Biosynthesis of Carnitine and 4-*N*-Trimethylaminobutyrate from Lysine

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The conversion of L-[U-¹⁴C]lysine into carnitine was demonstrated in normal, choline-deficient and lysine-deficient rats. In other experiments *in vivo* radioactivity from L-[4,5-³H]lysine and DL-[6-¹⁴C]lysine was incorporated into carnitine; however, radioactivity from DL-[1-¹⁴C]lysine and DL-[2-¹⁴C]lysine was not incorporated. Administered L-[Me-¹⁴C]methionine labelled only the 4-*N*-methyl groups whereas lysine did not label these groups. Therefore lysine must be incorporated into the main carbon chain of carnitine. The methylation of lysine by a methionine source to form 6-*N*-trimethyl-lysine is postulated as an intermediate step in the biosynthesis of carnitine. Radioactive 4-*N*-trimethylaminobutyrate (butyrobetaine) was recovered from the urine of lysine-deficient rats injected with [U-¹⁴C]lysine. This lysine-derived label was incorporated only into the butyrate carbon chain. The specific radioactivity of the trimethylaminobutyrate was 12 times that of carnitine isolated from the urine or carcasses of the same animals. These data further support the idea that the last step in the formation of carnitine from lysine was the hydroxylation of trimethylaminobutyric acid, and are consistent with the following sequence: lysine + methionine → 6-*N*-trimethyl-lysine → → 4-*N*-trimethylaminobutyrate → carnitine.

The ability of animal tissues to synthesize carnitine (3-hydroxy-4-*N*-trimethylaminobutyrate) was recognized many years ago. Fraenkel (1953) showed that carnitine content in the chick embryo increased severalfold during development. Methionine was the first compound shown to be involved in carnitine biosynthesis. Methyl-labelled methionine was slowly incorporated into the *N*-methyl groups of carnitine by the rat (Bremer, 1961; Strength *et al.*, 1965; Wolf & Berger, 1961), but the methyl acceptor was not determined. 4-Aminobutyrate was believed a likely precursor but its conversion into carnitine could not be demonstrated in the rat or mouse (Bremer, 1962; Lindstedt & Lindstedt, 1965). In the rat hydroxyproline, threonine, ornithine, acetate and glucose were not converted into carnitine (Wolf & Berger, 1961), and in the mouse 5-*N*-trimethylaminopentanoate or 6-*N*-trimethylaminohexanoate were not converted into carnitine (Lindstedt & Lindstedt, 1965). In mice, however, Lindstedt & Lindstedt (1965) found rapid hydroxylation of 4-*N*-trimethylaminobutyrate (butyrobetaine) to form carnitine; this reaction was catalyzed by the enzyme trimethylaminobutyrate (butyrobetaine) hydroxylase present in the soluble fraction of rat liver homogenates (Lindstedt, 1967).

Lysine was first shown to be a precursor of carnitine in lysine auxotrophs of *Neurospora crassa* (Horne *et al.*, 1971; Horne & Broquist, 1973); later, lysine was demonstrated to be a precursor of

carnitine in the rat (Tanphaichitr *et al.*, 1971; Tanphaichitr & Broquist, 1973). In the present study the role of lysine and methionine in the biosynthesis of carnitine was further defined. Lysine was found to be incorporated into both carnitine and 4-*N*-trimethylaminobutyrate in the rat and the results were consistent with the following sequence: lysine + methionine → 6-*N*-trimethyl-lysine → → 4-*N*-trimethylaminobutyrate → carnitine.

Materials and Methods

Materials

L-[U-¹⁴C]Lysine, DL-[6-¹⁴C]lysine, DL-[4,5-³H]lysine, L-[Me-¹⁴C]methionine and L-[Me-³H]methionine were purchased from Schwarz/Mann Radiochemicals, Orangeburg, N.Y., U.S.A. The DL-[1-¹⁴C]lysine was purchased from New England Nuclear Corp., Boston, Mass., U.S.A., and the DL-[2-¹⁴C]lysine, DL-[1-¹⁴C]carnitine and DL-[Me-¹⁴C]carnitine were from International Chemical and Nuclear Corp., Cleveland, Ohio, U.S.A. L-Carnitine chloride and the special rat diets were supplied by Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A. The lysine-deficient diet consisted of an amino acid test diet with lysine replaced by weight with sucrose, and the choline-deficient diet contained 20% (w/w) protein and not more than 0.6% methionine. The column resins AG-50 (X8;

