**Pseudomonas** species MA was grown with methylamine as a sole source of carbon and nitrogen enabling the total flow of carbon and nitrogen into this organism to be simultaneously monitored *in vivo* using $^{13}$C and $^{15}$N NMR. $^{13}$C Methylamine was rapidly and extensively incorporated into the methyl group of $N$-methylglutamate during high oxygenation of the cell suspension, but when the oxygenation rate was lower, a significant portion was also found in the methyl group of $\gamma$-glutamylmethylamide. At later times the carbon label was found in intermediates of the serine assimilation pathway, with glutamate derived from the tricarboxylic acid cycle being the most abundant product. Incorporation of $[^{15}\text{N}]	ext{methylamine}$ was only detected as $N$-methyl$[^{15}\text{N}]\text{glutamate}$, but when protein synthesis was inhibited, the label was also detected in the amino nitrogen of glutamate. When oxygenation rates were lower, the $^{15}$N-labeled methylamine was found in the methylamide group of $\gamma$-glutamylmethylamide in addition to being incorporated into $N$-methylglutamate. $\gamma$-Glutamylmethylamide formation was linked to the overall energy state of the cell and was not affected by inhibition of the carbon assimilation pathway. Neither 5-hydroxy-$N$-methylpyroglutamate nor $N$-methyl-$\alpha$-ketoglutarate were detected to any significant extent. A mechanism was proposed for the role of $\gamma$-glutamylmethylamide in the regulation of endogenous nitrogen supplies in this organism.

*Bacteria capable of methylamine utilization may be grouped into two types depending on the means of methylamine cleavage. Members of one group possess an enzyme that directly cleaves methylamine into formaldehyde and ammonia (8). Members of the other group including *Pseudomonas* MA do not possess such a direct cleavage enzyme and have a complex pathway of methylamine utilization (Fig. 1). Methylamine combines with glutamate in two different ways to produce $N$-methylated amino acids. In the formation of $N$-methylglutamate, methylamine displaces the original amino group of the glutamate molecule (1). The product is oxidized, resulting in the cleavage of the original methylamine carbon-nitrogen bond, regenerating glutamate, and producing formaldehyde, which is assimilated by the isocitrate lyase (+) variant of the serine pathway, providing the organism with both cell carbon and energy (10, 11).

A different reaction of methylamine with glutamate yields $\gamma$-glutamylmethylamide (12). The metabolism of $\gamma$-glutamylmethylamide within this organism is less clearly understood. No evidence exists for oxidative cleavage of the methylamine moiety in this compound, nor does there appear to be any mechanism for the transfer of the methylamine group to other metabolites. It is believed that the formation and metabolism of $\gamma$-glutamylmethylamide acts as a temporary storage sink for methylamine, but it may also have a role in attenuating the nitrogen flow into the central metabolism of the organism. Such a mechanism appears to be necessary due to the unsuitably high nitrogen:carbon ratio imposed by the growth medium.

In addition to these reactions with glutamate, methylamine can also react with $\alpha$-ketoglutarate, catalyzed by 5-hydroxy-$N$-methylpyroglutamate (HMPG)$^\dagger$ synthase (13). Although the end product of this reaction is 5-hydroxy-$N$-methylpyroglutamate (HMPG), it has been demonstrated (14) that $N$-methyl-$\alpha$-ketoglutarate is formed first and is also the true substrate for the reverse reaction, indicating that HMPG is in chemical equilibrium with $N$-methyl-$\alpha$-ketoglutarate. Whereas the role and function of $N$-methylglutamate is clearly defined, no function has yet been ascribed to these other $N$-methylated compounds in the metabolism or assimilation of methylamine.

The original metabolic studies with methylamine were done using traditional $^{14}$C pulse-chase techniques. In order to gain further insight into the roles of the $N$-methylated amino acids in this organism we used the *in vivo* NMR technique with both $[^{13}\text{C}]$- and $[^{15}\text{N}]$methylamine. In this study, the flow of $^{15}$N from methylamine was monitored in a conventional spectrometer, using similar time frames and acquisition conditions to those used in $^{13}$C NMR experiments. This enabled the data from both sets of experiments to be correlated, providing an additional insight into the roles of methylamine metabolism.

