In Vivo Studies of Pyridine Nucleotide Metabolism in Escherichia coli and Saccharomyces cerevisiae by Carbon-13 NMR Spectroscopy*

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Pyridine nucleotide metabolism has been studied in vivo in a prokaryotic (Escherichia coli) and a eukaryotic (Saccharomyces cerevisiae) system cultured in a medium containing carbon-13-labeled nicotinic acid, followed by NMR detection of the labeled organisms. Chemical exchange between oxidized and reduced nucleotides is found to be sufficiently slow on the NMR time scale to permit the observation of separate resonances corresponding to each redox state. The possibility of significant exchange broadening of reduced pyridine nucleotide resonances under some conditions was further evaluated based on comparative NMR studies utilizing organisms cultured in the presence of either [2-13C]nicotinate or [5-13C]nicotinate. Based on these experiments, it was concluded that broadening as a consequence of intermediate exchange is not significant. Although it was initially anticipated that the carbon-13 resonances arising from the di- and triphosphopyridine nucleotide pools could not be distinguished, the absence of observable resonances corresponding to reduced nucleotides in oxygenated yeast and E. coli cells suggests that the NMR method is fairly specific for determining the redox status of the diphosphopyridine nucleotide pool.

Studies of the effects of a variety of perturbations including variation of the oxygen supply, addition of ethanol, and addition of the oxidative phosphorylation uncoupler dinitrophenol have been carried out. Dramatic differences in the response of the catabolic reduction charge, CRC = [NADH]/([NADH] + [NAD\(^+\)]) between the yeast and E. coli cells are observed. The CRC values for the yeast undergo large changes in response to these perturbations which are not observed for the bacterial cells.

Oxidation/reduction reactions are central to all aspects of cellular metabolism, and the majority of these involve pyridine nucleotide coenzymes. As a consequence of the involvement of these coenzymes in enzyme-catalyzed reactions, the ratios of reduced to oxidized nucleotide, or “reduction charge” of the pools have been viewed as important metabolic regulatory parameters. In this context, Anderson and von Meyenburg (1) have defined CRC = [NADH]/([NADH] + [NAD\(^+\)]) and ARC = [NADPH]/([NADPH] + [NADP\(^+\)]).

We have recently pointed out that various aspects of pyridine nucleotide metabolism in general, and a “mean” reduction charge in particular, can be studied in vivo by carbon-13 NMR spectroscopy (2). The approach makes use of the salvage pathways or pyridine nucleotide cycles, and the addition of \(^{13}\)C-labeled nicotinate or nicotinamidase precursors to introduce the label into the dinucleotide pool. The present study represents the first in a series designed to evaluate the potentials and limitations of this approach for studying various aspects of in vivo pyridine nucleotide metabolism by this method.

MATERIALS AND METHODS

[2-13C]Nicotinic acid was synthesized from [methyl-3-13C]cyanoacetate (3) essentially as described by Bryson et al. (4). [5-13C]Nicotinic acid was synthesized from [1-13C]acetate (5) by a method previously described (6).

A nicotinate-requiring strain of Escherichia coli (ATCC 25788) was cultured on a minimal medium (7) containing \(^{13}\)C-labeled nicotinic acid (0.5 mM) and glucose (0.5%) or glycerol (1.0%). The organism was grown at 27°C in shake culture or under anaerobic conditions in a spinner flask. Anaerobic cultures were maintained at pH 6.5 with the addition of ammonium hydroxide. Saccharomyces cerevisiae (ATCC 18790) were cultured in a complex medium which contained yeast extract (Difco, 1%), Bacto-peptone (Difco, 2%), and glucose (2%), and was supplemented with \(^{13}\)C-labeled nicotinic acid (0.5 mM). The organism was grown in shake culture at 30°C.

The cells were harvested in exponential growth phase, washed to remove exocellular nicotinic acid, and resuspended at a density of approximately \(10^9\) cell/ml in phosphate buffer (pH 7.2) either with or without a carbon source. Cells were oxygenated in the spectrometer using a specially designed dual capillary system mounted concentri-

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‡ The abbreviations used are: CRC, catabolic reduction charge; ARC, anaerobic reduction charge.
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RESULTS AND DISCUSSION

