The Formation and Degradation of Carnitine in *Pseudomonas**

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ABSTRACT: Pathways involved in the biosynthesis and degradation of carnitine (3-hydroxy-4-trimethylaminobutyric acid) have been studied in a bacterial system. γ-Butyrobetaine (4-trimethylaminobutyric acid) is hydroxylated to carnitine by acetone powder prepared from a new *Pseudomonas* strain (*Ps. sp.* AK 1) in a reaction which requires molecular oxygen and occurs with a combination of Fe^{2+}, ascorbate, and a reduced nicotinamide–adenine dinucleotide phosphate regenerating system. The degradation of carnitine to glycine betaine occurs in the presence of oxidized nicotinamide–adenine dinucleotide (NAD^+), adenosine triphosphate (ATP), and coenzyme A (CoA), whereas trimethylaminoacetone is formed in the absence of ATP and CoA in a NAD^+-requiring reaction, presumably with the intermediate formation of 3-keto-4-trimethylaminobutyric acid.

The results from a series of experiments exclude a pathway from carnitine to glycine betaine involving betaine aldehyde as an intermediate.

Carnitine[1] occurs not only in mammalian tissues but also in several microorganisms, *e.g.*, *Neurospora*, *Streptococci*, and yeast (Fraenkel and Friedman, 1957). Although carnitine was identified as one of the main low-molecular nitrogen-containing compounds in muscle in 1905 (Gulewitsch and Krimberg, 1905; Kutscher, 1905), and although its important role in lipid metabolism has recently become apparent (for a review, see Fritz, 1964), very little is known about its biosynthesis and degradation. In rat and mouse, carnitine may be formed from γ-butyrobetaine (Bremer, 1962; Lindstedt and Lindstedt, 1961) in an oxygenase reaction (Lindstedt and Lindstedt, 1962; Lindstedt, 1967). In normal rats, labeled carnitine is recovered unchanged in the urine. In pregnancy, starvation, choline deficiency, and under other special dietary conditions, rats excrete β-methylcholine in the urine (Mehlman and Wolf, 1963; Khairallah and Wolf, 1965). Since carnitine is metabolized slowly in mammals, we have earlier studied a bacterial system which degrades carnitine to glycine betaine (Lindstedt and Lindstedt, 1961). The results from these studies suggested the possibility that carnitine might be formed from γ-butyrobetaine in a β oxidation.

The present work has shown that carnitine is degraded to trimethylaminoacetone in the presence of NAD^+ and to glycine betaine in the presence of NAD^+, ATP, and CoA by acetone powders from a new *Pseudomonas* strain capable of utilizing γ-butyrobetaine or carnitine as sole source of carbon and nitrogen. The same strain forms carnitine from γ-butyrobetaine in a reaction which is apparently of the oxygenase type.

Experimental Procedures

Chemicals.[2] Commercially available chemicals and enzymes were obtained from the following sources: 4-amino-3-hydroxybutyric acid, DL-carnitine chloride, 4-aminobutyric acid, methyl iodide, palladized charcoal (10%), and sodium DL-isocitrate from Fluka AG, Chemische Fabrik, Buchs, Switzerland; dimethylaminoacetone from K & K Laboratories, Inc., Plainview, N. Y.; dimethylaminoacetaldehyde diethyl acetal from Aldrich Chemical Co., Inc., Milwaukee, Wis.; glycine betaine chloride from Nutritional Biochemicals Corp., Cleveland, Ohio; [^{14}C]methyl iodide from The Radiochemical Centre, Amersham, Bucks, England; 4-amino[carboxy-^{14}C]butyric acid and [methyl-^{14}C]glycine betaine chloride from New England Nuclear Corp., Boston, Mass.; CoA, NAD^+, NADPH, isocitrate dehydrogenase ($NADP^+$ specific), and catalase from C. F. Boehringer & Soehne, GmbH Mannheim, West Germany; ATP from Sigma Chemical Co., St. Louis, Mo.; sodium ascorbate from Theodor Schuchardt GmbH and Co., München, West Germany; and silica gel G from E. Merck AG, Darmstadt, West Germany.

γ-Butyrobetaine, 4-dimethylaminobutyric acid hydrochloride, and DL-4-dimethylamino-3-hydroxybutyric acid were prepared as described previously (Lindstedt and Lindstedt, 1965a). Trimethylaminoacetone chloride was obtained by methylation of dimethylaminoacetone

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[1] Trivial names and abbreviations used: carnitine, 3-hydroxy-4-trimethylaminobutyric acid; γ-butyrobetaine, 4-trimethylaminobutyric acid; CoA, coenzyme A; ATP, adenosine triphosphate; $NADP^+$ and NADPH, oxidized and reduced nicotinamide–adenine dinucleotide phosphates; NAD^+, oxidized nicotinamide–adenine dinucleotide.

[2] Melting points were determined on a heating block and are uncorrected.

