Papadimitriov, J. M. (1973) *J. Pathol. 110*, 213.
Petty, H. R. (1985) *Mol. Immunol.* (in press).
Petty, H. R., Hafeman, D. G., & McConnell, H. M. (1980a) *J. Immunol. 125*, 2391.
Petty, H. R., Smith, L. M., Fearon, D. T., & McConnell, H. M. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6587.
Petty, H. R., Dereski, W., Francis, J., Fox, B., & Boxer, L. A. (1985) *Clin. Res.* (in press).
Pratt, D. S., Katilus, J., Dreisin, R. B., Robinson, H. M., & Schwarz, M. I. (1979) *J. Immunol. Methods 27*, 127.
Raschke, W. C., Baird, S., Ralph, P., & Nakoinz, I. (1978) *Cell (Cambridge, Mass.) 15*, 261.
Rifkind, R. A., Hsu, K. C., & Morgan, C. (1964) *J. Histochem. Cytochem. 12*, 131.
Sonada, S., Shigematsu, T., & Schlamowitz, M. (1973) *J. Immunol. 110*, 1682.
Spurr, A. R. (1969) *J. Ultrastruct. Res. 26*, 31.
Stahmann, M. A., Spencer, A. K., & Honold, G. R. (1977) *Biopolymers 16*, 1307.
Sundstrom, C., & Nilsson, K. (1976) *Int. J. Cancer 17*, 565.
Ternynck, T., & Avrameas, S. (1976) *Ann. Immunol. (Paris) 127C*, 197.
Thorell, J. I., & Larson, S. M. (1978) *Radioimmunoassay and Related Techniques*, Chapter 3, Mosby, St. Louis, MO.
Thrasher, S. G., Bigazzi, P. E., Yoshida, T., & Cohens, S. (1975) *J. Immunol. 114*, 762.
Titus, J. A., Sharrow, S. O., Connolly, J. M., & Segal, D. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 519.
Willingham, M. C., & Pastan, I. (1978) *Cell (Cambridge, Mass.) 13*, 501.
Wong, L., & Wilson, J. D. (1975) *J. Immunol. Methods 7*, 69.
Young, J. D. E., Unkeless, J. C., Kaback, H. R., & Cohn, Z. A. (1983a) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1636.
Young, J. D. E., Unkeless, J. C., Kaback, H. R., & Cohn, Z. A. (1983b) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1357.
Zuckerman, S. H., & Douglas, S. D. (1979a) *CRC Crit. Rev. Microbiol. 7*, 1.
Zuckerman, S. H., & Douglas, S. D. (1979b) *Annu. Rev. Microbiol. 33*, 267.

Formaldehyde Metabolism by *Escherichia coli*. Carbon and Solvent Deuterium Incorporation into Glycerol, 1,2-Propanediol, and 1,3-Propanediol†

Brian K. Hunter
Department of Chemistry, Queen's University, Kingston, Ontario, Canada
Kathryn M. Nicholls and Jeremy K. M. Sanders*
University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, United Kingdom
Received November 15, 1984

ABSTRACT: *Escherichia coli* were grown on 14.3% uniformly ^{13}C-labeled glucose as the sole carbon source and challenged anaerobically with 90% ^{13}C-labeled formaldehyde. The major multiply labeled metabolites were identified by ^{13}C NMR spectroscopy to be glycerol and 1,2-propanediol, and a minor metabolite was shown to be 1,3-propanediol. In each case, formaldehyde is incorporated only into the C_1 position. A novel form of ^{13}C NMR isotope dilution analysis of the major products reveals that all the 1,2-diol C_1 is formaldehyde derived but that about 40% of the glycerol C_1 is derived from bacterial sources. Glycerokinase converted the metabolite [1-^{13}C]glycerol to equal amounts of [3-^{13}C]glycerol 3-phosphate and [1-^{13}C]glycerol 3-phosphate, demonstrating that the metabolite is racemic. When [^{13}C]formaldehyde incubation was carried out in H_2O/D_2O mixtures, deuterium incorporation was detected by β- and γ-isotope shifts. The 1,3-diol is deuterium labeled only at C_2 and only once, while the 1,2-diol and glycerol are each labeled independently at both C_2 and C_3; C_3 is multiply labeled. Deuterium incorporation levels are different for each metabolite, indicating that the biosynthetic pathways probably diverge early.

Formaldehyde is a well-known antibacterial agent. However, we have recently shown that *Escherichia coli* can detoxify low concentrations of formaldehyde by metabolism to relatively innocuous products (Doddrell et al., 1984; Hunter et al., 1984). Using in vivo NMR[1] spectroscopy, we demonstrated the formation of formate, methanol, and three unidentified products; we now report that these unknowns are glycerol (1,2,3-propanetriol), 1,2-propanediol, and 1,3-propanediol. We present isotope labeling evidence concerning both the natural pool size of these products and the mechanism of their biosynthesis.

When *E. coli* suspensions are challenged with a 10 mM aqueous solution of ^{13}C-labeled formaldehyde, the resulting metabolism can be observed by ^{13}C NMR (Hunter et al., 1984) and by ^1H NMR (Doddrell et al., 1984). Further experiments have established that the previously described metabolism is mainly anaerobic unless air is actively bubbled through the suspension in the spectrometer (B. K. Hunter, unpublished results). The two major unidentified species give ^{13}C signals from the labeled carbon at 64 and 68 ppm; a minor species of variable intensity appears at 60 ppm. In proton-

† This work was supported by funds from the U.K. Science and Engineering Research Council (to K.M.N. and J.K.M.S.) and the Hickinbottom Bequest of the Royal Society of Chemistry (to J.K.M.S.).

[1] Abbreviations: ATP, adenosine 5'-triphosphate; d, doublet; NADH, reduced nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; ppm, parts per million; s, singlet.

0006-2960/85/0424-4148$01.50/0

coupled spectra, all three signals appear as triplets, J_{CH} ca. 140 Hz, demonstrating that the labeled carbon is present as a CH_2 group. A two-dimensional ^{13}C–^{1}H correlation experiment showed that the chemical shifts of those protons attached to the labeled carbon were in the region 3.3–3.7 ppm; each of the protons attached to the 64 and 68 ppm carbons is nonequivalent, implying the presence of a nearby chiral or prochiral center. The signals arising from the minor product were too weak for detailed analysis. Taken together with other experiments, the evidence suggested that the unknowns incorporate labeled formaldehyde in the form *C–$^{13}CH_2OH$, where *C denotes a chiral or prochiral center in the two major products. None of the metabolites is serine or any other obvious product of the tetrahydrofolate pathway. Essentially all of the metabolite signals visible in our previously reported in vivo spectra are due to secreted or diffusable molecules in the extracellular medium. The bacterial volume in these suspensions is too small to allow rapid detection of intracellular materials.

