Role of Lysine and e-N-Trimethyllysine in Carnitine Biosynthesis

I STUDIES IN NEUROSPORA CRASSA*

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SUMMARY

A lysine-carnitine precursor-product relationship was envisaged. To test this hypothesis a series of isotopic labeling experiments was conducted with Neurospora crassa lysine auxotrophs. Following growth of such mutants on a simple defined medium lacking carnitine but supplemented with variously labeled test carnitine precursors, biosynthesized carnitine was subsequently isolated from the mold mycelium by ion exchange chromatography and examined for radioactivity. Radioactivity from Dl-[6-14C]lysine and Dl-[4,5-3H]lysine was incorporated into carnitine without dilution of specific activity, whereas radioactivity from Dl-[1-14C]lysine and Dl-[2-14C]lysine was not found in biosynthesized carnitine in significant amounts. In an experiment employing [e-15N]lysine, it was demonstrated that the e-N atom of lysine becomes the nitrogen atom of carnitine; there was some exchange of the [e-15N]amino group of lysine with 15N in the medium during carnitine synthesis. e-N-[methyl-3H]Trimethyl-L-lysine was incorporated into carnitine without dilution of specific activity; and, as expected [methyl-3H]methionine also labeled carnitine, but with markedly less efficiency (0.23%) consistent with dilution by endogenous methionine. [1,14C]γ-Butyrobetaine (4-N-trimethylamino[1-14C]butyric acid), but not (4-amino[1-14C]butyric acid), was readily utilized (9.4%) for carnitine formation, in agreement with its established role as a carnitine precursor in animals. From these and other considerations it is postulated that in Neurospora the biogenesis of carnitine derives from the amino acids lysine and methionine in which either free or bound lysine is successively methylated to give e-N-trimethyllysine which is then cleaved in a series of as yet unknown transformations to lose carbon atoms 1 and 2 yielding γ-butyrobetaine. The latter compound is then hydroxylated at the β position to give carnitine as previously shown by other workers in the field.

Although it has been well established that γ-butyrobetaine is a precursor of carnitine in animals (1-3) and that methionine is the penultimate source of the N-methyl groups (4, 5), the biosynthetic origin of the butyrate carbon chain and γ-nitrogen atom of γ-butyrobetaine has been obscure. e-N-Trimethyllysine and δ-hydroxy-e-N-trimethyllysine have recently been discovered in diverse sources in nature (cf. Ref. 6 for references). Our attention was drawn to the structural features these compounds have in common with γ-butyrobetaine and carnitine, respectively, and led us to consider the possibility that γ-butyrobetaine, and consequently carnitine, might derive from lysine metabolism. To test the possibility of a lysine-carnitine precursor-product relationship, Neurospora crassa lysine auxotrophs were selected for study as such mutants could be grown in a carnitine-free synthetic medium with appropriately labeled test lysines. If the lysine-carnitine precursor-product relationship were true, the biosynthesized carnitine should have the same specific activity as the initial proffered isotopic lysine.

From experiments employing isotope incorporation and isotope dilution techniques, evidence will be presented that lysine, e-N-trimethyllysine, and γ-butyrobetaine are precursors of carnitine in Neurospora. The data support the conclusion that the entire lysine molecule, save C-1, C-2, and the α-N atom, is involved in the lysine → carnitine transformations. Brief accounts of certain of these studies have appeared (7-9).

EXPERIMENTAL PROCEDURE

Radioisotopes, Sources and Methods—L-[G-3H]Lysine, [H]-methyliodide, L-[methyl-3H]methionine, and γ-amino[1-14C]butyric acid were obtained from New England Nuclear. DL-[6-14C]-Lysine was purchased from Schwarz-Mann. DL-[1-14C]- and [2-14C]lysines were from Mallinckrodt Chemical Co. DL-[4,5-3H]Lysine was obtained by catalytic reduction with tritium gas (New England Nuclear) of DL-4,5-dehydrolysine. The latter was synthesized by reacting N-(4-bromo-2-butynyl)phthalimide (10) with ethyl sodium phthalimidomalonic ester (11) according to the procedures of Fink et al. (12). The [4,5-14H]lysine was purified on a Stein and Moore short column for basic amino acids (13), e-N[methyl-3H]Trimethyl-L-lysine was synthesized from α-acetyl-L-lysine (Calbiochem) and [H]methyliodide, and [1-14C]γ-butyrobetaine (4-N-trimethylamino[1-14C]butyric acid)
was synthesized from \(\gamma\)-amino\({}_{1-34}^{13}\)C]-butyric acid and CH\(_3\)I (Fisher), both according to the method of Mazzetti and Lemmon (14). Both compounds were radiochemically pure as judged by thin layer chromatography as described previously (7).