200–400 mesh; H^+ form), AG-1 (X8; 200–400 mesh; Cl^- form), and AG-11A8 (50–100 mesh) were purchased from Bio-Rad Laboratories, Richmond, Calif., U.S.A. The resin AG-50 (X8) was converted into the Na^+ form and the resin AG-1 (X8) was converted into the OH^- form by the methods suggested by the supplier. Chloroplatinic acid was obtained from Fisher Scientific Co., Cleveland, Ohio, U.S.A.

The 4-*N*-trimethylaminobutyrate and 6-*N*-trimethyl-lysine and their radioactive derivatives were synthesized as described by Cox & Hopfel (1973).

Animals

Male weanling rats of the Wistar strain weighing (45–55 g) were obtained from Carworth Farms, New City, N.Y., U.S.A. The animals were housed in wire bottom cages and fed on either Purina Lab Chow, the lysine-deficient diet, or the choline-deficient diet for the periods described. All rats were given radioactive substances by intraperitoneal injection in a solution of 0.9% NaCl neutralized with $NaHCO_3$. Rats maintained on the special diets were injected with the radioactive compounds in equal doses once daily for 4 days, and were then killed on the fifth day.

Isolation of carnitine

Carnitine was extracted by a modification of the method of Pearson & Tubbs (1964). The rats were killed by decapitation, and the carcasses prepared by removing the skin, feet, tail and intestinal tract. The remains were then homogenized and extracted twice with 5% (w/v) $HClO_4$ (total 6 ml/g wet wt. of tissue). After the removal of the protein precipitate, the supernatant was adjusted to pH 13 with saturated KOH and kept at room temperature for 1 h to hydrolyse the acid-soluble acylcarnitines. After neutralization with 70% (w/v) $HClO_4$ the extract was cooled in ice for at least 1 h and then centrifuged. The carnitine was further purified by one of two procedures: method I or method II.

Method I. The neutralized extract was freeze-dried; the residue was extracted with water and acidified to a pH of less than 3.0 with conc. HCl. The acid extract (15–20 ml) was chromatographed through a jacketed column (1 cm × 50 cm) of resin AG-50 (X8; Na^+ form) at 50°C, eluted with a citrate buffer gradient described by Piez *et al.* (1956) and modified by Horne *et al.* (1971); the mixing flask contained citrate buffer, pH 4.1 (0.25 M- Na^+), and the reservoir contained 0.25 M- $NaOH$. The effluent was collected in 2 ml fractions. As shown in Fig. 1, carnitine, 4-trimethylaminobutyrate and 6-*N*-trimethyllysine were well separated by this system of elution. Fractions containing carnitine were combined and further purified by chromatography through a column

Fig. 1. Elution pattern of carnitine (○), 4-*N*-trimethylaminobutyric acid (■) and 6-*N*-trimethyl-lysine (△) from a resin AG-50 column

A solution containing 10 mg of each compound in 5 ml of water was chromatographed through a column (1 cm × 50 cm) eluted with the citrate buffer gradient described in the Materials and Methods section and 2 ml fractions were collected. Carnitine was assayed enzymically (Marquis & Fritz, 1964); 4-trimethylaminobutyrate was assayed by the periodide method (Wall *et al.*, 1960), and trimethyl-lysine was determined by the ninhydrin assay (Moore & Stein, 1954).

(1.5 cm × 10 cm) of resin AG-1 (X8; OH^- form) eluted with water. The fractions containing carnitine were desalted on a column (1 cm × 50 cm) of resin AG-11 A8 eluted with water or with a column (1 cm × 50 cm) of resin AG-50 (X8) eluted with 1.5 M-HCl.