The following problems were addressed in the work described below: (1) what are the structures of these unknowns and (2) are they compounds that are produced naturally by unchallenged bacteria or are they only formed from formaldehyde? In order to answer these questions, we grew *E. coli* on glucose that was uniformly labeled with 14.3% ^{13}C. By challenging these bacteria with 90% [^{13}C]formaldehyde we hoped to generate multiply labeled metabolites. From the ^{13}C–^{13}C couplings we expected to locate the chemical shifts of those carbons *C adjacent to the formaldehyde-derived carbons, giving information on their chemical nature; from the coupling pattern of *C we expected to measure the ^{13}C enrichment at the CH_2OH group and hence determine, by this form of isotope dilution analysis, the proportion of metabolite derived from formaldehyde. Both of these aims were successfully achieved, and as a bonus, unexpected deuterium incorporation from the solvent used in the NMR experiments has provided evidence for divergent pathways in the biosynthesis of these metabolites from formaldehyde.

Experimental Procedures

Paraformaldehyde (91% ^{13}C) was obtained from Prochem and Merck Sharp & Dohme. ^{13}C-Labeled formaldehyde solutions were prepared either by heating the appropriate amount of paraformaldehyde in water at 115 °C in an autoclave for 30 min to give a solution that was 50 mM in formaldehyde or by heating the components in a sealed tube at 120 °C to yield a 200 mM solution. All solutions were assayed by using the sodium sulfite method (Walker, 1964). Uniformly enriched D-glucose (14.3 atom % ^{13}C) was from Merck Sharp & Dohme.

Glycerokinase (ATP:glycerol 3-phosphotransferase; EC 2.7.1.30) from *Candida mycoderma* was supplied by Sigma as a suspension containing 2 mg/mL protein and 90 units of enzyme activity/mg of protein. Other chemicals were supplied by Sigma, Aldrich, BDH, and Fisons and were analytical reagent grade.

^{13}C spectra were obtained at 100.6 MHz with a Bruker WH400 spectrometer. Spectra of bacterial cultures were acquired in 10 mm diameter tubes, with continuous, low-level, broad-band proton decoupling and 2-s relaxation delays as described previously (Hunter et al., 1984). Spectra of supernatants were generally acquired in 5-mm tubes by using 0.5 W of WALTZ decoupling (Shaka et al., 1983) and 45° (6-μs) pulses.

E. coli strain MU 352 from the Manchester University Collection of bacteria was supplied by Dr. M. Allwood, Addenbrookes Hospital, Cambridge, U.K. Tryptone soya agar was prepared from 15 g/L Oxoid agar no. 1 and 30 g/L Oxoid tryptone soya broth (TSB) in an appropriate volume of distilled deionized water. In most cases the bacteria were grown on Spizizen (1958) salts medium in which 0.2% ammonium sulfate was replaced by 0.1% ammonium chloride. This medium contains 0.5% w/v glucose; on occasion, the glucose concentration was reduced to 0.2%.

Bacteria were generally prepared for spectroscopy and formaldehyde challenge as described previously (Hunter et al., 1984), the standard solvent being 4:1 H_2O/D_2O. However, when cultures were grown on ^{13}C-enriched glucose, the procedure was slightly different. A 25-mL "starter" inoculum was added to 200 mL of salts and labeled glucose. Sodium citrate was omitted from the medium, but all other salts were present in their usual concentrations, as was glucose. The cells grew to stationary phase in about 8 h. They were harvested and washed as usual. The ^{13}C NMR spectrum at 100.6 MHz of the whole cells in 20% D_2O was recorded. [^{13}C]Formaldehyde was added to a concentration of 10 mM, and the metabolism was followed. When most of this first dose had been consumed, a second dose of the same size was added. When formaldehyde metabolism had almost stopped, the sample was centrifuged and the supernatant was concentrated by partial lyophilization. A ^{13}C spectrum of this concentrated supernatant was obtained, 66 500 transients being collected in 32K data points with a spectral width of 8065 Hz, the acquisition time being 2.03 s. Gaussian resolution enhancement and zero filling were used.

For the glycerokinase experiment, bacteria were prepared as above, [^{13}C]formaldehyde was added to the cell suspension to a concentration of 10 mM, and the mixture was allowed to stand for several hours before a second 10 mM dose was supplied and the suspension left to stand overnight. The sample was centrifuged and the supernatant solution lyophilized. The resulting solid was redissolved in 0.6 mL of 50 mM carbonate–bicarbonate buffer, pH 9.8, and 0.4 mL of D_2O. To half of this solution was added 3 mg (ca. 5 μmol) of NaATP. The ^{13}C NMR spectrum was recorded and 20 μL (i.e., 3.6 units) of enzyme added. Reaction occurred almost immediately. A total of 512 transients were recorded in 16K data points with a spectral width of 2016 Hz, the acquisition time being 4.1 s. Spectra were processed with a line broadening of 1 Hz.

For deuterium incorporation studies, bacteria were grown and harvested as usual. During washing, the final centrifugation was arranged so that bacteria were collected in three equally sized portions. These were then resuspended, each in 2 mL of an appropriate D_2O/H_2O mixture. Formaldehyde solution (200 mM) in H_2O was added to each sample to a concentration of 10 mM. The calculated deuterium content of these suspensions was adjusted for the formaldehyde addition. The mixtures were allowed to stand for 4 h. After this time a second formaldehyde dose of the same size as the first was added, and the suspension was left overnight. The suspensions were then centrifuged (in an Eppendorf microfuge) and the supernatants lyophilized. Each sample was redissolved in 0.5 mL of D_2O and filtered through a cotton plug before examination by NMR. Spectral widths of 4032 Hz were recorded in 16K data points, giving an acquisition time of 2.05 s. Spectra were usually processed with Gaussian resolution enhancement.