$^{13}$C Labeling—The introduction of the $^{13}$C label into the cellular pyridine nucleotide pools is based on the activity of the various pyridine nucleotide cycles (11). These cycles serve primarily as salvage pathways, but serve additional functions related to the activation of DNA ligase in bacteria, and the maintenance of the poly(ADP) ribose pool in higher organisms (12). The primary bacterial cycle is illustrated in Fig. 1, although a significant flux is thought to short-circuit the nicotinamide and nicotinate (13). In order to study cellular pyridine nucleotide metabolism, it is first necessary to establish the shifts of the metabolites corresponding to this cycle. Some relevant data currently exist (14); however, the shifts reported for NAD(P)H and NAD(P)$^+$ were obtained at pH 9.8 (9.2) and 2.0 (1.0), respectively, and shifts for other important metabolites were not included in this study. Carbon-13 chemical shifts for the pyridine moiety of these metabolites are summarized in Table I. From these data, several important conclusions can be drawn: (a) chemical shift differences for the pyridine carbons between the di- and triphosphopyridine nucleotides are negligible so that the separate intracellular pools cannot be distinguished based on shift differences. (b) The easily position to label, C-7, is of limited utility for the study of intracellular pyridine nucleotide reduction charge as a consequence of the similarity in chemical shift between the carboxyl resonance of free nicotinate and the carbamido resonance of NAD(P)H. As noted below, the free nicotinate levels in bacteria can be significant and time dependent so that determination of the reduced pyridine nucleotide levels becomes difficult or impossible. (c) In contrast with the data of Birdshall and Feeney (14), there are significant chemical shift differences between NMN$^+$ and NAD(P)$^+$ so that separate resonances can be resolved under standard conditions (Fig. 2). This discrepancy probably reflects the pH differences in the studies reported by Birdshall and Feeney (14): pH 2.0

Table I

| Metabolite | C-2 | C-3 | C-4 | C-5 | C-6 | C-7 |
|------------|-----|-----|-----|-----|-----|-----|
| Nicotinate | 149.87 | 133.21 | 138.58 | 124.83 | 151.17 | 173.85 |
| Nicotinamide | 148.29 | 129.70 | 137.04 | 124.98 | 152.48 | 170.79 |
| NAD$^+$ | 140.72 | 134.68 | 146.80 | 129.69 | 143.35 | 166.22 |
| NADP$^+$ | 140.83 | 134.63 | 146.74 | 129.70 | 143.34 | 166.12 |
| NADH | 139.15 | 101.25 | 22.86 | 106.22 | 125.12 | 173.62 |
| NMN | 140.81 | 134.94 | 147.12 | 129.62 | 144.01 | 166.76 |
| NaMN | 142.03 | 138.19 | 147.99 | 129.34 | 142.26 | 168.57 |
| NaAD | 141.52 | 137.94 | 147.86 | 129.32 | 142.39 | 168.03 |
| αNAD | 142.58 | 133.38 | 146.04 | 128.07 | 144.55 | 166.51 |

**Fig. 1.** The pyridine nucleotide cycle in E. coli which forms the basis for incorporation of labeled nicotinate into the pyridine nucleotide coenzymes (7). Some flux bypassing nicotinamide/nicotinate metabolites has also been postulated (12). NaAD, nicotinate adenine dinucleotide; NaMN, nicotinate mononucleotide; PRPP, 5-phospho-d-ribosylpyrophosphate; RP, d-ribose-5-phosphate.

**Fig. 2.** Downfield regions of the $^{13}$C NMR spectra. A, a 3:1 mixture of NMN:NAD$^+$; B, a 2:1 mixture of nicotinate mononucleotide (NaMN):NAD$^+$. These spectra demonstrate the existence of significant chemical shifts for many of the nicotinamide/nicotinate carbons between these metabolites.

for NADP$^+$, 2.8 for NMN$^+$. This result is not too surprising in view of NMR studies indicating that conformations with the adenine and nicotinamide moieties stacked predominate in solution (15). (d) From the standpoint of chemical shift, the C-2 carbon represents an attractive labeling target since the resonances close to 140 ppm fall in a relatively uncrowded...
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spectral window. However, the chemical shift difference between reduced and oxidized resonances is relatively small. Thus, the choice of optimal labeling strategy is not completely unambiguous.

**Relaxation Behavior**—An understanding of the relaxation behavior of intracellular metabolites is of fundamental importance for the analysis of metabolic behavior by NMR. In general, studies of intracellular pyridine nucleotides (16-18) and small metabolites (19, 20) have yielded relaxation parameters similar to those observed in buffered aqueous solutions. These results indicate that the intracellular microviscosity must be similar, within an order of magnitude, to the value for pure water. $T_1$ measurements of intracellular metabolites are hindered by the possibility of simultaneous metabolic transformations which will be superimposed with the time-dependent magnetization changes resulting from spin lattice relaxation. As discussed in the previous section, this problem can be dealt with to some extent by modifying the inversion recovery sequence so that data corresponding to each delay are accumulated in short blocks. In this way, more gradual changes in resonance intensity resulting from metabolic processes will be averaged over each different delay. Using this procedure, exponential recovery curves were obtained, and the spin lattice relaxation times obtained for the intracellular $[2-{^{13}}C]$ NAD* pool of *E. coli* at two temperatures are given in Table II. The longer $T_1$ values were actually somewhat longer than those reported for the C-6 resonance of NAD* at 0.5 or 0.68 M solutions, pH 7.0 (21). Riddle et al. (21) have shown that, as expected, the $^{13}$C relaxation mechanism is predominantly the $^{13}$C-$^1$H dipolar interaction for the protonated carbons, so that based on the similarities in $T_1$ values, this assumption is reasonable for the intracellular pool as well. In addition, based on the relatively short $T_1$ values for the NAD*, pulse delays can be selected in order to ensure essentially full relaxation of the methine carbon resonances.

