolism, raising the possibility of "cannizzarase"[2] (formaldehyde dismutase) activity. At an early stage we observed the detoxification of [D_2]formaldehyde in *E. coli*, using deuterium NMR, and ruled out dominant dismutase activity in that organism (Hunter et al., 1984) on the basis of the following logic.

A dismutase transferring a hydrogen atom from one formaldehyde molecule to another (either directly or via a tightly bound cofactor such as NAD) would generate [D_3]methanol and [D]formate from [D_2]formaldehyde in a cell system containing essentially no other deuterium:[3]

$$2CD_2(OH)_2 \rightarrow DCO_2H + CD_3OH + H_2O$$

The deuterium NMR spectrum of CD_3OH in water is a singlet, the coupling to the hydroxyl proton being washed out by exchange. The outcome of the independent operation of oxidizing and reducing enzymes, each using NADH or a similar cofactor, would be quite different:

$$CD_2(OH)_2 + NAD^+ \rightarrow DCO_2H + NADD$$

$$CD_2(OH)_2 + NADH \rightarrow CD_2HOH + NAD^+$$

The deuterium NMR signal of CD_2HOH is a doublet (J_{HD} = 1.7 Hz) by virtue of the geminal H–D coupling. This simple singlet vs doublet appearance is, therefore, a criterion for distinguishing dismutase activity from independent oxidation/reduction systems. This logic is valid provided that the pool of NADH is large enough not to be seriously perturbed by the NADD produced by the oxidation reaction above, and indeed, we showed some time ago that *E. coli* produced CD_2HOH as the major methanol isotopomer (Hunter et al., 1984).[4]

Kato et al. (1983) had previously reported that *P. putida* F61 was particularly tolerant to formaldehyde, growing satisfactorily with up to 60 mM in the growth medium. They also isolated and thoroughly studied (Kato et al., 1986) a formaldehyde dismutase from the same organism and showed that the hydrogen transfer is achieved via a tightly bound NAD. In this paper, we extend our deuterium NMR work not only to demonstrate the dominant dismutase activity in *P. putida* F61a and *S. aureus* but also to define a spectrum of substrate specificity in vivo, to determine the absolute stereochemistry of a cross-dismutation reaction, and to estimate an enzymic deuterium isotope effect. Our observations imply that the unusual formaldehyde resistance in *P. putida* F61a is a direct result of efficient dismutation acting as a route for detoxification.

The results reported here should be viewed as an attempt to explore the possibilities, and limitations, of in vivo deuterium NMR rather than a detailed study of a particular enzyme system, our approach being largely qualitative. After we completed this work, London et al. (1987) described their use of deuterium NMR to follow methionine metabolism in the intact rat; they also reviewed earlier in vivo work with this nucleus. For a general review of the use of NMR to monitor the fate of deuterium in biosynthetic processes see Abell (1986).

Experimental Procedures

Labeled materials were obtained from the following sources: D_2O (99%) from Aldrich, [D_2]paraformaldehyde (98%) from Merck Sharpe & Dohme, [^{13}C]paraformaldehyde (91% or 99%) from Prochem or Merck Sharpe & Dohme, respectively, $^{13}CH_3OH$ from Aldrich, $NaBD_4$ from Service des molécules marqués, France, and sodium formate (91.7% ^{13}C) from Prochem. Stock solutions of formaldehyde (600 mM to 1.5 M) were prepared by autoclaving an appropriate amount of paraformaldehyde in distilled water, with a drop of concentrated hydrochloric acid, in a sealed tube for 2 h. The formaldehyde solutions were sometimes neutralized with sodium hydroxide prior to assay with the standard Nash (1953) assay. Dried growth media were obtained from Oxoid. Other chemicals were obtained from Sigma, Aldrich, BDH, or Fisons.

Bacillus megatherium URL 2082, *Branhamella catarrhlis*, *Escherichia coli* NCIB 8797, *Staphylococcus aureus* (Oxford strain) NCTC 6571, and *Streptococcus mutans* were obtained as freeze-dried cultures from the Colworth culture collection of Unilever Research Ltd. *P. putida* F61a was obtained on a slope of brain heart infusion agar from Prof. N. Kato, Tottori University, Japan.

Most metabolic experiments were performed on bacteria harvested toward the end of exponential growth phase. NMR spectra were acquired on Bruker WM250 (62.6 MHz for ^{13}C), WH400 (100.6 MHz for ^{13}C, 61.4 MHz for ^{2}H), or AM500 (76.75 MHz for ^{2}H) instruments.

Procedure for NMR Assay of Detoxification. A bacterial growth inoculum was formed by aseptically transferring a scraping from a slope of the bacteria to 10 mL of broth: brain heart infusion for *P. putida* and *S. aureus*; tryptone soya for *E. coli* and *B. megatherium*; Todd Hewitt for *S. mutans*. *P. putida* were grown at 34 °C and all other bacteria at 37 °C in the dark, overnight to stationary phase. The inoculum (1 mL) was transferred to 150 mL or 1 L of similar sterile medium and allowed to grow, as above, until it had reached an optical density (540 nm) of about 1.2 (i.e., late exponential growth phase) in 8–24 h. The bacteria were harvested by centrifugation at 3500*g* for 15 min, at 4 °C. The pellet was resuspended in 0.2 M phosphate buffer (pH 6.5, 0.85% saline) and again centrifuged, as before. The pellet was stored on ice until required and then resuspended in buffer to give a bacterial cell concentration of about 10^{10} cells mL^{-1}.

[2] The base-catalyzed Cannizzaro reaction of aldehydes is $2RCHO \rightarrow RCO_2H + RCH_2OH$, a process involving direct transfer of hydrogen between the two aldehyde molecules. A true cannizzarase employing the same mechanism appears intuitively unlikely.

[3] The natural abundance of deuterium is only 0.016%. Its presence can, therefore, be safely ignored, except in water where its concentration is ~17 mM.

[4] Aeration experiments and observation of *S*-(hydroxymethyl)glutathione as an intermediate in formaldehyde oxidation (Mason et al., 1986a) also allowed us to rule out dismutation as a major pathway in *E. coli*.