Results

Culture Conditions. In a prior control experiment designed to optimize the use of labeled glucose, bacterial cultures are

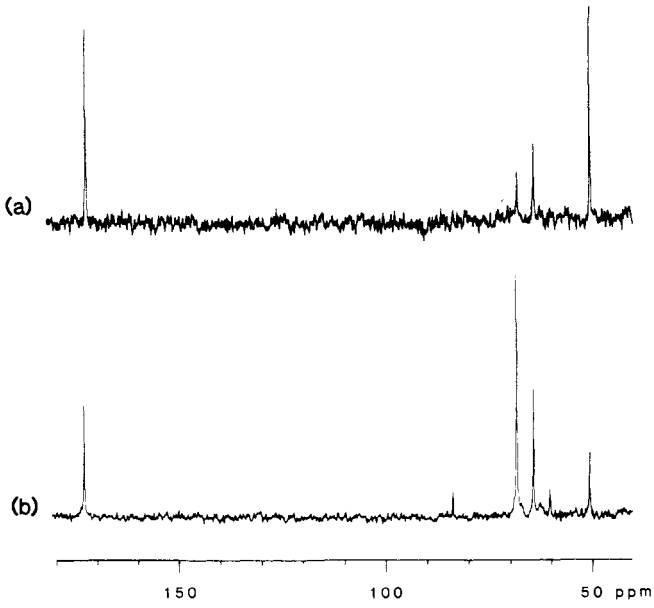

FIGURE 1: Influence of glucose concentration in the growth medium on the pattern of formaldehyde metabolism: 100.6-MHz ^{13}C NMR spectra of *E. coli* cultures that have been grown on either (a) 0.2% glucose or (b) 0.5% glucose then challenged with 10 mM [^{13}C]-formaldehyde.

grown on various concentrations of unlabeled glucose and then tested for formaldehyde-metabolism activity. Bacteria grown in low concentrations (0.2%) produced mainly methanol and formate (Figure 1a), but those grown on "normal" concentrations (0.5%) produced the unknowns more efficiently (Figure 1b). Detailed investigation showed that variations in growth medium and harvesting time lead to quantitative differences in the material flux along different detoxifying pathways but not to substantial qualitative variations (unpublished observations). For the work described here, labeled glucose was used at normal concentrations to produce 14.3% uniformly labeled bacteria. NMR analysis of the supernatant from the culture growth revealed that (a) all the labeled glucose had been consumed and (b) there was formate present, presumably formed via pyruvate in the usual way (Knappe et al., 1974).

Structure Determination. The anaerobic metabolism of 90% labeled formaldehyde by these labeled bacteria in H_2O/D_2O (4:1) was followed by NMR in the usual way. As expected, the background from bacterial signals was severe. After some hours, the suspension was centrifuged, and the supernatant containing the metabolites was concentrated by partial lyophilization. The ^1H NMR spectrum of this solution revealed the presence of formate that was labeled with ca. 90% ^{13}C, as judged by the relative intensities of the central singlet and ^{13}C satellites. This formate is, therefore, specifically the product of formaldehyde metabolism. The proton spectrum around, and to high field of, the water signal was too complex to interpret.

The ^{13}C NMR spectrum of the same solution showed that all of the methanol, and some of the product giving rise to the 68 ppm signal, had been lost in lyophilization. In addition to intense signals from formaldehyde, formate, and the two resonances at 64 and 68 ppm, there were numerous weak signals, including that at 60 ppm due to the minor metabolite. The important signals are shown in Figure 2 together with their eventual structural assignments. The 64 and 68 ppm signals showed the expected ^{13}C satellites, with an intensity corresponding to ca. 13% of the total signal and J values of 41.2 and 40.8 Hz, respectively, but they also show several additional, unexpected signals (see Figure 2). We show below that these arise from β- and γ-shifts due to incorporation of small amounts of deuterium in the solvent,[2] together with a further minor metabolite X. Inspection of the remainder of the spectrum revealed corresponding carbons at 73.5 (coupled to 64 ppm) and 69.3 ppm (coupled to 68 ppm). These are illustrated in Figure 3. The 69.3 ppm signal also shows splittings due to β-shifts, and the intensities of its ^{13}C satellites are distorted as a result of strong coupling to the satellites at 68 ppm.

It rapidly became apparent from consideration of these shifts and coupling constants that the major metabolites were glycerol (64 and 73.5 ppm) and 1,2-propanediol (68 and 69.3 ppm); the metabolite giving the 60 ppm signal was too dilute to reveal a C_2 signal, but given the above products, 1,3-propanediol seemed a reasonable guess. All three structures were supported by ^{13}C NMR spectra of authentic samples[3] and further corroborated (Figure 4) by comparing authentic ^1H chemical shifts with those derived from our earlier two-dimensional experiment (Hunter et al., 1984). The relationship of the different isotope patterns displayed by the C_2 carbons (Figure 3) to natural pool size is discussed below.

Identification of glycerol and definitive assignment of its C_1 signal at 63.9 ppm were confirmed by conversion in the NMR tube to glycerol 3-phosphate using glycerokinase and ATP. A supernatant was prepared by challenging *E. coli* in H_2O with [^{13}C]formaldehyde; ATP was added, and the pH was adjusted to 9.8. The resulting solution gave the spectrum shown in Figure 5a. Addition of glycerokinase gave rapid and complete conversion of glycerol to its 3-phosphate but no other visible change (Figure 5b). ^{13}C signals for both C_1 (63.7 ppm, s) and C_3 (66.3 ppm, d, $J_{CP} \approx 5$ Hz) of glycerol phosphate are present to approximately equal extents. As glycerokinase is known to phosphorylate stereospecifically the *pro-R* branch of glycerol (Alworth, 1972; Karnovsky et al., 1957), this experiment demonstrates a lack of any substantial stereoselectivity in the biosynthesis of glycerol from formaldehyde (see Figure 6). This experiment also reveals, 0.04 ppm downfield from glycerol, a minor signal that arises from another compound, X (see Figure 5b).

Deuterium Incorporation. It seemed likely that the addition lines seen in the C_1 signals (Figure 2) arose from deuterated molecules, the shifts from the main peaks being consistent with β-isotope incorporation.[2] This conclusion was confirmed by challenging *E. coli* that had been grown on normal glucose with [^{13}C]formaldehyde in water containing deuterium oxide at various concentrations. Figure 7 shows the C_1 region of each of the three carbon metabolites biosynthesized in solvent containing 0, 45, and 60 atom % deuterium. Changing the solvent deuterium level postbiosynthetically had no effect on the observed deuterium incorporations.

The 1,3-diol shows a pattern that is the simplest to interpret, showing only two lines when derived from partially deuterated solvent; these are due to undeuterated and mono-β-deuterated species. The β-shift is 0.057 ppm, and the level of incorporation is approximately equal to the deuterium level of the solvent. There is no evidence, either in the spectra shown or in others that have been obtained on several different occasions, for incorporation either of a second β-deuterium or of any

[2] Deuterium substitution on an adjacent carbon, i.e., $^{13}C-C-D$, gives an upfield "β-shift" of 0.04–0.1 ppm (Abell & Staunton, 1981). Substitution one carbon further away ($^{13}C-C-C-D$) leads to a γ-shift that is somewhat smaller.

[3] Small quantities of authentic diol or glycerol were added to a supernatant solution. The C_1 signals of authentic material and metabolite were coincident.