From the standpoint of the metabolic NMR studies, it is of primary importance to obtain accurate relative intensities. This requires that the nuclear Overhauser effect values be similar, that the resonances not be overpulsed, or that the resonances all be overpulsed to an equivalent extent. From the standpoint of pyridine nucleotide metabolism, it is of primary importance to obtain accurate ratios for the reduced and oxidized pyridine nucleotide resonances. In view of the similarity in $T_1$ values, this assumption is reasonable for the intracellular pool as well, as well as the similarities in molecular weight between the reduced and oxidized nucleotides, it is reasonable to assume that the spin lattice relaxation times for the nicotinamide methine carbons will be nearly identical for the two species. Thus, even if the spectrum is overpulsed, it is anticipated that the ratio of oxidized to reduced intensity will not be altered.

The above conclusion can be further tested by determining whether decreases in the oxidized nucleotide intensity are accompanied by quantitatively identical increases in the reduced resonance intensity. In practice, it may also be necessary to take into account the biosynthesis of other pyridine nucleotide metabolites. In all studies, we have found that, subject to the accuracy of the measurement, this assumption is valid. Thus, there is essentially conservation of resonance intensity as the distribution of intensity between oxidized and reduced resonances varies. In summary, there appear to be no significant problems of quantitating resonance intensity resulting from differences in $T_1$ or nuclear Overhauser effect values.

**Analysis of Chemical Exchange**—In contrast to the above conclusions, the possible effects of chemical exchange present a formidable analytical problem. Two types of exchange phenomena can be distinguished: (a) exchange between oxidized and reduced nucleotides; and (b) exchange with cellular enzyme-binding sites. In each case, the exchange rates may fall into the slow, intermediate, or fast limits. The effects of exchange between oxidized and reduced pyridine nucleotides are more readily analyzed and considered first. In the fast exchange limit, a single resonance will be observed with a chemical shift representing a weighted average of the fractional populations of oxidized and reduced nucleotides. In this case, the chemical shift would provide a measure of the reduction charge of the corresponding pyridine nucleotide pool. As discussed in detail in the following sections, perturbations which alter the reduction charge of the pools do not result in perturbations of the resonance shift, but instead lead to changes in the distribution of intensity observed between resonances corresponding to reduced and oxidized forms of the nucleotides. We can thus draw the important conclusion that, with the labeling strategy employed in these studies, exchange between oxidized and reduced pyridine nucleotides is not rapid on the NMR time scale.

In the intermediate exchange limit, the appearance of the spectrum is dependent on the chemical shift difference between oxidized and reduced forms. Such exchange behavior can be most directly characterized by varying the "NMR time scale" utilizing different carbon-13 labels. Thus, for the pyridine C-2 resonance, the chemical shift difference between reduced and oxidized forms is 1.5 ppm, while for C-5 it is 23 ppm. If the resonances corresponding to C-2 fall into the intermediate exchange limit, the C-5 resonances will tend to fall into the slow exchange limit, since $\Delta \nu$ is more than an order of magnitude greater. As discussed in more detail in the following sections, the C-2 and C-5 labeling studies have given equivalent results, suggesting that the rate for chemical exchange between the oxidized and reduced forms does not fall into the intermediate exchange limit.

As discussed in greater detail below, the data obtained for prokaryotic (*E. coli*) and eukaryotic (*S. cerevisiae*) systems indicate that the exchange between oxidized and reduced forms of the pyridine nucleotides is slow on the NMR time scale. Thus, separate resonances are typically observed for reduced and oxidized species. However, even in this limit, there are potential errors in the determinations reflecting exchange broadening of the resonances. In the steady state, the lifetimes of the intracellular oxidized and reduced nucleotides are inversely related to the fractional populations:

$$f_{\text{ox}} \tau_{\text{ox}} = f_{\text{red}} \tau_{\text{red}}$$  

where $f_{\text{ox}}$ and $f_{\text{red}} = 1 - f_{\text{ox}}$ are the fractional concentrations of oxidized and reduced nucleotides, respectively, and $\tau_{\text{ox}}$ ($\tau_{\text{red}}$) the lifetime of the oxidized (reduced) species. In general, for $f_{\text{ox}} \neq f_{\text{red}}$, the degree of exchange broadening will differ for the two resonances. For the diphosphopyridine nucleotides for which $f_{\text{ox}} >> f_{\text{red}}$, the exchange broadening of the reduced

| Table II | $^{13}$C spin lattice relaxation times of intracellular nicotinamide-2,$^{13}$C/NAD* |
|---------|----------------------------------|
| $T_1$   | $r_\text{ox}$, $T$ |
| ms      | °C |
| 300     | 19  |
| 250     | 32  |