Method II. In this method the resin AG-1 column adsorption was carried out before the extract was chromatographed on the AG-50 citrate buffer column. The neutralized extract (approx. 500 ml) was passed through a column (2.5 cm × 12 cm) of AG-1 (X8; OH^- form) and washed through with 50 ml of water. The effluent was acidified with HCl and freeze-dried; this material was extracted with 5 × 20 ml of hot ethanol, and the ethanol removed by evaporation. The resultant residue was taken up in 5 ml of water and chromatographed through an AG-50 (X8) column eluted with citrate buffer as described in method I. The carnitine fractions were desalted by using a column (1 cm × 50 cm) of AG-50 eluted with 1.5 M-HCl. On the desalting column, citrate was eluted in the first 60 ml and carnitine in 110–125 ml.

Radioactivity was measured by using a Packard Tri-Carb Model 3375 liquid-scintillation spectro-

meter. Samples were diluted to 0.5–1.0 ml with water and counted for radioactivity in 10 ml of scintillant composed of 16.7 g of 2,5-diphenyloxazole, 0.17 g of 1,4 bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 667 ml of toluene and 333 ml of Triton X-100. Carnitine was assayed enzymically by the method of Marquis & Fritz (1964).

Thin-layer chromatography

The carnitine which had been purified from each experiment was subjected to t.l.c. in four different systems. Cellulose plates (Eastman no. 6064) were used with the following solvent systems (R_F of carnitine is given): (A) methanol–conc. NH_3 (sp. gr. 0.88)–water (93:2:5, by vol.), $R_F = 0.55$; (B) propan-2-ol–ethyl methyl ketone–0.1 M-HCl (5:3:2, by vol.), $R_F = 0.45$. Two other solvent systems were used with silica-gel plates (Eastman no. 6061): (C) methanol–acetone–conc. HCl (5:45:2, by vol.), $R_F = 0.30$; (D) methanol–conc. NH_3 (sp. gr. 0.88) (4:1, v/v), $R_F = 0.4$. Carnitine was spotted along a 1.5 cm line in a relatively large amount (200 μg) to detect contaminants and determine the amount of radioactivity present. The solvent front was allowed to run 15 cm from the origin. Compounds were detected by spraying the plates with 1% (w/v) I_2 in ether followed by either 0.5% (w/v) ninhydrin in butanol or iodoplatinate (Sunshine, 1969). The chromatograms were cut into 1 cm strips, which were counted for radioactivity in scintillant vials with 1 ml of water and 10 ml of the Triton–toluene scintillant.

Deamination of carnitine and 4-N-trimethylamino-butylate

The amount of radioactivity in the 4-N-methyl groups of carnitine was determined by deamination in base, and the evolved trimethylamine was trapped in 1.5 M-HCl (Wolf & Berger, 1961). The 1.5 M-HCl trap was evaporated to dryness *in vacuo* and the trimethylamine hydrochloride was precipitated with 4% (w/v) chloroplatinic acid in ethanol. The trimethylamine–chloroplatinate precipitate was collected by centrifugation, washed with 5 ml of cold ethanol, and dried at 100°C *in vacuo*. A portion (5–10 mg) of the precipitate was weighed into a counting vial, dissolved in 1 ml of water and radioactivity was determined. The total amount of radioactivity in the methyl groups of carnitine was calculated from the specific radioactivity of the trimethylamine–chloroplatinate complex. Similar methods were used to deaminate trimethylaminobutylate, except that the radioactive compound plus carrier were boiled for 1 h in 50% (w/v) NaOH instead of 10% (w/v) NaOH.

Results

Development of methods

A sufficient amount of tissue (50–200 g wet wt.) was extracted to obtain enough carnitine for further studies. In a preliminary experiment an AG-50 column, eluted with the citrate buffer system, did not adequately separate carnitine from other lysine-labelled compounds. When an acid extract from animals injected with $[\text{U-}^{14}\text{C}]$ lysine was chromatographed through the AG-50 citrate buffer column, one of the radioactive peaks corresponded to the fractions containing carnitine. However, when these fractions were desalted and chromatographed on t.l.c. two radioactive spots were detected. Most of the radioactivity (80%) chromatographed on silica gel with carnitine (solvent system C; $R_F = 0.30$); the remaining 20% was associated with a compound detected only with the ninhydrin spray ($R_F = 0.55$). No carrier carnitine was added to the samples before t.l.c.