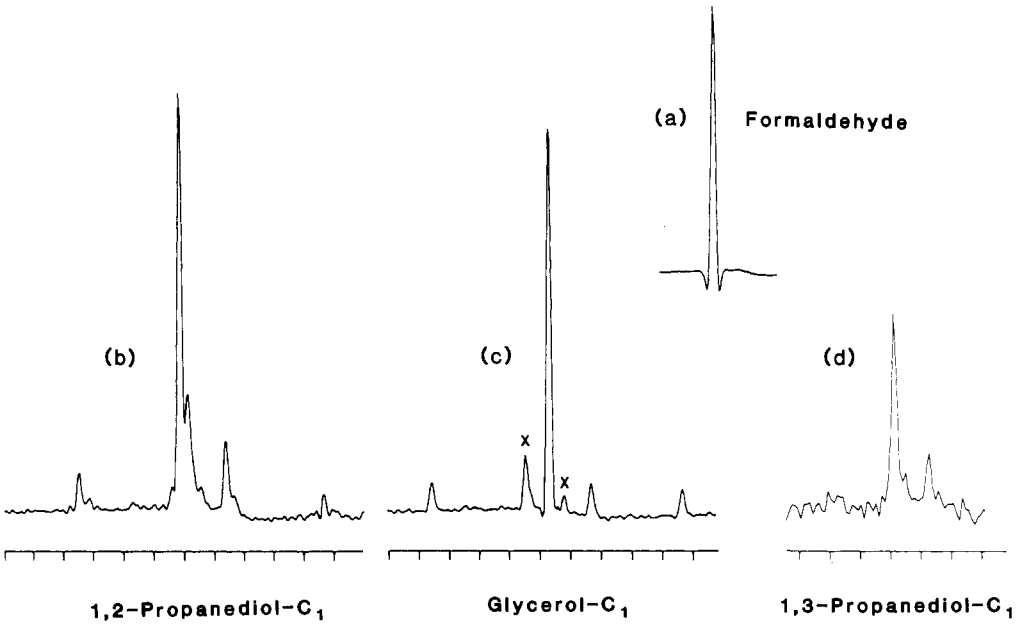

FIGURE 2: Selected formaldehyde-derived carbon signals (100.6 MHz) in supernatant obtained after challenging 14.3% ^{13}C-labeled *E. coli* in H_2O/D_2O (4:1) with 90% [^{13}C]formaldehyde: (a) residual formaldehyde, (b) 68 ppm signal, (c) 64 ppm signal, and (d) 60 ppm signal. In (c), X marks an additional, as yet unidentified, component. Scale markers are 5 Hz each. This spectrum required a 40-h acquisition (see Experimental Procedures).

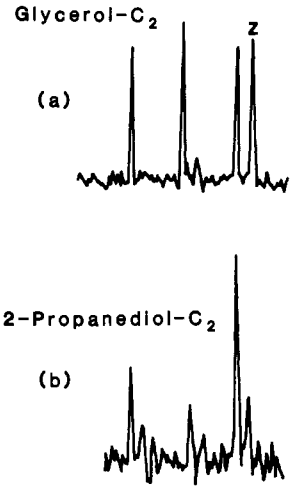

FIGURE 3: C_2 signals from the same spectrum as Figure 2: (a) multiplet at 73.5 ppm, due to glycerol C_2; (b) multiplet at 69.3 ppm, due to 1,2-propanediol C_2. Z arises from another species.

γ-deuterium. Approximately 0.2 ppm to high field of the 1,3-diol resonance is another minor unknown, Y, which shows essentially complete incorporation of a single β-deuterium. Y is observed only very occasionally, in low abundance; we have no further information on it.

The 1,2-diol clearly shows the presence of both β-shifted peaks ($\Delta\delta = 0.076$) and an ill-defined hump characteristic of multiple γ-shifted peaks ($\Delta\delta = 0.015$ per deuterium). The deuterium incorporation is only around 70% of that in the 1,3-diol or in the solvent. The simultaneous presence of unshifted, β-shifted, γ-shifted, and multiply shifted species demonstrates that the incorporations at C_2 and C_3 are independent. Note that β-shifted lines are detectably broader due to unresolved deuterium coupling.

Finally, it is apparent that there is actually two compounds contributing to the 64 ppm signals, only one of which can be glycerol; in the majority of our spectra of supernatants there is a minor species, labeled X, ca. 0.04 ppm to low field of the glycerol C_1. (This is most obvious in Figure 5b, where the glycerol signal has been shifted by phosphorylation.) The glycerol resonance in Figure 7 is accompanied by both β-shifted ($\Delta\delta = 0.070$) and γ-shifted ($\Delta\delta = 0.10$) peaks, with an incorporation of less than 50% of the solvent level. Again, β-shifted lines are broader. The minor unknown X has only a single β-shifted partner ($\Delta\delta = 0.063$) with incorporation essentially equal to the solvent level.

Discussion

Figure 8 summarizes all the metabolic processes for the detoxification of exogenous formaldehyde in *E. coli* that we have observed with in vivo NMR spectroscopy. We had previously identified methanol, formate, and bicarbonate and reduction and oxidation products, and in this paper we have demonstrated the incorporation of formaldehyde into glycerol and the two propanediols. In work to be described elsewhere we have shown that the metabolite, 68.9 ppm, that is produced under aerobic conditions is hydroxyacetone (acetol); 1,2-propanediol and hydroxyacetone can be interconverted by *E. coli* simply by changing oxygen tension (B. K. Hunter and F. Commadari, unpublished results).

Our results inevitably give rise to the following questions: (1) can the isotope incorporation patterns observed at C_2 tell us anything about the natural pool size of the formaldehyde metabolites, (2) what do the deuterium incorporation observations tell us about the biosynthetic routes and mechanisms involved, (3) what is the relationship of these observations to earlier knowledge of formaldehyde biochemistry, and (4) are we justified in describing these processes as detoxification?