\( \text{TI} \) (b) exchange with cellular enzyme-binding sites. In each case, the exchange rates may fall into the slow, intermediate, or fast limits. The effects of exchange between oxidized and reduced pyridine nucleotides are more readily analyzed and considered first. In the fast exchange limit, a single resonance will be observed with a chemical shift representing a weighted average of the fractional populations of oxidized and reduced nucleotides. In this case, the chemical shift would provide a measure of the reduction charge of the corresponding pyridine nucleotide pool. As discussed in more detail in the following sections, perturbations which alter the reduction charge of the pools do not result in perturbations of the resonance shift, but instead lead to changes in the distribution of intensity observed between resonances corresponding to reduced and oxidized forms of the nucleotides. We can thus draw the important conclusion that, with the labeling strategy employed in these studies, exchange between oxidized and reduced pyridine nucleotides is not rapid on the NMR time scale.

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resonance will typically be more than an order of magnitude greater than for the reduced resonance:

\[
\frac{v_{ox}}{v_{red}} = \frac{1}{\pi} \frac{r_{ox}}{r_{red}} + \frac{1}{\pi} \frac{f_{ox}}{f_{red}}
\]

where \(v_{ox}\) and \(v_{red}\) are the line widths of the oxidized and reduced resonances, and \(v_0\) the line width resulting from all effects other than chemical exchange. It is therefore possible that, at very high ratios of \(f_{ox}/f_{red}\), the reduced resonance may become unobservable as a consequence of this selective broadening effect. However, the data obtained to date for both the E. coli and S. cerevisiae systems, as well as for perfused hamster liver, indicate that even in the case of large \(f_{ox}/f_{red}\) values, no significant differential broadening is observed between reduced and oxidized resonances, so that it appears that the exchange rate between reduced and oxidized resonances is sufficiently low to rule out significant broadening in the slow exchange limit. It is important to note that the above arguments do not hold for the triphosphopyridine nucleotide pool for which the \(f_{ox}/f_{red}\) values are expected to be considerably lower. For this pool in the slow exchange limit, the broadening of the reduced resonance would be less extreme than the broadening of the oxidized resonance.

In summary, it appears that the data obtained to date are consistent with the conclusion that the exchange between oxidized and reduced pyridine nucleotides is sufficiently slow to result in separate resonances and to preclude significant broadening resulting from chemical exchange. However, although it appears unlikely, there remains some possibility that at very high \(f_{ox}/f_{red}\) ratios, exchange broadening effects may selectively increase the line width of the reduced resonance.

In addition to the chemical exchange of each pool between reduced and oxidized forms, two additional effects can be noted: (a) chemical exchange between NAD\(^+\) and NADP\(^+\) catalyzed by the enzyme NAD kinase (22); and (b) chemical exchange of the form

\[
\text{NADH} + \text{NADP}^+ \rightleftharpoons \text{NAD}^+ + \text{NADPH}
\]
catalyzed by transhydrogenases (23, 24). Since typically the di- and triphosphopyridine nucleotide pools are maintained at very different redox potentials, exchange of the first type is presumably slow and can be neglected. The reaction catalyzed by the transhydrogenase results in the interconversion of oxidized and reduced forms of each pool, and hence is covered by the discussion given above.

In contrast with the above discussion, exchange broadening resulting from the association with cellular enzymes can be significant and must be evaluated in practice largely by the process of elimination. The existence of enzyme-binding sites which are (primarily) also catalytic extends the complexity of the exchange problem to a consideration of four sites corresponding to uncomplexed reduced and oxidized nucleotides and to enzyme-complexed reduced and oxidized nucleotides. The central observation discussed in the following sections that is relevant in this respect is that there is an absence of observable resonances corresponding to reduced nucleotides for oxygenated cells of all types studied to date. This result indicates that either the levels of such reduced nucleotides are below detection threshold under such conditions, or that they are exchange broadened to a degree that renders them unobservable. Based on literature data from a wide variety of organisms reflecting \(a\) that the ratio of tri- to diphosphopyridine nucleotides is typically \(\geq 20\%\) (1, 25–29) and \(b\) the fact that the triphosphopyridine nucleotide pool is primarily (\(\geq 50\%\)) reduced, a resonance from NADPH should be detected. The absence of observable reduced resonance intensity suggests that exchange broadening may be a significant factor. Since, as discussed above, the ratio of \(f_{\text{NADPH}}/f_{\text{NADP}} < 1\), it may be concluded that the NADPH–NADP\(^+\) exchange would not broaden the NADPH resonance significantly; it is therefore likely that such exchange broadening reflects the interaction of NADPH with cellular enzymes. The NADPH resonances could be broadened either if there is complexation of a large fraction of the NADPH pool with enzymes, or if NADPH were in the slow exchange limit with enzyme-binding sites. In this regard, we note that two \(^{13}\text{C}\) resonances of [carboxamoido-\(^{13}\text{C}\)]NADPH are observed in the presence of the enzyme dihydrofolate reductase, reflecting slow chemical exchange (30). The selective broadening of the NADPH resonances may reflect the fact that the association constants \((K_a)\) for the reduced nucleotides are typically 2- to 100-fold higher at neutral pH than those for the oxidized nucleotides (31). However, as discussed in the following sections, under a variety of conditions, resonances corresponding to reduced pyridine nucleotides are readily observed.

