Utilization of L-Threonine by a Species of *Arthrobacter*

A NOVEL CATABOLIC ROLE FOR 'AMINOACETONE SYNTHASE'

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1. A species of *Arthrobacter* (designated *Arthrobacter* 9759) was isolated from soil by its ability to grow aerobically on L-threonine as sole source of carbon atoms, nitrogen atoms and energy; the organism also grew well on other sources of carbon atoms including glycine, but no growth was obtainable on aminoacetone or DL-1-aminopropan-2-ol. 2. During growth on threonine, ^{14}C from L-[U- ^{14}C]threonine was rapidly incorporated into glycine and citrate, and thereafter into serine, alanine, aspartate and glutamate. 3. With extracts of threonine-grown cells supplied with L-[U- ^{14}C]threonine, evidence was obtained of the NAD and CoA-dependent catabolism of L-threonine to produce acetyl-CoA plus glycine. Short-term incorporation studies in which [2- ^{14}C]acetate and [2- ^{14}C]glycine were supplied (a) to cultures growing on threonine, and (b) to extracts of threonine-grown cells, showed that the acetyl-CoA was metabolized via the tricarboxylic acid cycle and glyoxylate cycle whereas the glycine was converted into pyruvate via the folate-dependent 'serine pathway'. 4. The threonine-grown organism contained 'biosynthetic' threonine dehydratase and a potent NAD-linked L-threonine dehydrogenase but possessed no L-threonine aldolase activity. 5. Evidence was obtained that the acetyl-CoA and glycine produced from L-threonine had their immediate origin in the α -amino- β -oxobutyrate formed by the threonine dehydrogenase; the CoA-dependent cleavage of this compound was catalysed by an α -amino- β -oxobutyrate CoA-ligase, which was identified with 'aminoacetone synthase'. A continuous spectrophotometric assay of this enzyme was developed, and it was found to be inducibly synthesized only during growth on threonine and not during growth on acetate plus glycine. 6. By using a reconstituted mixture of separately purified L-threonine dehydrogenase and α -amino- β -oxobutyrate CoA-ligase (i.e. 'aminoacetone synthase'), L-[U- ^{14}C]threonine was broken down to [^{14}C]glycine plus [^{14}C]acetyl-CoA (trapped as [^{14}C]citrate). 7. There was no evidence of aminoacetone metabolism by *Arthrobacter* 9759 even though a small amount of this amino ketone appeared in the culture medium during growth on threonine.

Several species of bacteria are known to catabolize L-threonine by virtue of their possession of an NAD-linked threonine dehydrogenase. The product of this oxidation is α -amino- β -oxobutyrate, a highly unstable substance having a half-life of less than 1 min. at room temperature and pH 7 (Laver, Neuberger & Scott, 1959), which spontaneously decomposes to yield aminoacetone and carbon dioxide. For this reason interest has always been focussed on the subsequent metabolic fate of aminoacetone. Elliott (1959) suggested that it might be converted into methylglyoxal and thence into pyruvate, and indeed it was found that aminoacetone produced by *Pseudomonas* species during growth on DL-1-aminopropan-2-ol is utilized by this route (Higgins, Turner & Willetts, 1967).

α -Amino- β -oxobutyrate and thence aminoacetone are also produced by the condensation of acetyl-CoA with glycine, catalysed by 'aminoacetone synthase' (Elliott, 1958; Neuberger & Tait, 1962; Urata & Granick, 1963), which proceeds as follows:

Since the enzyme catalyses only reaction (1), it is unfortunate that it has acquired the name 'aminoacetone synthase'; even when it acts in this biosynthetic capacity it might more properly be

called α -amino- β -oxobutyrate synthase. Elliott (1959) proposed that 'aminoacetone synthase' catalyses the first in a series of reactions whereby aminoacetone is synthesized from acetyl-CoA and glycine only to be oxidized via methylglyoxal and pyruvate so as to regenerate the acetyl-CoA. Each turn of this cycle would completely oxidize one molecule of glycine and, although no conclusive proof of its operation has as yet been obtained, those who have subsequently studied 'aminoacetone synthase' have seemingly been content with this explanation of its metabolic role as a catalyst of the synthesis of aminoacetone from acetyl-CoA and glycine.

The present paper reports the presence of L-threonine dehydrogenase and 'aminoacetone synthase' in a species of *Arthrobacter* when it is growing aerobically on L-threonine as sole source of carbon atoms and energy, and presents evidence that these enzymes collaborate in catalysing the catabolism of threonine to acetyl-CoA and glycine. In this novel route of threonine catabolism 'aminoacetone synthase' functions as an α -amino- β -oxobutyrate CoA-ligase. Part of this work has been reported in a preliminary form (McGilvray & Morris, 1966).

MATERIALS AND METHODS

Maintenance and growth of organisms. The species of *Arthrobacter* employed in this work was isolated from soil by elective culture on a minimal salts medium containing L-threonine (25 mM) as sole source of carbon atoms, nitrogen atoms and energy. The isolate was kindly identified as an *Arthrobacter* species (possibly a strain of *Arthrobacter globiformis*) by Dr T. G. Mitchell and Dr J. M. Shewan of the Torry Research Station, Aberdeen, and it has been deposited in the National Collection of Industrial Bacteria (accession no. 9759). It is designated below as *Arthrobacter* 9759. It was maintained on slopes of threonine growth medium solidified with 1.5% of Ionagar no. 2 (Oxoid). Cultures were grown aerobically at 30°, and stored at 2°; they were subcultured weekly. For experimental use, organisms from the threonine-agar slopes were used to inoculate 1 l. of medium containing: 7 g. of K_2HPO_4 , 3 g. of KH_2PO_4 , 0.5 g. of NaCl, 0.1 g. of $MgSO_4 \cdot 7H_2O$, 0.02 g. of $CaCl_2$, 0.3 ml. of salts mixture 'Metals 44' (Cohen-Bazire, Sistrom & Stanier, 1957), and, as sole source of carbon and nitrogen atoms, 1.8 g. of L-threonine. The 1 l. amounts of this threonine medium in 2 l. conical flasks were pre-sterilized by autoclaving (15 lb./in.² for 15 min.). These cultures were incubated at 30° with constant shaking in a rotary incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) and the bacteria were harvested during their exponential phase of growth (at cell densities of 0.4–0.5 mg. dry wt./ml.) by centrifuging at 15000 g for 20 min. at 2°. For experiments on the incorporation of ¹⁴C from ¹⁴C-labelled substrates into growing organisms, the bacteria were resuspended to a cell density of about 10 mg. dry wt./ml. in fresh growth medium containing L-threonine (1 mg./ml.). For studies on the oxidation of substrates and for preparation of cell extracts the

organisms were washed with, and resuspended in, 10 mM-potassium phosphate buffer, pH 7.2.

Measurement of growth. Growth was followed turbidimetrically at 680 m μ with a Unicam SP. 600 spectrophotometer. The relation between E_{680} and dry weight was determined and was found to be linear up to an E_{680} value of about 0.8.

Incorporation of ¹⁴C from ¹⁴C-labelled substrates. The procedures used to study the incorporation of ¹⁴C from [¹⁴C]threonine, amino[¹⁴C]acetone, [2-¹⁴C]glycine and [2-¹⁴C]acetate into constituents of the ethanol-soluble fraction of organisms growing on L-threonine were similar to those described for *Pseudomonas* (Kornberg, 1958) and *Escherichia coli* (Kornberg, Phizackerley & Sadler, 1961) metabolizing acetate.

Assay and identification of labelled compounds. Isotopically labelled materials were located and identified on two-dimensional paper chromatograms by radioautography and co-chromatography with authentic unlabelled materials as described by Kornberg (1958). In addition, radioactive products were also analysed by high-voltage paper-strip electrophoresis in formic acid-acetic acid, pH 1.85, as described by Atfield & Morris (1961), with 60 cm. strip length, 6 kv and 25 ma for 40 min.

The radioactive zones of chromatograms and electrophoretograms located by radioautography were either (1) directly monitored by using a thin-window Geiger-Müller tube (General Electric Co.; type 2B2) and scaler, and correcting for background activity, or (2) cut out, folded and placed in glass vials containing 10 ml. of liquid-scintillation fluid [5 g. of 2,5-diphenyloxazole and 0.3 g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in 1 l. of A.R. toluene] and counted in a Packard model 4312 Tri-Carb spectrophotometer.

Preparation of cell extracts. Suspensions of freshly grown organisms (15 mg. dry wt./ml.) in 0.01 M-potassium phosphate buffer, pH 7.2, were subjected for 7–10 min. at 0–4° to the output of an MSE 60 W ultrasonic disintegrator operating at 1.5 A. The resultant extracts were centrifuged at 20000 g for 10 min. at 2° and the supernatant solutions thus obtained were used for experimental purposes.