The ^{13}C-satellite pattern of the C_2 carbons derived from labeled bacteria contains information on the overall $^{12}C/^{13}C$ ratio at C_1 and C_3 and so allows us to perform an isotope dilution analysis on both glycerol and 1,2-diol. Figure 9 shows schematic diagrams of how the patterns expected for C_2 of a three-carbon metabolite depend on the carbon sources used in biosynthesis. In this analysis "natural abundance" is 14% ^{13}C. In Figure 9a, C_1 is entirely bacterial in origin and only 14% labeled; C_2 would then be mainly a singlet. In Figure 9b, C_1 is entirely formaldehyde derived and 90% labeled; C_2 is therefore predominantly a doublet, split by coupling with the adjacent ^{13}C. In Figure 9c, both C_1 and C_3 are form-

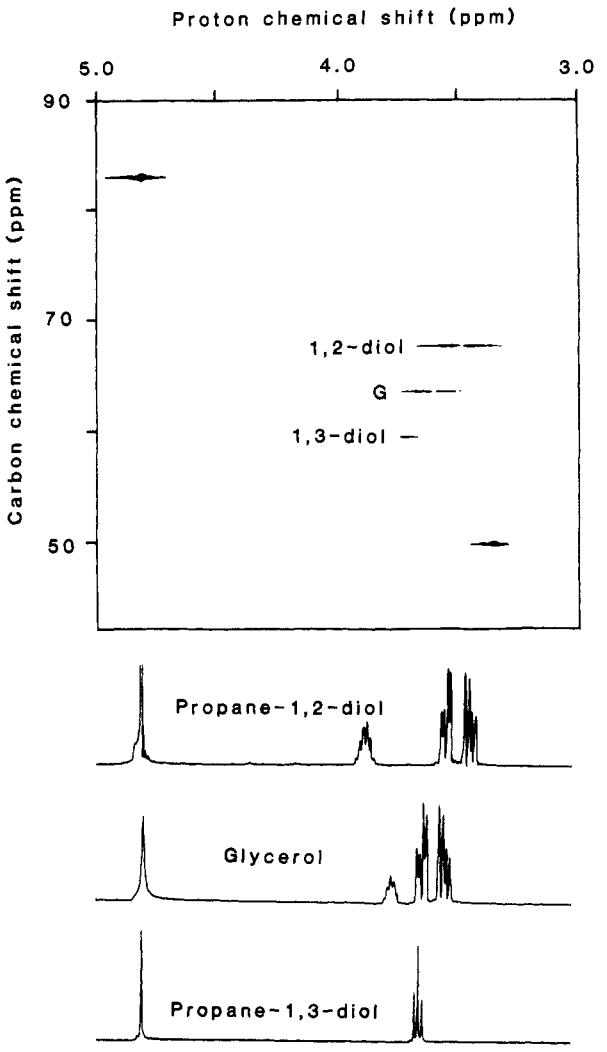

FIGURE 4: (Top) $^{1}H-^{13}C$ two-dimensional correlation spectrum of a supernatant [from Hunter et al. (1984)]; (bottom) 400-MHz ^{1}H NMR spectra of authentic samples of metabolites in D_2O. In the one-dimensional spectra, the 4.8 ppm signal is due to residual water, while in the two-dimensional spectrum the signal at the same shift is due to formaldehyde.

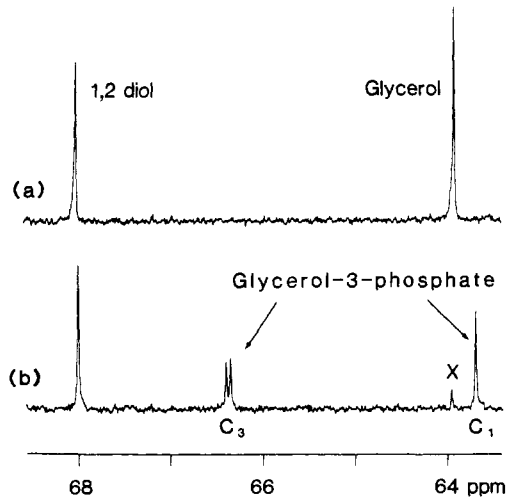

FIGURE 5: Conversion of [1-^{13}C]glycerol to glycerol 3-phosphate by glycerokinase: partial 100.6-MHz ^{13}C NMR spectrum of a supernatant, pH 9.8, (a) before the addition of glycerokinase and (b) after addition of enzyme.

aldehyde derived, and the bulk of the C_2 signal would be a triplet. Comparison of these three possibilities with the actual multiplets in Figure 3 reveals that, within the wide limits set

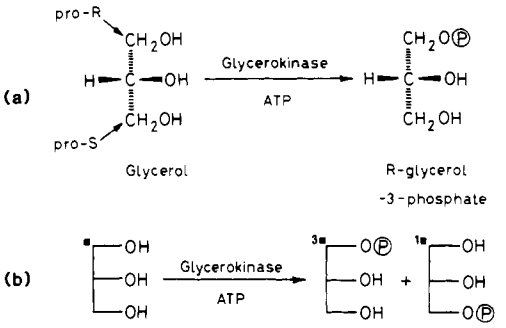

FIGURE 6: (a) Stereospecificity of glycerokinase and (b) lack of stereospecificity in glycerol biosynthesized in this work; product is labeled at C_1 and C_3.

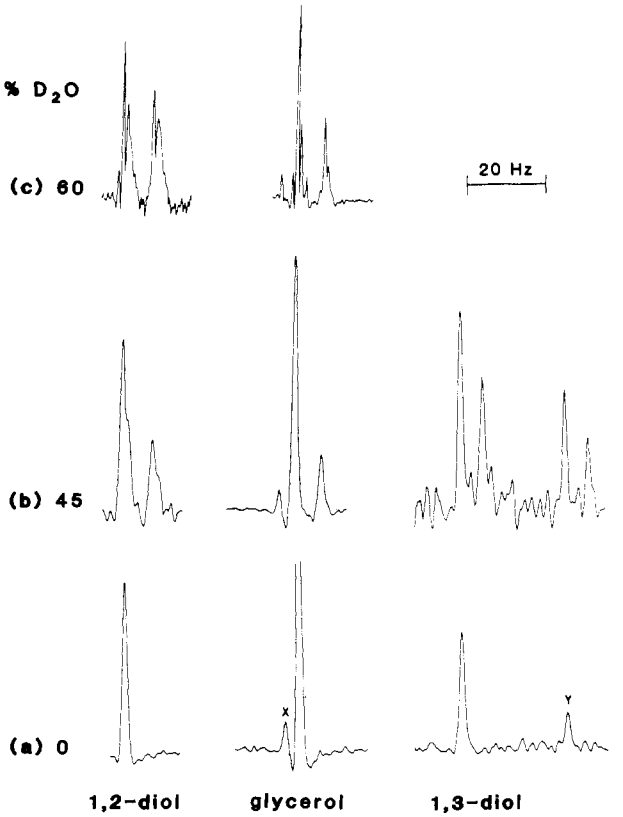

FIGURE 7: Effect of solvent deuterium incorporation on the C_1 signals of 1,2-propanediol, glycerol, and 1,3-propanediol. Deuterium content of solvent during biosynthesis was as shown. In experiment c, no 60 ppm signals were seen.

