***N*⁶-Trimethyl-lysine Metabolism**

3-HYDROXY-*N*⁶-TRIMETHYL-LYSINE AND CARNITINE BIOSYNTHESIS

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Rats injected with *N*⁶-[Me-³H]trimethyl-lysine excrete in the urine five radioactively labelled metabolites. Two of these identified metabolites are carnitine and 4-trimethylammoniobutyrate. A third metabolite, identified as 5-trimethylammoniopentanoate, is not an intermediate in the biosynthesis of carnitine; the fourth and major metabolite, *N*²-acetyl-*N*⁶-trimethyl-lysine, is not a precursor of carnitine. The remaining metabolite (3-hydroxy-*N*⁶-trimethyl-lysine) is converted into trimethylammoniobutyrate and carnitine by rat liver slices and into trimethylammoniobutyrate by rat kidney slices. In rat liver and kidney-slice experiments, radioactivity from DL-*N*⁶-trimethyl-[1-¹⁴C]lysine and DL-*N*⁶-trimethyl[2-¹⁴C]lysine was incorporated into *N*²-acetyl-*N*⁶-trimethyl-lysine and 3-hydroxy-*N*⁶-trimethyl-lysine, but not into trimethylammoniobutyrate or carnitine. A procedure was devised to purify milligram quantities of 3-hydroxy-*N*⁶-trimethyl-lysine from the urine of rats injected chronically with *N*⁶-trimethyl-lysine (100 mg/kg body wt. per day). The structure of 3-hydroxy-*N*⁶-trimethyl-lysine was confirmed chemically and by nuclear-magnetic-resonance spectrometry [Novak, Swift & Hoppel (1980) *Biochem. J.* **188**, 521–527]. The sequence for carnitine biosynthesis in liver is: *N*⁶-trimethyl-lysine → 3-hydroxy-*N*⁶-trimethyl-lysine → 4-trimethylammoniobutyrate → carnitine.

Carnitine (3-hydroxy-4-trimethylammoniobutyrate) is synthesized from the essential amino acids lysine and methionine in the rat (Tanphaichitr & Broquist, 1973; Cox & Hoppel, 1973a; Strength *et al.*, 1965) and in *Neurospora crassa* (Horne & Broquist, 1973). Protein-bound lysine is methylated in the 6-amino position to form *N*⁶-trimethyl-lysine (Paik & Kim, 1971), and this in turn is converted into carnitine via hydroxylation of 4-trimethylammoniobutyrate (butyrobetaine) (Cox & Hoppel, 1973b; Tanphaichitr & Broquist, 1973; Horne & Broquist, 1973) by the following sequence: trimethyl-lysine → trimethylammoniobutyrate → carnitine. The hydroxylation of trimethylammoniobutyrate has been studied in detail (Lindstedt *et al.*, 1970), but little is known about the conversion of trimethyl-lysine into trimethylammoniobutyrate.

In previous work presented in abstract form (Hoppel *et al.*, 1976), we showed that 3-hydroxy-*N*⁶-trimethyl-lysine is synthesized from trimethyl-lysine and is a precursor of carnitine. Hochalter & Henderson (1976), from tracer studies *in vivo*, proposed that glycine and 4-trimethylammonio-

butyraldehyde are in the pathway of carnitine biosynthesis. The aldehyde metabolite has not been structurally identified from biological samples. For *Neurospora crassa*, Kaufman & Broquist (1978) also reported 3-hydroxy-*N*⁶-trimethyl-lysine to be a precursor of carnitine. The proposed pathways for metabolism of trimethyl-lysine are shown in Scheme 1.

In rats, trimethyl-lysine is converted into at least three other metabolites in addition to trimethylammoniobutyrate and carnitine (Cox & Hoppel, 1973b, 1974a,b). One of these metabolites (I) has been tentatively identified as 5-trimethylammoniopentanoate, but this compound does not appear to be a precursor of carnitine (Cox & Hoppel, 1973b). The present study identified two other metabolites of trimethyl-lysine. The first, *N*²-acetyl-*N*⁶-trimethyl-lysine, is the major urinary metabolite of trimethyl-lysine, but it also is not a precursor of carnitine. The second metabolite, 3-hydroxy-*N*⁶-trimethyl-lysine (II_b), is an intermediate in the conversion of trimethyl-lysine into trimethylammoniobutyrate.

The use of n.m.r. spectroscopy to identify the

Scheme 1. Proposed pathways for the metabolism of trimethyl-lysine

structure of compound (II_b) as 3-hydroxy-trimethyl-lysine is reported in the following paper (Novak *et al.*, 1980).

Materials and Methods

[³H]Methyl iodide (sp. radioactivity 300 Ci/mol) and DL-[2-¹⁴C]lysine hydrochloride (sp. radioactivity 1.27 Ci/mol) were purchased from International Chemical and Nuclear Corp., Cleveland, OH, U.S.A. DL-[1-¹⁴C]Lysine hydrochloride (sp. radioactivity 6.36 Ci/mol) was purchased from New England Nuclear Corp., Boston, MA, U.S.A.

Radioactivity was measured as previously described (Cox & Hoppel, 1973a). Two citrate buffer systems were used in column chromatography, one a pH 4.1 citrate buffer containing 0.25 M-Na⁺ and the other a pH 5.8 citrate buffer containing 0.35 M-Na⁺. These buffers were prepared as described by Piez *et al.* (1956) and modified by Horne *et al.* (1971). All

chromatography was done at room temperature (20–23°C).