Determination of protein. Soluble protein was generally measured by the method of Lowry, Rosebrough, Farr & Randall (1951); a standard curve was prepared with crystalline bovine plasma albumin (Armour Laboratories, Eastbourne, Sussex).

Spectrophotometric procedures. All spectrophotometric assays were performed at 20 $\pm$ 1° in silica cuvettes (1.5 ml. volume, 1 cm. light-path) by using either an Optika model CF-4 or Unicam SP.800 recording spectrophotometer.

Preparation of amino[¹⁴C]acetone. Amino[¹⁴C]acetone was prepared enzymically from [¹⁴C]threonine and was purified by cation-exchange-column chromatography by a method based on that described by Elliott (1960) for the separation of aminoacetone from δ -amino-laevalic acid.

The incubation mixture contained (in 5 ml. final volume): 200 μ moles of tris-HCl buffer, pH 8, 20 μ moles of NAD⁺, extract of threonine-grown *Arthrobacter* 9759 (about 20 mg. of soluble protein) and L-[¹⁴C]threonine (50 μ C; 5.4 μ C/ μ mole). After incubation for 7 hr. at 30°, protein was precipitated with ethanol and removed by centrifugation. The clear supernatant was evaporated to dryness at 40° and was redissolved in 1.5 ml. of 0.1 M-sodium acetate buffer.

pH 4.9. At this stage, 0.2 mg. of 'carrier' aminoacetone hydrochloride was added and the radioactive solution applied to a short column of Amberlite IRC-50 resin that had been washed several times with 1 M-sodium acetate buffer, pH 4.9, and finally with water. Amino[U- 14 C]acetone was retained on the column and non-basic impurities were removed by washing the column with water. Then 2 M-HCl was applied to the column and the effluent was collected in 2 ml. fractions, which were assayed for their content of radioactivity and aminoacetone. Those fractions containing the radioactive aminoacetone were pooled and evaporated to dryness *in vacuo* at 30–40°. The residual solid was extracted with ethanol, the solution again evaporated to dryness and the amino[U- 14 C]acetone hydrochloride taken up in water.

Other radioactive materials. All other radioactive substances were obtained from The Radiochemical Centre, Amersham, Bucks. L-[U- 14 C]Threonine (10 μ C/ml.) and [2- 14 C]glycine (20 μ C/ml.) were purified by applying 2 ml. volumes to 15 cm. $\times$ 0.9 cm. columns of Dowex 50 (X8; H⁺ form). After removal of contaminating materials by washing the columns with 30 ml. amounts of water and 0.2 M-HCl, the 14 C-labelled amino acids were eluted with 0.4 M-HCl. The effluents were collected in 5 ml. fractions and those that contained the bulk of the radioactivity were in each case pooled and concentrated under vacuum at 30°. The pH was adjusted to 7 with NaOH and the concentration of amino acid was determined with ninhydrin reagent. The absolute purity of the ultimate products was confirmed by high-voltage paper electrophoresis and radioautography. [2- 14 C]Acetate was purified immediately before use by vacuum distillation as described by Kornberg (1958).

Other materials used. Aminoacetone hydrochloride was synthesized from chloroacetone and potassium phthalimide by the method of Gabriel & Colman (1902). Tetrahydropteroylglutamate was prepared by the catalytic hydrogenation of pteroylglutamic acid in acetic acid as described by Hatefi, Talbert, Osborn & Huennekens (1960). Thiol-CoA esters were prepared as described by Stadtman (1957). Acyl-CoA esters were estimated by the method described by Canovas & Kornberg (1966). Citrate synthase, L-lactate dehydrogenase and L-malate dehydrogenase from pig heart were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. All other reagents were of the highest purity commercially available.

Identification and assay of aminoacetone. (a) On chromatograms. Aminoacetone was detected on chromatograms by spraying with ninhydrin reagent (0.5% in acetone). Aminoacetone gave a characteristic yellow colour that slowly changed to purple on standing at room temperature. When only very small quantities of aminoacetone were produced enzymically, the compound was extracted and concentrated before chromatography. The incubated reaction mixture was deproteinized with ethanol, and the clear supernatant was evaporated to dryness *in vacuo* at 30°. The residue was redissolved in 5 ml. of 0.1 M-sodium acetate buffer, pH 4.9, and the solution was shaken for 10 min. with Amberlite IRC-50 that had previously been equilibrated with 1 M-sodium acetate buffer, pH 4.9, and washed with water. The resin was recovered by filtration and was washed with water before the bound aminoacetone was eluted with 2 M-HCl. The resultant solution was evaporated to dryness *in vacuo*, and the residue was redissolved in 95% ethanol; NaCl was removed by centrifugation. For identification purposes,

descending paper chromatography was performed at room temperature with ethanol-1 M-acetic acid-pyridine-water (95:10:3:3, by vol.) as solvent; authentic aminoacetone possessed R_F 0.48 in this system.

(b) In solution. A colorimetric assay procedure (Urata & Granick, 1963) was used in which reaction with acetylacetone was followed by colour development with a modified Ehrlich reagent.

Enzyme assays

'Aminoacetone synthase'. Because of the instability of α -amino- β -oxobutyrate this enzyme must be assayed in the synthetic direction with acetyl-CoA and glycine as substrates.

(a) Colorimetric method. The rate of aminoacetone production from acetyl-CoA and glycine was followed by a discontinuous assay procedure whereby the amounts of aminoacetone produced in various time-intervals were assayed colorimetrically (Urata & Granick, 1963).

(b) Spectrophotometric method. This method of assay was developed to minimize the product inhibition by CoA, which is a marked feature of the enzyme-catalysed reaction when this is assayed by the colorimetric method. In this spectrophotometric method, reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) by CoA released by condensation of acetyl-CoA with glycine is followed continuously as the rate of increase in E at 412 nm. Use of a suitable blank, consisting of reaction mixture minus glycine, corrects for CoA formation from acetyl-CoA by any glycine-independent acylase activity. The complete reaction mixture, in a 1.5 ml. cuvette (1 cm. light-path), contained, in 1 ml. final volume: 200 μ moles of potassium phosphate buffer, pH 7.5, 0.5 μ mole of acetyl-CoA, 0.1 μ mole of 5,5'-dithiobis-(2-nitrobenzoic acid), 300 μ moles of glycine (adjusted to pH 7.5), and enzyme-containing extract (0.3 mg. of soluble protein). This mixture, without glycine, was used as blank, and the reaction was started by the addition of the enzyme-containing extract to both cuvettes. Assuming ϵ_{412} of reduced 5,5'-dithiobis-(2-nitrobenzoic acid) to be 1.36×10^4 M⁻¹ cm.⁻¹, the specific activity was calculated as μ moles of CoA released/mg. of protein/hr.

Erythro- β -hydroxyaspartate aldolase. This enzyme was assayed by following the rate of pyruvate production from erythro-DL- β -hydroxy- β -methylaspartate (Kornberg & Morris, 1965).

Erythro- β -hydroxyaspartate dehydratase. This enzyme was assayed spectrophotometrically by coupling with an excess of a NADP-linked malate dehydrogenase and following oxidation of NADPH on addition of erythro-DL- β -hydroxyaspartate by the decrease in E at 340 nm. (Kornberg & Morris, 1965).

Citrate synthase (EC 4.1.3.7). This enzyme was assayed spectrophotometrically by following the oxaloacetate-dependent release of CoA from acetyl-CoA; the increase in E at 412 nm. was measured, as 5,5'-dithiobis-(2-nitrobenzoic acid) was reduced by liberated CoA (Srere, Brazil & Gonen, 1963).

The 'glycine-cleavage system'. The activity of this enzyme system was measured simultaneously by two methods: (1) by following the rate of production of 14 CO₂ from [1- 14 C]-glycine, and (2) by assaying the rate of formation of 5,10-methylene-H₄PteGlu* by a coupled spectrophotometric

* Abbreviation: H₄PteGlu, tetrahydropteroylglutamic acid.

method in which NADP-dependent 5,10-methylene- H_4 PteGlu dehydrogenase was used to convert 5,10-methylene- H_4 PteGlu into 5,10-methylidene- H_4 PteGlu and the product was assayed by making use of its distinctive absorption in acid solution at 355 $m\mu$.