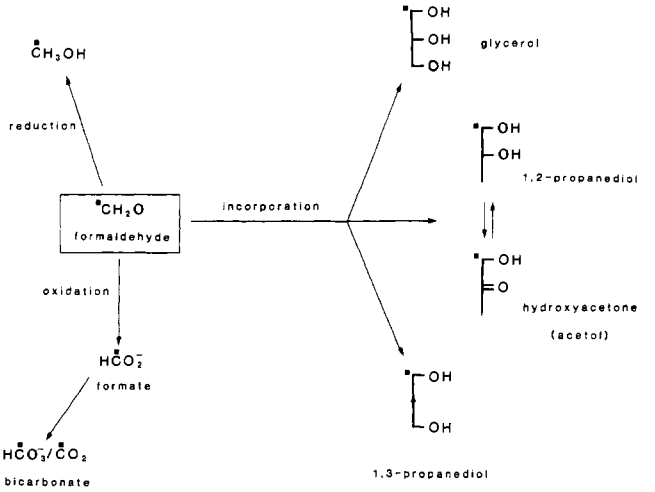

FIGURE 8: Summary of formaldehyde metabolic pathways observed in *E. coli* by in vivo NMR spectroscopy.

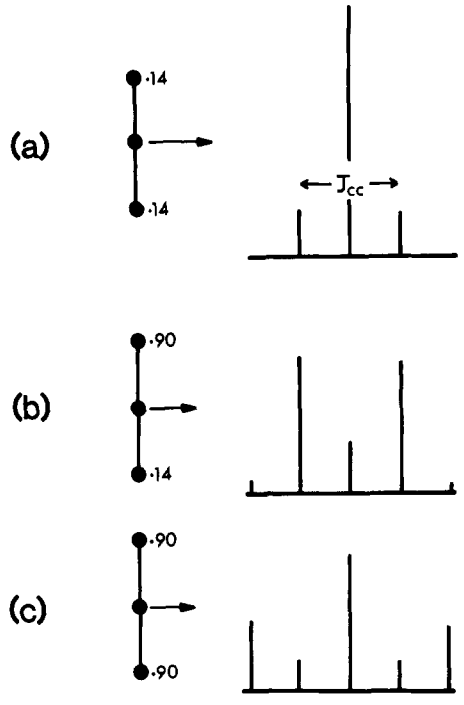

FIGURE 9: Schematic diagram of the dependence of the C_2 signal appearance on the isotopic composition at C_1 and C_3: (a) "natural product" is 14% labeled at C_1 and C_3; (b) C_1 is completely derived from formaldehyde and is therefore 90% labeled; (c) both C_1 and C_3 are formaldehyde derived.

by rather intrusive noise, approximately 90% of the 1,2-propanediol C_2 is split into a doublet, so *all* of the 1,2-propanediol C_1 appears to be formaldehyde derived; C_2 and C_3 are bacterially derived. This metabolite is therefore not synthesized by the bacteria under the experimental conditions except from exogenous formaldehyde. In contrast, the central line of the C_2 of glycerol is more intense than the individual satellite lines; analysis reveals that only some 60% of the glycerol is derived from formaldehyde, the remainder being "natural product". Given the central importance of glycerol derivatives in the cell, it is not too surprising that some free glycerol is present.

It should be pointed out that this type of isotope dilution analysis does not require the separation, isolation, or purification of the metabolite in question. *It does not even require identification of that metabolite.* It can be carried out on a supernatant or, in principle, in vivo. All that is required is a differential isotope labeling of "natural" and exogenous material sources. To the best of our knowledge, this method of dilution analysis is novel. It could clearly be extended quantitatively to give a measure of natural pool sizes. It should be pointed out that neither this nor any other dilution analysis will distinguish between the two possible types of "natural" products: those that are genuinely natural and are synthesized by the organism under normal conditions and "stress metabolites", which are produced in response to the exogenous drug but do not incorporate that drug.

With information in hand concerning assignment, deuterium incorporation, and minor components, it is possible to interpret fully the complex C_1 and C_2 multiplets observed in the experiment with ^{13}C-labeled bacteria. Figure 10 shows the C_1 signal of 1,2-propanediol in the form of a "stick" diagram, which is labeled with each of the expected isotopomers, and the actual spectrum from Figure 2. The stick diagram was constructed by using the isotope shifts and incorporations reported above, taking into account strong coupling in the satellites.[4] Because β-shifted lines are perceptibly broadened

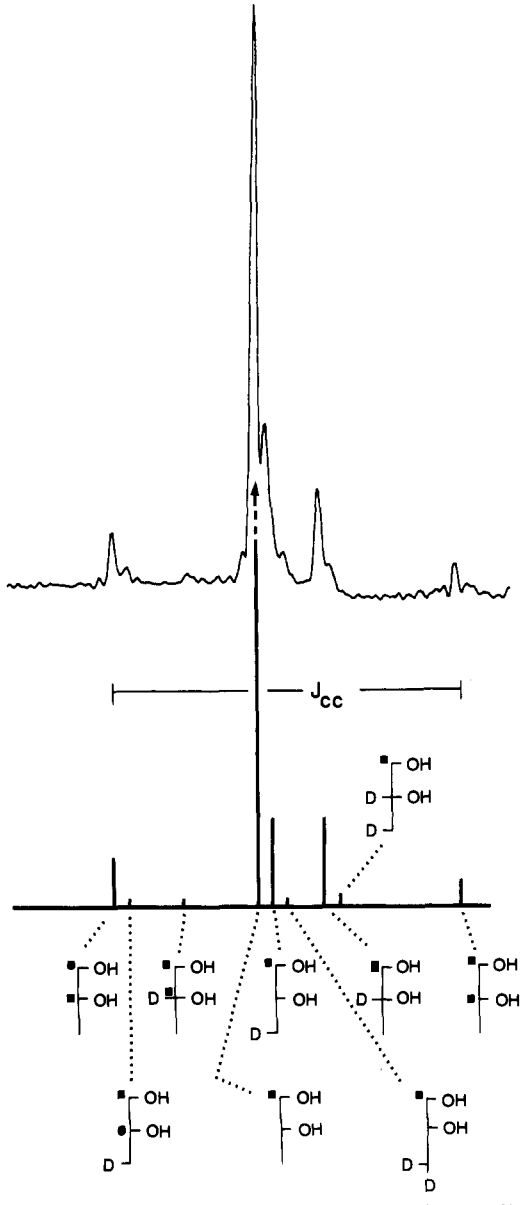

FIGURE 10: (Top) C_1 of 1,2-propanediol derived from 90% [^{13}C]-formaldehyde and 14% ^{13}C-labeled bacteria (Figure 2). (Bottom) Stick diagram showing the predicted positions and intensities of the contributions from the eight major isotopomers calculated to be present.

by unresolved coupling to deuterium, we may have slightly underestimated β-deuterium incorporation, but the error is likely to be very small. The agreement between calculated and actual spectra is excellent, with seven of the eight expected different isotopomers being assigned; only the signal from [2-^2H,1,2-^{13}C]-1,2-diol is absent, lost in the base-line noise. Similar agreement is reached for glycerol and the minor component X.