\([{1-15N}]\text{Lysine, Sources and Methods}--nL-\text{[e-15N]}\text{Lysine was synthesized employing potassium \([{25N}]\text{phthalimide (95.1 atom per cent excess 15N, Nuclear Equipment Chemical Co.) by the procedures of Fink et al. (12). Synthetic [e-15N]lysine ethyl ester was used to calculate the 15N enrichment of the synthetic [e-15N]lysine. This analysis showed that the product contained 89.6\% atom excess 15N. In an experiment to be described (Table IV) in which \(N. \text{crassa}\) strain 15069 was grown in medium containing \([e-15N]\)lysine, the mycelial proteins were subsequently hydrolyzed to release bound lysine which was isolated, converted to the ethyl ester, and analyzed for 15N enrichment as described above for synthetic \([e-15N]\)lysine. In this instance the ratio of the peaks at \(m/e \text{31 and 30} \text{ was first used to calculate the enrichment of the e-N atom of the bound lysine. The ratio of peaks at m/e 102 and 101 (corresponding to loss of the carboxylic moiety of lysine ethyl ester) and m/e 175 and 174 (the molecular ion) were then used to calculate the total 15N content of the lysine and by subtraction the 15N enrichment of the e-N atom of the lysine could be determined. The isolation and purification of bound \([15N]\)lysine is given in the legend of Table IV.

\(N. \text{crassa}\) Cultures, Source, and Maintenance—Cultures of \(N. \text{crassa}\) lysine auxotrophs strains 33933 and 15069 (Fungal Genetics Stock Center, Dartmouth College) were maintained on 2\% agar slants (Difco Neurospora culture medium) and transferred monthly. Mutant 33933 is blocked at an early step of the homocitrinate-amino-adipate pathway of lysine biosynthesis (16); mutant 15069 lacks saccharopine dehydrogenase (17) the final enzyme of this pathway.

Isolation of Carnitine from Neurospora Cultures, Assay Procedures—The experimental plan and specific procedures followed in much of this study are given in the legend of Fig. 1 in Reference 7, and in summary encompass the following steps: (a) growth of \(N. \text{crassa}\) cells in carnitine-free medium supplemented with lysine and variously labeled test precursors, (b) subsequent harvesting of the mycelium, (c) extraction of biosynthesized carnitine and hydrolysis of the O-acyl carnitine esters, (d) isolation of carnitine by cation exchange chromatography (18), and (e) determination of the radioactivity and carnitine content of appropriate column eluates. Radioactivity of these fractions, Step 6, was measured by removing 0.1-ml aliquots for liquid scintillation counting in a Packard Tri-Carb liquid scintillation spectrometer with the mixture employed previously (7). The efficiency of individual samples was determined by the external standard channels ratio method using standard solutions of toluene (New England Nuclear) quenched with chloroform. Carnitine was determined by carnitine acetyltransferase assay (19). From these data, the per cent incorporation of a given test isotope into biosynthesized carnitine could be made, and the specific activity of such carnitine could be determined. Figures depicting elution profiles of radioactivity versus carnitine acetyltransferase activity from Steps d and e above are included in only a few instances to conserve space.