The assay was performed in dual-side-arm Warburg manometer flasks at 30° under an atmosphere of N_2 . The complete system in the main compartment of the flask contained (in 2.3 ml. final volume): 100 μ moles of potassium phosphate buffer, pH 7.5, 5 μ moles of [^{14}C]glycine (0.2 μ C/ μ mole), 0.3 μ mole of NAD^+ , 0.3 μ mole of $NADP^+$, 1 μ mole of H_4 PteGlu (as a solution in 0.5 ml. of 0.1 M-phosphate buffer, pH 7.5, containing 50 mM-2-mercaptoethanol), 0.2 μ mole of pyridoxal 5'-phosphate and 10 μ moles of Benzyl Viologen. One side arm contained 0.5 ml. of cell extract (approx. 3 mg. of protein) and the other side arm contained 0.2 ml. of 3 M-HCl; the centre well contained filter paper impregnated with 0.2 ml. of 6 M-NaOH. After the extract had been tipped in to start the reaction, the flasks were incubated with shaking at 30° for 3 hr. before the HCl was tipped in from the second side arm to (a) stop the reaction, (b) liberate bound $^{14}CO_2$ and (c) produce that acid pH at which 5,10-methylidene- H_4 PteGlu demonstrates its distinctive absorption. After a further 20 min. incubation, the contents of the centre well were removed and carbonate was completely precipitated from the solution as $Ba^{14}CO_3$, whose radioactivity was measured after plating at infinite thickness on aluminium planchets. The acidified contents of the main compartment were centrifuged to remove precipitated protein and E of the clear supernatant was measured at 355 nm. against a reaction blank in which preheated extract (100° for 10 min.) had been used. The total quantity of 5,10-methylidene- H_4 PteGlu that had been formed was then calculated assuming its ϵ_{355} to be $2.5 \times 10^4 M^{-1} cm^{-1}$.

Glyoxylate carboligase. This enzyme was assayed manometrically by measuring the rate of anaerobic release of CO_2 from glyoxylate as described by Kornberg & Gotto (1961).

Isocitrate lyase (EC 4.1.3.1). This enzyme was assayed spectrophotometrically in imidazole-HCl buffer, pH 6.8, by following the increase in E at 324 nm. due to the formation of glyoxylate phenylhydrazone from *threo*- D_3 -isocitrate as described by Kornberg (1965).

Malate synthase (EC 4.1.3.2). This enzyme, like citrate synthase, was assayed spectrophotometrically by using 5,5'-dithiobis-(2-nitrobenzoic acid) and following the increase in E at 412 nm. consequent on the glyoxylate-dependent release of CoA from acetyl-CoA.

L-Serine dehydratase (EC 4.2.1.13). This enzyme was assayed by following the rate of production of pyruvate from L-serine by using a procedure based on the Friedemann & Haugen (1943) assay for keto acids. The reaction mixture, in a test tube, contained in 1 ml. final volume: 50 μ moles of tris-HCl buffer, pH 8.2, 10 μ moles of $MgCl_2$ and cell extract (0.3 mg. of soluble protein). The same system but with cell extract heated before use at 100° for 15 min. was used as the reaction blank. The tubes were incubated at 30° and the reaction was started by the addition of 50 μ moles of L-serine (adjusted to pH 8.2) to each tube. After 15 min. the reaction was stopped by the addition of 0.33 ml. of 0.1% 2,4-dinitrophenylhydrazine in 2 M-HCl. After a further 10 min. at 30°, 2 ml. of 2 M-NaOH was added and the mixture was centrifuged. The developed colour was read against the blank at

445 $m\mu$. ϵ_{445} of pyruvate 2,4-dinitrophenylhydrazone in this assay was assumed to be $1.8 \times 10^4 M^{-1} cm^{-1}$ and the specific activity was calculated as μ moles of pyruvate produced/mg. of protein/hr.

L-Serine hydroxymethyltransferase. The activity of this enzyme was assayed in the direction of 5,10-methylene- H_4 PteGlu formation from H_4 PteGlu and L-serine. A coupled spectrophotometric assay was employed that relied on endogenous 5,10-methylene- H_4 PteGlu dehydrogenase to convert 5,10-methylene- H_4 PteGlu into 5,10-methylidene- H_4 PteGlu, whose distinctive absorption at 355 $m\mu$ could be measured.

The assay was performed aerobically with an assay mixture containing (in 10 ml.): 500 μ moles of potassium phosphate buffer, pH 7.5, cell extract (about 3 mg. of soluble protein), 1 μ mole of pyridoxal 5'-phosphate, 5 μ moles of NAD^+ , 5 μ moles of $NADP^+$, 4 μ moles of H_4 PteGlu (as a solution in 2 ml. of 0.1 M-phosphate buffer, pH 7.5, containing 100 μ moles of 2-mercaptoethanol) and 10 μ moles of $MgCl_2$. The reaction was started by the addition of 100 μ moles of L-serine and 1 ml. samples were withdrawn into 2 ml. of 0.23 M-HCl at 1 min. intervals for 8 min. Precipitated protein was removed by centrifuging and E of the supernatant was measured at 355 nm. against a blank in which the serine was added after acidification with HCl. ϵ_{355} of 5,10-methylidene- H_4 PteGlu was assumed to be $2.5 \times 10^4 M^{-1} cm^{-1}$ and the specific activity was calculated as μ moles of 5,10-methylidene- H_4 PteGlu produced/mg. of protein/hr.

L-Threonine aldolase (EC 4.1.2.5). This enzyme was assayed by measuring acetaldehyde production consequent on the aldol cleavage of L-threonine, by trapping the acetaldehyde released in this reaction as its semicarbazone as described by Greenberg (1962). The assay was performed (a) spectrophotometrically, by following the increase in E at 224 nm., and (b) by using L-[^{14}C]threonine and measuring the radioactivity of the semicarbazone.

L-Threonine dehydratase (EC 4.2.1.16). This enzyme was assayed by a colorimetric method wherein α -oxobutyrate produced from L-threonine was measured as described by Datta (1966).

L-Threonine dehydrogenase. (a) Colorimetric assay. In this discontinuous test-tube assay, the 'ultimate' product (i.e. aminoacetone) was measured by the colorimetric method of Urata & Granick (1963). The reaction mixture contained, in 0.5 ml. final volume: 100 μ moles of tris-HCl buffer, pH 7.5, 0.5 μ mole of NAD^+ and cell extract (about 0.1 mg. of soluble protein). The same mixture but with cell extract heated at 100° for 15 min. was used as the blank. The tubes were incubated at 30° and the reaction was started by the addition of 20 μ moles of L-threonine (neutralized). After 30 min. incubation the reaction was stopped by the addition of 1 ml. of 0.3 M-trichloroacetic acid and, after centrifuging to remove precipitated protein, the concentration of aminoacetone in the clear supernatant was assayed colorimetrically. The specific activity was calculated as μ moles of aminoacetone produced/mg. of protein/hr.

(b) Spectrophotometric method. This involved following the increase in E at 340 nm. consequent on the L-threonine-dependent reduction of NAD^+ . With extracts that possessed high NADH-oxidase activity this assay should be performed anaerobically, but, in general, it was found that the measured aerobic initial velocity was also the true reaction rate.

The reaction mixture, in a 1.5 ml. silica cuvette (1 cm. light-path), contained in 1 ml. final volume: 200 μ moles of tris-HCl buffer, pH 8.5, 0.5 μ mole of NAD⁺ and cell extract (0.3 mg. of soluble protein). A reference cuvette contained the same mixture and the reaction was started by the addition of 30 μ moles of L-threonine (adjusted to pH 8.5) to the test cuvette. The rate of increase in *E* at 340 nm. was followed at room temperature. ϵ_{340} of NADH was assumed to be $6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ and the specific activity was calculated as μ moles of NAD⁺ reduced/mg. of protein/hr.

RESULTS

Growth of the organism on L-threonine and other sources of carbon atoms. *Arthrobacter* 9759 grew well under aerobic conditions at 30° on a defined basal medium containing L-threonine as sole source of carbon atoms, nitrogen atoms and energy. Indeed, although the lag before commencement of growth was slightly greater in threonine medium than in the same medium containing glucose as sole source of carbon atoms, the ultimate rates and final extents of growth of the organism on both media were similar (Fig. 1). Growth on L-threonine and on other amino acids was not enhanced by NH₄⁺ ions, and, in contrast with the behaviour of another strain of *A. globiformis* (Morris, 1960), biotin was not required for growth. The optimum growth temperature was 25–30°, although quite good growth occurred at temperatures as low as 15° and as high as 33°. The degree of aeration markedly affected growth; no growth of *Arthrobacter* 9759 occurred in any medium under strictly

anaerobic conditions, and in static aerobic culture the organisms frequently clumped on the surface of the liquid medium. Best growth was obtained in shaken liquid cultures or on the surface of agar-solidified media.