The C_2 multiplets shown in Figure 3 can also be interpreted. We would not expect to see the C_2 signals from glycerol or 1,2-diol molecules that contain deuterium at C_2 because such carbons are split into three lines by deuterium coupling and lack any protons to aid relaxation or give NOE's. They are likely, therefore, to be too weak to observe. However, we would expect to see β-shifted C_2 resonances from molecules that contain deuterium at C_3. These are the molecules that show

[4] This second-order effect is calculated to move the apparent chemical shift by 3 Hz toward the C_2 satellites (Emsley et al., 1963). When this is taken into account, there is no detectable isotope effect of ^{13}C at C_2 on the chemical shift of C_1 or vice versa.

γ-shifted C_1 signals. Glycerol biosynthesized in 20% deuterium oxide experiences too little 3-deuteration to reveal β-shifted peaks at C_2, but the 1,2-diol resonance clearly shows 20% deuteration at C_3, the β-shift being 0.05 ppm.

The multiple and random deuterium incorporation at C_3 of the 1,2-diol is consistent with a simple solvent exchange via enolization, assuming that C_2 is a carbonyl or equivalent group at some point in the biosynthesis. Since we know that the 1,2-diol is interconvertible with hydroxyacetone under the experimental conditions, it is reasonable to speculate that hydroxyacetone is in fact the first product, being then reduced to diol under anaerobic conditions. This would explain the C_3 labeling pattern. The reduction itself gives a high level of deuteration at C_2, but both the 1,3-diol results we discuss below and other, unpublished experiments throw some doubt on whether this is a simple NADH (or equivalent) reduction.

If hydroxyacetone is the precursor to the 1,2-diol, then a plausible biosynthetic scheme can be proposed. It is known (Doelle, 1975) that enterobacteria produce 2,3-butanediol (butylene glycol); this reaction involves the adduct of acetaldehyde and thiamin pyrophosphate attacking a pyruvate molecule, this condensation being followed by decarboxylation and reduction. If, instead, the acetaldehyde–thiamin adduct condensed with formaldehyde, then the product would be hydroxyacetone.

It is less easy to write an equivalent scheme for glycerol. Deuterium incorporation at both C_2 and C_3 is substantially less than in the 1,2-diol, and we have been unable to detect the analogous dihydroxyacetone. At this stage we can only say that because the deuterium incorporation is different for the two metabolites, it is likely that they are produced by pathways that diverge quite early.

In the experiment using ^{13}C-labeled *E. coli* the apparent deuterium incorporation into glycerol should be corrected for the fact that the 40% that is not derived from formaldehyde was probably synthesized before any deuterium was added. To do this, we must calculate what proportion of the 1-^{13}C-labeled glycerol (which is what we are observing) is formaldehyde derived. Ninety percent of the formaldehyde-derived molecules will be labeled, which is approximately 54% of the entire glycerol population, while 14% of the remaining molecules will be labeled, which is 5.6% of the entire population (0.4 × 14%). It seems, therefore, that some 54/60 of the [1-^{13}C]glycerol is formaldehyde derived. Given the very approximate incorporation and isotope dilution results obtained in this work, the necessary correction for deuterium incorporation is insignificant. In general, however, the correction would become crucial if this approach were used with molecules with a much higher proportion of "natural" product. Where "natural abundance" organisms are used, essentially all the ^{13}C is derived in any case from the added compound, and no correction will be necessary.

The most puzzling incorporation pattern is that for the 1,3-diol. This material is produced, unreliably, in very small amounts. Nevertheless, it is clear that effectively only one C_2 deuterium is incorporated but that it is incorporated with extremely high efficiency. It is possible that the differential C_2 deuteration seen between the three three-carbon products is a result of different kinetic isotope effects for steps that all use, for example, the same partially deuterated reducing agent. An alternative is that the C_2 deuterium in 1,3-diol arises by a reductive process that produces either a radical or an anion at C_2 and that this reactive intermediate acquires a proton from the solvent. This scheme is attractive because it explains both the very high deuteration and the fact that only one deuterium is acquired. Such a process could also be occurring for C_2 in the 1,2-diol.

To our knowledge, the one-carbon incorporation that we have described has not been observed before. 1,2-Propanediol is a known metabolite of fucose (Chen et al., 1983), but that is presumably not relevant here. 1,3-Propanediol appears to be unknown in *E. coli*, while the normal biosynthetic route to glycerol bears little resemblance to that involved in this work. However, formaldehyde is apparently ubiquitous in cells, either as the free species or as an adduct with tetrahydrofolate, and there is in principle an incorporation pathway through tetrahydrofolate to serine and many other cell components. Previous work with low levels of [^{14}C]formaldehyde revealed no sign of folate-derived products (Roberts et al., 1955), but nonspecific incorporation into amino acids and nucleic acids was observed. The natural concentration of formaldehyde is submillimolar (Heck et al., 1982); the concentrations with which we challenge *E. coli* are about 100-fold higher and near the limit that the bacteria will tolerate. It appears, then, that the usual pathways of formaldehyde incorporation cannot process large quantities of formaldehyde.[5]

We conclude, therefore, that the large amounts of formaldehyde that we impose upon the cultures are susceptible to attack by a variety of different enzymes with imperfect substrate specificity. We have, for example, tentatively assigned the production of 1,2-propanediol to the enzyme system that normally produces 2,3-butanediol. This may be regarded as a case of "mistaken identity" by the enzyme or, more appropriately, as chemical promiscuity on the part of the highly reactive formaldehyde molecule. This combination of mistaken identity and promiscuity constitutes the detoxifying defense mechanism that we have previously discussed (Hunter et al., 1984).

ACKNOWLEDGMENTS

We are grateful to Dr. B. E. McCarry (McMaster University) for the original suggestion that glycerol might be one of the metabolic products.

Registry No. Formaldehyde, 50-00-0; formaldehyde-^{13}C, 3228-27-1; glycerol, 56-81-5; 1,2-propanediol, 57-55-6; 1,3-propanediol, 504-63-2; glucose, 50-99-7.

REFERENCES

Abell, C., & Staunton, J. (1981) *J. Chem. Soc., Chem. Commun.*, 856–858.

Alworth, W. L. (1972) *Stereochemistry and Its Application in Biochemistry*, pp 115–118, Wiley-Interscience, New York.