Pyridine Nucleotide Metabolism in E. coli—As discussed under "Materials and Methods," the pyridine nucleotide studies in E. coli utilized a nicotinic acid-requiring mutant (5), ATCC 23788. The mutant was used in order to ensure complete labeling of the pool, although it is probably not essential since it has been reported that nicotinate represses quinolinate synthesis (32), and induces enzymes for its utilization up to 100-fold (33). Incorporation of exogenous labeled nicotinate also forms the basis of the pyridine nucleotide assay procedure of Andersen and von Meyenberg (1), which utilizes [carboxy-\(^{14}\text{C}\)]nicotinate. The proton-decoupled \(^{13}\text{C}\) NMR spectrum of the cells grown aerobically after harvest and resuspension in medium and observed under anaerobic conditions reveals two resonances which are assigned to free nicotinate and to oxidized pyridine nucleotide (Fig. 3A). Based on the ratios of NAD\(^+\)/NADPH reported in the literature for E. coli (1, 26, 29), the latter resonance is assumed to correspond almost completely to NAD\(^+\). The absence of a resolved resonance corresponding to reduced pyridine nucleotides can be interpreted in several ways: (a) under the conditions of the present study, the total concentration of reduced pyridine nucleotide is too low to permit direct observation; (b) chemical exchange between oxidized and reduced pyridine nucleotides is sufficiently rapid to permit only the observation of a single resonance with a chemical shift equal to the weighted average of the reduced and oxidized forms; (c) chemical exchange between reduced and oxidized pyridine nucleotides is intermediate on the NMR time scale so that the resonance corresponding to the reduced nucleotides is selectively broadened (Equations 1 and 2) (as discussed in the previous section, this interpretation can explain the selective broadening of the NADH, but not the NADPH resonance); (d) resonances of the reduced pyridine nucleotides are selectively broadened as a consequence of chemical exchange with enzyme-binding sites; (e) ratios of oxidized/reduced pyridine nucleotides in the literature which are based on extraction techniques are not applicable under the growth conditions used or are incorrect, and in particular give excessively high ratios of reduced/oxidized nucleotide.

Several of the possibilities noted above are readily tested. For an intermediate exchange rate as suggested by (c) above,
the NMR time scale can be changed by observation of carbons with different values of δ (oxidized − reduced). Thus, for C-2, the value of δ is 1.5 ppm, while for C-5 the value is 23 ppm. 13C spectra of cells cultured in medium which contained either [2-13C] or [5-13C]nicotinate are shown in Fig. 3, A and B, respectively. The results for both studies are identical, and we believe that these data tend to rule out the possibility of intermediate chemical exchange between oxidized and reduced pyridine nucleotides as noted above in (c). The second alternative explanation can be evaluated by observation of the spectrum under conditions which alter the reduced/oxidized pyridine nucleotide ratios. Several perturbations were introduced with this objective including addition of ethanol (Fig. 3C) and observation of anaerobically grown cells (Fig. 3D). Spectra obtained in both cases were equivalent to spectra obtained for cells grown aerobically on glucose and observed either aerobically or anaerobically.

As discussed previously (2), we were ultimately able to produce a more highly reduced pyridine nucleotide pool by culturing E. coli aerobically on glycerol as the sole carbon source and observing the cells by NMR in the glycerol-containing medium anaerobically (Fig. 4, A and B). Under these conditions, a separate resonance corresponding to reduced pyridine nucleotide is observed. In principle, this resonance may contain a significant contribution from both NADPH and NADH. However, quantitation of the spectra indicates that the increased intensity of the reduced resonance is matched by a reduced intensity of the oxidized resonance. Since the latter is ascribed primarily to NAD⁺, the former presumably arises from NADH. This is also consistent with the assumption that since the triphosphopyridine nucleotide pool is believed to be highly reduced under all conditions, increased reduction of this pool would not be expected to produce dramatic spectral changes. In conclusion, this series of experiments suggests that: (a) exchange is slow enough to permit observation of separate resonances from reduced and oxidized nucleotides; and (b) triphosphopyridine nucleotide resonances are not observed, either as a consequence of very low concentrations under the conditions of the present studies, or as a consequence of complexation with cellular enzyme-binding sites. As a consequence of these conclusions, we arrive at the important result that the NMR measurement appears...
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to be selective for the diphosphopyridine nucleotides, and can provide an in vivo value for the CRC, subject to the sensitivity limitations of the method.