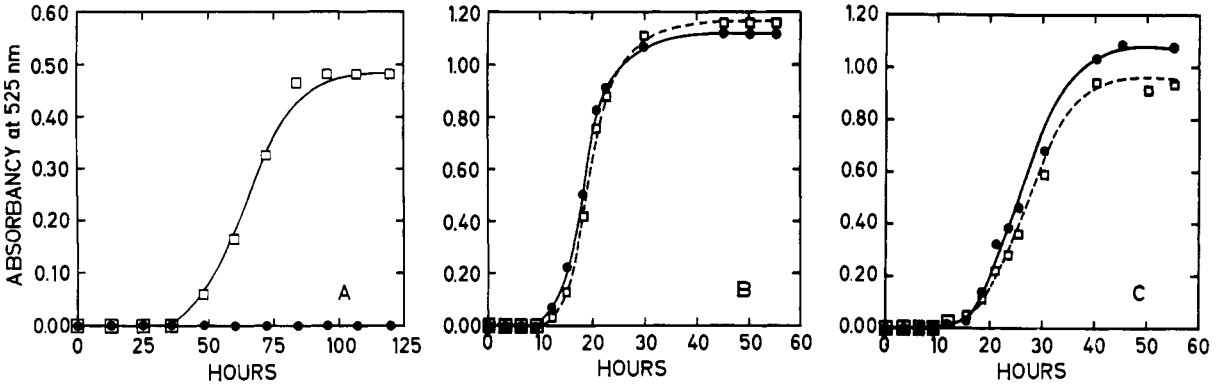


FIGURE 1: Growth curves for *Ps. aeruginosa* NCTC A 7244 (A) and *Pseudomonas sp.* AK 1 (B,C) in medium 1, containing (A) (□——□) carnitine, (●——●) γ-butyrobetaine, (B) carnitine, and (C) γ-butyrobetaine. In B and C, ●——● represents growth of bacteria which had been adapted to the substrate by two precultures and □——□ represents the growth of bacteria which had previously been grown on a glucose–ammonium chloride medium. All media were initially 0.02 M in substrate.

with methyl iodide in the following way. Dimethylaminoacetone (26 mmoles) was dissolved in 15 ml of ethanol, the solution was cooled in ice water, and methyl iodide (30 mmoles) was added. The reaction mixture was allowed to stand for 15 hr and then taken to dryness. The residue was dissolved in water and filtered through a column of Dowex 1-X2 (200–400 mesh, Cl^- form). The combined filtrate and washings were taken to dryness and the remaining trimethylaminoacetone chloride was crystallized from ethanol–diethyl ether. The crystals were hygroscopic and the gold salt and picrate were prepared; tetrachloroaurate, mp 141–142°, lit. (Major and Cline 1932) mp 139.5°; picrate, mp 151–152°, lit. (Gibson *et al.*, 1942) mp 149–150°. Trimethylaminoacetaldehyde diethyl acetal iodide was obtained by methylation of dimethylaminoacetaldehyde diethyl acetal with methyl iodide as described above and recrystallized from ethanol–diethyl ether, mp 114–115°, lit. (Fourneau and Chantalou, 1945) mp 115°. Trimethylaminoacetaldehyde diethyl acetal iodide was filtered through Dowex

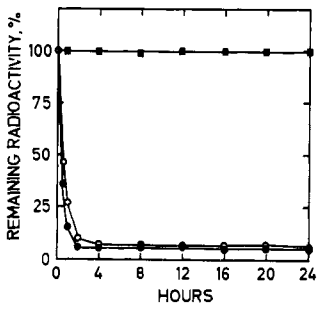


FIGURE 2: Recovery of radioactivity in nonvolatile matter in centrifuged medium after incubation of *Pseudomonas sp.* AK 1 with [methyl-^{14}C]trimethylaminoacetone (■——■, bacteria adapted to carnitine), R-[methyl-^{14}C]carnitine (○——○, bacteria adapted to carnitine), and [methyl-^{14}C]γ-butyrobetaine (●——●, bacteria adapted to γ-butyrobetaine). Six microcuries (0.4 μmole) of each compound was incubated with the cells in 10 ml of medium 1 (about 10^9 cells/ml).

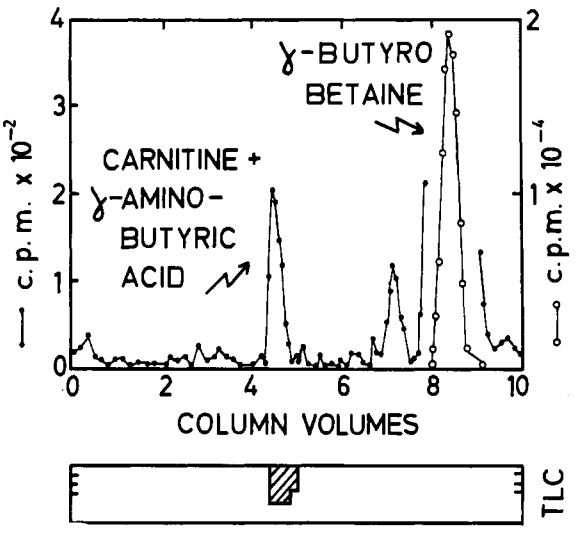


FIGURE 3: Fractionation on a column (diameter 1.4 cm, height 50 cm) of Dowex 50-X8 of a trichloroacetic acid extract of medium and cells after incubation of *Pseudomonas sp.* AK 1 with 10^{-4} M [carboxy-^{14}C]γ-butyrobetaine. Fractions of 1 ml had been removed at 30-min intervals and cooled. The fractions had been recombined and 15 mg of DL-carnitine had been added before chromatography. The upper diagram shows the distribution of radioactivity in the ion-exchange chromatogram and the lower diagram the localization of carnitine with thin layer chromatography.