A second column chromatography step was then used to purify carnitine further. The anion-exchange resin AG-1 (OH^- form) binds most amino acids, including lysine, but excludes quaternary amines such as carnitine, trimethylaminobutylate and trimethyl-lysine. Therefore carnitine fractions from the AG-50 citrate buffer column were washed through a column of resin AG-1 and desalted (method I). In the desalted fraction all radioactivity co-chromatographed with carnitine in the four t.l.c. systems, and no ninhydrin-positive material was detected. A minor non-radioactive contaminant was found with the I_2 spray in some cases. After the addition of carrier the radioactive carnitine was crystallized six times from ethanol without change in specific radioactivity. Since the compounds of primary interest were excluded from the AG-1 resin, this adsorption procedure was carried out before chromatography on the AG-50 resin citrate buffer column (method II). The AG-50 chromatogram of Fig. 2(c) shows that prior adsorption with AG-1 resin eliminates any overlap by other radioactive compounds into the carnitine peak (cf. Figs. 2a and 2b).

Conversion of uniformly labelled lysine into carnitine

The conversion of lysine into carnitine *in vivo* was shown in normal, choline-deficient and lysine-deficient rats (Table 1). When a rat fed on a normal diet was given $[\text{U-}^{14}\text{C}]$ lysine (Fig. 2a) the isolated carnitine was labelled, but at a low specific radioactivity (120 d.p.m./ μmol). For further studies *in vivo* a system was developed which more efficiently labelled carnitine from injected $[\text{U-}^{14}\text{C}]$ lysine. Rats fed on a choline-deficient diet show an increased turnover of carnitine (Mehlman *et al.*, 1971) and incorporate methyl-labelled methionine into carnitine at a faster

Fig. 2. Chromatography of carcass extracts from animals injected with L-[U- 14 C]lysine

The samples were chromatographed on columns (1cm $\times$ 50cm) of AG-50 resin and eluted with the citrate buffer system. (a) The extract was obtained from one male rat weighing 274 g and fed on Purina Lab Chow. The rat was injected with 9 μ Ci/100g body wt. daily for 4 days and the carnitine purified by method I (see the text). (b) The carcass extract was prepared by method I from two rats weighing 152 g and 148 g. The animals were kept for 41 days on the choline-deficient diet and injected with 8.25 μ Ci/100g daily for the last 4 days. (c) The carcass extract obtained from two rats weighing 31 g each which had been kept on a lysine-deficient diet for 24 days and given 25 μ Ci/100g daily for 4 days. Carnitine was purified by method II. $\square$, Radioactivity; $\circ$, carnitine.

rate than do normal animals (Strength *et al.*, 1965). Thus two choline-deficient rats were given L-[U- 14 C]lysine daily for 4 days at about the same dose as was given to normal rats. Carnitine isolated from these animals had a specific radioactivity of 570 d.p.m./ μ mol (Fig. 2b).

Acute choline supplementation increased the concentration of carnitine in the tissues of choline-deficient rats (Corredor *et al.*, 1967); therefore two choline-deficient animals were given L-[U- 14 C]lysine (8.25 μ Ci/100g) daily for 4 days and in addition

70 μ mol of choline chloride/100g body wt. on each of the last 3 days. This choline supplementation resulted in an increase of 50% in extractable carnitine, but the specific radioactivity decreased to 310 d.p.m./ μ mol (Table 1). Since two combined carcasses were used in these experiments and there was an adequate amount of carnitine recovered, acute choline supplementation of choline-deficient animals offered no advantage as a model.

In an attempt to decrease the influx of dietary lysine, and therefore decrease the dilution of administered [14 C]lysine, rats were kept on a lysine-deficient diet. Two of these animals were given 100 μ Ci of L-[U- 14 C]lysine/100 g, a dose three times that given to the normal rat, and the specific radioactivity of the isolated carnitine was 2290 d.p.m./ μ mol (Table 1). Because the lysine-deficient and choline-deficient states led to higher specific radioactivity of carnitine after the injection of labelled lysine, both of these models were used in further studies.