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$^1$The abbreviations used are: HMPG, 5-hydroxy-$N$-methylpyroglutamate; NOE, nuclear Overhauser enhancement.
unique insight into the carbon and nitrogen flow from methylamine in this organism.

**MATERIALS AND METHODS**

*Cell Growth and Cell Extract Preparation—Pseudomonas MA* was grown in a minimal salts medium supplemented with 50 mM methylamine, pH 7.2-7.5 (15). 1-Liter cultures were incubated overnight at 30 °C in 2-liter shake flasks equipped with baffles. The cells were harvested by centrifugation at 4 °C and resuspended in ice-cold minimal medium + 50 mM potassium phosphate, pH 7.6, to a final cell density of 255 mg dry weight/ml. This suspension, 2.5 ml, was used for each NMR experiment. I5N- or I3C-labeled methylamine hydrochloride was added to a final concentration of 150 mM after the cells had reached thermal equilibrium in the probe. The cells were oxygenated at a rate of 12 or 25 ml/min by means of a thin polyethylene tube extending to the bottom of the sample. Oxygen bubbling may have been the cause of the somewhat broad resonances observed in the 13C spectra because of disruption of sample homogeneity and paramagnetic relaxation of the observed nuclei. Although this broadening was less than desirable we were still able to obtain valuable information from the spectra. Oxygenation rates were monitored with an in-line Gilson flow meter. A coaxially mounted 5-mm tube containing 1 M H3NO3 in D2O was used as a reference and lock for 15N experiments, whereas a similar arrangement containing neat C6D6 was used for 13C experiments. Relative intensity values for 13C resonances were obtained by comparing integrals of the relevant resonances with that of the reference deuterobenzene. Where overlap of two resonances occurred, a curve-fitting program was used to calculate the contributions from each signal. This procedure enabled relative intensities of the same metabolite to be compared in different experiments, since the acquisition parameters were identical in each experiment. However, due to the differential effects of the spectral parameters on the signals from the individual carbons and the different degree of nuclear Overhauser enhancement, it is not possible to obtain absolute concentration data within a given experiment for each compound.

Cell extracts were prepared by rapidly mixing the NMR sample with 2.5 ml of 30% perchloric acid and immersing the mixture in liquid nitrogen. The mixture was taken through two freeze-thaw cycles and the pH adjusted to 7.0 by addition of 5 M KOH. The cell debris and the potassium perchlorate precipitate produced were pelleted by centrifugation at 13,000 × g for 10 min. The supernatant was freeze-dried and resuspended in water containing 30% D2O and

**FIG. 1.** Pathways of methylamine utilization in *Pseudomonas* species MA. Enzymes depicted are: 1, N-methylglutamate synthase; 2, γ-glutamylmethylamide synthetase; 3, N-methylglutamate dehydrogenase; 4, 5-hydroxy-N-methylpyroglutamate synthase; 5, serine hydroxymethyltransferase; 6, proposed γ-glutamylmethylamide transaminase. Reaction 7 is nonenzymatic.

**FIG. 2.** *A,* time course of [13C]methylamine metabolism by a suspension of *Pseudomonas* MA cells at a rate of oxygenation of 25 ml/min. Spectra were obtained with 2000 scans, the free induction decay zero-filled to 16 K and processed with 6 Hz of line broadening. The probe temperature was maintained at 30 °C throughout the experiment. The times shown represent the midpoint of the interval for each set of accumulations. Each spectrum took 13 min to collect; the interval between sets was 2 min. *B,* metabolism of [15N]methylamine by a suspension of *Pseudomonas* MA with 25 ml/min oxygenation rate. Spectra were obtained with 820 scans, the free induction decay zero-filled to 16 K and processed with 4 Hz of line broadening. Experimental conditions and acquisition times were as given for *A.*
When the oxygenation rate was reduced from 25 to 10 ml/min (Fig. 3A), a new resonance was observed in addition to N-methylglutamate. This was identified as belonging to γ-glutamylmethylamide (Fig. 3B). An additional resonance slightly downfield of methylamine was also detected, which could not be assigned on the basis of its chemical shift because of coinciding resonances from other compounds, (see Table I) but was postulated to be that of γ-glutamylmethylamide.