N⁶-[Me-³H]Trimethyl-lysine (sp. radioactivity 76.8 Ci/mol) was synthesized and purified as previously described (Cox & Hoppel, 1973b).

Synthesis of C-1- and C-2-labelled trimethyl-lysine

To synthesize specifically labelled trimethyl-lysine, a method was adapted for synthesizing trimethyl-lysine directly from lysine. In this synthesis lysine was complexed with copper to block the 2-amino group (Neuberger & Sanger, 1943), and the 6-amino group was methylated with dimethyl sulphate to form trimethyl-lysine. The trimethyl-lysine we synthesized by published techniques by the lysine-copper-complex method (Takemoto *et al.*, 1964) was not chromatographically pure as indicated by t.l.c. Therefore the following modification of this method was devised. L-Lysine hydrochloride (1.82 g) and 3.32 g of CuCO₃·Cu(OH)₂ were added

to 18 ml of water and the mixture was boiled under reflux for 5 min. After centrifugation and cooling to room temperature, 2.55 ml (3.8 g) of dimethyl sulphate was added slowly to the supernatant with enough 10% (w/v) NaOH (in 1 ml portions) to keep the reaction pH between 7.5 and 8.5. The reaction was stirred for 1 h at room temperature and the pH was maintained between 7.5 and 8.5 by the periodic addition of 10% NaOH. The solution was then placed on a column (5 cm $\times$ 12 cm) of AG-1 (X8; OH⁻ form); the trimethyl-lysine was washed through the column with 100 ml of water, and 10 ml fractions were collected. Fractions that were ninhydrin-positive (tested by spotting a sample on paper and spraying with 4% ninhydrin in butanol) were pooled and neutralized with concentrated HCl. This solution was chromatographed on a column (2.2 cm $\times$ 50 cm) of AG-50 (X8; Na⁺ form) by eluting 2 litres of pH 5.8 citrate buffer (0.35 M-Na⁺). After 1 litre of eluent passed through the column, 15 ml fractions were collected and the ninhydrin-positive fractions were acidified to pH 3 and desalted by using a column (2.5 cm $\times$ 15 cm) of AG-50 (X8; H⁺ form). The latter column was washed first with 150 ml of 1 M-HCl to remove citrate and then with 300 ml of 4 M-HCl to elute the trimethyl-lysine. Ninhydrin-positive fractions were pooled, evaporated to a glass, triturated with diethyl ether and dried *in vacuo* over KOH. This trimethyl-lysine chromatographed as one spot, as detected by ninhydrin and iodoplatinate sprays (Cox & Hoppel, 1973b) in three thin-layer systems (for details, see below). The *p'*-hydroxyazobenzene-*p*-sulphonate salt was crystallized from water and its melting point was 226–228°C (Kakimoto & Akazawa, 1970).

DL-*N*⁶-Trimethyl[1-¹⁴C]lysine and DL-*N*⁶-trimethyl[2-¹⁴C]lysine were synthesized by the method adapted for synthesis of non-radioactive trimethyl-lysine from lysine. In 1 ml of water 250 μ Ci of DL-[1-¹⁴C]lysine hydrochloride (sp. radioactivity 6.36 Ci/mol) or DL-[2-¹⁴C]lysine hydrochloride (sp. radioactivity 1.27 Ci/mol) was boiled with an excess of CuCO₃, Cu(OH)₂, and the supernatant was treated for 1 h with a 3-fold excess of dimethyl sulphate. The reaction pH was kept between 7.5 and 8.5 by adding 5–10 μ l amounts of 10% (w/v) NaOH. After the reaction period, the solution was passed over a column (1.5 cm $\times$ 8 cm) of AG-1 (X8; OH⁻ form) and eluted with water. After neutralization with HCl, the radioactive fractions were concentrated and further purified on a column (1 cm $\times$ 50 cm) of AG-50 (X8; Na⁺ form) eluted with 500 ml of pH 5.8 citrate buffer (0.35 M-Na⁺). Fractions containing the major radioactive peak (trimethyl-lysine) were pooled and desalted on a column (1 cm $\times$ 50 cm) of AG-50 (X8; H⁺ form). Citrate was washed from the column with 150 ml of 1 M-HCl, and the labelled trimethyl-lysine was eluted

with 100 ml of 4 M-HCl. Fractions containing radioactivity were pooled and evaporated to remove HCl. The yield of labelled trimethyl-lysine was 40–50%. Both *N*⁶-trimethyl[1-¹⁴C]lysine and *N*⁶-trimethyl[2-¹⁴C]lysine co-chromatographed with carrier trimethyl-lysine made from *N*²-acetyl-lysine (Cox & Hoppel, 1973b) in the following thin-layer systems: cellulose plates (Eastman 6064) with methanol/NH₄OH 28% (w/v)/water (93:2:5, by vol.) as solvent; cellulose (Eastman 6064) with isopropyl alcohol/ethyl methyl ketone/0.1 M-HCl (51:31:2, by vol.) as solvent; silica gel (Eastman 6061) with methanol/acetone/conc. HCl (5:45:2, by vol.) as solvent.

Each of the labelled compounds (50 000 d.p.m.) was chromatographed on thin-layer plates and the only detectable area of radioactivity migrated with the appropriate carrier. For AG-50 column chromatography, 5 $\times$ 10⁶ d.p.m. of the labelled compound was chromatographed and the only detectable radioactivity eluted with the carrier. Recoveries were quantitative. Therefore the radioactive trimethyl-lysine contained less than 0.05% radioactive impurities.