The high CRC reflected in Fig. 4B represents a significant deviation from the normal value, reflecting the fact that E. coli will not grow anaerobically using glycerol as the sole source of carbon and energy (34). Under such anaerobic conditions, an alternative exogenous hydrogen acceptor such as fumarate or nitrate must be supplied (35, 36). If the cells are not allowed to ferment for an excessive period of time under these conditions, the subsequent addition of fumarate restores the CRC value to a lower, more typical level (Fig. 4C). The fumarate is gradually consumed with the concomitant production of succinate (Fig. 4D). Thus, the observation of a reduced pyridine nucleotide resonance in E. coli can be achieved for relatively short periods only with the creation of an environment which will not support growth.

Pyridine Nucleotide Metabolism in S. cerevisiae: Effects of Oxygen Deprivation—The proton-decoupled $^{13}$C NMR spectra obtained for the yeast cells S. cerevisiae differ significantly from the spectra obtained for the bacterial cells from the standpoint of the intensity of the background resonances arising from unlabeled cellular materials (Fig. 5, A and B). As shown previously for studies of Candida utilis (37), the $^{13}$C spectrum for unlabeled cells is dominated by resonances which can be assigned to polysaccharides and monosaccharides (60–110 ppm) and to unsaturated fatty acids presumably arising primarily from storage triglycerides. Consequently, $^{13}$C-labeling studies must be designed to minimize overlap with these background resonances. $^{13}$C spectra of S. cerevisiae grow as described under "Materials and Methods" with [2-$^{13}$C] or [5-$^{13}$C]nicotinate in the medium are shown in Fig. 5, A and B, respectively. As is readily apparent from these spectra, the background resonances exhibit intensities similar in magnitude to the intensity of the $^{13}$C-enriched pyridine nucleotide resonances. The C-2 label is optimal from the standpoint of having minimal overlap with the background resonances; however, the resolution between reduced and oxidized resonances is limited (Table I). In contrast, the oxidized pyridine nucleotide resonance observed with the [5-$^{13}$C]pyridine nucleotide resonance overlaps the fatty acid olefinic carbon resonances, making quantitation difficult.

In sharp contrast with the results described above for E. coli, a reduced pyridine nucleotide resonance is readily observed in the S. cerevisiae, the intensity of which is sensitive to the addition of various metabolites and to oxygen deprivation. This behavior is illustrated in Fig. 6 corresponding to a series of studies in which O$_2$ and N$_2$ were alternately supplied to the bubbler. There is a transition from a spectrum in which the reduced pyridine nucleotide resonance is below noise level (Fig. 6B), to a spectrum with roughly equal intensities for the oxidized and reduced peaks coincident with the transition from O$_2$ to N$_2$ (Fig. 6C). The spectrum reverts to the original pattern upon resumption of O$_2$ bubbling (Fig. 6E). Quantitation of the resonances suggests that total intensity of reduced + oxidized nucleotides is maintained nearly constant so that the reduced resonance presumably corresponds to NADH (Fig. 7).

As in the studies with E. coli discussed above, we are again faced with an interpretational problem relating to the assignment of the resonances to di- and triphosphopyridine nucleotide pools and, in this case, to mitochondrial and cytosolic pools as well. The absence of a reduced pyridine nucleotide resonance in oxygenated cells, coupled with the assumption that the triphosphopyridine nucleotide pool is $\geq$50% reduced, suggests that the levels of triphosphopyridine nucleotides either are too low to permit their detection using this technique, or are selectively broadened as a consequence of complexation with cellular enzyme-binding sites. In either case, the NMR data are then interpretable in terms of the diphosphopyridine nucleotide pool and presumably provide a measure of CRC = $[\text{NADH}]/([\text{NADH}]+[\text{NAD}^+]$).

There are reports of both large and small changes in CRC value under anaerobic conditions. For example, it has been reported that the NAD$^+$/NADH ratio of the cytoplasmic pool falls from 1000:1 to 400:1 in yeast made anaerobic (38). Such changes would be well below those observed in the present studies. Alternatively, the NAD$^+$/NADH ratio in another eukaryote, Tetrahymena pyriformis, dropped from 10:1 to 0.23:1 in the transition from a shaken to a static culture (27). In view of the broad range of values reported in the literature, it does not appear possible at this time to ascribe the observed NMR signals to either the cytosolic or mitochondrial pool, or to some weighted average. Additional studies with isolated mitochondria may provide information useful in separating these two pools. One possible hypothesis is based on the identification of the behavior of the mitochondrial pool with the behavior observed in the E. coli, in which case the observed changes would be interpreted to correspond to CRC changes of the cytosolic pool.

In addition to the changes in the reduced/oxidized ratio, the spectra show evidence of formation of an additional resonance 1.6 ppm downfield of the NAD$^+$ peak. Based on the data in Table I, this resonance is tentatively assigned to either nicotinate mononucleotide or to nicotinate adenine dinucleotide (deamino-NAD$^+$). The latter metabolite has been reported to accumulate in eukaryotes synthesizing NAD$^+$ from nicotinate (39).