RESULTS

Contribution of Lysine Carbon Atoms to Carnitine Formation—Table I summarizes a series of experiments in which the degree of utilization of various isotopically labeled lysines by \(N. \text{crassa}\) lysine auxotrophs for carnitine biosynthesis was studied. We have previously reported (7, 8) that \(nL-[6-14C]\)lysine, but not \(nL-[1,14C]\)lysine, was incorporated into carnitine by \(N. \text{crassa}\) strain 33933. The coincidence or absence of radioactivity in carnitine fractions following isolation of carnitine by ion exchange chromatography in these instances are given in Figs. 1 and 3 of Ref. 7 and are summarized as Experiments 1 and 4 of Table I herein. Two additional trials with \(nL-[6-14C]\)lysine were conducted (Experiments 2 and 3, Table I). The specific activity data in all three experiments illustrate that a moiety of lysine which includes C-6 is incorporated into carnitine without dilution. Experiment 3, Table I, which involved \(N. \text{crassa}\) mutant 15069 is worthy of note as this mutant lacks saccharopine dehydrogenase. Thus, intermediates of the homocitrinate-amino-adipate pathway of lysine biosynthesis that might possibly arise

TABLE I

| Experiment no. | Isotope | Specific activity* | Incorporated | Specific activity of carnitine |
|----------------|---------|-------------------|--------------|-------------------------------|
|                | µCi/mmol| µCi/l             | %            | µCi/mmol                      |
| nL-[6-14C]lysine | 1.25 | 500 | 0.22 | 1.20 | 1.25 |
| 2              | 0.53 | 290 | 0.09 | 0.52 | 0.33 |
| 3              | 0.19 | 150 | 0.10 | 0.17 | 0.19 |
| nL-[15N]lysine | 0.58 | 150 | 0.006 | 0.01 | 0.0 |
| nL-[14C]lysine | 0.25 | 100 | 0.01 | 0.02 | 0.0 |
| nL-[5,5-14C]lysine | 0.19 | 74.5 | 0.12 | 0.17 | 0.14 |
| nL-[15N]lysine | 0.19 | 74.5 | 0.16 | 0.16 | 0.14 |
| 8              | 1.25 | 250 | 0.15 | 0.80 | 0.60 |

* Calculated on the basis of the L component of the radioactive nL-lysine and on the L-lysine (0.2 mmole) given to the mold for growth purposes.

* Calculated on the hypothetical basis that C-3, C-4, C-5, and C-6 of lysine indeed contribute C-1, C-2, C-3, and C-4 of the butyric acid carbon chain of carnitine, respectively.

When the results of Table I were plotted as a function of specific activity of carnitine versus that of lysine, it now seems that the somewhat higher specific activity of the carnitine (1.65 µCi per pmol) versus the L-lysine, reported previously (7), then attributed to variations of salt concentration, was probably due to destruction of carnitine during desalting procedures. The values reported above were calculated from the amounts of radioactivity and carnitine obtained directly from the carnitine fractions of the modified Piez column.
ultimately contribute appreciably to the l-carbon pool which and C-6 of lysine indeed contribute the butyrate carbon chain of lysine. The observed specific activities of the biosynthesized carnitine. Such a postulation demands the loss of 2 hydrogen atoms from C-3 of lysine and loss of 1 hydrogen atom from C-5 of carnitine. Table I, the "anticipated specific activity" of biosynthesized carnitine was calculated on the postulation that C-3, C-4, C-5, and C-6 of lysine indeed contribute the N-methyl groups of carnitine.

Experiment was repeated and the data from these trials, together with the findings with DL-[6-3H]lysine (Experiments 1 to 3, Table I) or with results to be presented in trials with L-[4,5-3H]lysine or L-[G-3H]lysine (Experiments 6 to 8, Table I). It is possible that, in the lysine catabolism of N. crassa, C-1 and C-2 of the lysine molecule may ultimately contribute appreciably to the 1-carbon pool which in turn would be expected to label the N-methyl groups of carnitine.

Fig. 1. Ion exchange chromatography of N. crassa 33933 mycelial extract following growth on DL-[4,5-3H]lysine (A) or DL-[2-14C]lysine (B). For experimental details, see "Experimental Procedure" and Reference 7.

via reversal of this pathway are not involved in the lysine → carnitine transformations.