Conversion of specifically labelled lysines into carnitine

Having established that [U- 14 C]lysine was converted into carnitine, we used specifically labelled lysines to determine which carbon fragment was the actual precursor. Carnitine isolated from choline-deficient rats given DL-[6- 14 C]lysine had a specific radioactivity of 260 d.p.m./ μ mol, and the carnitine isolated from animals given L-[4,5- 3 H]lysine had a specific radioactivity much higher than would be expected for non-specific chemical exchange of hydrogen atoms (Table 2). However, no significant label could be found in carnitine after the administration of DL-[1- 14 C]lysine or DL-[2- 14 C]lysine (Table 2). Thus in choline-deficient animals the C-4, C-5 and C-6 of lysine were incorporated into carnitine, whereas C-1 and C-2 were not incorporated.

Deamination of carnitine

Carnitine from the rats injected with [U- 14 C]lysine or [6- 14 C]lysine was deaminated to determine if the lysine label was incorporated into the 4-N-methyl groups of the main carbon chain. The deamination liberated the methyl groups as trimethylamine, which was precipitated as the chloroplatinate, and its specific radioactivity was determined. No significant amount of radioactivity was found in the liberated trimethylamine (Table 3) whereas reference [Me- 14 C]carnitine gave 95–105% recovery of label in the methyl groups and [carboxy- 14 C]carnitine yielded 0%. Therefore label from lysine must be present in the main carbon chain of carnitine.

Methionine and lysine as precursors of carnitine

The contributions of methionine and lysine to carnitine biosynthesis were determined simultane-

Table 1. *Carnitine isolated from rats given L-[U-¹⁴C]lysine*

Expt. A, further details are given in Fig. 2(a); Expt. B, further details are given in Fig. 2(b); Expt. C, the carnitine was extracted from the carcasses of two rats (145 g and 140 g) which were kept on a choline-deficient diet for 60 days. These animals were injected daily with 70 μ mol of choline chloride/100 g during the last three days of administering radioactive lysine. Expt. D, further data in Fig. 2(c).

Diet	Amount of radioactivity injected (μ Ci/4 days per 100 g wt.)	Carnitine extracted (μ mol/100 g of carcass)	Carnitine specific radioactivity (d.p.m./ μ mol)
Expt. A, Purina Lab Chow	36	63	120
Expt. B, choline-deficient	33	33	570
Expt. C, choline-deficient (choline supplemented)	33	52	310
Expt. D, lysine-deficient	100	45	2290

Table 2. *Conversion of specifically labelled lysines into carnitine*

For each experiment two weanling rats were raised for 40 days on a choline-deficient diet and then injected intraperitoneally for 4 days with equal daily doses of labelled lysine. The animals weighed 120–150 g when they were killed.

Labelled lysine injected	Amount of radioactivity injected (μ Ci/4 days per 100 g wt.)	Carnitine extracted (μ mol/100 g of carcass)	Carnitine specific radioactivity (d.p.m./ μ mol)
DL-[1- ¹⁴ C]Lysine	34	32	20
DL-[2- ¹⁴ C]Lysine	37	30	30
L-[4,5- ³ H]Lysine	410	28	5030
DL-[6- ¹⁴ C]Lysine	33	32	260

Table 3. *Deamination of carnitine*

Expt. A, further details of experimental conditions are given in Fig. 2(b); Expt. B, further details given in Table 1 (Expt. C). Expt. C, further details given in Table 2; Expt. D, details given in Fig. 2(c); Expt. E, details given in Fig. 2(a).

Diet	Compound injected	Carnitine specific radioactivity (d.p.m./ μ mol)	Amount of radioactivity in methyl groups (%)
Expt. A, choline-deficient	L-[U- ¹⁴ C]Lysine	570	3
Expt. B, choline-deficient	L-[U- ¹⁴ C]Lysine choline supplement	310	4
Expt. C, choline-deficient	DL-[6- ¹⁴ C]Lysine	260	0
Expt. D, lysine-deficient	L-[U- ¹⁴ C]Lysine	2290	2
Expt. E, Purina Lab Chow	L-[U- ¹⁴ C]Lysine	120	4

ously. A pair of lysine-deficient rats were injected with both [U-¹⁴C]lysine and [Me-³H]methionine and the carnitine was purified by method II (Fig. 3). A comparison between Figs. 3 and 2(c) showed several radioactive peaks derived from [³H]methionine; however, carnitine was still well separated from the other radioactive compounds. Demethylation of this carnitine revealed that [³H]methionine label was

incorporated solely into the 4-*N*-methyl groups whereas the [¹⁴C]lysine label was incorporated into the main carbon chain (Table 4). A similar experiment was done by using choline-deficient rats injected with [4,5-³H]lysine and [Me-¹⁴C]methionine. Carnitine purified from these animals was subjected to demethylation and again only methionine label was incorporated into the 4-*N*-methyl groups (Table 4).