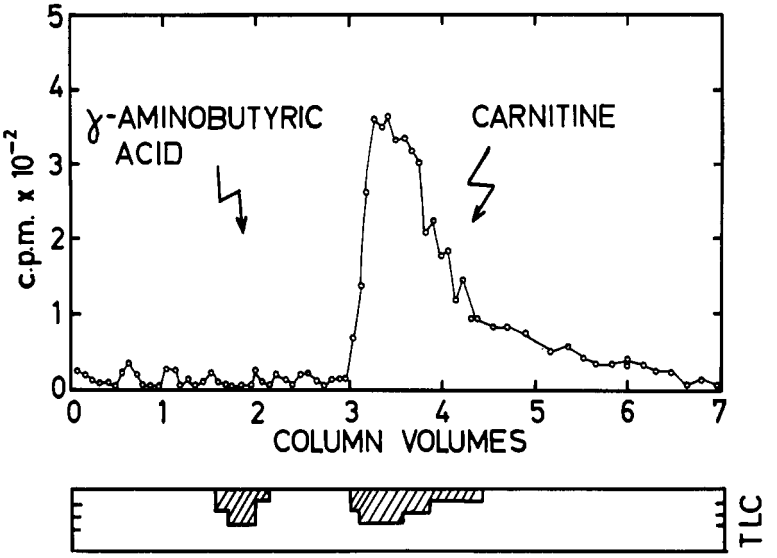


FIGURE 4: Fractionation on a column (diameter 1.6 cm, height 50 cm) of Duolite C-3 of "carnitine–γ-aminobutyric acid fraction" isolated by chromatography on Dowex 50-X8 (Figure 3). γ-Aminobutyric acid (15 mg) had been added before chromatography. The upper diagram shows the distribution of radioactivity in the ion-exchange chromatogram and the lower diagram the localization of γ-aminobutyric acid and carnitine with thin layer chromatography.

1-X2 (200–400 mesh, Cl^- form), and the resulting chloride was treated with concentrated hydrochloric acid at room temperature as described by Fischer (1893). As judged by thin layer chromatography this resulted in complete liberation of trimethylaminoacetaldehyde chloride (glycine betaine aldehyde).

Labeled Compounds. The preparation of R-[methyl-^{14}C]carnitine and [carboxy-^{14}C]γ-butyrobetaine has been described previously (Lindstedt and Lindstedt, 1964, 1965a). [Methyl-^{14}C]trimethylaminoacetone, DL-[methyl-^{14}C]carnitine, and [methyl-^{14}C]γ-butyrobetaine were obtained by treating a tenfold excess of the corresponding dimethylamines with [^{14}C]methyl iodide in 90% aqueous methanol. This gave 90–95% isotopic yield of methyl-labeled quaternary amines with specific radioactivities of 15–30 mc/mmole. For instance, DL-[methyl-^{14}C]carnitine (0.5 mc, 15 mc/mmole) was prepared by mixing 300 μmoles of DL-4-dimethylamino-3-hydroxybutyric acid and 200 μmoles of barium hydroxide with 33 μmoles of [^{14}C]methyl iodide in a glass tube cooled in liquid nitrogen. Aqeuous methanol (5 ml of 90%) was added and the tube was sealed in a flame. It was left at room temperature for 6 days with occasional shaking and the content was then taken to dryness at a water bath temperature below 50°. The residue was dissolved in 0.1 M sodium hydroxide and left for 2 hr at room temperature for hydrolysis of any methyl ester which might have been formed. The mixture was then acidified with 0.1 M hydrochloric acid and chromatographed on a 100-ml column of Dowex 50-X8 (200–400 mesh, H^+ form) which was eluted with 1 M hydrochloric acid. The fractions containing radioactive material which appeared after approximately 4.5 bed volumes of effluent were pooled and taken to dryness with a rotatory evaporator at a bath temperature below 50°. In order to establish that no 4-trimethylaminocrotonic acid had been formed as the result of acid-catalyzed dehydration of carnitine during this procedure a small amount was rechromatographed on a Dowex 50 column; isotope was found only in the carnitine peak. Further confirmation of the chemical and isotopic purity of the carnitine was obtained by thin layer and paper chromatography. [Methyl-^{14}C]trimethylaminoacetaldehyde diethyl acetal chloride (0.1 mc, 30 mc/mmole) was prepared by adding 30 μmoles of dimethylaminoacetaldehyde diethyl acetal in 0.5 ml of ethanol to 3.3 μmoles of [^{14}C]methyl iodide in a glass tube cooled in liquid nitrogen. The tube was sealed and left at room temperature for 20 hr. After lyophilization, the residue was dissolved in water and iodide ions were exchanged for chloride by passage through a column of Dowex 1-X2 (200–400 mesh, Cl^- form). The aldehyde was obtained by acid hydrolysis of the acetal. When a sample of the aldehyde was chromatographed on a column of Dowex 1-X2 (200–400 mesh, HSO_3^- form) all the radioactive material was eluted with water after about 1.5 bed volumes, whereas glycine betaine would have been eluted after about 0.8 bed volume.

Bacteriological Technique and Incubating Conditions. The experiments were performed with two strains of the genus *Pseudomonas: Pseudomonas aeruginosa* NCTC A 7244, and a new strain isolated from a sweet water aquarium in the home of T. Midtvedt. This strain was isolated in the following way. Aquarium content (5 ml) was added to 100 ml of medium 1 (2.65 g of KH_2PO_4, 5.43 g of $Na_2HPO_4 \cdot 2H_2O$, 75 mg