When N. crassa 33933 was grown with DL-[2-14C]lysine, the carnitine elution profile following ion exchange chromatography of the mycelial extract had perceptible radioactivity, Fig. 1B. But it is clear from the data of Table I, that the dilution of specific activity of either DL-[1-14C]lysine (Experiment 4) or DL-[2-14C]-lysine (Experiment 5) into carnitine is very marked (3% and 12-fold, respectively) in contrast to the findings with DL-[6-3H]lysine (Experiments 1 to 3, Table I) or with results to be presented in trials with L-[4,5-3H]lysine or L-[G-3H]lysine (Experiments 6 to 8, Table I). It is possible that, in the lysine catabolism of N. crassa, C-1 and C-2 of the lysine molecule may ultimately contribute appreciably to the 1-carbon pool which in turn would be expected to label the N-methyl groups of carnitine.

Fig. 1A illustrates the coincidence of radioactivity with carnitine fractions following ion exchange chromatography of mycelial extracts of N. crassa 33933 grown with DL-[4,5-3H]lysine. This experiment was repeated and the data from these trials, together with an experiment with L-[G-3H]lysine, are all summarized in Table I (Experiments 6 to 8). As indicated in Footnote b of Table I, the "anticipated specific activity" of biosynthesized carnitine was calculated on the postulation that C-3, C-4, C-5, and C-6 of lysine indeed contribute the butyrate carbon chain of carnitine. Such a postulation demands the loss of 2 hydrogen atoms from C-3 of lysine and loss of 1 hydrogen atom from C-5 of lysine. The observed specific activities of the biosynthesized carnitine in these instances (Experiments 6 to 8, Table I) are consistent with these assumptions, indeed the values are even slightly higher than anticipated. These differences may not be significant or, if so, could imply an isotope effect. In any event the utilization of DL-[4,5-3H]lysine in carnitine biosynthesis provides important evidence for the incorporation of C-4 and C-5 of lysine into carnitine, since the pattern of retention of the tritium label implies the participation of the respective carbon atoms in carnitine formation. Unfortunately lysine isotopically labeled at C-3 was unavailable for testing as a carnitine precursor in these studies.

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\text{\textit{e-N-Trimethyllysine and \gamma-Butyrobetaine as Precursors of Carnitine}}
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Evidence from isotope dilution experiments for involvement of e-N-trimethyllysine and \gamma-butyrobetaine in carnitine biosynthesis in Neurospora

Columns 1 and 2 indicate the specific activity and test level of the isotopic lysines on which Neurospora crassa 33933 was grown either with or without unlabeled test carnitine precursor (Column 3). The results tabulated in Columns 4, 5, and 6 are calculated from the amount of carnitine biosynthesized as determined by carnitine acetyltransferase assay and radioactivity of such carnitine following isolation by ion exchange chromatography (see "Experimental Procedure" and Reference 7 for details).

| Specific activity | Unlabeled test precursor | Biosynthesized carnitine |
|------------------|--------------------------|--------------------------|
|                  | Total                     | Yield                    | Incorporated specific activity \(\mu\text{mole}\) | Dilution specific activity % |
| e-N-Trimethyllysine | None                      | 0.50                     | 0.12                    | 0.16                    | 93 |
| L-[4-3H]lysine   | 0.19 74.5                 | 1.26                     | 0.02                    | 0.01                    | 93 |
| L-[G-3H]lysine   | 0.75 150                  | 0.35                     | 0.15                    | 0.30                    | 82 |

\(a\) Millicurie per mmole.
substrate for carnitine synthesis. This experiment was repeated with very similar results as shown in Experiment 2, Table III. The utilization of [6-14C]lysine (e.g. 0.22%, Experiment 1, Table I) for carnitine biosynthesis versus ε-N-[methyl-3H]trimethyllysine (e.g. 19%, Experiment 2, Table III) reflects the diverse functions of lysine for growth of N. crassa, only one of which is participation in carnitine formation, whereas trimethyllysine is a unique lysine derivative with more limited functions in metabolism, one of which is to serve directly in carnitine biosynthesis.