Table 4. *Source of the main carbon chain and methyl groups of carnitine*

Expt. A, carcasses were combined from two rats (198g and 182g) which were kept for 50 days on the choline-deficient diet. Expt. B, carcasses were combined from two rats (52g and 57g) which were kept on the lysine-deficient diet for 24 days (see also Fig. 3).

Diet	Labelled compound injected	Amount of radioactivity injected ($\mu\text{Ci}/4$ days per 100g wt.)	Carnitine extracted ($\mu\text{mol}/100$ g of carcass)	Carnitine specific radioactivity (d.p.m./ μmol)	Amount of radioactivity in methyl group (%)
Expt. A, choline-deficient	DL-[4,5- ^3H]Lysine	260	35	1700 [^3H]	0
	L-[Me- ^{14}C]Methionine	26		3850 [^{14}C]	91
Expt. B, lysine-deficient	L-[U- ^{14}C]Lysine	94	43	2250 [^{14}C]	4
	L-[Me- ^3H]Methionine	540		24600 [^3H]	98

Fig. 3. AG-50 chromatography of a carcass extract from two lysine-deficient rats injected with L-[U- ^{14}C]lysine and L-[Me- ^3H]methionine

The extract was prepared by method II, washed through an AG-1 (OH^- form) column followed by resin AG-50 chromatography with the citrate buffer elution system. For experimental conditions see Table 4. Δ , ^3H Radioactivity; $\blacksquare$, ^{14}C radioactivity; $\circ$, carnitine.

Conversion of lysine into 4-N-trimethylaminobutyrate

Trimethylaminobutyrate (butyrobetaine) was not isolated from carcass extracts of animals given radioactive lysine since only a small amount of radioactivity was present in the area where trimethylaminobutyrate was eluted from the AG-50 resin citrate buffer column. In contrast with the findings of Tanphaichitr & Broquist (1973), a significant amount of radioactive trimethylaminobutyrate was recovered from the urine of lysine-deficient rats injected with [U- ^{14}C]lysine. This urinary 4-trimethylaminobutyrate was iodoplatinate-positive and ninhydrin-negative and chromatographed on cellulose t.l.c. plates (system A) in a manner identical with synthetic 4-trimethylaminobutyrate ($R_f = 0.5$). The

Table 5. *Conversion of labelled lysine into carnitine and 4-trimethylaminobutyrate*

Labelled metabolites were isolated by method II from two weanling rats which had been maintained for 27 days on a lysine-deficient diet and then injected with $84.5 \mu\text{Ci}$ of L-[U- ^{14}C]lysine/100g daily for the last four days. The urine was collected daily into 1 ml of 0.1M-HCl, and the four samples were pooled. To determine the specific radioactivity of trimethylaminobutyrate in the urine, the appropriate citrate buffer column fractions were desalted, and approx. $15 \mu\text{g}$ of trimethylaminobutyrate was further purified by t.l.c. on cellulose by using solvent system A. The compound was eluted from the t.l.c. plate with 0.5 ml of 1.0M-HCl and assayed by the periodide reaction (Wall *et al.*, 1960). After dissolving the periodide-quaternary amine precipitate in ethylene dichloride, a portion was determined spectrophotometrically with 4-trimethylaminobutyrate as a standard, and radioactivity was also determined on a portion.