A variety of L-amino acids other than L-threonine as well as other low-molecular-weight carbon compounds supported the aerobic growth of *Arthrobacter* 9759; a selection of these, together with a list of some substances not utilized as sole sources of carbon atoms for growth, is recorded in Table 1. A rather surprising finding was that, although washed suspensions of the organism were capable of oxidizing acetate, the organism grew slowly and poorly when provided with acetate as sole source of carbon atoms. It was also noteworthy that neither aminoacetone nor DL-1-amino-propan-2-ol was utilized as sole source of carbon atoms and nitrogen atoms, and that, although no growth was obtainable on compounds usually metabolized via glyoxylate (i.e. ethanediol, glycolate), the organism grew well on glycine, and still better on an equimolar mixture of glycine plus acetate.

L-Threonine-utilizing enzymes in cell-free extracts. The specific activities of key threonine-utilizing enzymes were assayed in cell-free extracts of *Arthrobacter* 9759 grown (a) on pyruvate and (b) on L-threonine. No L-threonine aldolase activity was detectable in either extract. L-Threonine dehydratase activity was present in both extracts at

Fig. 1. Growth of *Arthrobacter* 9759 on glucose or L-threonine as sole source of carbon atoms. Organisms from a nutrient-agar slope culture were inoculated into liquid medium (see the Materials and Methods section) containing either 10 mM-glucose (○) or 15 mM-L-threonine (●) as sole source of carbon atoms. Growth was followed turbidimetrically during aerobic incubation in flasks shaken at 30°.

Table 1. Growth of *Arthrobacter* 9759 on various sources of carbon and nitrogen atoms

The compounds were tested (at 15 mM final concentration) as sole supplements to the minimal salts medium (see the Materials and Methods section) with or without NH₄Cl (3 g./l.). Aerobic incubation was in shaken flasks at 30°.

Compounds supporting rapid growth	Compounds supporting slow growth	Compounds not supporting growth
Sources of carbon		
D-Glucose	Acetate	Ethanediol
Glycerol	Citrate	Glycollate
DL-Lactate	L-Malate	
α -Oxoglutarate*	Succinate	
Pyruvate*		
Sources of carbon and nitrogen		
L-Alanine		DL-Allothreonine
L-Aspartate		Aminoacetone*
L-Glutamate		DL- α -Aminobutyrate
Glycine		DL-1-Aminopropan-2-ol
Glycine plus acetate		
DL-Homoserine		
L-Serine		
L-Threonine		

* Sterilized by membrane filtration.

similar specific activities, i.e. 3.5 and 5.2 μ moles of α -oxobutyrate produced/mg. of protein/hr. respectively in extracts from pyruvate- and threonine-grown cells. Since its activity in both extracts was more than 90% inhibited by L-isoleucine (0.33 mM), it was concluded that the assayed activity was due solely to the 'biosynthetic' threonine dehydratase of the organism that is concerned with the synthesis of L-isoleucine (Umbarger & Davis, 1962).

The most significant finding was that L-threonine dehydrogenase activity was present only in the extract of threonine-grown cells (at a specific activity of 9.2 μ moles of NADH produced/mg. of protein/hr.). Subsequent examination of extracts of the organism prepared after growth on a wide range of substances showed that production of this enzyme was specific to growth on L-threonine.

This discovery of a potent L-threonine dehydrogenase in extracts of threonine-grown *Arthrobacter* 9759 suggested that aminoacetone might be an intermediate in the catabolism of threonine by this organism. The ability of *Arthrobacter* 9759 to produce and metabolize aminoacetone was therefore investigated.

Production of aminoacetone during growth on L-threonine. A culture was harvested in its late exponential phase of growth on L-threonine (15 mM) and the spent medium was examined for its content of amino ketones by the colorimetric procedure of Urata & Granick (1963). Aminoacetone was found to be present, but in only very low concentration (0.072 mM).

Table 2. *Formation of aminoacetone from L-threonine by dialysed extracts of threonine-grown Arthrobacter 9759*

Cell-free extract of threonine-grown organisms was dialysed at 2° for 24 hr. against two changes (1000 vol. each) of 10 mM-potassium phosphate buffer, pH 8, and was then incubated (2.4 mg. of protein/ml.) in 1 ml. total volume with: 200 μ moles of tris-HCl buffer, pH 8.0, 1 μ mole of NAD⁺ and 10 μ moles of L-threonine. NAD⁺ was omitted, and 1 μ mole of CoA and 0.02 μ mole of pyridoxal 5'-phosphate were added where indicated. After 10 min. incubation at 30° the reaction was stopped by adding 1 ml. of 0.3 M-trichloroacetic acid. A portion of each mixture was then colorimetrically assayed for its content of aminoacetone (see the Materials and Methods section). Each result represents the average of duplicate findings.

Additions to complete system	Aminoacetone formed (μ moles)	% of yield in complete system
None	4.0	(100)
None but minus NAD ⁺	0.6	14
CoA	2.2	54
CoA plus pyridoxal 5'-phosphate	1.7	43

Formation of aminoacetone from threonine by cell extracts. Only extracts derived from threonine-grown organisms were capable of producing aminoacetone from added L-threonine. By using dialysed extracts at pH 8 it was found that NAD⁺ was an obligatory requirement for aminoacetone production and that addition of CoA (1 mM), with or without pyridoxal 5'-phosphate (0.02 mM), markedly decreased the yield of aminoacetone (Table 2). Best production of aminoacetone was achieved with aged and CoA-depleted extracts with high concentrations of added NAD⁺.

Non-utilization of aminoacetone by the organism. *Arthrobacter* 9759 was unable to grow on a medium in which aminoacetone was provided as sole source of carbon atoms (Table 1). That washed suspensions of threonine-grown cells did not oxidize added aminoacetone hydrochloride was demonstrated by manometric investigation of oxygen consumption, and by oxygen electrode studies. Nor did extracts of threonine-grown organisms catalyse the disappearance of aminoacetone (measured colorimetrically) when aerobically incubated at 30° in phosphate buffer, pH 7.5. The cell extract was provided at a final concentration of 3 mg. of soluble protein/ml. and was supplemented with magnesium chloride (0.5 mM), and with all combinations of the following cofactors (whose use was suggested by the known routes of aminoacetone metabolism in other organisms): NAD⁺, NADP⁺, α -oxoglutarate, pyruvate, ATP, CoA, glutathione and H₄PteGlu. Cell extracts of threonine-grown organisms provided with these supplements were also incubated with amino[U-¹⁴C]acetone; after 30 min. at 30°, reaction was stopped by the addition of ethanol (to 80%, v/v) and samples of the ethanol-soluble fraction were analysed by two-dimensional paper chromatography and radioautography. Although more than one radioactive component was present on all radioautograms, those radioautograms produced from the experimental samples were identical with the radioautogram obtained when boiled cell extract was used.

Thus although during its growth on threonine *Arthrobacter* 9759 produces an enzyme that catalyses the formation of α -amino- β -oxobutyrate from L-threonine, it cannot metabolize the aminoacetone that would be formed by the spontaneous decarboxylation of this labile intermediate. In an attempt to resolve this paradox and to discover the function of L-threonine dehydrogenase in this organism, the route of threonine utilization was directly investigated by using radiotracer techniques.

Incorporation of ¹⁴C from L-[U-¹⁴C]threonine during growth. When L-[U-¹⁴C]threonine was added to cultures of *Arthrobacter* 9759 growing aerobically on L-threonine as sole source of carbon atoms, ¹⁴C was rapidly incorporated into both the ethanol-

Fig. 2. Variation with time of the percentage distribution of ^{14}C incorporated from L-[U- ^{14}C]threonine by *Arthrobacter* 9759 growing on L-threonine as sole source of carbon atoms, into glycine plus serine (●), glutamate (■), citrate (▲) and aspartate (○). L-[U- ^{14}C]Threonine ($1.4\text{ }\mu\text{moles}$; $7.4\text{ }\mu\text{C}/\mu\text{mole}$) was added to 10 ml. of a culture of *Arthrobacter* 9759 (10 mg. dry wt./ml.) growing aerobically on L-threonine (1 mg./ml.). Samples (1 ml.) of culture were withdrawn at intervals into 3 ml. of hot ethanol and the distribution of ^{14}C among the ethanol-soluble components was analysed by two-dimensional paper chromatography and radioautography; radioactivity was directly assayed with a Geiger-Müller tube (see the Materials and Methods section). The total radioactivity (minus background and residual [^{14}C]threonine) fixed on the chromatograms after 4, 8, 14, 26, 45 and 65 sec. incubation was 153, 367, 720, 1786, 2328 and 3199 counts/min. respectively.

soluble and protein fractions. The incorporation of ^{14}C into the ethanol-soluble fraction increased linearly with time for the duration of the experiment (60–90 sec.).