Samples of 3.5 mL were prepared for ^{13}C NMR from resuspended bacteria (0.5 mL), D_2O (0.5 mL for lock), and [^{13}C]formaldehyde solution to a final concentration of 5, 10, 20, or 40 mM, buffer being used to make up the final volume. Samples were placed in 10 mm o.d. tubes, and ^{13}C NMR spectra were acquired at 100.6 MHz with deuterium lock, but without spinning. Generally, 8K data points were acquired across 250 ppm. The data were zero filled once and line broadened or resolution enhanced prior to Fourier transformation. $^{13}CH_2(OH)_2$ was used as an internal reference at 83.2 ppm. A 70° pulse was applied with 2-s relaxation delay, giving a repetition time of 2.5 s. Originally, gated proton broad-band decoupling was applied to avoid heating, but more recently, we used WALTZ decoupling (Shaka et al., 1983).

Samples for deuterium NMR were prepared in a similar way to those for ^{13}C NMR, but [D_2]formaldehyde replaced [^{13}C]formaldehyde and D_2O was replaced by extra buffer. Spectra were acquired unlocked at 61.4 MHz, with the deuterium-observe coil of a triple resonance probe which had been tuned for deuterium observation. Optimum resolution and sensitivity were obtained from 3.5-mL samples in 10 mm o.d. tubes. The magnet was shimmed on a sample of 3.5 mL of hexafluorobenzene (C_6F_6) with ^{19}F lock; this standard was removed and replaced with a bacterial sample in the same size tube and set to spin at an identical rate. Further shimming was carried out on the proton water FID observed through the decoupler coil. Resolution was much enhanced by spinning the samples at 25–30 Hz. Generally 4K data points were acquired over 16 ppm; 90° pulses were applied with a repetition time of 3 s. Proton-decoupled spectra were obtained by irradiation on the water resonance. Spectra were zero filled twice to 16K and resolution enhanced.

In many cases, successive spectra were acquired on a 5–20 min time scale to follow the time course of metabolism. After complete metabolism of the initial dose of formaldehyde, further formaldehyde was sometimes added and metabolism again monitored. Where particularly high resolution was required, suspensions were centrifuged after metabolism, and the supernatants were examined by NMR.

Biosynthesis of Methyl Formate in S. aureus. A suspension (3.5 mL) of late exponential phase *S. aureus* was produced, as described above for [^{13}C]formaldehyde metabolic studies; 1 M $^{13}CH_3OH$ (18 μL, final concentration 5 mM), 1 M [^{13}C]formate (20 μL, 5 mM), and 1.4 M [D_2]formaldehyde (54 μL, 20 mM) were added. A series of ^{13}C spectra was then obtained at 62.6 MHz. In a separate experiment $^{13}CH_3OH$ was added together with [D_2]formaldehyde and the metabolism was observed by ^2H NMR. To verify the identity of the methyl formate, authentic material was added to a suspension of *S. aureus* which contained some $^{13}CH_3O_2{}^{13}CH$ from metabolism of [^{13}C]formaldehyde.

Isotope Effect for H vs D Transfer. P. putida and *S. aureus* were grown to late exponential growth phase and prepared for ^2H NMR as described above. [^{13}C]- or [^{12}C]formaldehyde and [D_2]formaldehyde were added in various ratios, but the total formaldehyde concentration was kept constant at 30 mM. These samples were incubated overnight at 37 °C. They were spun down, and the cell-free supernatants were examined by ^{13}C NMR at 62.6 MHz and by ^2H NMR at 61.4 or 76.75 MHz. Deuterium spectra were obtained with 60° pulses and 3-s recycle time to ensure complete relaxation and reliable signal intensities. In parallel experiments, metabolism was monitored, as it occurred, in the spectrometer. Supernatants were obtained from these samples and examined by NMR, to show that the ratio of signal intensities was the same in the supernatants as in the suspensions. As the deuterium line widths for the various methanol isotopomers were different, spectra used in quantification were processed without apodization.

Simulated spectra for different values of k_H/k_D were obtained from a computer program which modeled simple second-order reactions: the model assumed that all the reducing equivalents are derived from formaldehyde itself and that any secondary effects from H → D or ^{12}C → ^{13}C substitution are negligible. The program cyclically performed four second-order reactions with small time increments until the formaldehyde was exhausted or reached a predetermined level:

$$\frac{d[CH_3OH]}{dt} = k_H[CH_2O]^2$$

$$\frac{d[CDH_2OH]}{dt} = k_D[CH_2O][CD_2O]$$

$$\frac{d[CD_2HOH]}{dt} = k_H[CH_2O][CD_2O]$$

$$\frac{d[CD_3OH]}{dt} = k_D[CD_2O]^2$$

Final simulated results were compared with experimental intensities until an appropriate value for k_H/k_D was found.

Cross-Dismutation. Metabolism experiments were performed as above, mixtures of [D_2]formaldehyde (10–20 mM) and a range of other aldehydes (10–40 mM) being used with deuterium NMR observation. Solutions of synthetic monodeuteriated alcohols (RCHDOH) were prepared by reacting $NaBD_4$ in phosphate buffer, pH 7, with the appropriate aldehyde; products were generally not isolated.

Stereochemistry of Benzyl Alcohol. Racemic [1-D]benzyl alcohol was prepared and isolated as follows: benzaldehyde (200 μL, 1.9 mmol) and $NaBD_4$ (100 mg, 2.4 mmol) in H_2O (2 mL) were stirred for 24 h. TLC ($CHCl_3$/silica) showed benzyl alcohol to be the only product. The reaction mixture was acidified with HCl to destroy excess $NaBD_4$. The mixture was extracted twice with $CHCl_3$, and the solvent was removed under reduced pressure, to give pure racemic [1-D]benzyl alcohol. ^2H NMR showed a peak at 4.5 ppm.

This racemic [D]benzyl alcohol (2 μL, 20 μmol) was dissolved in a mixture of glycine buffer (1 mL, 0.2 M) and distilled water (2 mL), and the pH was adjusted to pH 8.7 with a few drops of 0.5 M sodium hydroxide. NAD^+ (50 mg, 70 μmol) and acetaldehyde (30 μL, 700 μmol) were added, and the pH was again adjusted to 8.7. The ^2H NMR spectrum was acquired at 61.4 MHz. LADH (6 units in 600 μL of distilled water) was added to the mixture, the pH was again verified as 8.7, and NMR spectra were acquired.