Isolation of radioactive metabolites of trimethyl-lysine from urine

A 130 g Wistar rat (obtained from Carworth Farms, New City, NY, U.S.A.) was injected intraperitoneally with a total of 550 μ Ci of *N*⁶-[Me-³H]trimethyl-lysine (sp. radioactivity 76.8 Ci/mol) by giving four equal doses 3 h apart (Cox & Hoppel, 1973b). Urine was collected in 1 ml of 0.1 M-HCl during the injection period and for another 18 h after the last injection. The urine was evaporated to dryness, taken up in a small amount of water and applied to a column (1.5 cm $\times$ 12 cm) of AG-1 (X8; OH⁻ form). The column was then washed with water, and 5 ml fractions were collected. Those fractions containing radioactivity (fractions 1–5) were then pooled, neutralized with conc. HCl, and placed on a column (1 cm $\times$ 50 cm) of AG-50 (X8; H⁺ form) and eluted with a linear HCl gradient (150 ml of 1.5 M-HCl in the mixing flask and 150 ml of 4.0 M-HCl in the reservoir); 5 ml fractions were collected (Fig. 1a). In this experiment, 90% of the injected radioactivity was recovered from the column [2% in carnitine, 11% in 4-trimethyl-ammoniobutyrate, 30% in peak II (160–205 ml of effluent) and 48% in *N*⁶-trimethyl-lysine]. Urinary peak II plus the first fractions of the trimethyl-lysine peak (shown in Fig. 1a) were pooled, evaporated and rechromatographed on a similar AG-50 (X8) column with HCl gradient elution (Fig. 1b). This additional chromatographic step showed the presence of a small peak I (not delineated in the first chromatographic step), peak II and trimethyl-lysine.

Fig. 1. *Chromatographic isolation and purification of trimethyl-lysine metabolites in urine* Radioactivity (—) was determined in 5 μ l portions of each fraction, and carnitine (---) was assayed enzymically (Marquis & Fritz, 1964). See the text for details of the chromatographic isolation.

Previously, we found that t.l.c. of urinary peak II indicated that two trimethyl-lysine metabolites were present (Cox & Hoppel, 1973b). Therefore the fractions containing peak II (Fig. 1b) were pooled, evaporated to dryness and taken up in a small amount of water. This sample was chromatographed on a column (1 cm $\times$ 50 cm) of AG-50 (X8; Na⁺ form) previously equilibrated with a pH 4.1 citrate buffer containing 0.25 M-Na⁺. The radioactive compounds were eluted from the column with a gradient of increasing pH established by using a flask containing 150 ml of pH 4.1 citrate buffer and a reservoir of 150 ml of 0.25 M-NaOH. The column was eluted at room temperature (20–23°C) and 5 ml fractions were collected (Fig. 1c). This chromatographic step separated peak II into two components: (1) a peak (II_a) eluted early by the citrate gradient; (2) a later peak containing a mixture of compound (II_b) and trimethyl-lysine. The fractions containing peak II_b plus trimethyl-lysine (Fig. 1d) were pooled and chromatographed on a column (1 cm $\times$ 50 cm) of AG-50 (X8; H⁺ form) with the HCl gradient system described previously in Figs. 1(a) and 1(b). This desalting step separated compound (II_b) from trimethyl-lysine (Fig. 1d). Peak II_b was evaporated to dryness and taken up in a small volume of water for further characterization. Chromatography of peak II_a on an AG-50 column with the HCl gradient system showed it to chromatograph as a single peak.

Peak I obtained from the column effluent illustrated in Fig. 1(b) was further purified on the citrate buffer column (see above), where it was separated from a small amount of contaminating peak II_a (Fig. 1e). Rechromatography of peak I on an AG-50 column with an HCl gradient revealed that only one peak of radioactivity was present.

These three trimethyl-lysine metabolites (peaks I, II_a and II_b) were further analysed by t.l.c., where each chromatographed as a single radioactive component (Fig. 2). The isolated compounds contained less than 0.05% radioactive impurities (Cox & Hoppel, 1973b) and were considered to be sufficiently pure to test as precursors of carnitine in the liver-slice system.

Isolation and purification of milligram quantities of unlabelled compound (II_b)

Three separate batches of compound (II_b) were prepared. Twelve male Wistar rats (150–200 g each) were injected intraperitoneally daily with trimethyl-lysine at a dose of 100 mg/kg body wt. Urine was collected daily into 1 ml of 1 M-HCl. For the first two preparations, experiments continued for 3 weeks, at the end of which time the urines were pooled and evaporated with a rotary evaporator, and the residue was extracted three times with 50 ml of warm ethanol. The ethanol fraction was evaporated and the residue was dissolved in water. Purification of compound (II_b) was performed in a manner analo-

Fig. 2. Thin-layer chromatogram of radioactive peaks I (a), II_a (b) and II_b (c)

The radioactive metabolites were purified from the urine of a rat injected with [*Me*-³H]trimethyl-lysine as described in the Materials and Methods section and Fig. 1. Chromatography was carried out by using cellulose plates with a solvent of methanol/NH₄OH 28% (w/v)/water (93:2:5, by vol.), and radioactivity was determined on 1 cm strips. Carrier 5-*N*-trimethylammoniopentanoate (25 μg) was added to the peak I spot, and the carrier was detected by spraying with iodoplatinate reagent. No other spots were seen on any of the chromatograms.