Response of Pyridine Nucleotide Reduction Charge to Other Perturbations—In addition to the response to hypoxic condi-

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![Image: Proton-decoupled $^{13}$C NMR spectra of yeast (S. cerevisiae) cells cultured on a medium containing (A) [2-$^{13}$C] nicotinate and (B) [5-$^{13}$C]nicotinate. In addition to the resonances corresponding to the labeled NAD(P)$^+$, resonances corresponding to the C-1 of trehalose(a-D-glycopyranosyl-a-D-glycopyranoside) and to C-1 and C-2 of ethanol are observed in the spectra.](image-url)
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A) ANAEROBIC

B) $O_2$ BUBBLED

C) $N_2$ BUBBLED

D) $O_2$ BUBBLED

**Fig. 6. Cycling of the pyridine nucleotide reduction charge in response to alternate bubbling with $O_2$ and $N_2$.** The steady increase in the reduction charge (ascribed to the CRC as discussed in the text) under anaerobic conditions is apparent from A. Subsequent bubbling with $O_2$ (B) leads to a rapid (<several minutes) reoxidation of the pool so that no significant resonance intensity corresponding to the oxidized nucleotides is apparent. Substitution of $N_2$ (C) again leads to a progressively increased reduction of the pyridine nucleotide pool, while $O_2$ bubbling (D) again produces a highly oxidized pool.

Additions discussed above, the NMR reduction charge in *S. cerevisiae* was also found to be sensitive to other perturbations. Addition of ethanol leads to a transient increase in the reduction charge (Fig. 8). This response presumably reflects the reduction of the diprophosphorpyridine nucleotide pool as a consequence of the alcohol dehydrogenase (cytosolic) and the acetaldehyde dehydrogenase (mitochondrial) reactions. Effects of alcohol on the catabolic reduction charge have been widely evaluated in mammalian liver (40,41) based on observations of the metabolic flux.

The pyridine nucleotide reduction charge has also been reported to be sensitive to the addition of uncouplers of oxidative phosphorylation (42). This behavior was tested in anaerobic cells which exhibit a high reduction charge (Fig. 9). Addition of 2,4-dinitrophenol results in a rapid drop in the NADH/NAD$^+$ ratio as anticipated (42).

**CONCLUSIONS**

The magnetic resonance approach developed in the present series of studies represents an alternative to the use of fluorescence measurements for the characterization of intracellular pyridine nucleotide metabolism (25, 43, 44). Although the latter technology offers a significant sensitivity advantage, there are a number of important advantages to the NMR measurements which include: 1) the observation of both reduced and oxidized nucleotides as well as other metabolites such as free nicotinate; 2) the capability of making observations in cells containing other strongly fluorescent molecules such as hemoglobin or myoglobin which would interfere with the reduced pyridine nucleotide determination; and 3) the potential for simultaneous characterization of the pyridine nucleotide reduction charge, which can be an important metabolic regulatory parameter, and the metabolic flux using $^{13}$C-labeled metabolites. The latter aspect of these studies can be of particular value for evaluating the regulatory role of the pyridine nucleotides in cellular metabolism.

In applying either the fluorescence or the NMR approach, the actual physical state of the molecules under observation modulates the observation. Such effects provide a basis for characterizing the physical behavior of intracellular metabolite pools, but can also lead to errors in quantitation if not adequately taken into account. Thus, it has been established that enzyme-complexed NAD(P)H is more fluorescent than uncomplexed NAD(P)H (45). This result reflects primarily the different conformational distributions of free (more probably folded) and enzyme-complexed (unfolded) species, in conjunction with the quenching of the NAD(P)H fluorescence.
FIG. 7. Quantitation of the experiment illustrated in Fig. 6. We note that there is approximate conservation of intensity of the reduced and oxidized resonances, this conclusion being limited by the incomplete resolution of the peaks.

FIG. 8. Effects of the addition of 10 mM ethanol on the NMR reduction charge observed in yeast. Levels of NAD(P), NAD(P)H, and a third metabolite, tentatively identified as nicotinate mononucleotide (NaMN) in response to an addition of 10 mM ethanol at 80 min are indicated. Each spectrum represents a 20-min accumulation.

by the adenine base (46). Consequently, changes in fluorescence resulting from increased reduced pyridine nucleotide levels are not readily separated from changes resulting from increased enzyme complexation. In the case of NMR, the broader resonances associated with enzyme-complexed metabolites will be unobservable relative to the sharper resonances arising from uncomplexed metabolites. From the standpoint of metabolic regulation, the latter situation is preferable since kinetic and thermodynamic analysis of metabolic regulation is based on concentrations of free cellular metabolites. From this standpoint, the NMR measurement may also be superior to extraction techniques which can give erroneously high values for metabolites reflecting both free and enzyme-complexed forms. A limitation of this nature has been reported in studies of cellular ADP levels, reflecting the fact that a large fraction of the extracted ADP is believed to be derived from actin-complexed ADP and hence is not representative of the free nucleotide pool (47). More generally,
the acid (base) lability of the reduced (oxidized) pyridine nucleotides represents a serious practical difficulty in the quantitation of intracellular pools using the extraction approach, and may account for much of the variability found in the literature (48, 49).