P. putida, grown to late exponential phase, were harvested by centrifugation, washed, and resuspended in buffer (3 mL, pH 6.5) to a concentration of 10^9 cells mL^{-1}. [D_2]Formaldehyde (16 μL, 1.4 M, 20 μmol) and benzaldehyde (10 μL, 100 μmol) were added, and then the suspension was kept overnight at room temperature. The supernatant was added to glycine buffer (1 mL, 0.2 M) and water (1 mL), and the pH was adjusted to 8.8. NAD^+ (50 mg, 70 μmol) acetaldehyde (30 μL, 700 μmol) were added, and the pH was checked. A ^2H NMR spectrum was obtained. LADH (6 units in 600 μL of water) was added, and the mixture was again observed by deuterium NMR. To prove that CH_3CHDOH was derived from the benzyl alcohol and not from CD_3OH, the experiment was repeated with CD_3OH in place of benzyl alcohol.

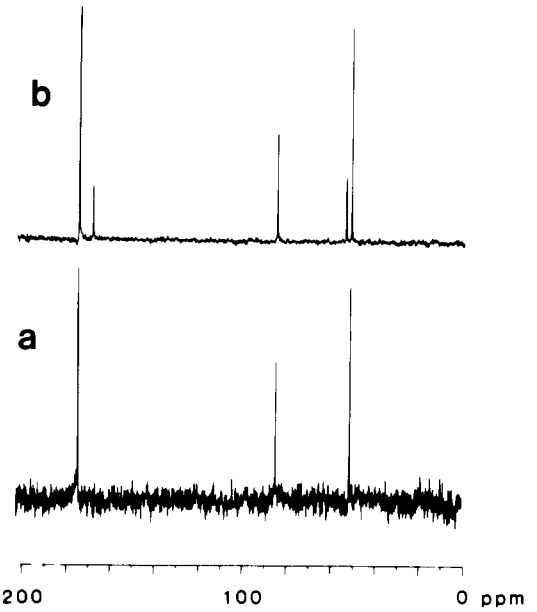

FIGURE 1: 100.6-MHz ^{13}C proton-decoupled NMR spectra of anaerobic, actively metabolizing suspensions of (a) *P. putida* and (b) *S. aureus* acquired immediately after challenge with 10 mM [^{13}C]-formaldehyde. The spectra represent the sum of 100 and 500 transients, respectively, processed with 10-Hz exponential broadening. Residual formaldehyde appears at 83.2 ppm, methanol at 50 ppm, and formate at 172 ppm.

RESULTS

Detoxification Pathways. Initial studies to delineate pathways were carried out by ^{13}C NMR. When an anaerobic suspension of *P. putida* F61a grown to late exponential phase on brain heart infusion broth and resuspended in phosphate buffer (pH 6.5) at room temperature was challenged with [^{13}C]formaldehyde (10 mM), metabolism was found to be much faster than that for *E. coli*. Only two products were formed: formate and methanol (Figure 1a). Ten millimolar [^{13}C]formaldehyde was consumed within 15 min and up to 60 mM could be completely consumed, without apparent cell death. The formate and methanol peaks grew at similar rates, consistent with the possibility of dismutase activity.

When an anaerobic suspension of *S. aureus* (NCTC 6571, Oxford strain) in buffer (pH 6.5, 37 °C) was challenged with [^{13}C]formaldehyde (10 mM), methanol and formate were again observed in roughly equal amounts, as shown in Figure 1b. Two additional peaks, which had not been observed in other organisms, grew at 165.9 and 52.8 ppm at equal rates. The new signals were a doublet and quartet, respectively, in proton-coupled ^{13}C spectra. The corresponding signals in deuterium spectra, resulting from metabolism of [D_2]formaldehyde, appeared at 8.1 and 3.8 ppm in the intensity ratio 1:3. The new product was therefore readily identified as the ester methyl formate, a conclusion supported by addition of authentic material.

In order to test whether this ester was biosynthesized from formic acid and methanol, we carried out a variety of mixed feeding experiments using formaldehyde and differently labeled methanol and formic acid. For example, feeding [D_2]formaldehyde plus [^{13}C]methanol led to ^{13}C incorporation only into the methyl group of the ester, whereas [D_2]formaldehyde plus [^{13}C]formic acid led to no ^{13}C incorporation at all. Thus both carbons of methyl formate are formaldehyde derived, one being incorporated via methanol. These conclusions were fully supported by complementary deuterium incorporation experiments and lead to the conclusion that methyl formate is actually the product of enzymic oxidation of the methanol hemiacetal of formaldehyde—see Discussion.

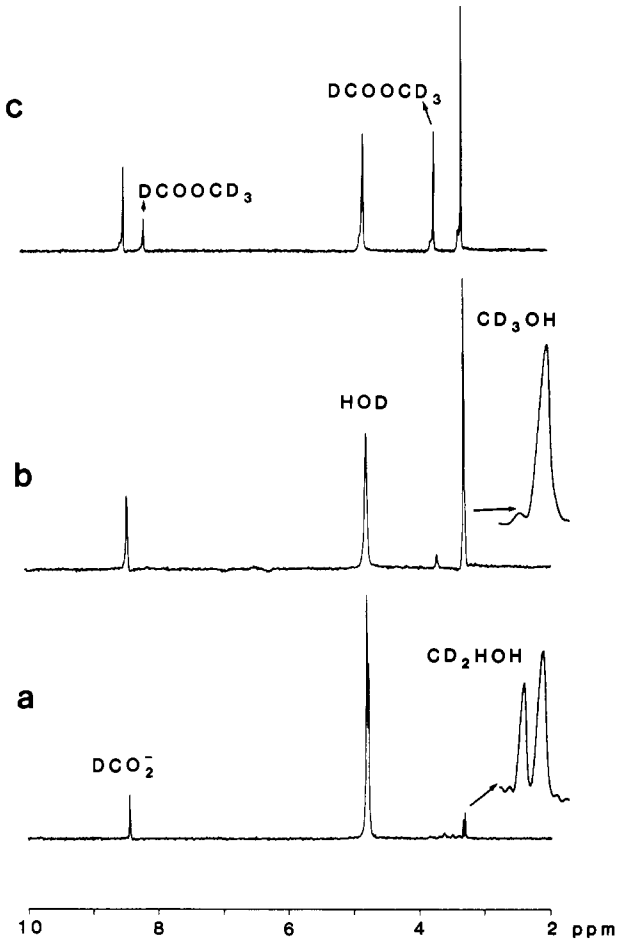

FIGURE 2: 61.4-MHz proton-coupled deuterium NMR spectra of suspensions of (a) *E. coli*, (b) *P. putida*, and (c) *S. aureus* acquired during anaerobic metabolism of 10 mM [D_2]formaldehyde. 100 transients were acquired over 5 min; Gaussian resolution enhancement was applied.