It seemed likely that the origin of the N-methyl groups of ε-N-trimethyllysine for carnitine biosynthesis would be methionine, particularly since it has been shown that the penultimate source of the N-methyl groups of carnitine in the rat is methionine (4, 5). The data of Fig. 2A show that radioactivity derived from the methyl group of methionine is associated with the carnitine fraction, but the extent of incorporation of radioactivity in this instance was only 0.23% (Experiment 3, Table III). Furthermore, the specific activity of the [methyl-3H]methionine was reduced over a 100-fold in the biosynthesized carnitine (Table III). This finding undoubtedly reflects the demand for the methyl group of methionine for diverse reactions of 1-carbon metabolism and that the methyl group of methionine is constantly being resynthesized de novo from nonradioactive 1-carbon precursors. In any event, it is clear that the incorporation of ε-N-[methyl-3H]trimethyllysine into carnitine (Experiments 1 and 2, Table III), is direct and does not involve demethylation and subsequent reutilization of the methyl groups via methionine by some other biosynthetic mechanism for carnitine synthesis. The data of Experiments 1 to 3 of Table III taken together suggest that methionine is the penultimate source of the methyl groups of carnitine in Neurospora, but that ε-N-trimethyllysine is the direct metabolite concerned in carnitine biosynthesis. The specific activity and test levels of the variously labeled test carnitine precursors on which Neurospora crassa 33933 was grown. The results tabulated in the last two columns of the table were calculated from the amount of carnitine biosynthesized as determined by carnitine acetyltransferase assay and radioactivity of such carnitine following isolation by ion exchange chromatography (see “Experimental Procedure” and Reference 7 for details).

| Experiment No. | Isotope | Incorporated | Specific activity carnitine |
|----------------|---------|--------------|-----------------------------|
| 1              | ε-N-[methyl-3H]trimethyllysine | 43 | 114 | 16 | 27.2 |
| 2              | ε-N-[methyl-3H]trimethyllysine | 43 | 34 | 19 | 10.2 |
| 3              | ε-N-[methyl-3H]methionine | 116 | 100 | 0.23 | 0.85 |
| 4              | [1-14C]γ-butyrobetaine | 1.64 | 12 | 9.4 | 0.84 |
| 5              | γ-Amino[1-14C]butyrate | 1.64 | 10 | 0 | 0 |

**Fig. 2.** Ion exchange chromatography of Neurospora crassa 33933 mycelial extracts following growth on L-[methyl-3H]methionine (A) or ε-N-[methyl-3H]trimethyl-L-lysine (B). For experimental details, see “Experimental Procedure” and Reference 7.

**Fig. 3.** Ion exchange chromatography of Neurospora crassa 33933 mycelial extract following growth on [1-14C]γ-butyrobetaine (A) or γ-amino[1-14C]butyric acid (B). For experimental details, see “Experimental Procedure” and Reference 7.
In this instance, the reduction of specific activity of [1-14C]γ-butyrobetaine was only about 50%, and the incorporation of radioactivity was 9.4% (Experiment 4, Table III). These data thus illustrate that γ-butyrobetaine is an excellent precursor of carnitine in *Neurospora*, in accord with the findings in animals that γ-butyrobetaine (γ, 3), but not γ-amino butyrate (1), is a precursor of carnitine, and make untenable the early postulation that γ-butyrobetaine might arise via successive methylation of γ-amino butyrate (20). These data are consistent rather with the view that γ-butyrobetaine derives from ε-N-trimethyllysine metabolism.

**Origin of Nitrogen Atom of Carnitine**—Based on the efficient incorporation of ε-N trimethyllysine into carnitine (Experiments 1 and 2, Table III) coupled with the pattern of lysine carbon atoms utilized for carnitine biosynthesis (Table I), it was attractive to speculate that the ε-N-atom of either free or bound lysine is successively methylated yielding ε-N-trimethyllysine and that the nitrogen atom of carnitine thus initially derives from the ε-N atom of lysine. To test this hypothesis [1-15N]lysine containing 69.6% 15N atom excess was prepared (cf. “Experimental Procedure”) diluted to 44.8% 15N atom excess with [14N]lysine, and *N. crassa* strain 15069 grown on carnitine-free medium containing such lysine. Mutant 15069 lacking saccharopine dehydrogenase was selected for this experiment to eliminate the possibility of lysine precursors figuring in the interpretation of the results. Carnitine was subsequently isolated from the mold mycelium and analyzed for 15N content as described in the legend of Table IV. The experiment depends on the alkaline degradation of carnitine to trimethylamine and analysis of the latter by combined gas-liquid chromatography-mass spectrometry.