Distribution of ^{14}C from L-[U- ^{14}C]threonine. The distribution of ^{14}C among the labelled components of the ethanol-soluble fractions was analysed by two-dimensional chromatography and radioautography. Label from L-[U- ^{14}C]threonine rapidly appeared in glycine, serine, citrate, glutamate and aspartate (Fig. 2). Although most of the radioactive products were well separated during chromatography, the areas occupied by glycine and serine overlapped and initially only the sum of their radioactivity was measured (Fig. 2). In the sample taken at the earliest time (4 sec.) glycine plus serine contained by far the greatest proportion (about 96%) of the radioactivity remaining on the chromatograms. In subsequent samples this proportion of label in glycine plus serine fell rapidly, until at 65 sec. it was insignificant. The only other radioactive product detectable in the 4 sec. sample was citrate, which accounted for about 4% of the total ^{14}C incorporated. The time-course of incorporation of ^{14}C into citrate showed an initial rise to 15.8% at 14 sec. and a rapid decline

thereafter. The proportion of incorporated ^{14}C that was present in aspartate changed with time in a very similar fashion to citrate, whereas the proportion of incorporated ^{14}C found in glutamate increased markedly with time (Fig. 2). After 65 sec. the distribution of label was as follows: glycine plus serine, <1%; citrate, 0.3%; glutamate, 67%; α -oxoglutarate, 4.4%; alanine, 8.6%; aspartate, 3.7%; malate, 0.6%; succinate plus fumarate, 0.2%. No labelled aminoacetone was detected in any sample.

When the radioactive material that chromatographed in the glycine and serine areas was eluted and analysed by high-voltage paper electrophoresis and radioautography, it was found that in the first (4 sec.) sample only glycine was present; radioactive serine first appeared in the second (8 sec.) sample. It thus appeared that glycine (96% of incorporated ^{14}C) and citrate (4% of incorporated ^{14}C) were the very first stable radioactive products of L-[^{14}C]threonine utilization by the growing organism. This early production of labelled citrate (before any labelling of malate or aspartate was noticeable) signalled the still earlier production of [^{14}C]acetate. Certainly, the early labelling pattern disclosed by these studies confirmed that L-[U- ^{14}C]threonine was not metabolized via aminoacetone and subsequently by way of methylglyoxal and pyruvate.

Utilization of L-[U- ^{14}C]threonine by cell extracts. When L-[U- ^{14}C]threonine (5 mM; $0.15\text{ }\mu\text{C}/\mu\text{mole}$) was incubated in phosphate buffer, pH 7.5, with an extract of threonine-grown organisms (0.8 mg. of protein/ml.) and NAD^+ (1 mM), ^{14}C was extensively incorporated into several products, including glycine and aminoacetone. Omission of NAD^+ from the reaction mixture particularly curtailed production of glycine and aminoacetone. The relative proportions of these substances in the final mixture varied with reaction conditions, and it seemed that there was an inverse relationship between the amounts of aminoacetone and glycine that were formed. In particular, use of aged extracts and high concentrations of NAD^+ favoured formation of aminoacetone, seemingly at the expense of glycine. Optimum conditions for production of glycine from threonine were determined with dialysed extract. Besides the obligatory requirement for NAD^+ (best satisfied by a NAD^+ -regenerating system consisting of pyruvate plus lactate dehydrogenase, EC 1.1.1.27), it was found that provision of CoA was essential; GSH could not substitute for CoA, and iodoacetamide was inhibitory (Table 3). Though EDTA (1 mM) inhibited glycine production, excess of magnesium chloride (10 mM) was somewhat inhibitory, as were added ATP (5 mM) and pyridoxal 5'-phosphate (0.1 mM).

Since the ^{14}C -incorporation experiments with

Table 3. *Formation of glycine from L-threonine by dialysed extracts of threonine-grown Arthrobacter 9759*

The dialysed extract was prepared as described in Table 2. Incubation mixtures contained, in 1 ml. final volume: dialysed extract (about 1.4 mg. of protein), 25 μ moles of potassium phosphate buffer, pH 7.5, 5.1 μ moles of L-[U- 14 C]threonine (0.15 μ C/ μ mole), 0.4 mg. of NAD $^{+}$, an NAD $^{+}$ -regenerating system consisting of 5 μ moles of sodium pyruvate and lactate dehydrogenase (10 μ g. of protein), 0.6 μ mole of CoA, a CoA-regenerating system consisting of 5 μ moles of oxaloacetate and mammalian citrate synthase (4 μ g. of protein), 10 μ moles of MgCl $_2$, 5 μ moles of ATP and 0.1 μ mole of pyridoxal 5'-phosphate. Cofactors were omitted and 20 μ moles of iodoacetamide, 1 μ mole of EDTA and 0.5 μ mole of GSH were added as indicated. A boiled-extract control was also prepared. After 30 min. incubation at 30° the reaction was stopped by adding 3 ml. of ethanol, and the distribution of 14 C among the components of the ethanol-soluble fraction was analysed by high-voltage paper electrophoresis and radioautography. Radioactivity in glycine and on the remainder of the electrophoretogram was measured by using a scintillation spectrometer (see the Materials and Methods section).

Omissions from complete system	Radioactivity in glycine (% of total incorporated 14 C)
(1) None	9.5
(2) None but with boiled extract	0
(3) NAD $^{+}$	0.4
(4) CoA	3.0
(5) Pyridoxal 5'-phosphate	11.5
(6) ATP	11.3
(7) MgCl $_2$ but with EDTA added	2.0
(8) As (4) but with GSH added	2.9
(9) As (4) but with iodoacetamide added	1.0

growing cultures supplied with L-[U- 14 C]threonine suggested early production of [14 C]acetate, an attempt was made to trap any [14 C]acetyl-CoA that might be formed from L-[U- 14 C]threonine by cell extracts. The method used was essentially that of Kornberg *et al.* (1961) whereby [14 C]acetyl-CoA is trapped either as citrate (by supplying oxaloacetate and making use of the endogenous citrate synthase), or as malate (by supplying glyoxylate and using endogenous malate synthase). The incubation mixtures contained, in phosphate buffer, pH 7.5, L-[U- 14 C]threonine, crude extract of threonine-grown cells (1 mg. of protein/ml.) and NAD $^{+}$ (1 mM), supplemented in turn with (a) no addition, (b) oxaloacetate (2 mM), (c) glyoxylate (2 mM) and (d) L $_5$ -isocitrate (5 mM). After 30 min. incubation at 30° the distribution of radioactivity among the ethanol-soluble components of each mixture was analysed. In sample (a), which had contained only the basal constituents, the bulk of incorporated

Fig. 3. Variation with time of the percentage distribution of 14 C incorporated from [2- 14 C]acetate by *Arthrobacter* 9759 growing on L-threonine as sole source of carbon atoms into glutamate (■), citrate (▲) and aspartate (○). Sodium [2- 14 C]acetate (6 μ moles; 5.4 μ C/ μ mole) was added to 10 ml. of a culture of *Arthrobacter* 9759 (about 10 mg. dry wt./ml.), which was growing exponentially at the expense of L-threonine. Samples were removed and analysed as described in Fig. 2. The total radioactivity (minus background) fixed on the chromatograms after 25, 45, 60, 90 and 120 sec. incubation was 169, 474, 740, 987, and 2046 counts/min. respectively.

radioactivity was found in glycine and aminoacetone. In the sample containing added oxaloacetate there was extensive labelling of citrate, whereas in both remaining samples, to which had been added either glyoxylate or isocitrate, there was extensive labelling of malate. Besides confirming that acetyl-CoA was an important product of L-threonine degradation by these extracts, these findings showed that extracts of threonine-grown organisms possessed citrate synthase, malate synthase and isocitrate lyase activities.

Incorporation of 14 C from [2- 14 C]acetate during growth on L-threonine. When [2- 14 C]acetate was supplied to a culture of *Arthrobacter* 9759 growing aerobically at 30° on L-threonine, 14 C was slowly incorporated into the ethanol-soluble fraction. The distribution of 14 C among the labelled components of this fraction was analysed by two-dimensional paper chromatography and radioautography (Fig. 3). In the earliest sample to show significant incorporation of 14 C (25 sec.), the bulk of the radioactivity was present as glutamate (57% of total), citrate (25.4%) and aspartate (4.6%). In subsequent samples this distribution changed; the proportion of the total incorporated 14 C present in citrate decreased swiftly whereas that in glutamate increased. Yet again, so little radioactive malate, fumarate and succinate were produced that it was not possible to determine whether, as a result of the

Table 4. Incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{acetate}$ by extracts of threonine-grown *Arthrobacter* 9759

Incubation mixtures contained (in a total volume of 1 ml.): 25 μmoles of potassium phosphate buffer, pH 7.5, 5 μmoles of MgCl_2 , 5 μmoles of ATP, 0.1 μmole of CoA, 2.5 μmoles of GSH, 5.1 μmoles of sodium $[2\text{-}^{14}\text{C}]\text{acetate}$ (0.3 $\mu\text{C}/\mu\text{mole}$), extract of threonine-grown cells (containing 1.4 mg. of protein) and, as additional substrates where indicated, 2 μmoles of oxaloacetate, 2 μmoles of glyoxylate and 5 μmoles of $\text{L}_\alpha\text{-isocitrate}$. After 30 min. incubation at 30° the reaction was stopped by addition of 3 ml. of ethanol, and the radioactively labelled components of the ethanol-soluble fraction were analysed by two-dimensional paper chromatography, radioautography and measurement of the radioactivity with a Geiger-Müller tube.