gous to the system of chromatography shown in Fig. 1. For this large-scale-purification scheme, the size of the AG-1 (X8; OH⁻ form) column was increased to the dimensions 2.5 cm × 15 cm. The AG-50 cation-exchange column again had the dimensions 1.0 cm × 50 cm. In both the first and second experiments, approx. 20 mg of dried powder of compound (II_b) was obtained; this was chromatographically pure on thin-layer and column chromatography. In the third experiment, the rats were injected daily for 6 weeks, and the isolation procedure was modified. Urine in 250 ml portions was treated with 3% (w/v) HClO₄ and centrifuged. The pH of the supernatant was adjusted to 3.0–4.5 and the solution was

chromatographed on a column (3.7 cm × 10 cm) of AG-50 (X8; H⁺ form). This column was washed with 200 ml of water followed by 200 ml of 1 M-pyridine, and both eluates were discarded. The resin was then washed with 300 ml of 4 M-NH₄OH and 10 ml fractions were collected. Fractions containing trimethyl-lysine metabolites (130–240 ml) were pooled and evaporated. This initial step quickly removed large quantities of unwanted material, and it was not necessary to evaporate the urine before chromatography. Isolation and purification of compound (II_b) was done as discussed above. The yield of compound (II_b) in this last experiment was 40 mg.

Incubations

The methods for preparing, incubating and extracting radioactive metabolites from rat tissue slices were as previously described (Cox & Hoppel, 1974a,b).

The specific radioactivity of [*Me*-¹⁴C]trimethyl-ammoniobutyrate was determined by converting trimethylammoniobutyrate to carnitine with butyrobetaine hydroxylase and isolation of carnitine by cation-exchange chromatography as previously described (Cox & Hoppel, 1973b). The amount of [¹⁴C]carnitine was determined by radioenzymic analysis using [³H]acetyl-CoA (Bohmer *et al.*, 1974) with dual-label liquid-scintillation counting.

Results

Metabolism of C-1-labelled and C-2-labelled trimethyl-lysine by rat kidney and liver slices

When trimethyl-[1-¹⁴C]- or [2-¹⁴C]-lysine was incubated with liver and kidney slices, no radioactivity was incorporated into carnitine. However, ³H label from the methyl groups of L-N⁶-[*Me*-³H]trimethyl-lysine was incorporated into carnitine in liver slices only (Table 1). The finding that radioactivity from trimethyl-lysine specifically labelled to the C-1 or C-2 positions was not incorporated into carnitine *in vitro* is consistent with results *in vivo* using C-1- or C-2-labelled lysine. Label in the C-4–C-6 positions of lysine was incorporated into carnitine *in vivo* (Cox & Hoppel, 1973a; Tanphaichitr & Broquist, 1973).

Consistent with the proposed scheme of carnitine biosynthesis, radioactivity from C-1- or C-2-labelled trimethyl-lysine was not incorporated into 4-trimethylammoniobutyrate by liver or kidney slices (Table 1). When the acid extracts from these incubations were chromatographed on AG-50 columns, a small amount of radioactivity derived from the labelled compounds was eluted near 4-trimethylammoniobutyrate. However, t.l.c. (on cellulose plates with methanol/NH₄OH/water as solvent) of the AG-50 column fractions demonstrated that 4-trimethylammoniobutyrate (*R_F* 0.5) did

not contain radioactive label derived from either the C-1- or C-2-labelled trimethyl-lysine, but some radioactive compound having an R_F of 0.3 was present. The incorporation of N^6 -[Me - 3H]trimethyl-lysine into trimethylammoniobutyrate is greater in kidney slices than in liver slices (Table 1) and is consistent with previous results (Cox & Hopfel, 1974b).

Liver and kidney slices incorporated both C-1 and C-2 of trimethyl-lysine into peak II (Table 1). The proportion of DL- N^6 -trimethyl[1- ^{14}C]lysine or DL-

N^6 -trimethyl[2- ^{14}C]lysine incorporated into peak II was about one-half the amount of (Me - 3H)-labelled L-trimethyl-lysine incorporated into this peak in the same incubations (Tables 1 and 2). This would be expected if only one isomer of trimethyl-lysine were active as a precursor of the peak II compounds. If we assume only one-half of the ^{14}C label of DL-trimethyl-lysine to be biologically active, the ratio of (Me - 3H)-derived label to (1- ^{14}C)- or (2- ^{14}C)-derived label is the same for peak II and the trimethyl-lysine peak. T.l.c. of peak II isolated from

Table 1. *Metabolism of N^6 -trimethyl[1- ^{14}C]- or -[2- ^{14}C]-lysine plus N^6 -[Me - 3H]trimethyl-lysine by rat liver and kidney slices*

Liver or kidney slices (0.5 g) were incubated with L- N^6 -[Me - 3H]trimethyl-lysine (sp. radioactivity 78.6 $\mu Ci/\mu mol$) plus either DL- N^6 -trimethyl[1- ^{14}C]lysine (sp. radioactivity 6.36 $\mu Ci/\mu mol$) or DL- N^6 -trimethyl[2- ^{14}C]lysine (1.27 $\mu Ci/\mu mol$) as described in the Materials and Methods section. Kidney slices were incubated with (a) 4.86 μCi of [3H]trimethyl-lysine plus 9.87 μCi of trimethyl[1- ^{14}C]lysine or (b) 3.0 μCi of [Me - 3H]trimethyl-lysine plus 5.28 μCi of trimethyl[2- ^{14}C]lysine. Liver slice incubations contained (c) 12.1 μCi of [Me - 3H]trimethyl-lysine plus 10.2 μCi of trimethyl[1- ^{14}C]lysine or (d) 11.2 μCi of [3H]trimethyl-lysine plus 4.5 μCi of trimethyl[2- ^{14}C]lysine. After incubation, carriers were added to the acid extracts, and the latter were passed over an AG-1 (OH⁻ form) column (1.5 cm $\times$ 10 cm) eluted with water. The fractions containing radioactivity were chromatographed over columns (1 cm $\times$ 50 cm) of AG-50 (X8; H⁺ form) eluted with the HCl gradient system.