of $MgSO_4 \cdot 7H_2O$, 4.5 mg of $MnCl_2 \cdot 4H_2O$, 5 mg of $FeSO_4 \cdot 7H_2O$, and 10 mg of $Na_2S_2O_3 \cdot 5H_2O$) containing 0.02 M carnitine chloride which had been neutralized with potassium hydroxide before addition to the medium. This and other media containing carnitine or γ-butyrobetaine were adjusted to pH 7.0 and sterilized by filtration through a Millipore type GS filter. After aerobic incubation at 37° for 24 hr, an aliquot of 0.5 ml was again incubated with 50 ml of medium 1 containing 0.02 M γ-butyrobetaine chloride. The next incubation was made on plates of 1.5% agar and medium 1 containing 0.02 M γ-butyrobetaine. The growth on these plates consisted of only one kind of colonies. The bacteria thus isolated were small Gram-negative rods which grew only under aerobic conditions. They were further identified by conventional techniques (Cowan and Steel, 1965; Skerman, 1959; Cruickshank, 1960). Growth curves were obtained as follows. Two successive incubations were made in 10 ml of medium 1 with 0.02 M substrate. The last culture was centrifuged and the pellet was washed twice with medium 1. The sediment was then suspended in such a volume of medium 1 that the absorbancy of the suspension was 0.140 ± 0.005 at 525 nm (10^8–10^9 living cells/ml). After a 10^2-fold dilution (in the experiment shown in Figure 1A a 10^4-fold dilution was used) 0.1 ml was used to inoculate 10 ml of 0.02 M substrate in medium 1 in calibrated test tubes. The tubes were shaken in a water bath at 37° and turbidity was measured at 525 nm.

Incubations with labeled compounds were carried out with bacteria which had been adapted by three transfers to media containing 0.02 M DL-carnitine or γ-butyrobetaine. The bacteria from a 40-ml culture were collected by centrifugation at the end of the logarithmic growth phase, washed three times with medium 1, and then resuspended in the original volume of medium 1. For radioactivity measurements, incubations were centrifuged and aliquots were evaporated on aluminum planchets.

Acetone-dried cells of strain AK 1 were prepared from bacteria which had been grown for 72 hr in 5 l. of medium 1 containing 0.1 M carnitine or γ-butyrobetaine and 0.01 M ammonium succinate. These bacteria were collected by centrifugation, washed three times with medium 1 and two times with a 0.9% sodium chloride solution, resuspended in 10 ml of distilled water, and poured into 300 ml of acetone which had been cooled to −15°. The precipitate was washed three times with acetone at −15°, filtered, and kept at −15° in a desiccator over concentrated sulfuric acid. About 1 g of dried cells was obtained from a 5-l. culture. Acetone-dried cells (5 mg) were incubated with the labeled substrates and cofactors in 0.1 M Tris-HCl buffer at pH 7.8 or 0.1 M potassium phosphate buffer at pH 7.4. The incubations were done in 5-ml test tubes with a glass bead by shaking in a water bath at 37°.

Incubations with argon as gas phase were carried out in Thunberg tubes. After addition of the complete system (see legend to Table II) except for γ-butyrobetaine, the tubes were cooled in ice, evacuated to about 10 torr, and refilled with argon, containing less than 5 ppm of oxygen. The procedure was repeated five times, γ-butyrobetaine was added, and the tubes were evacuated and refilled with argon another five times. Two sets of control incubations were included; one was kept in air during the whole experiment and one was flushed with argon as above, and air was then introduced. The results were the same in these two types of control incubations.

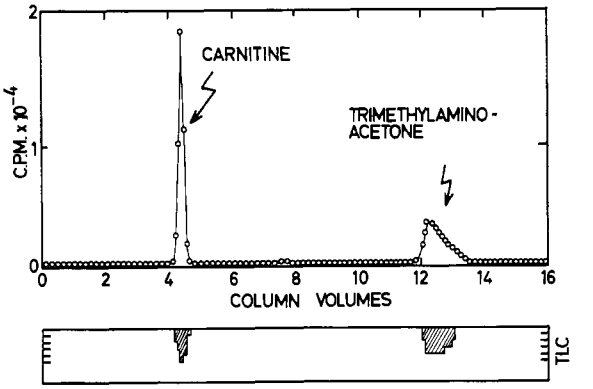


FIGURE 5: Fractionation on a column (diameter 1.6 cm; height 50 cm) of Dowex 50-X8 of the supernatant after incubation for 6 hr at 37° of DL-[methyl-^{14}C]-carnitine (4.5×10^{-4} M) with 5 mg of acetone-dried cells of *Pseudomonas sp.* AK 1 in 0.5 ml of 0.1 M potassium phosphate buffer at pH 7.4 in the presence of 2 μmoles of NAD^+. Fifteen milligrams of carnitine and trimethylaminoacetone had been added before chromatography. The upper diagram shows distribution of radioactivity in the ion-exchange chromatogram and the lower diagram localization of carnitine and trimethylaminoacetone by thin layer chromatography.

Analytical Methods. Descending paper chromatography was carried out on Munkfell No. 312 filter paper for 12–15 and 4–6 hr, respectively, in 96% ethanol–25% ammonia (95:5). The distribution of radioactive material on the paper strips was determined in a scanner operating in the proportional region with an efficiency for ^{14}C of about 10%. Quaternary ammonium compounds were made visible by dipping the papers in a solution of iodine in diethyl ether. Typical R_F values were: carnitine and 4-trimethylaminobutyric acid 0.19, glycine betaine 0.32, and trimethylaminoacetone and glycine betaine aldehyde 0.58.