$10^{-2} \times \text{Radioactivity (counts/100 sec.)}$

Additions	Citrate	Malate	Succinate	α -Oxoglutarate	Aspartate	Glutamate
None	0	0	0	0	0	0
Oxaloacetate	14.4	6.03	16.2	11.3	0	17.5
Glyoxylate	2.25	25.6	6.56	1.04	8.57	0.43
Isocitrate	1.07	13.8	3.12	0	3.26	0.22

Table 5. Activities of key enzymes of the glyoxylate cycle in *Arthrobacter* 9759 growing on various sources of carbon atoms

Cell-free extracts of organisms harvested during exponential growth were assayed for their content of isocitrate lyase, malate synthase and citrate synthase as described in the Materials and Methods section. Specific activities are expressed as μmoles of substrate transformed/mg. of protein/hr.

Carbon source during growth	Specific activity		
	Isocitrate lyase	Malate synthase	Citrate synthase
L-Threonine	13.0	4.1	13.7
Acetate*	20.8	3.3	48.6
Glycine	0.1	2.9	18.0
Glycine plus acetate (15 mM each)	7.2	4.5	16.5

* Slow and sparse growth only.

operation of a glyoxylate cycle, malate could have been labelled before succinate.

Incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{acetate}$ by extracts of threonine-grown cells. When $[2\text{-}^{14}\text{C}]\text{acetate}$ was supplied to an extract of threonine-grown *Arthrobacter* 9759 supplemented with ATP, CoA, Mg^{2+} and GSH, there was little incorporation of ^{14}C into the ethanol-soluble fraction unless oxaloacetate or glyoxylate or isocitrate had also been supplied. The pattern then obtained of distribution of incorporated ^{14}C among intermediates of the tricarboxylic acid cycle plus aspartate and glutamate (Table 4) confirmed the presence in extracts of threonine-grown cells of the key enzymes of the tricarboxylic acid cycle and glyoxylate cycle.

Enzymes of the glyoxylate cycle in L-threonine-grown organisms. The activities of citrate synthase, isocitrate lyase and malate synthase were assayed in

extracts of *Arthrobacter* 9759 grown on a number of different sources of carbon atoms, and on L-threonine (Table 5). Isocitrate lyase was apparently inducibly synthesized by this organism during its rather poor growth on acetate and its rapid growth on L-threonine. Malate synthase was present in extracts of threonine-grown organisms in quite high activity, but, like citrate synthase, was seemingly more constitutively formed by the organism (Table 5).

Subsequent metabolism of glycine. No glycine was excreted by the organism during its growth on L-threonine, and only insignificantly small amounts of $[^{14}\text{C}]\text{glyoxylate}$ were produced when $[2\text{-}^{14}\text{C}]\text{glycine}$ was incubated with extracts of these cells. Further, these extracts possessed no glyoxylate carboligase, erythro- β -hydroxyaspartate aldolase or erythro- β -hydroxyaspartate dehydratase activities, so could not have metabolized glyoxylate via the glycerate pathway (Kornberg & Gotto, 1961) or by way of the β -hydroxyaspartate pathway (Kornberg & Morris, 1965). It was found, however, that a small but significant evolution of $^{14}\text{CO}_2$ occurred when $[1\text{-}^{14}\text{C}]\text{glycine}$ was incubated with an extract of threonine-grown organisms (about 2 mg. of protein/ml.), and that this decarboxylation was greatly enhanced by the addition of pyridoxal 5'-phosphate and H_4PteGlu . Further investigation with dialysed extracts disclosed the stimulatory effect of Benzyl Viologen, NAD^+ and NADP^+ (Table 6), and measurement of 5,10-methyldiyn- H_4PteGlu formation confirmed the supposition that glycine oxidation in these extracts was initiated by the 'glycine-cleavage reaction' (Sagers & Gunsalus, 1961). This 'glycine-cleavage system' was present in extracts of *Arthrobacter* 9759 obtained after growth on L-threonine, or glycine, or acetate plus glycine, but was not found in extracts of pyruvate-grown cells. This was further evidence that glycine was a major product of L-threonine catabolism and suggested

Table 6. Activity of the 'glycine-cleavage system' in threonine-grown *Arthrobacter* 9759

The activity of the 'glycine-cleavage system' was assayed in dialysed extracts of threonine-grown organisms by concurrent assay of $^{14}\text{CO}_2$ release from $[1-^{14}\text{C}]$ glycine and of 5,10-methyldiylne- H_4PteGlu formation. For the methods employed and the concentrations of cofactors used see the Materials and Methods section.

System	$^{14}\text{CO}_2$ released		5,10-Methyldiylne- H_4PteGlu produced	
	(counts/100sec.)	(% of yield from complete system)	(nmoles/ml.)	(% of yield from complete system)
Complete	3802	(100)	87	(100)
Complete but with boiled extract	336	9	—	—
Complete minus glycine	—	—	20	23
Complete minus pyridoxal 5'-phosphate	463	12	25	29
Complete minus NAD^+	2451	65	64	74
Complete minus NADP^+	2539	67	10	11
Complete minus H_4PteGlu	395	10	5	6
Complete minus Benzyl Viologen	1522	40	—	—

Table 7. Activities of key enzymes involved in the production of glycine from L-threonine and in the subsequent metabolism of glycine in *Arthrobacter* 9759 grown on various sources of carbon atoms

Cell-free extracts obtained from exponentially growing organisms were assayed for activities of the enzymes listed, by the methods described in the Materials and Methods section. The activity of the glycine-cleavage system was measured by $^{14}\text{CO}_2$ release from $[1-^{14}\text{C}]$ glycine and relative specific activities only are listed for this enzyme (assuming its specific activity in extracts of glycine-grown cells to be 100). Other specific activities are expressed as μmoles of substrate transformed/mg. of protein/hr.

Carbon source for growth	Specific activity				
	L-Threonine dehydrogenase	'Aminoacetone synthase'	Glycine-cleavage system	L-Serine hydroxymethyltransferase	L-Serine dehydratase
Pyruvate	Nil	Nil	(7.9)	0.70	Nil
Acetate	Nil	Nil	—	0.94	Nil
Glycine	Nil	Nil	(100)	1.00	40.3
Glycine plus acetate	Nil	Nil	(43.5)	0.82	15.1
L-Threonine	9.2	4.2	(50.7)	1.02	11.1

that it was further metabolized in cells growing on threonine via the serine pathway previously shown to account for glycine utilization by *Arthrobacter globiformis* (Morris, 1963; Jones & Bridgeland, 1966). Key enzymes of this pathway are L-serine hydroxymethyltransferase and L-serine dehydratase, and both were found to be present in adequately high activity in extracts of L-threonine-grown *Arthrobacter* 9759. L-Serine hydroxymethyltransferase was constitutively synthesized (Table 7), but L-serine dehydratase, though also produced by the organism when growing on glycine, or on acetate plus glycine, was not present in cells grown on other sources of carbon atoms. Similar findings have been made with *Arthrobacter globiformis*, and *Arthrobacter* 9759 further resembled the type species in that its L-serine dehydratase demonstrated abnormal kinetic behaviour compatible with homotropic activation by L-serine (Jones & Bridgeland, 1966).

Incorporation of ^{14}C from $[2-^{14}\text{C}]$ glycine during

growth on L-threonine. When $[2-^{14}\text{C}]$ glycine was added to cultures of *Arthrobacter* 9759 growing on L-threonine medium, ^{14}C was rapidly incorporated into the ethanol-soluble fraction of the cells. An analysis of the distribution of ^{14}C among the components of this fraction (by two-dimensional paper chromatography, high-voltage paper electrophoresis and radioautography) showed that radioactivity had been rapidly incorporated into serine, alanine, citrate, aspartate and glutamate (Fig. 4); there was no evidence of $[^{14}\text{C}]$ glyoxylate formation. In the earliest sample (5sec.), 80% of the total incorporated ^{14}C was present in serine. In subsequent samples the proportion of ^{14}C present as serine decreased rapidly, and after 36sec. this compound could hardly be detected on radioautograms. Alanine and glutamate became progressively more heavily labelled as time progressed, until at 5min. more than 90% of the total incorporated ^{14}C was present in glutamate.