Tissue	Labelled compound added	Isolated metabolites (% of added radioactivity)			
		Carnitine	4-Trimethyl-ammoniobutyrate	Peak I	Peak II
Kidney	(a) L- N^6 -[Me - 3H]Trimethyl-lysine	0	7.10	0.26	1.06
	Plus DL- N^6 -trimethyl[1- ^{14}C]lysine	0	0	0.03	0.63
	(b) L- N^6 -[Me - 3H]Trimethyl-lysine	0	5.27	0.46	2.13
	Plus DL- N^6 -trimethyl[2- ^{14}C]lysine	0	0	0.32	1.20
Liver	(c) L- N^6 -[Me - 3H]Trimethyl-lysine	1.0	0.34	0.33	0.33
	Plus DL- N^6 -trimethyl[1- ^{14}C]lysine	0	0	0.01	0.16
	(d) L- N^6 -[Me - 3H]Trimethyl-lysine	1.0	0.38	0.33	0.33
	Plus DL- N^6 -trimethyl[2- ^{14}C]lysine	0	0	0.17	0.17

Table 2. *Metabolism of (Me - 3H)-labelled N^6 -trimethyl-lysine versus carbon-chain-labelled N^6 -trimethyl-lysine in liver and kidney slices*

Kidney slices were incubated with DL- N^6 -trimethyl[1- ^{14}C]lysine or DL- N^6 -trimethyl[2- ^{14}C]lysine plus L- N^6 -[Me - 3H]trimethyl-lysine in dual-label experiments. The amounts of 3H and ^{14}C label in the peak AG-50 column fraction of the various metabolites were compared. Details of the experimental conditions are given in Table 1. Abbreviations used: [3H]TML, L- N^6 -[Me - 3H]trimethyl-lysine; DL-[^{14}C]TML, DL- N^6 -trimethyl[1- ^{14}C]lysine or DL- N^6 -trimethyl[2- ^{14}C]lysine; L-[^{14}C]TML, L- N^6 -trimethyl[1- ^{14}C]lysine or L- N^6 -trimethyl[2- ^{14}C]lysine.

Tissue	Labelled compound added	N^6 -Trimethyl-lysine		Peak I $^3H/^{14}C$ ratio	Peak II $^3H/^{14}C$ ratio
		[3H]TML DL-[^{14}C]TML	[3H]TML L-[^{14}C]TML		
Kidney	(a) L- N^6 -[Me - 3H]Trimethyl-lysine plus DL- N^6 -trimethyl[1- ^{14}C]lysine	0.41	0.82	—	0.83
	(b) L- N^6 -[Me - 3H]Trimethyl-lysine plus DL- N^6 -trimethyl[2- ^{14}C]lysine	0.45	0.90	0.81	0.87
Liver	(c) L- N^6 -[Me - 3H]Trimethyl-lysine plus DL- N^6 -trimethyl[1- ^{14}C]lysine	1.03	2.05	—	2.20
	(d) L- N^6 -[Me - 3H]Trimethyl-lysine plus DL- N^6 -trimethyl[2- ^{14}C]lysine	2.32	4.64	4.60	4.50

the kidney-slice experiments demonstrated that about 15% of both labels chromatographed with an R_F of 0.2 (II_b) and 85% with an R_F of 0.6 (II_a). The above results indicate that the quaternary amine group and the complete six-carbon-unit chain of N^6 -trimethyl-lysine are incorporated into the compounds in peak II.

In both kidney- and liver-slice incubations, label from N^6 -trimethyl[2- ^{14}C]lysine was incorporated into peak I; no incorporation was found in this peak when N^6 -trimethyl[1- ^{14}C]lysine was incubated (Table 1). In the latter case, a very small amount of label in the peak I fractions appeared to be caused by spread from peak II (results not shown). Similarly to what occurred at peak II, the label from DL- N^6 -trimethyl[2- ^{14}C]lysine was incorporated into peak I at about one-half the proportion of label from L- N^6 -[Me- 3H]trimethyl-lysine (Table 2). Again, assuming that only L- N^6 -trimethyl-lysine is biologically active, the ratio of 3H label to ^{14}C label in peak I and N^6 -trimethyl-lysine remains constant. Our experiments indicated that the quaternary ammonio group of N^6 -trimethyl-lysine and five carbon atoms of the main carbon chain are incorporated into peak I.

Trimethyl-lysine metabolites as precursors of carnitine

The radioactive compounds (peaks I and II) isolated from the urine of the rat injected with tritiated trimethyl-lysine (as described in the Materials and Methods Section) were tested as precursors of carnitine using a rat liver slice system.

Peak I (trimethylammoniopentanoate)

When 1 μCi of peak I was incubated with 0.5 g (wet weight) of liver slices from normal rats. 0.17% of the label was converted into carnitine. When 3H -labelled peak I material was incubated together with synthetic 5-[Me- ^{14}C]trimethylammoniopentanoate (Cox & Hoppel, 1973b), approximately the same proportion of 3H and ^{14}C was incorporated into carnitine (results not shown). However, in the liver-slice system, peak I and 5-trimethylammoniopentanoate showed only minimal activity as carnitine precursors compared with trimethyl-lysine or 4-trimethylammoniobutyrate.