Thin layer chromatography was performed on silica gel G with methanol–25% ammonia (50:50), methanol–25% ammonia (75:25), and 25% hydrochloric acid–acetone–methanol (10:90:10) as mobile phases (Eneroth and Lindstedt, 1965; Lindstedt and Lindstedt, 1965b). Quarternary ammonium compounds were localized with iodine vapor, and primary amines were localized by spraying the plates with a 1% solution of ninhydrin in ethanol for plates developed in the basic media or

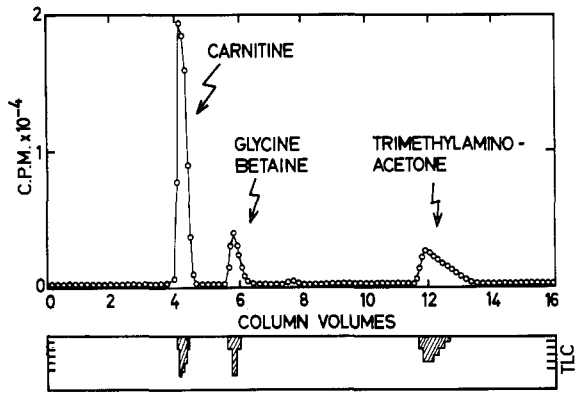


FIGURE 6: Fractionation on a column (diameter 1.6 cm; height 50 cm) of Dowex 50-X8 of the supernatant after incubation for 6 hr at 37° of DL-[methyl-^{14}C]-carnitine (4.5×10^{-4} M) with 5 mg of acetone-dried *Pseudomonas sp.* AK 1 in 0.5 ml of 0.1 M potassium phosphate buffer at pH 7.4, containing 2 μmoles of NAD^+, 2 μmoles of ATP, 0.5 μmole of CoA, and 2 μmoles of Mg^{2+}. Fifteen milligrams of carnitine, glycine betaine, and trimethylaminoacetone had been added before chromatography. The upper diagram shows the distribution of radioactivity on the ion-exchange chromatogram and the lower diagram the localization of the compounds with thin layer chromatography.

in ethanol–pyridine (97:3) for plates developed in the acid medium. For radioactivity measurements, silica gel from plates treated only with iodine vapor was scraped off and extracted with 80% methanol in water. The extracts were taken to dryness on glass planchets.

Ion-exchange chromatography was performed on columns of Dowex AG 50-X8 (200–400 mesh, H^+ form) which were eluted with 1 M hydrochloric acid. Carnitine and 4-aminobutyric acid were separated on columns of Duolite C-3 (200–400 mesh, H^+ form, purchased as Bio-Rex 40 from Bio-Rad Laboratories, Inc., Richmond, Calif.) which were eluted with 2 M hydrochloric acid. Glycine betaine aldehyde was isolated on a column of Dowex 1-X2 (200–400 mesh, HSO_3^- form) which was eluted with water. The isotope content of the chromatographic fractions was determined on aliquots which were evaporated in glass dishes and counted in a flow counter (Frieseke & Hoepfner GmbH, Erlangen-Bruck, West Germany) with an efficiency for ^{14}C of about 40%.

Results

Classification of the Isolated Bacterial Strain. Based on the characteristics of the isolated bacteria which are given in Table I, they were classified as belonging to the genus *Pseudomonas* and will be designated *Pseudomonas sp.* AK 1.

Growth Curves. The results of experiments in which the growth of *Pseudomonas sp.* AK 1 and *Ps. aeruginosa* NCTC A 7244 were compared in different media are presented in Figure 1. *Ps. aeruginosa* NCTC A 7244 could utilize carnitine, but not γ-butyrobetaine, as the sole source of carbon and nitrogen (Figure 1A), whereas strain AK 1 utilized both carnitine (Figure 1B) and γ-butyrobetaine (Figure 1C). The same growth response was obtained with bacteria that had been adapted to carnitine and γ-butyrobetaine as with bacteria which had previously been grown on glucose and ammonium chloride (Figure 1B,C). The growth curves in the glucose–ammonium chloride medium were identical irrespective of the previous adaptation of the bacteria. Trimethylaminoacetone was not used as a substrate by either *Ps. aeruginosa* NCTC A 7244 or by *Pseudomonas sp.* AK 1.

TABLE I: Characters of *Pseudomonas sp.* AK 1.

Positive Tests	Negative Tests
Mobility; oxidase and catalase activity; growth on McConkey agar and utilization of citrate; acid production[a] from xylose, dextrose, galactose, and mannose; and production and diffusion of pigment	Growth at 5 and 42°; hydrolysis of gelatin and starch; reduction of nitrate; urease activity; and acid production from rhamnose, lactose, maltose, sucrose, raffinose, inulin, glycerol, inositol, and sorbitol

[a] Acid production was determined by pH measurements; in no case was gas production observed.

Figure 2 shows the disappearance of radioactivity when [methyl-^{14}C]carnitine, [methyl-^{14}C]γ-butyrobetaine, and [methyl-^{14}C]trimethylaminoacetone were incubated with living cells of *Ps. sp.* AK 1. Carnitine and γ-butyrobetaine were metabolized, but not trimethylaminoacetone.

Formation of Carnitine from γ-Butyrobetaine in Living Cells of Pseudomonas sp. AK 1. [Carboxy-^{14}C]γ-butyrobetaine (2 μc, 10^{-4} M) was incubated for 5 hr with bacteria adapted to growth on γ-butyrobetaine. Aliquots were taken for determination of radioactivity at 30-min intervals. After 2 hr 7% of the original amount of isotope remained in the uncentrifuged medium and on paper chromatography; less than 10% of the radioactivity appeared at the position of γ-butyrobetaine and carnitine, the remainder at the origin. The fractions removed at different times were recombined and deproteinized by addition of an equal volume of 10% trichloroacetic acid in water. After centrifugation, the sediments were washed twice with 5% trichloroacetic acid. The combined supernatant fractions were chromatographed together with carnitine on columns of Dowex 50 (Figure 3). The radioactivity put onto the column was recovered in three peaks; one metabolite was eluted together with carnitine which had been