Under both these sets of conditions, there was no significant production of formaldehyde, formate, or bicarbonate from the oxidation of N-methylglutamate. This indicates that this step is tightly coupled to subsequent steps that involve the metabolism of formaldehyde. Because of the high toxicity of formaldehyde, such a mechanism is obligatory for the organism under these growth conditions to prevent any accumulation of this product. This was identified in the low-field region in the spectra depicted in Figs. 2A and 3A remain unassigned but are most likely carboxylates of tricarboxylic acid cycle-derived metabolites. Low levels of label incorporation into these positions (3) and possible metal ion binding are causes of the low intensities of these resonances when compared to other label sites such as C-4 of glutamate. Their more prominent appearance in Fig. 2 compared with the other spectra supports our contentions that under these conditions the carbon flow into the tricarboxylic acid cycle is maximal.

The flow of label from methylamine into γ-glutamylmethylamide could not be properly monitored with 13C NMR, since the 13C chemical shift of the N-methyl group lies very close to that of methylamine and several other 13C resonances of tricarboxylic acid cycle as well as the N-methyl amino acids. The 15N shifts, however, are readily separable (see Table I), and by this method, the methylamine moiety can be unambiguously traced into both of the N-methylated amino acids. Fig. 2B shows the metabolism of 15N-labeled methylamine under highly oxygenated conditions. The N-methylglutamate resonance was the only predominant metabolite to be observed, indicating that under these conditions, the bulk of the methylamine was metabolized immediately through the oxidative pathway and into the tricarboxylic acid cycle. Although oxidation of N-methylglutamate results in the formation of 15N-labeled glutamate, this metabolite was not detected in our experiment. This was probably due to a rapid exchange and dilution of the label into other amino acids by transamination, and the ultimate incorporation of the label into proteins rendering it undetectable with our spectral parameters.

When 15N methylamine was introduced to poorly oxygenated cells (Fig. 3B), a new resonance was observed in addition to N-methylglutamate. This was identified as belonging to the amido nitrogen of γ-glutamylmethylamide (Table I). It was observed only after significant accumulation of N-methylglutamate had occurred, and its occurrence was relatively
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A transient. Its disappearance coincided with a brief increase in the methylamine resonance, which may indicate a pathway was operating for the conversion of the methylamide group of $\gamma$-glutamylmethylamide back to methylamine. In some spectra, a transient resonance at about 1 ppm upfield from the $\gamma$-glutamylmethylamide was detected, which only appeared after a significant accumulation of $\gamma$-glutamylmethylamide. This was tentatively assigned as the N-methyl-$\alpha$-ketoglutarate resonance (see Fig. 5C).

Fig. 5A shows $^{13}$C NMR spectra taken from cells treated with 50 $\mu$g/ml tetracycline at low oxygenation. Here, labeled N-methylglutamate accumulated as in the untreated cells, but the flow of carbon into the tricarboxylic acid cycle was impaired as seen by a greater fraction of label in the C3 metabolites derived from serine when compared with the tricarboxylic acid cycle products, i.e. glutamate (see Fig. 6, A and B). The appearance of free formaldehyde and formate indicated a shunting of carbon through a linear C1 oxidation pathway. The C1 oxidation pathway products were detected under both low and high (spectra not shown) oxygenation rates when tetracycline was present, indicating that the coupling of N-methylglutamate oxidation to formaldehyde assimilation had been disrupted. A similar result was obtained in these organisms when methotrexate was added to the cell suspension (data not shown). The oxygenation rate also had a marked effect on the flow of carbon through the C1 oxidation pathway. When low rates were used, the principal products detected were bicarbonate and formate, with only minor amounts of formaldehyde. However, during high oxygenation rates, high levels of formaldehyde were detected with little of the other products observed (see Fig. 7). Absolute amounts of formate and bicarbonate are undoubtedly higher than they appear in these spectra due to pulse saturation and low NOE. Also, during low oxygenation in the presence of tetracycline, a strong peak downfield of the methylamine resonance was observed. This resonance did not originate from any tricarboxylic acid cycle reactions, since under these conditions, very little labeling of any tricarboxylic acid cycle-derived metabolites occurred. On the basis of these observations, we believe that this resonance belonged to the methyl group of $\gamma$-glutamylmethylamide.