Dismutase Activity? In order to check whether our proposed method for identifying methanol isotopomers was viable, we looked first at *E. coli*, where the oxidation process is known not to involve dismutation (Hunter et al., 1984; Mason et al., 1986a). Figure 2a shows the result of challenging a suspension of *E. coli* NCIB 8797 with [D_2]formaldehyde. Product methanol (3.3 ppm) clearly occurs as an asymmetric doublet (J_{HD} = 1.7 Hz), proving that formation of CD_2HOH is the dominant process as expected. In numerous experiments over a period of years, the doublet was consistently asymmetric as we noted previously (Hunter et al., 1984). It had been suggested that the extra intensity of the upfield half of the doublet could be caused by a small amount of CD_3OH, shifted by a chemical shift isotope effect. We have now confirmed this: proton decoupling collapsed the doublet to produce a major singlet, together with a minor peak 0.02 ppm (1.2 Hz) upfield, corresponding to CD_3OH. Identification of the latter signal was proved by addition of authentic CD_3OH.

Other peaks are observed at 8.4 ppm (DCO_2^-) and 4.8 ppm. There are hints between 3 and 4 ppm of the three-carbon alcohol metabolites previously identified (Hunter et al., 1985); however, extensive proton coupling and quadrupolar relaxation produce broad, ill-defined peaks for these resonances. At room temperature and neutral pH, HOD and $CD_2(OH)_2$ are virtually coincident at 4.8 ppm. The concentration of HOD in water is about 17 mM, and this often obscures the residual [D_2]formaldehyde, but for this particular study, this was not

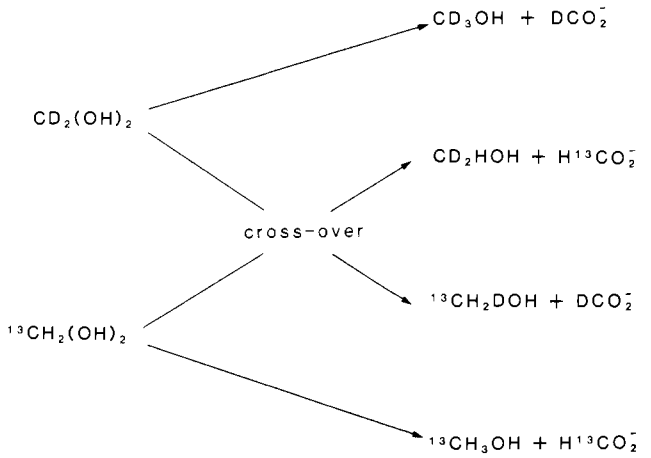

FIGURE 3: Cross-dismutation reaction scheme for measurement of k_H/k_D for hydride transfer.

a problem. These spectra are much better resolved than those in our earlier work (Hunter et al., 1984) because an improved deuterium-observe probe was available.

If a suspension of *P. putida* was challenged with 10 mM $CD_2(OH)_2$, methanol was observed as a singlet in the proton-coupled spectrum (Figure 2b). This is the result predicted for the action of a dismutase producing CD_3OH. There is just a hint of a minor downfield signal due to CD_2HOH. Similarly, when a suspension of *S. aureus* was challenged with 10 mM [D_2]formaldehyde, the product methanol was again predominantly a singlet in the proton-coupled spectrum, proving that CD_3OH was produced (Figure 2c). A small CD_2HOH peak is observed 1.2 Hz downfield. Additionally, peaks from methyl formate are seen at 8.4 and 3.6 ppm. So, in *P. putida* and *S. aureus*, CD_3OH and DCO_2^- are produced by dismutation, but the ratio of intensities is usually found to be greater than 3:1, presumably because some formate is lost by further oxidation to CO_2.

S. mutans also produce only CD_3OH and DCO_2^-, while *B. catarrhlis* gave a 1:1 mixture of CD_2HOH and CD_3OH; however, metabolism of 10 mM formaldehyde in these organisms was both slow and incomplete, and they were not further investigated.

Deuterium Isotope Effect. A simple mixed-isotope experiment using [^{13}C]formaldehyde and [D_2]formaldehyde was carried out in order to detect cross-dismutation (Figure 3). The ^{13}C label was added both to allow direct ^{13}C observation and to increase the dispersion in the deuterium spectrum by introducing C–D couplings. Crossover was indeed observed, and Figure 4 shows the result of adding a 1:2 mixture of $CD_2(OH)_2$ and $^{13}CH_2(OH)_2$ to a suspension of *S. aureus*. Three of the predicted methanol isotopomers, i.e., CD_3OH, CD_2HOH (0.02 ppm downfield), and $^{13}CDH_2OH$ (0.04 ppm downfield, J_{CD} = 22 Hz), are clearly observed by deuterium NMR (Figure 4b). The fourth isotopomer, $^{13}CH_3OH$, together with $^{13}CDH_2OH$ (0.2 ppm upfield, J_{CD} = 22 Hz) is observed in the corresponding ^{13}C NMR spectrum of the same sample (Figure 4a). A similar isotope distribution is also observed in the methyl group of methyl formate.