Carboxylation of pyruvate by cell extracts. When

Fig. 4. Variation with time of the percentage distribution of ^{14}C incorporated from $[2\text{-}^{14}\text{C}]\text{glycine}$ by *Arthrobacter* 9759 growing on L-threonine into serine (●), glutamate (■), alanine (△), aspartate (○) and citrate (▲). $[2\text{-}^{14}\text{C}]\text{glycine}$ ($2\text{ }\mu\text{moles}$; $10\text{ }\mu\text{C}/\mu\text{mole}$) was added to 10 ml. of a culture of *Arthrobacter* 9759 growing exponentially on L-threonine. Samples (1 ml.) were withdrawn at intervals into 3 ml. of hot ethanol and the distribution of ^{14}C among the ethanol-soluble components was analysed by the methods reported in Fig. 2, but with the additional use of high-voltage paper electrophoresis to separate serine from residual glycine. The total radioactivity (minus background and residual $[^{14}\text{C}]\text{glycine}$) fixed on the chromatograms after 5, 15, 25, and 36 sec. incubation was 575, 1423, 1916 and 2394 counts/min. respectively.

pyruvate was incubated in phosphate buffer, pH 7.5, with $\text{NaH}^{14}\text{CO}_3$, extract of threonine-grown cells, malate dehydrogenase (EC 1.1.1.37) and high concentrations of NADH, malate isolated from the reaction mixture was highly radioactive. The fixation of $^{14}\text{CO}_2$ by this pyruvate carboxylation system (which presumably yields $[^{14}\text{C}]\text{oxaloacetate}$) was dependent on the addition of ATP, and was stimulated by acetyl-CoA. As in *Arthrobacter globiformis* (Bridgeland & Jones, 1967) it would appear that the reaction was catalysed by pyruvate carboxylase (EC 6.4.1.1) allosterically activated by acetyl-CoA.

Presence of 'aminoacetone synthase' in threonine-grown organisms. The discovery that in threonine-grown *Arthrobacter* 9759 L-threonine is cleaved to acetyl-CoA plus glycine, and the inability to demonstrate any stable non-volatile intermediates in this reaction, might suggest the involvement of an L-threonine aldolase (yielding acetaldehyde plus glycine). Yet this enzyme was not present in extracts of threonine-grown cells. The possibility was therefore considered that the $\alpha\text{-amino-}\beta\text{-oxobutyrate}$ produced by L-threonine dehydro-

genase could be the immediate source of the acetyl-CoA plus glycine; this would require its CoA-dependent cleavage in a reaction catalysed by an $\alpha\text{-amino-}\beta\text{-oxobutyrate}$ CoA-ligase, which could be identical with that enzyme hitherto only reported to catalyse the reverse reaction, i.e. 'aminoacetone synthase'. Since the lability of $\alpha\text{-amino-}\beta\text{-oxobutyrate}$ prevented its use as substrate in a direct investigation of the $\alpha\text{-amino-}\beta\text{-oxobutyrate}$ CoA-ligase activity of extracts of *Arthrobacter* 9759, the presence and properties of this enzyme had first to be established by employing its previous method of assay which measures the rate of formation of aminoacetone from acetyl-CoA plus glycine (Urata & Granick, 1963).

Incubation of acetyl-CoA in phosphate buffer, pH 7.5, with a high concentration of glycine and cell extract from threonine-grown *Arthrobacter* 9759 resulted in the formation of an amino ketone. The product of this reaction, which was obtained only when concentrations of glycine greater than 0.05 M were employed, was identified as aminoacetone and was assayed colorimetrically by the method of Urata & Granick (1963). No aminoacetone was produced when either acetyl-CoA or glycine was omitted from the reaction mixture, nor when boiled cell extract was used. Although the omission of acetyl-CoA prevented development of the true aminoacetone colour in the assay, a different though similar colour was produced. Further investigations showed that this was due to non-enzymic reaction of glycine (in high concentration) with the components of the assay mixture. The absorption spectrum of this coloured product was similar to that produced by aminoacetone in that it possessed a peak and shoulder separated by 30 nm. , but the whole spectrum was displaced towards the ultraviolet end by 17 nm. (Fig. 5). Thus the proper control in these colorimetric assays was provided by reaction mixture minus acetyl-CoA. Only a small amount of aminoacetone was formed enzymically from acetyl-CoA and glycine in these assays. In a typical reaction mixture containing, in 1 ml. final volume, extract of threonine-grown cells (0.9 mg. of protein), acetyl-CoA ($0.7\text{--}0.9\text{ }\mu\text{mole}$) and glycine ($100\text{ }\mu\text{moles}$), only $20\text{--}30\text{ nmoles}$ of aminoacetone were produced in 30 min. at 30° . When the time-course of aminoacetone synthesis was investigated, it was found that the reaction, though initially quite rapid, slowed down very quickly; over half of the aminoacetone formed in 25 min. of incubation was in fact produced in the first minute of reaction.

This behaviour was also demonstrated with partially purified preparations of 'aminoacetone synthase' free of other acetyl-CoA-cleaving enzymes (McGilvray & Morris, 1969), so that the rapid decrease in the rate of aminoacetone production could not be attributed to removal of substrate

Fig. 5. Absorption spectra of colours given in the Urata & Granick (1963) amino ketone assay by (1) authentic aminoacetone, (2) the product formed enzymically from acetyl-CoA (0.9 mM) plus glycine (0.1 M), and (3) glycine (0.1 M). Blanks for (1) and (3) were prepared with water in place of the reactants, but the blank for (2) was prepared from incubation mixture minus acetyl-CoA.

acetyl-CoA by competing reactions. Aminoacetone synthesis from acetyl-CoA plus glycine was in fact markedly inhibited by free CoA; the following percentage inhibition was observed when CoA was added to the reaction mixture: 35% at 0.13 mM; 59% at 0.27 mM; 96% at 1 mM. Other thiol compounds were also inhibitory, e.g. at 10 mM L-cysteine and glutathione caused 98% and 72% inhibition respectively, whereas iodoacetamide (1 mM) increased the yield of aminoacetone by 50%.

When similar experiments were performed with extracts of pyruvate-grown *Arthrobacter* 9759, no aminoacetone was formed.

'Aminoacetone synthase' in extracts of threonine-grown cells. A continuous spectrophotometric assay for 'aminoacetone synthase' was developed in which 5,5'-dithiobis-(2-nitrobenzoic acid) was reduced by the free CoA released by the condensation of acetyl-CoA with glycine; the reaction was followed by the increase in E at 412 nm. (Materials and Methods section). The removal of free CoA by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) was beneficial to the assay since it virtually eliminated end-product inhibition by CoA. With crude extracts, some CoA was formed from acetyl-CoA by irrelevant acylase activity, so that a blank was always employed containing the test reaction mixture minus glycine. No activity was observed with boiled extracts, and preincubation of the extract with 5,5'-dithiobis-(2-nitrobenzoic acid) for 10 min. before the addition of acetyl-CoA and glycine caused no inhibition of subsequent activity. Under the reaction conditions described in the

Materials and Methods section, the initial velocity of the reaction was directly proportional to the concentration of extract used, up to approx. 0.1 mg. of protein/ml., and it was found that the specific activity of 'aminoacetone synthase' in crude extracts of L-threonine-grown *Arthrobacter* 9759 was 4–4.5 μ moles of CoA released/mg. of protein/hr.

To confirm that the activity observed in the spectrophotometric assay was indeed due to 'aminoacetone synthase', the stoichiometry of the reaction was studied. After assays in which added glycine had caused the release of 77 and 78 nmols of CoA from acetyl-CoA, the aminoacetone produced in each cuvette was assayed and was found to equal 62 and 60 nmols respectively. Although the aminoacetone produced was in both cases only some 80% of the CoA released, this discrepancy could be attributed to side reactions, including some dimerization of aminoacetone. It was concluded that the assayed activity could indeed be identified with 'aminoacetone synthase'; this was confirmed by partially purifying the enzyme from these extracts (McGilvray & Morris, 1969).

Induced synthesis of L-threonine dehydrogenase and 'aminoacetone synthase'. With the use of the most sensitive method of spectrophotometric assay and optimum reaction conditions, no 'aminoacetone synthase' activity could be detected in extracts of *Arthrobacter* 9759 after growth on any source of carbon atoms other than L-threonine (Table 7). Of particular interest was the finding that no 'aminoacetone synthase' activity was possessed by extracts of the organism obtained after growth on acetate plus glycine. When organisms that had been harvested during exponential growth on pyruvate were transferred to a medium containing 0.03% yeast extract and L-threonine (20 mM), growth recommenced after a short lag (2 hr.), during which time there was rapid synthesis of both L-threonine dehydrogenase and 'aminoacetone synthase' (Fig. 6). When the pyruvate-grown culture was transferred into fresh pyruvate medium neither of these enzymes was produced.