Although the selective broadening of the NMR resonances corresponding to enzyme-complexed metabolites may be viewed as beneficial, the intermediate exchange rates which can broaden resonances corresponding to "free" metabolites represent a significant potential limitation of the method. As discussed in this study, chemical exchange between free and oxidized nucleotides appears not to present a serious problem in the studies carried out to date, with such exchange believed to be "slow" on the NMR time scale. However, the absence of significant reduced pyridine nucleotide resonances in oxygenated E. coli and S. cerevisiae cells suggests the possibility of a significant exchange broadening effect. Thus, based on literature values, the reduced resonance should correspond to 10–20% of the intensity of the oxidized resonances. Such reduced resonances would reflect primarily the NADPH pool. The absence of such resonances in the aerobically grown and observed cells, coupled with the observation of significant reduced resonances in the S. cerevisiae under a variety of conditions, is most readily interpreted on the assumption that the NADPH resonances are selectively broadened as a consequence of complexation with cellular enzymes. In this event, reduced resonances which are observed in S. cerevisiae upon oxygen starvation, addition of ethanol, or other perturbations correspond primarily to NADH so that the NMR is measuring a parameter which is most nearly related to the state of the diphostopyridine nucleotides and hence provides a measure of the CRC. This conclusion suggests that the NMR method provides a good complement to the fluorometric approach which, as a consequence of the larger fractional contribution of the NADPH to the reduced nucleotide pool, is more sensitive to the status of the triphosphopyridine nucleotide pool.

The dramatic differences observed between the prokaryotic E. coli and the eukaryotic S. cerevisiae cells in these studies were largely unanticipated. Wimpenny and Firth (50) had reported the CRC in Klebsiella aerogenes and E. coli rising to 0.6. More recently, Bautista et al. (26) have reported NADH/NAD⁺ ratios of 0.19 and 0.46 for glucose- and acetate-grown E. coli, respectively, corresponding to CRC values of 0.16 and 0.32. Based on the interpretation that the NMR measurements are providing a CRC value, the present results are closest to the 0.05 value reported by Andersen and von Meyenburg (1).

These results raise several important points regarding the regulatory role of the pyridine nucleotides. By definition, a regulatory metabolic parameter must be sensitive to some set of environmental perturbations in order to mediate the appropriate (regulated) cellular response. Clearly, a static parameter cannot fulfill a regulatory role. Thus, a parameter which is itself tightly regulated cannot be considered to be regulatory. The data presented here suggest that while the catabolic reduction charge may readily be interpreted as a regulatory parameter in S. cerevisiae, this may not be the case in E. coli. Two alternatives exist to this interpretation. (a) In E. coli, the CRC may be regulating at values outside the detection range of the NMR study, e.g. the sensitive range may be for CRC values bounded by 0.01 and 0.1 which could not be reliably measured. However, the data obtained by Andersen and von Meyenburg (1) tend to eliminate this possibility since they have similarly found that the CRC values were nearly always maintained close to 0.05. (b) A second possibility is that the regulation may be so sensitive that very small changes in the CRC, for example from 0.05 to 0.08, have a significant regulatory impact. This interpretation has been advanced to explain the analogous behavior of the adenylate energy charge which is nearly invariant across the nutritional range from adequacy of substrate to near starvation (51). Thus, Atkinson and Walton (51) argue that the steepness, at intersection, of the ATP production and utilization curves leads to a high sensitivity to this parameter.

It is interesting in the context of the above discussion to evaluate the proposed regulatory role of the anabolic reduction charge in determining the flux through the glyoxylate shunt. Machado and co-workers (26, 27) have suggested that the higher reduction charges which characterize growth on an acetate carbon source inhibit isocitrate dehydrogenase, consequently favoring the cleavage of isocitrate by isocitrate lyase, the committed enzyme of the shunt. However, more recent data obtained by LaPorte and Koshland (52) indicate a primary role for enzyme phosphorylation of isocitrate dehydrogenase in determining the flux through the shunt.

The question of regulatory versus regulated behavior is clearly one of degree and not subject to simple interpretation. Regardless of the significances of the large differences in behavior of the CRC between E. coli and S. cerevisiae, it is still necessary to develop an understanding of the differing molecular mechanisms, K₎ values, NADH-dependent enzymes, etc. which from a mechanistic standpoint lead to the dramatic differences in CRC behavior. Additionally, it is important to determine whether the present results are confined to the microorganisms observed or whether they reflect more fundamental prokaryotic/eukaryotic differences.

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