$^{15}$N NMR spectra of cells exposed to tetracycline (Fig. 5, B and C) showed prolonged accumulation of N-methylglutamate compared to the untreated cells, which is in good agreement with the $^{13}$C NMR data. During low oxygenation rates, the $\gamma$-glutamylmethylamide resonance accumulated over time and did not disappear in contrast to the result with the untreated cells. When the oxygenation rate was increased, this metabolite was not detected in vivo and was only a minor feature in the cell extract spectrum (Fig. 8). The appearance of glutamate labeled in its nitrogen was detected when the tetracycline-treated samples were highly oxygenated, even though the glutamate pool was low as seen by $^{13}$C NMR (see Fig. 5A). This suggests that nitrogen incorporation into proteins was hindered, resulting in the accumulation of the label in amino acids. These observations are consistent with the inhibitory effect of tetracycline on the binding of amino-acyl tRNA thus preventing protein synthesis from amino acids (18).

**DISCUSSION**

$^{15}$N NMR was successfully used to monitor the fate of $^{15}$N-labeled methylamine in a suspension of methylamine-grown *Pseudomonas* MA cells. The acquisition times and experimental conditions for the spectra were the same as for $^{13}$C NMR experiments, enabling the flow of the $^{15}$N label to be monitored over the same time course as the $^{13}$C methylamine carbon.

In previous *in vivo* $^{15}$N NMR studies, metabolism of the cells within the probe was kept to a minimum, and spectra were obtained after various periods of external incubation with the $^{15}$N precursor. In our study, the label was continuously monitored within a suspension of actively metabolizing cells enabling us to correlate our $^{15}$N spectra with those...
obtained from the $^{13}$C experiments. As a result, we were able conclusively to assign resonances for the methyl groups of $N$-methylglutamate and $\gamma$-glutamylmethylamide in the $^{13}$C NMR spectra despite the fact that several other resonances had similar chemical shifts. Also this method allowed a direct comparison of the $^{15}$N label to that of $^{13}$C as a means of monitoring in vivo metabolic processes.

Overall, the $^{15}$N NMR spectra had poorer signal-to-noise ratios than the $^{13}$C spectra. Our experiment, based on a single pulse sequence with continuous broadband decoupling, is most sensitive to small mobile molecules containing protonated nitrogens (6). This is generally the case for most amino acid nitrogens, although there are some exceptions (such as the imido nitrogen of arginine, for instance). Under these conditions, the NOE gain from proton decoupling results in a one-tenth of the $T_1$ value of a nucleus to prevent saturation of key metabolites produced during methylamine metabolism by Pseudomonas MA. In other organisms, there are high physiological concentrations of certain amino acids, for example alanine in many species of fungi, and glutamate in sporulating yeast and within heart tissue. These types of amino acid pools are potentially accessible to a $^{15}$N NMR.

In summary, although, using the same procedure as for $^{13}$C, the range of nitrogen-containing functional groups that can be observed in vivo is narrow, it does include most of the amino acid nitrogens. Our study demonstrated that nitrogen flow-through these metabolites can be readily monitored with this approach, provided that their concentration is sufficiently large enough to be detectable. Although the detection limit for $^{15}$N-labeled metabolites was higher than for the corresponding $^{13}$C-labeled compounds, it was well below the levels of key metabolites produced during methylamine metabolism by Pseudomonas MA.
study using a similar approach to ours, since previous studies with $^{13}$C NMR have shown that they represent a major portion of the overall metabolite pool (3, 4, 21).