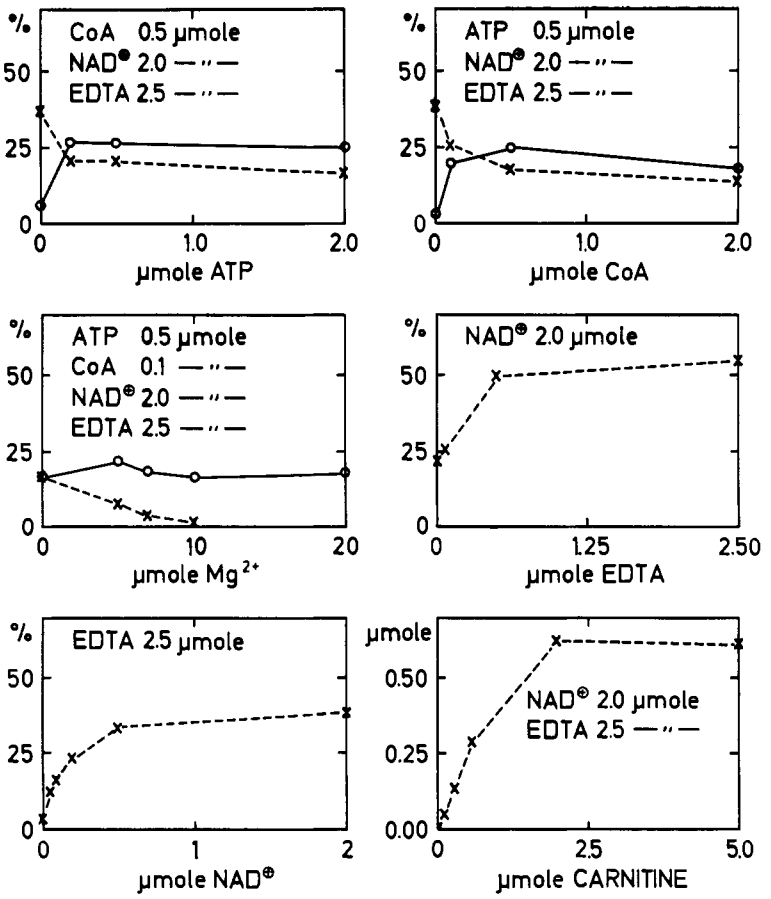


FIGURE 7: Formation of trimethylaminoacetone (×---×) and glycine betaine (○——○) under different conditions of incubation. The reaction mixture contained 5 mg of acetone-dried cells of *Pseudomonas sp.* AK 1, 2 µmoles (0.8 µc) of DL-[methyl-^{14}C]carnitine (except for the experiment in which the carnitine concentration was varied), and other additions as shown in the figure, in 0.5 ml of 0.1 M potassium phosphate buffer at pH 7.4. The temperature was 37° and the time of incubation 2 hr. The per cent conversion of carnitine to metabolites was calculated from radiochromatograms of the incubation mixture.

added before the chromatography and a second at the expected position of 4-dimethylaminobutyric acid. 4-Aminobutyric acid would have been eluted close to carnitine under these conditions (Lindstedt and Lindstedt, 1965b). In order to establish the identity of the radioactive material with carnitine it was rechromatographed, together with authentic 4-aminobutyric acid, on a column of Duolite C-3 (Lindstedt and Lindstedt, 1965b). As shown in Figure 4, the ^{14}C-containing material migrated together with carnitine. This was also the case when the material was chromatographed on paper. The radioactive carnitine accounted for only about 0.5% of the labeled material which had been added to the column. Addition of unlabeled DL-carnitine to the media did not increase the yield of labeled carnitine from [carboxy-^{14}C]γ-butyrobetaine. A similar chromatogram was also obtained from an experiment with [methyl-^{14}C]γ-butyrobetaine. With this substrate no radioactivity was found at the positions of glycine betaine or trimethylaminoacetone (see below).

Formation of Carnitine from γ-Butyrobetaine by Acetone-Dried Cells of Pseudomonas sp. AK 1. A series of incubations were performed with labeled γ-butyrobetaine and acetone powder prepared from cells of strain AK 1 under conditions which would favor a β oxidation and under conditions which would favor an oxygenase type of reaction and which have been found optimal for the hydroxylation of γ-butyrobetaine to carnitine in preparations of rat liver (Lindstedt and Lindstedt, 1962; Lindstedt, 1967). The incubations mixtures were deproteinized with 10% trichloroacetic acid and fractionated on columns of Dowex 50-X8. In the presence of NAD^+, ATP, and CoA less than 0.4% of the initial radioactivity was eluted at the positions of carnitine, trimethylaminoacetone, and glycine betaine (see below). With NADPH, ascorbate, and Fe^{2+} about 50% conversion of 2 µmoles of γ-butyrobetaine to carnitine was obtained with 5 mg of bacterial powder and with air as the gas phase, whereas less than 0.4% conversion was obtained with argon as the gas phase (Table II).

TABLE II: Conversion of γ-Butyrobetaine to Carnitine with Acetone Powder of *Pseudomonas sp.* AK 1.[a]

Expt	Cofactors	Gas Phase	Conversion to Carnitine (%)
a	NAD^+, ATP, CoA	Air	<0.4
b	NADPH, ascorbate, Fe^{2+}	Air	49
	NADPH, ascorbate	Air	29
	NADPH, Fe^{2+}	Air	2.7
	Ascorbate, Fe^{2+}	Air	0.7
	NADPH	Air	<0.4
	Fe^{2+}	Air	<0.4
	NADPH, ascorbate, Fe^{2+}	Argon	<0.4
	NADPH, ascorbate, Fe^{2+} (boiled enzyme)	Air	<0.4

[a] The incubation mixture contained in expt a: 2 μmoles of NAD^+, 0.5 μmole of ATP, 0.1 μmole of CoA, 5 μmoles of Mg^{2+}, 2.5 μmoles of EDTA, and 100 μmoles of potassium phosphate buffer at pH 7.4 in a total volume of 0.78 ml; and in expt b: 0.3 μmole of NADPH, 10 μmoles of sodium ascorbate, 0.1 μmole of $FeSO_4$, 1.3 μmoles of Mg^{2+}, 25 μmoles of KCl, 5 μmoles of sodium DL-isocitrate, 100 μmoles of Tris-HCl buffer at pH 7.8, 0.01 mg of isocitrate dehydrogenase, and 1.0 mg of catalase in a total volume of 1.0 ml. About 5 mg of acetone-dried cells of *Pseudomonas sp.* AK 1 were incubated at 37° for 2 hr with 2 μmoles (0.36 μc) of [methyl-^{14}C]γ-butyrobetaine.