In experiments where equimolar [H_2]- and [D_2]formaldehyde were used to challenge *P. putida* and *S. aureus*, it was always clear from ^{13}C spectra that [$^{13}CH_3OH$] > [$^{13}CH_2DOH$] and from deuterium spectra that [CD_2HOH] > [CD_3OH] ≈ [$^{13}CH_2DOH$], indicating a significant kinetic isotope effect. Quantification of the relative amounts was achieved by a combination of cut-and-weigh and simulation and was made easier at 76.75 MHz where all the contributing

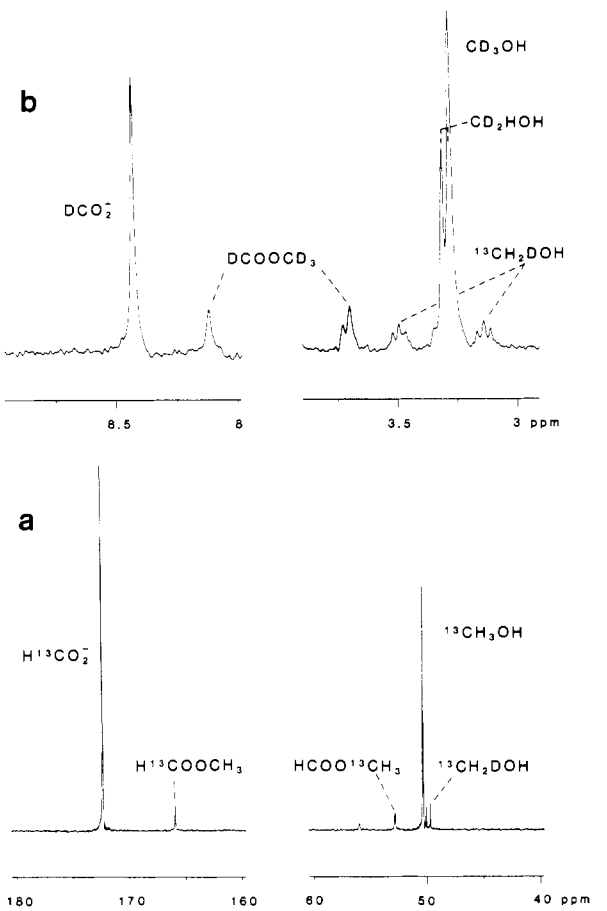

FIGURE 4: (a) 62.6-MHz ^{13}C and (b) 61.4-MHz deuterium NMR spectra demonstrating cross-dismutation of a 1:2 mixture of [D_2]- and [^{13}C]formaldehyde (total 30 mM) by *S. aureus*.

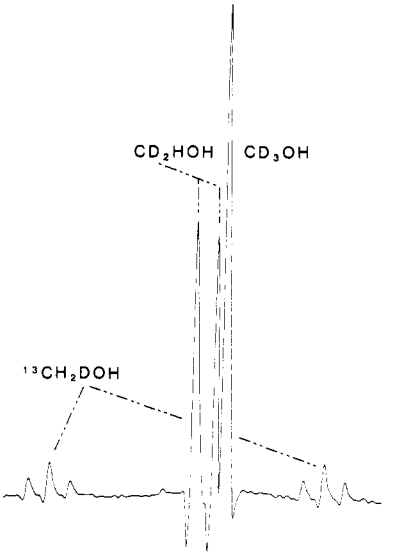

FIGURE 5: Resolution-enhanced 76.75-MHz proton-coupled deuterium NMR spectrum (1500 transients) of the supernatant resulting from challenge of *P. putida* with a mixture of [D_2]formaldehyde (10 mM) and [^{13}C]formaldehyde (20 mM).

signals are separated (Figure 5).

Comparison of experimental results with computer simulations using a range of isotope effects (see Experimental Procedures) gives an estimate of 3.7 ± 0.5 for k(H transfer)/k(D transfer).

Dismutase Substrate Specificity in Vivo. We investigated the specificity of dismutase activity in suspensions of *P. putida*

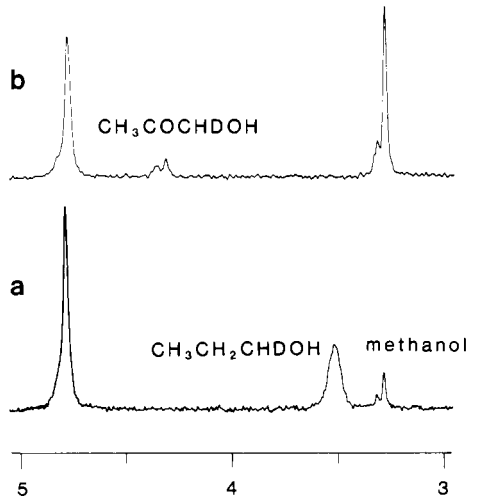

FIGURE 6: Partial 61.4-MHz deuterium NMR spectra (100 transients) of suspensions of *P. putida* challenged with a mixture of 10 mM [D_2]formaldehyde and of a second aldehyde at 20 mM: (a) propionaldehyde; (b) pyruvaldehyde.

and *S. aureus*: mixtures of an aldehyde (20 mM) and [D_2]formaldehyde (10 mM) were added to anaerobic suspensions of *P. putida* in phosphate buffer. Formate (not shown) and methanol were observed, together with, in some cases, deuteriated alcohols. These alcohols were derived from the aldehydes (RCHO), by deuteride transfer from [D_2]-formaldehyde:

$$CD_2(OH)_2 + RCHO \rightarrow DCO_2^- + RCDHOH$$

Figure 6a shows the results of propionaldehyde cross-dismutation. The chemical shift of the deuterium signal (3.5 ppm) matches that expected for propanol, the signal being broad due to unresolved proton–deuterium coupling. Moreover, addition of synthetic [1-D]propanol (produced by the reduction of propionaldehyde with $NaBD_4$) enhanced the signal. In a series of experiments, the [D_2]formaldehyde concentration was held constant at 10 mM, while the propionaldehyde concentration was varied between 10 and 40 mM. Increasing the propionaldehyde concentration led to increases in the proportion of [D]formate and [D]propanol formed, relative to methanol. Furthermore, the major methanol isotopomer formed under all conditions was D_3 rather than D_2. These results demonstrate that formaldehyde is heavily favored as the hydride "donor" and that propionaldehyde is the preferred "acceptor".

In the case of pyruvaldehyde (Figure 6b), the new product signal is an asymmetric doublet (4.3 ppm, J_{HD} = 2.7 Hz). Such a large coupling constant, corresponding to a J_{HH} of around 18 Hz, is typical of a geminal coupling adjacent to a carbonyl group, and the corresponding proton chemical shift of hydroxyacetone C_1 matches well. The alternative reduction product, [2-D]hydroxypropionaldehyde, has a chemical shift of 4.0 ppm and an expected $^3J_{HD}$ of only 1.1 Hz. Taken together, these data demonstrate the exclusive reduction of the aldo and not the keto group of pyruvaldehyde and hence the formation of [1-D]hydroxyacetone. The peak asymmetry may result from the formation of some [1,1-D_2]hydroxyacetone.