In this study, we confirmed the conclusions of previous work that $N$-methylglutamate and $γ$-glutamylmethyramide are key metabolites in methylamine utilization by Pseudomonas MA and confirmed directly the amino group replacement mechanism for the synthesis of $N$-methylglutamate that was previously deduced by mass spectroscopic analysis (1). We have shown that in relation to the other metabolites, the intracellular steady-state concentration of $N$-methylglutamate is large and in our experiment was apparently only limited by the depletion of methylamine within our sample. The change in oxygenation rate did, however, have an effect on its rate of oxidation, resulting in a reduction of label flow into the icl$^-$/pathway. This indicates that the $N$-methylglutamate dehydrogenase step is the rate-determining step of the overall metabolism with respect to oxygen levels. This step results in the generation of formaldehyde, which rapidly reacts, as $N^6,N^{10}$-methylene-FH$_4$, with glycine to produce
serine catalyzed by serine hydroxymethyltransferase (10). Most glycine is formed by the transamination of glyoxylate and since no formaldehyde or any other C1 metabolites were observed under normal conditions, we can assume that there is always sufficient glycine available to assimilate the formaldehyde. When oxygen is scarce, the reduced tricarboxylic acid cycle activity ultimately leads to a reduction in the capacity to synthesize glycine, which in turn results in a decreased capacity for formaldehyde fixation. This would necessitate a decrease in the amount of formaldehyde generated from the oxidation of N-methylglutamate in order to prevent the accumulation of this toxic C1 metabolite hence N-methylglutamate increases.

The coupling of N-methylglutamate oxidation to C1 assimilation was disrupted by the presence of tetracycline. This was an unexpected finding, since our original reason for using this antibiotic was to stop the incorporation of amino-acids into proteins, which would result in accumulation of nitrogen into amino acid pools thereby making them more accessible to observation by 15N NMR. A similar approach was used in an 15N NMR study of nitrogen metabolism in Neurospora crassa, where cycloheximide was used for the same purpose (7). In our experiment, the carbon was also diverted from potential pathways of amino acid synthesis to a C1 oxidation pathway involving formate and bicarbonate (carbon dioxide), neither of which has a strong role in amino acid metabolism. As a result, we did not see strong labeling of any additional amino acid pools other than those observed in the absence of tetracycline. A linear pathway of formaldehyde oxidation has not previously been reported for this organism but is common in many other methylophors where it is the major energy-producing pathway (22). Energy production in Pseudomonas MA is via a cyclic pathway involving enzymes of the assimilation pathway and the tricarboxylic acid cycle (17). However, substantial levels of separate NAD-linked dehydrogenases specific for both formaldehyde and formate are induced in methylamine-grown Pseudomonas MA compared with succinate-grown cells. This could point to an alternative energy-producing pathway or the pathway may simply be present as a means of detoxification as has recently been postulated for methylotrophic yeast (23). The lack of substantial amounts of formate occurring under normal conditions tends to support the latter hypothesis.

Although N-methylglutamate was always found in high amounts during methylamine metabolism, γ-glutamylmethy-
ammonia was also found with $^{14}$C radioisotope methodology during initial studies on this and a related organism (1, 12), we can assume that the conditions within the low oxygenation NMR experiment most closely resemble those found in a culture flask. In a typical NMR sample, the cell density is usually 20-40-fold greater, so the oxygenation rate becomes an important factor in maintaining cell metabolism. The physiological role of $\gamma$-glutamylmethylamide in this organism is not clear, but it appears to be produced in response to lowered oxygenation conditions. It could have been produced under either low oxygenation conditions. It could be made in response to a decrease in methylamine metabolism through the N-methylglutamate dehydrogenase pathway, providing the methylamine with another metabolic sink. This hypothesis was not supported by the tetracycline inhibition experiments, where the carbon flow into the tricarboxylic acid cycle was blocked in the presence of the antibiotic under both high and low oxygenation rates, resulting in both cases in an accumulation of only N-methylglutamate. $\gamma$-Glutamylmethylamide was observed only during low oxygenation rates, indicating that its synthesis was probably not related to the levels of the other observable metabolites. The other key factor that is affected by the oxygenation rate is the overall energy state of the cell, which in *Pseudomonas* MA can affect the uptake of methylamine (24). Since the nitrogen/carbon ratio of methylamine is much higher than the generally accepted value for cell biomass (~1:4), there must be a mechanism for attenuating the intracellular nitrogen levels. It is known that *Pseudomonas* MA does not utilize exogenous ammonia in the presence of methylamine (25). The oxidation of N-methylglutamate generates free ammonia; since the levels of N-methylglutamate are high, this step is probably a major contributor to the endogenous ammonia pool. In contrast, further metab-
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