When Fe^{2+} was omitted from the system the conversion to carnitine was 29%. Omission of ascorbate or the NADPH-regenerating system resulted in a low hydroxylating activity, *i.e.*, less than 3% conversion of γ-butyrobetaine to carnitine (Table II). Carnitine was the only metabolite of γ-butyrobetaine which was detected in these experiments.

Formation of Glycine Betaine from Carnitine by Living Cells of Pseudomonas sp. AK 1. R-[Methyl-^{14}C]carnitine (6 μc, 0.2 μmole) was incubated with 10 ml of medium 1 containing a suspension of bacterial cells and aliquots were taken after different time intervals (see Figure 2). The incubation mixtures were fractionated by ion-exchange and paper chromatography. Small amounts of labeled glycine betaine, *i.e.*, about 0.1% of the initial radioactivity, were found during the first 0.5 hr of incubation but not later. No labeled trimethylaminoacetone was found though 0.01% of the initial amount of radioactivity in this region of the chromatograms would have been detected.

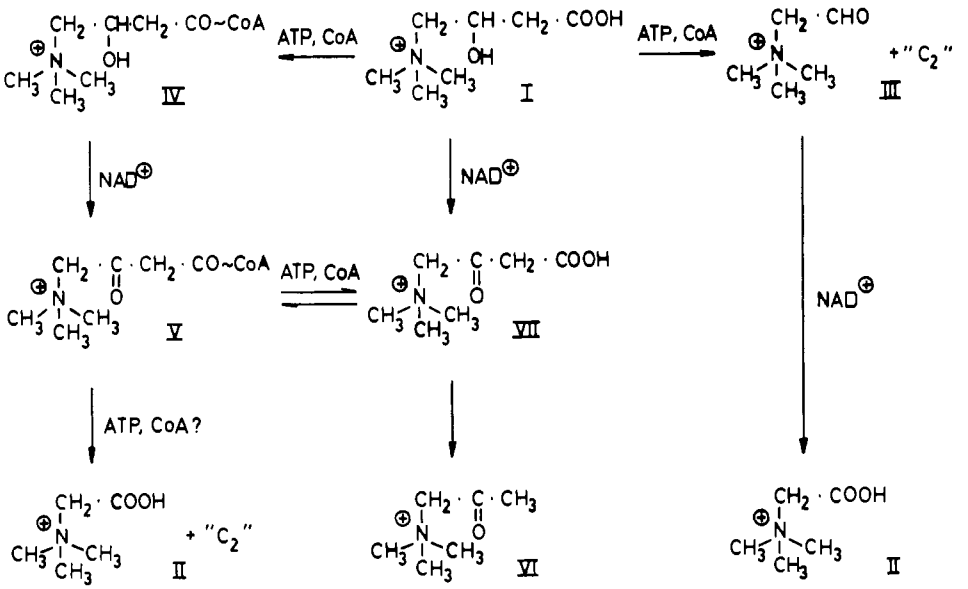
Conversion of Carnitine to Trimethylaminoacetone and Glycine Betaine by Acetone-Dried Cells of Pseudomonas sp. AK 1. Acetone-dried cells of strain AK 1 (5 mg) were first incubated with DL-[methyl-^{14}C]-carnitine (2 μc, 4.5×10^{-4} M) for 6 hr at 37° in 0.5 ml of phosphate buffer (pH 7.4) containing 2 μmoles of NAD^+. A paper chromatogram of the medium showed the presence of two labeled compounds with the same R_F values as carnitine and trimethylaminoacetone. Ion-exchange chromatography (Figure 5) on Dowex 50 showed two labeled compounds emerging together with authentic carnitine and trimethylaminoacetone.

In another experiment the incubation was carried out in the presence of NAD^+ (2 μmoles), CoA (0.5 μmole), and Mg^{2+} (2 μmoles). In this case three labeled compounds appeared on both the paper chromatograms and in the ion-exchange chromatograms (Figure 6). These compounds coincided with carnitine, glycine betaine, and trimethylaminoacetone. The fractions eluted from the ion-exchange column at the position of trimethylaminoacetone (50,000 cpm) were combined, nonradioactive trimethylaminoacetone chloride (100 mg) was added, and the tetrachloroaurate was prepared. The specific radioactivity of the tetrachloroaurate was about 150 cpm/mg and remained unchanged after two recrystallizations from 1 M hydrochloric acid. Nonradioactive glycine betaine chloride (100 mg) was added to the fractions eluted at the position of glycine betaine (32,000 cpm), and the tetrachloroaurate was prepared. The specific radioactivity of this was about 100 cpm/mg and was unchanged after two recrystallizations. No degradation of carnitine was observed in incubations with boiled acetone powders. In separate experiments it could be established that trimethylaminoacetone and glycine betaine were not metabolized under the incubating conditions which were used. The enzymic activity responsible for the formation of trimethylaminoacetone could not be extracted with phosphate buffer.