On addition of benzaldehyde and [D_2]formaldehyde to *P. putida*, a new signal gradually appeared at 4.6 ppm, due to [1-D]benzyl alcohol. The absolute stereochemistry of this transformation is described below. We have also observed cross-dismutation between [D_2]formaldehyde and *n*-butyraldehyde or acrolein, giving [1-D]butanol (3.6 ppm) and [1-D]allyl alcohol (4.6 ppm), respectively:

$$CD_2(OH)_2 + CH_3CH_2CH_2CHO \rightarrow DCO_2H + CH_3CH_2CH_2CHDOH$$

$$CD_2(OH)_2 + CH_2{=}CHCHO \rightarrow DCO_2H + CH_2{=}CHCHDOH$$

The chemical shifts match those quoted in the literature, and product identities were confirmed by adding authentic synthetic 1-D alcohols. Acetone, crotonaldehyde, trichloroacetaldehyde, D,L-glyceraldehyde, and isobutyraldehyde did not show any evidence for cross-dismutation. Similar specificity was observed for *S. aureus*.

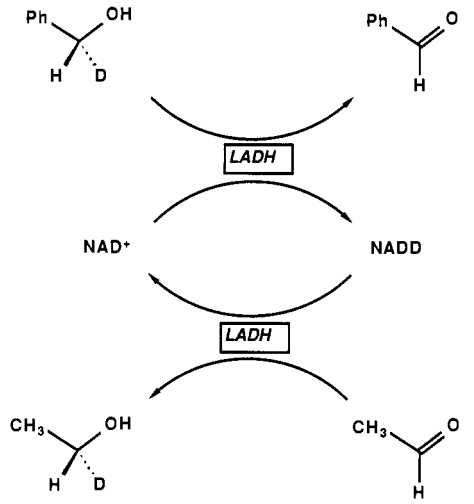

FIGURE 7: Reaction scheme for determination of the stereochemistry of [1-D]benzyl alcohol.

Simultaneous challenge of *P putida* or *S. aureus* with a mixture of ethanol and [D_2]formaldehyde led to a mixed oxidoreduction process, a major product being [1-D]ethanol (3.6 ppm, quintet, $^2J_{HD}$ = 1.4 Hz, $^3J_{HD}$ = 0.7 Hz). There was no evidence in either organism for the formation of ethyl formate, a product which might have been expected by analogy with the mixed methanol/formaldehyde results described above.

Stereochemistry. We wished to determine the stereochemistry of benzyl alcohol produced by dismutation of benzaldehyde and formaldehyde, partly to prove that the reaction is indeed a biological process. Our approach involved a novel use of deuterium NMR to determine the fate of deuterium in the acetaldehyde-linked LADH/NAD^+-catalyzed oxidation of benzyl alcohol. This process, which is summarized in Figure 7, has a known stereochemistry and has been used to prepare deuteriated alcohols of defined stereochemistry (Battersby et al., 1975). Thus, deuterium from (*R*)-[1-D]benzyl alcohol ultimately appears in [1-D]ethanol via deuteriated NADH (NADD); by contrast, the destination for deuterium from (*S*)-[1-D]benzyl alcohol is benzaldehyde, PhCDO. A simple deuterium NMR spectrum of the reaction mixture should allow assignment of stereochemistry without isolation of any of the components or products.

We first confirmed the feasibility of this approach with racemic, i.e., chemically produced, [1-D]benzyl alcohol. The deuterium NMR spectrum of an aqueous solution of NAD^+, acetaldehyde, and [D]benzyl alcohol (Figure 8a) is dominated by HOD at 4.8 ppm, but also has a clear signal due to the alcohol at 4.6 ppm. Addition of LADH gives, as expected, both [D]ethanol and [D]benzaldehyde (Figure 8b). Repetition of this experiment with a crude supernatant from *P. putida* shows (Figure 9) that the deuterium is delivered exclusively to ethanol (3.7 ppm). The arrow shows the expected position

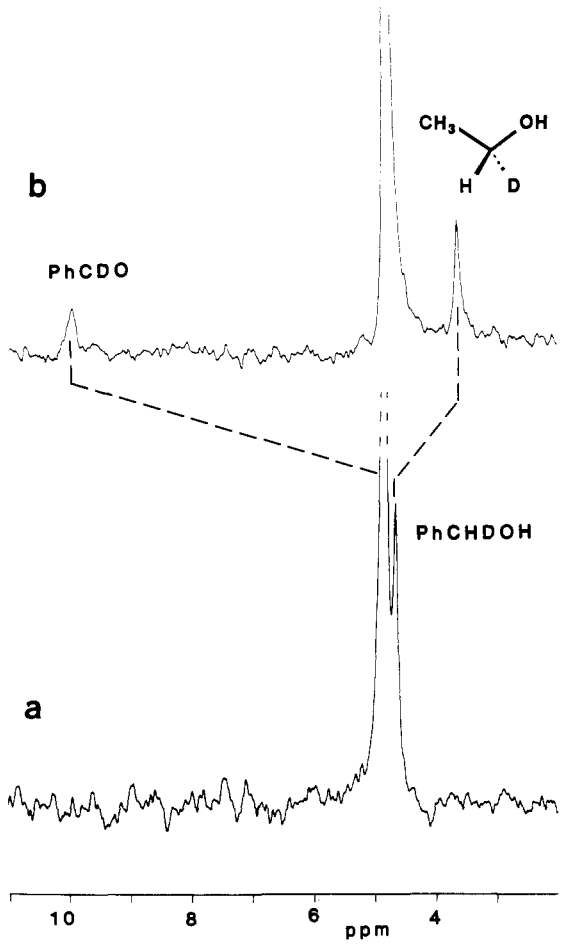

FIGURE 8: (a) 61.4-MHz deuterium NMR spectrum (400 transients) of an aqueous mixture of NAD^+, *rac*-[1-D]benzyl alcohol, and acetaldehyde. (b) The same after addition of LADH and incubation.