We tentatively identified peak I as trimethylammoniopentanoate (Cox & Hoppel, 1973b) on the basis of thin-layer- and column-chromatographic behaviour, and crystallization. A proposed pathway for the formation of trimethylammoniopentanoate is shown in Scheme I. An amino acid oxidase has been purified from *Neurospora crassa* that converts trimethyl-lysine into 2-oxo-6-trimethylammoniohexanoate (Lê & Villanueva, 1978). A similar enzyme in rat liver (and kidney) may oxidatively deaminate trimethyl-lysine. The 2-oxo acid meta-

bolite would be susceptible to decarboxylation during the acidic conditions used to prepare tissue samples for chromatography. Trimethylammoniopentanoate was not investigated further because it most likely is a by-product and was not considered to be an intermediate in carnitine biosynthesis.

Peak II_a (N^2 -acetyl- N^6 -trimethyl-lysine)

Peak II_a was incubated with liver slices. On two separate occasions, incubations of 3–5 μCi of this labelled compound did not show production of any new radioactively labelled metabolites under conditions in which 0.02% conversion into carnitine could have been detected. Therefore, peak II_a appeared to be inert as a carnitine precursor in the rat-liver-slice system.

The II_a sample chromatographed on thin-layer plates was iodoplatinate-positive and ninhydrin-negative and had an R_F value similar to synthetic N^2 -acetyl- N^6 -trimethyl-lysine (available as an intermediate in trimethyl-lysine synthesis from N^2 -acetyl-lysine). Hydrolysis of compound (II_a) in 6 M-HCl at 105°C for 20h resulted in quantitative conversion into trimethyl-lysine. Radioactive compound (II_a) was diluted with synthetic N^2 -acetyl- N^6 -trimethyl-lysine, and the mixture co-chromatographed on column (AG-50) and thin-layer-plate systems. Acid hydrolysis resulted in conversion of the mixture into trimethyl-lysine. Neither peak II_a nor N^2 -acetyl- N^6 -trimethyl-lysine was affected by alkaline hydrolysis. Rat liver slices were incubated with [Me- 3H]trimethyl-lysine and 2 μCi of [^{14}C]acetate. Chromatography of the acid extract of this incubation showed ^{14}C radioactivity only in peak II, and purified compound (II_a) contained both 3H and ^{14}C . This dual-labelled compound (II_a) co-chromatographed with N^2 -acetyl- N^6 -trimethyl-lysine on t.l.c. Acid hydrolysis resulted in the loss of ^{14}C , whereas the 3H radioactivity was recovered as trimethyl-lysine. The compound appears to be an acyl derivative of trimethyl-lysine and is probably the N^2 -acetyl derivative. Because the compound was not a precursor of carnitine, further investigation of the structure was not undertaken.

Peak II_b (3-hydroxy- N^6 -trimethyl-lysine)

When liver slices were incubated with purified peak II_b , 5.35% of the added radioactivity was converted into carnitine, and 6.72% was recovered as trimethylammoniobutyrate (Fig. 3a). T.l.c. on cellulose plates and methanol/ NH_4OH /water as solvent was used to confirm the identity of these II_b metabolites. No other metabolites were noted in the AG-50 HCl gradient elution chromatogram. Incubations of boiled liver slices with peak II_b demonstrated that less than 0.5% of the label chromatographed outside the II_b peak on an AG-50 column eluted with the HCl gradient. To further

Fig. 3. Chromatography of an acid extract from liver slices incubated with purified peak II_b .

(a) Liver slices were incubated with $2.49 \mu\text{Ci}$ of ^3H -labelled peak II_b purified from the urine of a rat injected with N^6 -[Me - ^3H]trimethyl-lysine. After incubation, the liver extract was chromatographed over a column ($1.0\text{ cm} \times 50\text{ cm}$) of AG-50 (X8; H^+ form) eluted with the HCl gradient system. Fractions (5 ml) were collected. Carrier carnitine and 4-trimethylammoniobutyrate (----) were assayed as previously described (Cox & Hoppe, 1973a) and radioactivity (—) was determined on 0.5 ml portions. (b) The experiment in (a) was repeated, but 5 mg of trimethylammoniobutyrate was added to the incubation. After incubation, 10 mg of carnitine and an additional 5 mg of trimethylammoniobutyrate were added to the acid extract before chromatography.

investigate the mechanism through which this urinary metabolite of trimethyl-lysine was converted into carnitine, labelled compound (II_b) was incubated with liver slices in the presence of 5 mg of unlabelled 4-trimethylammoniobutyrate. As illustrated in Fig. 3(b), carnitine from this incubation contained only 0.06% of the added label. However, the amount of radioactivity in the isolated 4-trimethylammoniobutyrate had increased to 9.37% . Similarly to what has occurred in other isotope-dilution experiments (Cox & Hoppe, 1974b), we found the total conversion of label into carnitine and 4-trimethylammoniobutyrate (9.43%) to be less than that without the unlabelled trap (12%). Nevertheless, these results demonstrate that peak II_b (3-hydroxy- N^6 -trimethyl-lysine) is readily converted into carnitine by liver slices, and that 4-trimethylammoniobutyrate is an intermediate in this conversion.