The converse transfer of a threonine-grown inoculum to pyruvate medium caused an immediate decrease in the customary high specific activity of both threonine dehydrogenase and 'aminoacetone synthase' in these cells. Though the total threonine dehydrogenase activity decreased, the total 'aminoacetone synthase' in the culture (specific activity $\times$ cell density) remained constant. During continued growth on fresh L-threonine medium, both enzymes retained their high starting specific activities.

Production of acetyl-CoA plus glycine from L-threonine by a reconstituted mixture of purified enzymes. In a reaction mixture containing oxaloacetate (5 mM), zinc sulphate (0.1 mM) and the full complement of cofactors required for the production

Fig. 6. Induced synthesis of L-threonine dehydrogenase and 'aminoacetone synthase'. *Arthrobacter* 9759 was grown in a medium containing pyruvate as sole source of carbon atoms. Harvested organisms were transferred to fresh medium containing L-threonine (15 mM) and yeast extract (0.03%). Growth was followed turbidimetrically (▲) during aerobic incubation at 30° and samples of cells (6 mg. dry wt.) were removed at intervals for assay of their L-threonine dehydrogenase (●) and 'aminoacetone synthase' (○) activities as described in the Materials and Methods section. The specific activity of each enzyme is expressed as μ moles of product formed/mg. of protein/hr.

of glycine from L-threonine by cell extracts (Table 3), purified L-threonine dehydrogenase (McGilvray & Morris, 1969) formed no radioactive glycine and no radioactive citrate from L-[U- 14 C]threonine. However, electrophoretograms of the final reaction mixture on radioautography disclosed the presence of traces of aminoacetone (mainly in the form of its dimer). No radioactive products were formed under the same reaction conditions when L-[U- 14 C]-threonine was incubated with purified 'aminoacetone synthase' (McGilvray & Morris, 1969). But, when a mixture of both purified enzymes was employed (i.e. L-threonine dehydrogenase plus 'aminoacetone synthase'), considerable quantities of labelled glycine and citrate were produced from L-[U- 14 C]threonine and no amino[14 C]acetone could be detected. There was no utilization of L-[U- 14 C]-threonine when the mixture of enzymes was boiled before use.

DISCUSSION

During growth of *Arthrobacter* 9759 on L-threonine as sole source of carbon atoms, the amino acid is oxidatively degraded to yield equimolar quantities of acetyl-CoA and glycine. The acetyl-CoA is metabolized via the tricarboxylic acid cycle and

glyoxylate cycle whereas the glycine forms pyruvate via the folate-dependent 'serine pathway'. The route of threonine catabolism in this organism is therefore that summarized in Scheme 1.

The novel feature of this metabolic route is the way in which the α -amino- β -oxobutyrate produced by L-threonine dehydrogenase is not spontaneously decarboxylated, but is instead cleaved in a CoA-dependent reaction to produce acetyl-CoA plus glycine. That this reaction is catalysed by the 'aminoacetone synthase' of *Arthrobacter* 9759 was evident from two findings.

(1) 'Aminoacetone synthase' was produced only during growth on L-threonine; the enzyme was not present in cells grown on an equimolar mixture of acetate and glycine, though these are the growth conditions most likely to have warranted its formation had its role been a 'synthetic' one in the oxidation of glycine in the cycle of reactions proposed by Elliott (1960). Further, synthesis of aminoacetone from acetyl-CoA plus glycine would seem to be gratuitous, since the organism could produce aminoacetone more directly from L-threonine, and in any event aminoacetone was not utilized by *Arthrobacter* 9759 (Table 1) and was not catabolized by suspensions and extracts of threonine-grown cells.

(2) Amino[14 C]acetone only was produced from L-[U- 14 C]threonine when this was incubated with purified L-threonine dehydrogenase plus NAD⁺, Zn²⁺, CoA and pyridoxal phosphate. Addition of purified aminoacetone synthase led to the formation of [14 C]acetyl-CoA and [14 C]glycine at the expense of amino[14 C]acetone production. Further, no alternative mechanism could explain the very rapid production of acetate plus glycine from L-threonine that was disclosed by the 14 C-incorporation studies performed with growing cultures supplied with L-[U- 14 C]threonine. L-Threonine aldolase, which might have served this function by producing from L-threonine an equimolar mixture of acetaldehyde plus glycine, was not present in threonine-grown cells.

The utilization of glycine via the folate-dependent serine pathway has previously been reported in aerobic coryneform organisms (Morris, 1963). Thus the discovery that our 'threonine isolate' metabolized the amino acid by this pathway instead of via glyoxylate and the glycerate pathway, or via the β -hydroxyaspartate pathway, supported the other taxonomic evidence that it is a species of *Arthrobacter*. The further finding that key enzymes of this pathway were present in high activity in extracts of L-threonine-grown cells supported the more direct evidence from tracer studies with L-[14 C]threonine and [14 C]glycine that glycine was an early product of threonine catabolism and that it was further metabolized via serine to yield pyruvate.

The presence of isocitrate lyase in *Arthrobacter* 9759 grown on L-threonine and its absence from extracts of glycine-grown cells was an indication of the production of major amounts of 'acetate' from L-threonine. This was confirmed by the more direct demonstration of [^{14}C]acetyl-CoA production from L-[U- ^{14}C]threonine. Since malate synthase was also present in the threonine-grown organisms it is likely that the glyoxylate cycle operates during growth on L-threonine and performs its usual anaplerotic function whereby C_4 dicarboxylic acids are formed from acetate. At first sight it would seem that, since glycine is a concomitant product with acetyl-CoA of L-threonine catabolism, and that this glycine is convertible into pyruvate and thence by carboxylation into oxaloacetate, operation of the glyoxylate cycle is gratuitous. Indeed, one might not expect isocitrate lyase to be formed in these circumstances, since synthesis of this enzyme is repressed by a direct product of pyruvate

metabolism (Kornberg, 1966). Yet, from every two molecules of L-threonine catabolized, two molecules of acetyl-CoA but only one molecule of pyruvate are produced (since two molecules of glycine must enter the serine pathway to yield one molecule of pyruvate). Growth of the organism on L-threonine is from this point of view the equivalent of its growth on an equimolar mixture of acetate and glycine, so that it was reassuring to find a similar isocitrate lyase activity in extracts of cells grown on an equimolar mixture of acetate plus glycine. It is conceivable that maintenance of a large pool of glutamate in the organism constitutes a drain on its oxaloacetate supply that, when exacerbated by the presence of excess of acetyl-CoA, becomes so great that oxaloacetate synthesis from pyruvate (derived from glycine) must be supplemented by ancillary production via the glyoxylate cycle.

The employment by *Arthrobacter* 9759 of 'amino-acetone synthase' to catalyse α -amino- β -oxo-

Scheme 2.

butyrate catabolism does not necessarily mean that in other circumstances this enzyme cannot be concerned with the biosynthesis of aminoacetone, though the high K_m for glycine of the enzyme from *Arthrobacter* 9759 (McGilvray & Morris, 1969) suggests that it is unlikely that it has any synthetic role in this organism. Nor does it mean that all organisms that use L-threonine dehydrogenase to initiate L-threonine utilization must simultaneously employ an 'aminoacetone synthase' to catalyse the degradation of the product α -amino- β -oxobutyrate. It is perfectly feasible that organisms capable of rapidly producing pyruvate from aminoacetone might dispense altogether with an α -amino- β -oxobutyrate CoA-ligase during their growth on L-threonine and rely instead on the non-enzymic decarboxylation of α -amino- β -oxobutyrate to yield aminoacetone. It could be argued that the catabolic employment of 'aminoacetone synthase' in a pathway of L-threonine utilization is likely only in organisms capable of efficiently utilizing both acetyl-CoA and glycine. Although *Arthrobacter* species with their ability to metabolize glycine via the serine pathway might appear to be especially favoured in this respect, it is conceivable that other organisms capable of rapidly forming glyoxylate from glycine might employ a malate synthase to produce malate from acetyl-CoA plus glyoxylate. These organisms would presumably not produce isocitrate lyase since they could transform threonine into malate via a pleasingly simple four-step pathway (Scheme 2). Therefore the discovery that 'aminoacetone synthase' can operate catabolically as an α -amino- β -oxobutyrate CoA-ligase, and its then necessary reliance on L-threonine dehydrogenase for the supply of its substrate α -amino- β -oxobutyrate, means that the role of 'aminoacetone synthase' in other organisms possessing threonine dehydrogenase activity must be re-evaluated. Systems wherein threonine can serve as a source of glycine in the absence of threonine aldolase activity should certainly be re-examined in the light of these findings.

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