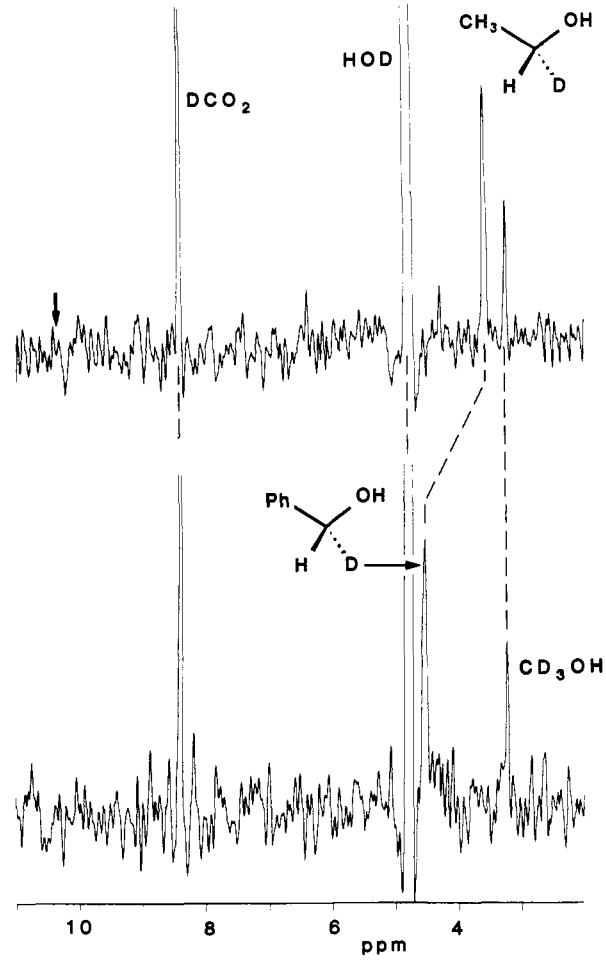

FIGURE 9: Determination of the stereochemistry of [1-D]benzyl alcohol produced by *P. putida* from [D_2]formaldehyde and benzaldehyde. (a) Deuterium spectrum (100 transients) of the supernatant. (b) The same solution after incubation with LADH and acetaldehyde. Arrow marks the expected position of PhCDO.

of the PhCDO signal; no such signal is observed, so the biologically produced benzyl alcohol is *R*.

In an analogous experiment, the deuterium in propanol produced biosynthetically from propionaldehyde and [D_2]-formaldehyde was also efficiently transferred to ethanol by the LADH system.

Discussion

This work has opened up several new areas of investigation. We have shown that deuterium NMR can be used to follow the fate of hydrogen in metabolic processes, to distinguish between different classes of enzyme and measure enzymic kinetic isotope effects in whole cells,[5] and to determine the absolute stereochemistry of metabolic products that are chiral only by virtue of their isotopic labeling. We discuss first the power and limitations of the NMR approach used in this work, placing particular emphasis on the deuterium aspects; second, we discuss the specific results on formaldehyde dismutase.

The main factors that make NMR an attractive tool for metabolic work are that it allows monitoring of live cells in real time and that it does not require prior knowledge—or prejudice—of the processes taking place: all the major small-molecule products will be detected in the experiment, whether we expect them or not. It is therefore relatively easy to detect novel biochemistry, as we have shown here and earlier (Hunter et al., 1985). In many cases these positive features are offset by the low inherent sensitivity of NMR. As a rough guide, products must accumulate to 10^{-4}–10^{-3} M if detection is to be rapid. If we consider the use of deuterium NMR as a method for monitoring metabolism of labeled exogenous materials, then the attractions are several:

(i) The low natural abundance of deuterium allows very high levels of enrichment and negligible background other than the 17 mM HOD in solvent water; this attraction is shared by tritium NMR. Proton NMR monitoring of unlabeled exogenous material is relatively easy in body fluids (Nicholson et al., 1985) but often more difficult in whole cells where the background signals may be intrusive or overwhelming. ^{13}C-labeling allows one to follow carbon signals directly or to observe selectively those protons coupled to the label (Cavanagh et al., 1988; Doddrell et al., 1984), but these approaches follow the fate of the carbon rather than the proton.

(ii) There is no complication from using highly radioactive samples within the NMR spectrometer, unlike the corresponding tritium experiment.

(iii) Deuterium generally relaxes rapidly, making it relatively easy to obtain reliable intensities.

Further attractions become obvious if one is interested in more subtle aspects of the biochemistry than merely monitoring the time course of a process:

(iv) Deuterium in this context is a bulk label rather than a tracer, unlike tritium. The importance of this distinction is apparent in the present work: we were able to characterize the operation of a dismutase in whole cells by virtue of 100% labeling and the resulting absence of coupling to protons. We

[5] We have also used ^{13}C NMR spectroscopy to measure an enzymic kinetic isotope effect in vivo (Mason et al., 1987a,b).

are also able to estimate kinetic isotope effects. The corresponding experiments with tritium would require isolation of the products, radiocounting, and uncertainties about how isotope effects might affect interpretation of the results.

(v) We have been able, by the use of the acetaldehyde-linked LADH assay, to determine the absolute stereochemistry of a metabolic product of the dismutase. This novel approach to measuring a well-established cyclic assay process does not require isolation of product and would not need high-field equipment. In both respects it appears much superior to the superficially more direct proton NMR assay of isolated product NADH (Barrow et al., 1986). Our method would also be appropriate with tritium.

Deuterium NMR is, however, no panacea for metabolic studies and suffers from several major problems by comparison with proton and tritium NMR: first, the low magnetogyric ratio ($\gamma_H:\gamma_D:\gamma_T$ = 1:0.15:1.07) gives deuterium a low sensitivity,[6] small effective chemical shift range, and proton–couplings that rarely exceed 2 Hz; second, the quadrupole moment associated with deuterium leads to severe line broadening for all signals except those in small molecules or mobile chains of larger molecules (Sanders & Hunter, 1987b). These properties of the nucleus will mean, as London et al. (1987) point out, that selection of appropriate metabolic processes for study will be critical to the success of this approach, although the likely advent of higher field magnets operating at 15–22 T, corresponding to ^1H frequencies of 600–1000 MHz and deuterium frequencies of 90–150 MHz, should help the dispersion and sensitivity problems greatly.