3-hydroxy- N^6 -trimethyl-lysine (II_b) as an intermediate in carnitine biosynthesis; isotope-dilution experiments

Isotope-dilution experiments with 3-hydroxy- N^6 -trimethyl-lysine (II_b) in liver and kidney slices, with [Me - ^{14}C]trimethyl-lysine as a substrate, are shown in Table 3. Rat liver slices incubated with [Me - ^{14}C]trimethyl-lysine incorporated 0.97% of the radioactivity into carnitine and 0.20% into 4-trimethylammoniobutyrate (Table 3). When 2.5 mg of non-radioactive trimethylammoniobutyrate was added to liver slices, the conversion of [Me - ^{14}C]trimethyl-lysine into carnitine was decreased, and the radioactivity accumulated in the trimethylammoniobutyrate trap. When 2.5 mg of 3-hydroxy- N^6 -trimethyl-lysine (II_b) was incubated with the liver slice, the conversion of [Me - ^{14}C]trimethyl-lysine into carnitine was decreased, and the radioactivity accumulated in trimethylammoniobutyrate. The specific radioactivity of carnitine and trimethyl-

Table 3. Metabolism of [^{14}C]trimethyl-lysine by liver and kidney slices in the presence of trapping compounds. In each experiment 0.5 g of liver or kidney slices was incubated with $7.9 \mu\text{Ci}$ of N^6 -[Me - ^{14}C]trimethyl-lysine for 2 h . The metabolites were separated by using a 1 cm diameter column of AG-50 (H^+ form) eluted with a 1.5 – 4.0 M gradient of HCl . Peak I corresponds to the region where trimethylammoniopentanoate elutes; peak II contains metabolites (II_a) and (II_b).

Tissue	Additions	Isolated metabolites (% of added radioactivity)				
		Carnitine	Trimethyl-ammoniobutyrate	Peak I	Peak II	Trimethyl-lysine
Liver	None	0.97	0.20	0.50	0.40	97.03
	Trimethylammoniobutyrate (2.5 mg)	—	0.87	0.25	0.27	96.92
	Metabolite (II_b) (2.5 mg)	0.28	1.11	0.48	0.46	96.97
Kidney	None	—	8.26	0.39	2.46	88.15
	Metabolite (II_b) (2.5 mg)	—	6.61	0.47	3.02	89.33

ammoniobutyrate was 2850 d.p.m./nmol in the tracer study, but with addition of unlabelled compound II_b, this was decreased to 260 d.p.m./nmol. Thus the decrease in incorporation of radioactivity was accompanied by an increased overall synthesis. There was no trapping of radioactivity in 3-hydroxy-*N*⁶-trimethyl-lysine. When rat kidney slices were used, the addition of 2.5 mg of 3-hydroxy-*N*⁶-trimethyl-lysine to the incubation medium also resulted in no trapping of radioactivity in 3-hydroxy-*N*⁶-trimethyl-lysine in peak II_b.

The fact that 3-hydroxy-*N*⁶-trimethyl-lysine did not trap radioactivity from [¹⁴C]trimethyl-lysine seems inconsistent with the proposed scheme for carnitine biosynthesis, but the accumulation of radioactivity in trimethylammoniobutyrate (with decreased specific radioactivity) instead of in peak II_b seems to indicate that compound II_b-derived trimethylammoniobutyrate was the trapping product. As shown in Figs. 3(a) and 3(b), II_b is readily converted into trimethylammoniobutyrate and carnitine. To examine the possibility that II_b does not accumulate to a sufficient degree inside hepatocytes to effectively trap radioactivity from trimethyl-lysine, the washed slice and medium were separated before chromatography. As shown in Table 4, when tracer amounts of (*Me*-³H)-labelled II_b were incubated with rat liver slices, one-half the synthesized carnitine and 15% of the trimethylammoniobutyrate were retained in the washed slice at the end of the incubation. The addition of 2.5 mg of unlabelled compound II_b to the incubation resulted in a 41% decrease in overall conversion of radioactive compound II_b (from 11.3% to 6.7%) (Table 4). The amount of compound II_b retained in the tissue slice at the end of the incubation was 3.6% of the starting radioactivity. Control incubations done with ¹²⁵I-labelled albumin showed the extracellular space in

the washed liver slices averaged 1.97% of the added ¹²⁵I-labelled albumin in six experiments. When the amount of compound II_b in the liver slice was corrected for the extracellular space, there was shown to be only a small amount that accumulates in the hepatocyte and is not converted into trimethylammoniobutyrate and carnitine. The lack of marked dilution of the conversion of compound II_b into products is consistent with the above hypothesis.

When 2.5 mg of trimethylammoniobutyrate was added to an incubation with radioactive trimethylammoniobutyrate, there was a marked decrease (95%) in conversion of radioactivity into carnitine with an increase in slice trimethylammoniobutyrate radioactivity (Table 4). This increase in slice trimethylammoniobutyrate is consistent with the trapping experiments with this compound.

Discussion

Is 3-hydroxytrimethyl-lysine (II_b) an intermediate in carnitine biosynthesis?

Trimethyl-lysine has been demonstrated to be converted into compound (II_b) in the intact rat, and compound (II_b) is recovered in the urine (Cox & Hoppel, 1973b). In addition, trimethyl-lysine is converted into compound (II_b) in both rat liver slices and rat kidney slices (Cox & Hoppel, 1974a,b). When tracer amounts of 3-hydroxy-*N*⁶-trimethyl-lysine are incubated with rat liver slices, radioactivity is readily converted into trimethylammoniobutyrate and carnitine. In isotope-dilution experiments with liver slices, trimethylammoniobutyrate is an intermediate in the conversion of compound (II_b) into carnitine. When incubated with rat kidney slices, compound (II_b) is readily converted into trimethylammoniobutyrate. These data are sugges-

Table 4. Metabolism of ³H-labelled metabolite (II_b) and [¹⁴C]trimethylammoniobutyrate by rat liver slices; distribution of substrate and products in the medium and washed slice

In each experiment 0.5 g of rat liver slice was incubated with 1.2 μCi of ³H-labelled metabolite (II_b) or 2.4 μCi of [¹⁴C]trimethylammoniobutyrate for 2 h. When metabolite (II_b) was the substrate, the metabolites were separated as described in Table 3. In the experiments with trimethylammoniobutyrate, the acid extracts were chromatographed on a column (1 cm × 50 cm) of AG-50 (X8; H⁺ form) eluted with 2 M-HCl.