Chen, Y.-M., Lin, E. C. C., Ros, J., & Aguilar, J. (1983) *J. Gen. Microbiol. 129*, 3355–3362.

Cornish, A., Nicholls, K. M., Hunter, B. K., Aston, W. J., Higgins, I. J., & Sanders, J. K. M. (1984) *J. Gen. Microbiol. 130*, 2565–2575.

Doddrell, D. M., Nicholls, K. M., & Sanders, J. K. M. (1984) *FEBS Lett. 170*, 73–75.

Doelle, H. W. (1975) *Bacterial Metabolism*, 2nd ed., Academic Press, London.

Emsley, J. W., Feeney, J., & Sutcliffe, L. H. (1963) *High Resolution Nuclear Magnetic Resonance Spectroscopy*, pp 310–320, Pergamon, Oxford.

[5] Similar results are obtained when the methanol oxidation pathway of methylotrophic bacteria is observed by in vivo NMR spectroscopy; it is known that a proportion of the formaldehyde formed is incorporated into the cell mass, but only the bulk oxidation products can be detected by NMR (Cornish et al., 1984).

Heck, H. d'A., White, E. L., & Casanova-Schmitz, M. (1982) *Biomed. Mass Spectrom. 9*, 347–353.
Hunter, B. K., Nicholls, K. M., & Sanders, J. K. M. (1984) *Biochemistry 23*, 508–514.
Karnovsky, M. L., Hauser, G., & Elwyn, D. (1957) *J. Biol. Chem. 226*, 881–890.
Knappe, J., Blaschkowski, H. P., Grotner, P., & Schmitt, T. (1974) *Eur. J. Biochem. 50*, 253–263.
Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. T., & Britten, R. J. (1955) *Studies of Biosynthesis in Escherichia coli*, Carnegie Institute of Washington, Publication 607, Washington, DC.
Shaka, A. J., Keeler, J. H., Frenkiel, T., & Freeman, R. (1983) *J. Magn. Reson. 52*, 335–338.
Spizizen, J. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 1072–1078.
Walker, J. F (1964) *Formaldehyde*, 3rd ed., Reinhold, New York.

Role of Superoxide in the N-Oxidation of *N*-(2-Methyl-1-phenyl-2-propyl)hydroxylamine by the Rat Liver Cytochrome P-450 System†

John D. Duncan,‡,§ Emma W. Di Stefano,‡ Gerald T. Miwa,‖ and Arthur K. Cho*,‡

Department of Pharmacology, UCLA School of Medicine, Center for the Health Sciences, Los Angeles, California 90024, and Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065

Received May 11, 1984; Revised Manuscript Received December 7, 1984

ABSTRACT: The N-oxidation of *N*-(2-methyl-1-phenyl-2-propyl)hydroxylamine (*N*-hydroxyphentermine, MPPNHOH) and the N-hydroxylation of 2-methyl-1-phenyl-2-propylamine (phentermine) by reconstituted systems that contained cytochromes P-450 purified from rat liver microsomes were demonstrated. The oxidation of MPPNHOH, but not of phentermine, could also be mediated by a superoxide and hydrogen peroxide generating system that contained xanthine and xanthine oxidase. Superoxide dismutase completely inhibited the oxidation of MPPNHOH by the xanthine/xanthine oxidase system and inhibited by 70% the oxidation mediated by a reconstituted cytochrome P-450 oxidase system. The majority of the microsomal oxidation was inhibited by an antibody raised against the major isozyme of cytochrome P-450 purified from livers of phenobarbital-pretreated rats. 2-Methyl-2-nitroso-1-phenylpropane (MPPNO) was found to be an intermediate in the overall oxidation of MPPNHOH to 2-methyl-2-nitro-1-phenylpropane ($MPPNO_2$). Superoxide dismutase appeared to inhibit the first step, the conversion of MPPNHOH to MPPNO. These observations are accounted for by a sequence of two mechanistically distinct P-450-mediated oxidations. In the first reaction, N-hydroxylation of phentermine occurs by a normal cytochrome P-450 pathway. The formed hydroxylamine then uncouples the cytochrome P-450 system to generate superoxide and hydrogen peroxide. The superoxide oxidizes MPPNHOH to MPPNO which is then oxidized to $MPPNO_2$, the ultimate product. This superoxide-mediated oxidation represents another pathway for N-oxidation by cytochrome P-450.

Previous studies in this laboratory have shown that phentermine (2-methyl-1-phenyl-2-propylamine; $MPPNH_2$)[1] is oxidized to *N*-hydroxyphentermine (MPPNHOH) and that MPPNHOH is further oxidized to 2-methyl-2-nitro-1-phenylpropane ($MPPNO_2$) (Sum & Cho, 1979; Maynard & Cho, 1981) by rat liver microsomal preparations. The N-hydroxylation of $MPPNH_2$ appeared to be a normal cytochrome P-450 catalyzed reaction in that it is a two-electron oxidation and was inhibited by carbon monoxide and DPEA (Sum & Cho, 1977). The oxidation of MPPNHOH to $MPPNO_2$ was also inhibited by carbon monoxide and DPEA (Sum & Cho, 1979, Maynard & Cho, 1981) but differed from the first oxidation in its sensitivity to superoxide dismutase. Further studies with rat and rabbit liver microsomes (Maynard & Cho, 1981; Cho et al., 1982) indicated that MPPNHOH caused a NADPH-dependent increase in H_2O_2 and that superoxide was responsible for the oxidation of MPPNHOH to $MPPNO_2$ by liver microsomes. Thus, MPPNHOH appeared to uncouple cytochrome P-450 to generate the superoxide that subsequently oxidized MPPNHOH.

The present investigation was initiated to obtain direct evidence for cytochrome P-450 involvement in the reaction by incubating MPPNHOH with reconstituted systems containing the major cytochrome P-450 isozyme from either pheno-

† Portions of this research were supported by U.S. PHS Grants GM26024 and CA09030.
‡ UCLA School of Medicine.
§ Present address: Behring Diagnostics, Division of American Hoechst Corp., La Jolla, CA 92037.
‖ Merck Sharp & Dohme Research Laboratories.

[1] Abbreviations: $MPPNH_2$, 2-methyl-1-phenyl-2-propylamine; MPPNHOH, *N*-(2-methyl-1-phenyl-2-propyl)hydroxylamine; MPPNO, 2-methyl-2-nitroso-1-phenylpropane; $MPPNO_2$, 2-methyl-2-nitro-1-phenylpropane; PB, phenobarbital; 3MC, 3-methylcholanthrene; PC, dilauroylphosphatidylcholine; DPEA, *N*-(2,4-dichloro-6-phenylphenoxy)ethylamine; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Me_2SO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography.

0006-2960/85/0424-4155$01.50/0