The effects of different cofactors on the conversion of carnitine to glycine betaine and to trimethylaminoacetone are shown in Figure 7. The formation of trimethylaminoacetone required NAD^+, was stimulated by EDTA in concentrations from 10^{-4} to 100×10^{-4} M, but was inhibited by the addition of Mg^{2+}. Glycine betaine was found when ATP and CoA had been added, and increased concentrations of these cofactors increased the formation of glycine betaine with a concomitant decrease in the formation of trimethylaminoacetone.

Exclusion of Betaine Aldehyde as an Intermediate in the Degradation of Carnitine. A possible pathway to glycine betaine could involve the initial formation of glycine betaine aldehyde. [Methyl-^{14}C]glycine betaine aldehyde (0.2 μmole, 6 μc) was incubated with 5 mg of acetone-dried cells of strain AK 1 and 2 μmoles of NAD^+ for 2 hr. Complete oxidation to glycine betaine was obtained, as shown by paper chromatography of the incubation mixture. A NAD^+-free system was obtained by suspension of the acetone powder in phosphate buffer, centrifugation, and repeated washings of the sediment with phosphate buffer. The supernatant was filtered through a column of Sephadex G-25, and the protein and salt fractions were collected. These preparations were incubated, sepa-

SCHEME I: Possible Pathways for the Degradation of Carnitine.



rately and in combination, together with labeled carnitine, ATP, CoA, Mg^{2+}, and EDTA in different concentrations, with and without the addition of unlabeled betaine aldehyde. No labeled betaine aldehyde could be detected. Glycine betaine and trimethylaminoacetone were formed when NAD^+ was added to the reconstituted system.

Discussion

Carnitine (I) (Scheme I) could be degraded to glycine betaine (II) either by an initial cleavage of the carbon chain to betaine aldehyde (III) and to a two-carbon fragment followed by an oxidation of the aldehyde, or by an initial oxidation of the CoA ester (IV) to a ketone (V) which is then cleaved to glycine betaine and a C_2 fragment. A reaction where betaine aldehyde is formed would be analogous to the cleavage of citric acid to oxaloacetic acid and acetate or to oxaloacetic acid and acetyl-CoA. The first of these reactions is catalyzed by the metal ion dependent citratase, isolated from several microorganisms (for a review, see Daron and Gunsalus, 1962), and the second reaction is catalyzed by an enzyme which occurs in animal tissues and requires ATP, Mg^{2+}, and CoA (Srere and Lipmann, 1953). No betaine aldehyde could be demonstrated in the experiments with acetone-dried cells and no degradation of carnitine was obtained in the absence of NAD^+, and thus there was no evidence for a pathway of carnitine degradation which involves betaine aldehyde as an intermediate.

Trimethylaminoacetone (VI) was formed in good yield when carnitine was incubated with acetone powder of strain AK 1 in the presence of NAD^+, and a probable intermediate would be 3-keto-4-trimethylaminobutyric acid (VII) which would be expected to decarboxylate rapidly in water solution. No radioactive trimethylaminoacetone could be demonstrated when living cells of strain AK 1 were incubated with carnitine, and under these conditions the substrate was completely metabolized. Strack and co-workers (1962, 1964) isolated small amounts of trimethylaminoacetone from 3- to 16-day-old cultures of *Ps. aeruginosa* or *Pseudomonas ovalis* growing in a carnitine medium.

Linneweh in 1929 advanced the hypothesis that carnitine was an intermediate in the β oxidation of γ-butyrobetaine. In *Pseudomonas sp.* AK 1 small amounts of radioactive carnitine could be demonstrated in incubations of living cells with radioactive γ-butyrobetaine, and γ-butyrobetaine was efficiently converted to carnitine by acetone-dried cells. The present demonstration of glycine betaine formation from carnitine by bacterial enzymes in the presence of NAD^+, ATP, and CoA suggested a β-oxidation mechanism for the degradation of carnitine and at first seemed to support the hypothesis of Linneweh, but no carnitine was formed from γ-butyrobetaine in the presence of these cofactors. As the results were obtained with acetone-dried cells they do not necessarily exclude that γ-butyrobetaine is degraded by a β-oxidation mechanism in the intact cell. With the acetone-dried cells carnitine was formed from γ-butyrobetaine in a reaction which was of the monooxygenase type (Hayaishi, 1964) since it occurred only in the presence of molecular oxygen and with a combination of Fe^{2+}, ascorbate, and NADPH. This indicates that different enzymatic mechanisms are involved in the formation and in the degradation of carnitine.

The results obtained with *Ps. aeruginosa* NCTC A 7244 agree with this concept. This strain utilizes carnitine for growth, but not γ-butyrobetaine, and it forms

glycine betaine from carnitine (Lindstedt and Lindstedt, 1961). Although no studies of cofactor requirements were made with this strain it appears likely that glycine betaine is formed from carnitine by the same mechanism in the two *Pseudomonas* strains. Had carnitine formation from γ-butyrobetaine occurred by the same mechanism, γ-butyrobetaine would have been a substrate for *Ps. aeruginosa* NCTC A 7244. As this was not the case it also appears that there was no hydroxylation of γ-butyrobetaine to carnitine by a hydroxylase of the same type as in *Pseudomonas sp* AK 1.

The hydroxylase reaction demonstrated in *Pseudomonas sp.* AK 1 is similar to that in rat liver (Lindstedt and Lindstedt, 1961; Lindstedt, 1967). So far hydroxylation of γ-butyrobetaine by this mechanism is the only established biosynthetic pathway to carnitine.

Acknowledgment

We are indebted to Grycksbo Pappersbruks AB, Grycksbo, Sweden, for a generous supply of filter paper for chromatography.

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