		Isolated metabolites (% of added radioactivity)		
		Carnitine	Trimethyl-ammoniobutyrate	Metabolite (II _b)
³ H-labelled metabolite (II _b)	Slice	0.7	1.5	3.6
	Medium	0.7	8.4	85.2
+ Metabolite (II _b) (2.5 mg)	Slice	0.24	1.48	4.23
	Medium	0.26	4.67	87.9
[¹⁴ C]Trimethylammoniobutyrate	Slice	14.65	4.27	
	Medium	6.91	67.31	
+ Trimethylammoniobutyrate (2.5 mg)	Slice	0.84	9.26	
	Medium	0.23	82.97	

tive of the sequence of metabolites shown in Scheme 1.

We then used traditional isotope-dilution experiments with unlabelled 3-hydroxy-*N*⁶-trimethyl-lysine (II_b) to complete the identification of this metabolite in the metabolic pathway of carnitine biosynthesis. Unexpectedly, compound (II_b) does not trap trimethyl-lysine radioactivity, but there is enhancement of the radioactivity chromatographing with trimethylammoniobutyrate and a decreased conversion into carnitine. The percentage of radioactivity from trimethyl-lysine converted into trimethylammoniobutyrate and carnitine was only slightly decreased with the large amounts of compound (II_b) present. Therefore, we determined the specific radioactivity of the products and found a similar decrease in both trimethylammoniobutyrate and carnitine. Thus there is dilution of the isotope, but in the 'wrong' intermediate. A similar lack of trapping of radioactivity in the metabolite (II_b) was observed in rat kidney slices. These data for the rat contrast with the results obtained by Kaufman & Broquist (1978) in *N. crassa*. These investigators observed that addition of 3-hydroxy-*N*⁶-trimethyl-lysine resulted in a marked decrease in incorporation of trimethyl-lysine radioactivity into both trimethylammoniobutyrate and carnitine. Specific radioactivities were not reported to distinguish between dilution or inhibition.

Although it is possible that compound (II_b) is not on the main pathway of carnitine biosynthesis from trimethyl-lysine, there is evidence to argue against this interpretation. First, compound (II_b) is a 6-carbon-chain metabolite of trimethyl-lysine still possessing the primary amine and the quaternary amine. Secondly, compound (II_b) is rapidly converted into trimethylammoniobutyrate and carnitine. Thirdly, the fact that the chromatogram in the isotope-dilution experiment with compound (II_b) is similar to the chromatogram from the isotope-dilution experiment using trimethylammoniobutyrate suggests that the trapping of the isotope occurs at trimethylammoniobutyrate and not at compound (II_b). Finally, the similar decrease in specific radioactivity of trimethylammoniobutyrate and carnitine is consistent with isotope dilution.

One plausible explanation for the lack of definitive isotope dilution at compound (II_b) is that there may exist a difference in the rate-limiting steps in this metabolic pathway. With tracer amounts of trimethylammoniobutyrate, approx. 22% of the radioactivity is converted into carnitine. When substrate amounts of trimethylammoniobutyrate (similar to those used in the trapping experiments) are added, the conversion of [³H]trimethylammoniobutyrate is markedly decreased (95%), so that approx. 1.1% is converted into carnitine, although there is an increase in the absolute amount of trimethyl-

ammoniobutyrate converted. In a similar series of experiments, the conversion of radioactively labelled compound (II_b) into trimethylammoniobutyrate and carnitine is decreased by 41% by the addition of substrate amounts of unlabelled compound (II_b). This suggests that the intracellular concentration of compound (II_b) during the isotope-dilution experiments is below saturation for the conversion of compound (II_b) into trimethylammoniobutyrate and carnitine. We performed experiments in which we examined the distribution of substrates between the medium and the slices during these incubations. With the addition of unlabelled trimethylammoniobutyrate, there was an accumulation of radioactive trimethylammoniobutyrate within the hepatocyte. When unlabelled compound (II_b) was added to an incubation with ³H-labelled compound (II_b), the amount of radioactively labelled compound (II_b) retained within the slice did not change. Thus the rapid conversion of compound (II_b) into trimethylammoniobutyrate and carnitine in these studies suggests that the rate-limiting step in compound (II_b) metabolism is the uptake by the hepatocyte. Compound (II_b) therefore does not accumulate within the hepatocyte, but is rapidly converted into trimethylammoniobutyrate. Because of the increased conversion into trimethylammoniobutyrate, there is trapping of the radioactive isotope in this compound with a subsequent decrease in the amount of radioactivity derived from trimethyl-lysine in carnitine. Thus compound (II_b) is an active intermediate in the conversion of trimethyl-lysine into trimethylammoniobutyrate. The identity of this metabolite as 3-hydroxy-*N*⁶-trimethyl-lysine has been established by using n.m.r. spectroscopy (Novak *et al.*, 1980).

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