Material	Specific radioactivity (d.p.m./ μmol)
Carnitine in carcass	3750
Carnitine in urine	3590
Trimethylaminobutyrate in urine	44 340
^{14}C label in 4-N-methyl groups of trimethylaminobutyrate in urine	0

specific radioactivity was about 12 times that of carnitine isolated from the carcass or urine of these same animals (Table 5). In addition, deamination showed that the [U- ^{14}C]lysine was not incorporated into the methyl groups (Table 5). By using this same technique of deamination, it was shown that synthetic 4-[Me- ^{14}C]trimethylaminobutyrate yielded 95–105% of the radioactivity in the methyl groups

whereas 4-trimethylamino[carboxy- ^{14}C]butyric acid yielded 0%.

Discussion

When rats were given radioactive lysine, the carnitine isolated from animals raised on diets deficient in lysine or choline had a much higher specific radioactivity than the carnitine recovered from animals fed on a normal diet. In choline deficiency this would be expected, since the turnover of carnitine is increased, and there is a decreased body pool of carnitine (about half that in control rats) which would dilute newly synthesized carnitine to a smaller extent (Mehlman *et al.*, 1971). The amount of carnitine extracted from the carcasses of rats fed on a lysine-deficient diet was one-quarter lower than from normal animals (Table 1); nothing is known about the turnover of carnitine under these conditions. However, other changes in lysine metabolism have been observed during lysine deficiency. After 1 week on a lysine-deficient diet, rats metabolized $[\text{U-}^{14}\text{C}]$ lysine to CO_2 , blood sugar, glycogen and lipid at one-half the rate in normally fed rats, yet the incorporation of labelled lysine into liver protein was almost doubled (Yamashita & Ashida, 1969). This latter point may be significant, since the only known route of 6-*N*-trimethyl-lysine synthesis (a proposed intermediate in carnitine biosynthesis) is via the methylation of protein-bound lysine by *S*-adenosylmethionine (Paik & Kim, 1971).

Earlier investigations in rats could not establish lysine as a precursor of carnitine (Wolf & Berger, 1961). In a normally fed rat we recovered 0.005% of the administered L- $[\text{U-}^{14}\text{C}]$ lysine in the carcass carnitine, in choline-deficient rats the recovery in carnitine was 0.014%, and in the lysine-deficient animals it was 0.026%. Considering this low conversion, it is not surprising that Wolf & Berger (1961) did not demonstrate incorporation of radioactive lysine into carnitine when they injected rats with only $0.9\mu\text{Ci}$ of $[\text{U-}^{14}\text{C}]$ lysine/100g body wt. in a shorter term (24h) experiment.

Our findings demonstrated that C-4, C-5 and C-6 of lysine were converted by the rat into carnitine, but C-1 and C-2 were not. Similar findings have been reported by Tanphaichitr & Broquist (1973). Further, the lysine label in carnitine was not in the 4-*N*-methyl groups and therefore was part of the main carbon chain. The studies with both labelled lysine and methionine showed that carnitine was synthesized by the addition of methyl groups from methionine to a carbon chain originating from lysine. It may be that lysine itself was the methyl-group acceptor, and methionine entered the pathway of carnitine biosynthesis at the level of 6-*N*-trimethyl-lysine synthesis. This would be consistent with the following sequence: lysine + methionine $\rightarrow$ 6-*N*-trimethyl-lysine $\rightarrow$ 4-*N*-trimethylaminobutyrate $\rightarrow$ carnitine.

Trimethylaminobutyrate, a proposed intermediate in the above scheme, has been identified in human and dog urine (Broekhuysen & Deltour, 1961) and was rapidly hydroxylated to form carnitine in the rat (Lindstedt & Lindstedt, 1961). This reaction was found to be catalysed by a hepatic enzyme, 4-trimethylaminobutyrate (butyrobetaine) hydroxylase, which showed a high degree of substrate specificity (Lindstedt, 1967). Trimethylaminobutyrate has been established as a metabolite of 6-*N*-trimethyl-lysine in yeast (Horne & Broquist, 1973) and in the rat (Cox & Hoppel, 1973; Tanphaichitr & Broquist, 1973). We have demonstrated that its four-carbon chain is derived from lysine. After the administration of radioactive lysine, 4-trimethylaminobutyrate was found to have a higher specific radioactivity than carnitine. This is consistent with a precursor-product relationship for these compounds and further supports the scheme of carnitine biosynthesis outlined above.

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