Another potential disadvantages of deuterium is that it is usually used as the source of the field/frequency lock for the spectrometer. In this work we generally worked with the spectrometer unlocked. For short-term accumulations this generally creates no problem because the drift rate *in hertz* is only 0.15 as great as that for protons, so any frequency shifts are well within the line width. On occasion, some files from a kinetic run have to be discarded because of a sudden frequency jump, but in our experience this is preferable to the loss of resolution that usually results from use of an internal capillary containing fluorinated material for an ^{19}F lock.

The very high resolution obtained in—and crucial to—this work is a result of two factors. The first is that methanol is a very small molecule, tumbling rapidly and therefore allowing deuterium to relax relatively slowly. The second is that all the products we have observed are extracellular, having been secreted into the medium.

One final point concerning the use of deuterium or tritium labeling applies to any method of detection via isotopes. The operation of large isotope effects on one important branch of a multiple-choice pathway can lead to "metabolic switching" into the branches that do not experience large effects. This effect, which could well be misleading, has been reported in several systems (Baldwin et al., 1987; Gordon et al., 1987; Lu et al., 1984).

Turning to the formaldehyde dismutases themselves, the question arises as to whether the in vivo properties implied by the NMR results correspond to the properties of the isolated enzyme (Kato et al., 1983). In this example at least, the agreement is good. We find essentially the same substrate specificity in the cross-dismutation experiments, indicating that transport of the various substrates into the cell is not rate limiting. This will not necessarily be the case for all such studies with other enzymes.

[6] Sensitivity is proportional to γ^3 (Sanders & Hunter, 1987a).

Combining our results with those of Kato et al. (1986), it is clear that the mode of action of *P. putida* formaldehyde dismutase can be summarized as follows, the NAD^+/NADH remaining bound to the same enzyme molecule throughout:

$$\text{donor-C}\mathit{H}(\text{OH})_2 + \text{NAD}^+ \rightarrow \text{donor-CO}_2\text{H} + \text{NAD}\mathit{H} + \text{H}^+$$

$$\text{acceptor-CHO} + \text{NAD}\mathit{H} + \text{H}^+ \rightarrow \text{acceptor-CH}\mathit{H}\text{OH} + \text{NAD}^+$$

The "donor" aldehyde is written in the hydrate form, but may well be in the form of enzyme-bound hemiacetal or thiohemiacetal. In the case of cross-dismutation between formaldehyde and other aldehydes, it is clear from our results that formaldehyde is heavily favored as the hydride "donor" and that the longer chain aldehyde is the preferred "acceptor". There is an obvious correlation here with the degree of hydration, as might be expected from the mechanism above, but whether this is a causal relationship is not clear (Abeles & Lee, 1960). Our observed kinetic isotope effect of around 4 is in the range expected for transfer of H to NAD (Cook & Cleland, 1981). The *R* stereochemistry of benzyl alcohol and propanol produced by the dismutase is identical with that of LADH, so the biosynthetically introduced deuterium is fully removed and transferred to ethanol.

The similarity between our *P. putida* and *S. aureus* results is intriguing, implying the presence of a hitherto unknown dismutase in the latter organism. It is, of course, possible that the dismutase activity we detect in both organisms belongs to a previously known dehydrogenase; dismutation is a known reaction, for example, of liver alcohol dehydrogenase under certain conditions (Battersby et al., 1975; Gupta, 1970). However, the unusual properties of the isolated *P. putida* dismutase, particularly the strongly bound NAD/NADH cofactor, would appear to make this unlikely. The natural function of these dismutases is not clear, but our results do indicate strongly that this particular strain of *P. putida* owes its unusual formaldehyde resistance to the very efficient detoxification achieved by the dismutase.

Our labeling results for methyl formate in *S. aureus* are consistent with, but do not prove, a mechanism in which methyl formate is actually the product of enzymic oxidation of the methanol hemiacetal of formaldehyde:

$$\text{CH}_2\text{O} + \text{CH}_3\text{OH} \rightarrow \text{CH}_3\text{OCH}_2\text{OH} \xrightarrow{[\text{O}]} \text{CH}_3\text{OCHO}$$

The isotope distribution within the methyl group of product methyl formate shows that the methanol is itself the product of dismutation rather than of an independent reductase. We have been unable to detect the hemiacetal in aqueous solution by mixing methanol and formaldehyde at the millimolar levels used for the metabolic experiments, but it is readily observed at higher concentrations, the methyl and methylene groups resonating at 56 and 87 ppm, respectively. This mechanism for methyl formate production was proposed some time ago for horse liver alcohol dehydrogenase (Abeles & Lee, 1960; Gupta, 1970).

The metabolic production of both methanol and formate from formaldehyde is remarkably widespread: we have observed it in most of the bacterial species we have studied and in a range of plant tissues such as potato (Mason et al., 1986b) and germinating cress seeds (unpublished).[7] It may be that

[7] The only exceptions in our experience are methylotrophic organisms utilizing methanol as sole carbon source (Cornish et al., 1984) and mammalian cells (unpublished), both of which employ exclusively oxidative pathways.

this balance of oxidation and reduction ensures a constant $NAD^+/NADH$ ratio. The extent to which the simultaneous oxidation and reduction pathways operate via apparent dismutation ranges from effectively 100% in *P. putida* and *S. aureus* to 30% in potato (Mason et al., 1986b) to less than 10% in *E. coli*. In the case of *E. coli*, the small amount of CD_3OH produced may well result from labeling the NAD pool rather than from a specific dismutase; indeed, if dismutase activity could be ruled out, this approach would provide a method for estimating the size of the NAD pool in the living cell.

The results presented here indicate that high-resolution deuterium NMR spectroscopy has great potential as a tool for exploring enzymology in vivo. The experimental requirements are largely trivial,[8] and the results are straightforward to interpret. In favorable cases, such as those we have discussed here, deuterium NMR provides a uniquely powerful yet subtle probe of enzyme pathways, kinetics, mechanisms, and stereochemistry.

ACKNOWLEDGMENTS

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Registry No. $[D_2]$-CH_2O, 1664-98-8; D_2, 7782-39-0; formaldehyde dismutase, 85204-94-0.

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[8] Apart from a suitable spectrometer!