The 70-e.v. spectrum of trimethylamine is published (21). The base peak at m/e 58 (C3H3N)⁺ corresponds to loss of one hydrogen from the molecular ion at m/e 59. Since the large peak at M-1 would interfere with the calculation of the 15N enrichment, the spectra were taken at 13 e.v. to minimize the M-1 base peak. The ratio of the peaks 60:59 was used to calculate a 20.3% enrichment in 15N in the trimethylamine and thus in the biosynthesized carnitine (Table IV).

Since [6-14C]lysine was incorporated into carnitine without dilution (Table I), it was surprising that the ε-N atom of lysine should be diluted in contributing to carnitine biosynthesis. It seemed important to establish the 15N content of the lysine of the cell proteins to serve as a control for the experiment. Accordingly, the *N. crassa* mycelium was hydrolyzed to release bound lysine which was isolated and analyzed for 15N enrichment as described in Table IV and under “Experimental Procedure.” As is shown in Table IV, no significant dilution of 15N into lysine of the mycelial proteins occurred by exogenous 15N of the medium. Hence it is concluded that in the lysine carnitine transformations a unique lysine intermediate exists which is in an environment such that the ε-N atom can readily exchange with nitrogen sources in the medium. This latter point will be more fully discussed below.

**DISCUSSION**

By using techniques of isotope incorporation and isotope dilution, it has been shown that a moiety of lysine is involved in the biosynthesis of carnitine in *N. crassa* lysine auxotrophs. About 0.2% of the lysine requirement of these mutants is demanded for the synthesis of carnitine which is presumably only needed in catalytic quantities by the cell. From the labeling patterns presented it seems reasonable that C-3, C-4, C-5, and C-6 together with N-6 of lysine constitute the butyric acid carbon chain and γ-N atom of carnitine, respectively, as shown in Fig. 4. Fig. 4 visualizes that either free or protein-bound lysine is successively methylated, yielding free or protein bound ε-N-trimethyllysine. If ε-N-trimethyllysine is indeed formed from protein-bound lysine, as for example by an appropriate S-adenosyl-methionine:protein-lysine methyltransferase (cf. Ref. 6 for reference), such bound trimethyllysine may then be released to give free ε-N-trimethyllysine. The latter is then subject to a series of reactions referred to as cleavage (Fig. 4), to yield γ-butyrobetaine which is then hydroxylated, finally giving carnitine.

The possibility that steps of carnitine biosynthesis involve bound lysine intermediates may figure in an explanation for the data of Table IV in which the course of the lysine carnitine transformations the ε-N atom of lysine exchanged with nitrogen sources in the medium. It is known that in the formation of desmosine a polyfunctional amino acid involved in cross-linking in elastin (22), protein-bound lysine, is oxidatively deaminated, yielding protein-bound 5-semialdehyde. If protein-

**TABLE IV**

| Material analyzed by gas-liquid chromatography-mass spectrometry | Atom excess found | Atom excess expected |
|---------------------------------------------------------------|------------------|---------------------|
| L-lysine in growth medium                                     | 44.8             |                     |
| Carnitineb from *N. crassa* 15069 grown on [1-15N]lysine     | 40.3             | 44.8                |
| Lysine from *N. crassa* 15069 mycelial protein                | 42.4             | 44.8                |

* Analyzed as the lysine ethyl ester.
  b Analyzed following degradation to trimethylamine.

*Neurospora crassa* strain 15069 was grown for 4 days in 0.1-liter volumes of carnitine-free synthetic medium supplemented with [1-15N]lysine·HCl (36.6 mg, 0.2 mM, 44.8% excess 15N in the ε position). Carnitine was isolated from extracts of mold mycelium by ion exchange chromatography (see “Experimental Procedure” and Reference 7 for details). Carnitine fractions were pooled and applied to a column, 1.2 × 15 cm, of AG 50W-X8 (200 to 400 mesh, H⁺ form). The column was washed with 100 ml of water, the carnitine was eluted with 1 N HCl, and such eluates were pooled and evaporated to dryness. Fifteen milliliter of 20% NaOH were added, and the solution was refluxed (24 hours) during which time a slow stream of nitrogen was swept through the flask into 20 ml of 2 N HCl to trap liberated trimethylamine. The HCl was evaporated; the trimethylamine hydrochloride dissolved in 20 ml of water, placed in a bottle with a conical bottom, and fitted with a septum. Just prior to assay by combined gas-liquid chromatography-mass spectrometry, 5 μl of 8 N NaOH was added to regenerate free trimethylamine. The 15N enrichment of the trimethylamine was assayed using an LKB combined gas chromatograph, mass spectrometer with an 18-inch column of 26%, Pennwalt 223 and 1%; KOH on Gas ehrm R (Pennwalt Co.). The oven was kept at 65°C, and the carrier gas (helium) flow rate was 2.5 ml per min. Following the isolation of [15N]carnitine above, the residual mycelium was hydrolyzed (0.5 N HCl, 24 hours), HCl was removed by flash evaporation, and the lysine in the hydrolysate was isolated by Stein and Moore ion exchange chromatography (13). The lysine fractions were pooled and desalted by applying to a column, 1.25 × 25 cm, of AG 50W-X8 (H⁺ form) and eluting with 300 ml of water and sufficient 1 N ammonia. The ammoniacal eluate was taken to dryness by flash evaporation; the lysine residue was converted to the ethyl ester (15) and analyzed by combined gas-liquid chromatography-mass spectrometry as outlined under “Experimental Procedure.”
Fig. 4. A postulated scheme of carnitine biosynthesis.

bound lysine consigned for carnitine synthesis is in an environment where such oxidation could take place, reamination of protein-bound aminoadipic semialdehyde by nitrogen donors in Neurospora cells would regenerate the lysine-carnitine precursor and account for the dilution of [1-14C]lysine in the experiment of Table IV. It is clear that the apparent exchange of the 14C atom of lysine with nitrogen sources in the cell is limited in Neurospora growth and metabolism, as [1-14C]lysine of the cell proteins in general was not significantly exchanged with 14N sources in the cell (Line 3, Table IV). It should be pointed out that in these studies the Neurospora cells were harvested 1 to 2 days after maximum growth was obtained to assure maximal carnitine synthesis. Hence, in this latter period, an opportunity is still being provided for exchange of 14N into carnitine but not into the mycelial proteins which could account in part for the findings of Table IV.

The nature of the postulated "cleavage reaction" (Fig. 4), wherein e-N-trimethyllysine is metabolized to γ-butyrobetaine, forms the basis of ongoing research in our laboratory. No evidence for a strict precursor-product relationship between e-N-trimethyllysine and γ-butyrobetaine was presented herein, as the data were not significant exchanged with 14N. Neurospora cells would regenerate the lysine-carnitine precursor where such oxidation could take place, reamination of protein-bound aminoadipic semialdehyde by nitrogen donors in Neurospora cells would regenerate the lysine-carnitine precursor and account for the dilution of [1-14C]lysine in the experiment of Table IV. It is clear that the apparent exchange of the 14C atom of lysine with nitrogen sources in the cell is limited in Neurospora growth and metabolism, as [1-14C]lysine of the cell proteins in general was not significantly exchanged with 14N sources in the cell (Line 3, Table IV). It should be pointed out that in these studies the Neurospora cells were harvested 1 to 2 days after maximum growth was obtained to assure maximal carnitine synthesis. Hence, in this latter period, an opportunity is still being provided for exchange of 14N into carnitine but not into the mycelial proteins which could account in part for the findings of Table IV.

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In preliminary studies, the hydroxylation of γ-butyrobetaine to carnitine in a cell-free extract of N. crassa 53963 was shown (25). The system had the same requirements as the rat liver hydroxylase system as recently described by Lindstedt et al. (26), namely oxygen, α-ketoglutarate, Fe++, and ascorbate. Further evidence for a commonality in steps of carnitine biogenesis between the fungal system described herein and the rat will be apparent from an accompanying paper (23) which establishes that lysine and e-N-trimethyllysine are precursors of carnitine in the rat as well.

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