Biochem. J. (1976) **156**, 449–458
Printed in Great Britain

Bacterial Catabolism of Threonine

THREONINE DEGRADATION INITIATED BY L-THREONINE–NAD$^+$ OXIDOREDUCTASE

By STEPHEN C. BELL* and JOHN M. TURNER
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

(*Received* 30 *December* 1975)

1. Isolates representing seven bacterial genera capable of growth on L-threonine medium, and possessing high L-threonine 3-dehydrogenase activity, were examined to elucidate the catabolic route. 2. The results of growth, manometric and enzymic experiments indicated the catabolism of L-threonine by cleavage to acetyl-CoA plus glycine, the glycine being further metabolized via L-serine to pyruvate, in all cases. No evidence was obtained of a role for aminoacetone in threonine catabolism or for the metabolism of glycine by the glycerate pathway. 3. The properties of a number of key enzymes in L-threonine catabolism were investigated. The inducibly formed L-threonine 3-dehydrogenase, purified from *Corynebacterium* sp. B6 to a specific activity of about 30–35 μmol of product formed/min per mg of protein, exhibited a sigmoid kinetic response to substrate concentration. The half-saturating concentration of substrate, $[S]_{0.5}$, was 20mM and the Hill constant (h) was 1.50. The K_m for NAD$^+$ was 0.8mM. The properties of the enzyme were studied in cell-free extracts of other bacteria. 4. New assays for 2-amino-3-oxobutyrate–CoA ligase were devised. The K_m for CoA was determined for the first time and found to be 0.14mM at pH8, for the enzyme from *Corynebacterium* sp. B6. Evidence was obtained for the efficient linkage of the dehydrogenase and ligase enzymes. Cell-free extracts all possessed high activities of the inducibly formed ligase. 5. L-Serine hydroxymethyltransferase was formed constitutively by all isolates, whereas formation of the 'glycine-cleavage system' was generally induced by growth on L-threonine or glycine. The coenzyme requirements of both enzymes were established, and their linked activity in the production of L-serine from glycine was demonstrated by using extracts of *Corynebacterium* sp. B6. 6. L-Serine dehydratase, purified from *Corynebacterium* sp. B6 to a specific activity of about 4 μmol of product formed/min per mg of protein, was found to exhibit sigmoid kinetics with an $[S]_{0.5}$ of about 20mM and $h = 1.4$. Similar results were obtained with enzyme preparations from all isolates. The enzyme required Mg^{2+} for maximum activity, was different from the L-threonine dehydratase also detectable in extracts, and was induced by growth on L-threonine or glycine.

Although L-threonine–NAD$^+$ oxidoreductase (L-threonine 3-dehydrogenase, EC 1.1.1.103) has been shown to initiate catabolism of the amino acid by an *Arthrobacter* species (McGilvray & Morris, 1969) and two *Pseudomonas* species (Lessie & Whiteley, 1969; Blackmore & Turner, 1971), its metabolic role in other bacteria is uncertain. Catabolic L-threonine dehydratase (EC 4.2.1.16; Umbarger & Brown, 1957) and L-threonine aldolase (EC 2.1.2.1; Morris, 1969) initiate dissimilation in other species. A survey of a large number of isolates capable of growth on L-threonine as the sole or major source of carbon and nitrogen has revealed that the majority possessed inducibly synthesized L-threonine 3-dehydrogenase of high activity but low dehydratase and aldolase activities (S. C. Bell, T. R. G. Gray & J. M. Turner, unpublished work). The route of L-threonine catabolism in bacteria representative of the genera to which the isolates were assigned has now been investigated. Evidence for the route and some properties of the enzymes involved are reported below.

* Present address: Department of Biochemistry, University of Strathclyde, Glasgow G1 1XW, Scotland, U.K.

Materials and Methods

Micro-organisms and media

All bacteria were originally isolated from soil samples by virtue of their ability to grow on L-threonine as the major source of carbon and nitrogen. Each isolate was identified and given a genus name together with a strain designation (S. C. Bell, T. R. G. Gray & J. M. Turner, unpublished work). Most

strains described have now been lodged with the National Collection of Industrial Bacteria and given the following accession numbers: *Achromobacter* sp. F14 (N.C.I.B. 11092), *Alcaligenes* sp. N2 (N.C.I.B. 11094), *Bacillus* sp. N5 (N.C.I.B. 11095), *Brevibacterium* sp. P2 (N.C.I.B. 11100) and B10 (N.C.I.B. 11101), *Corynebacterium* sp. B6 (N.C.I.B. 11220) and *Flavobacterium* sp. P4 (N.C.I.B. 11103).

Bacteria were maintained on nutrient broth and cultivated on semisynthetic media. The basal salts medium contained (per litre) 7.0g of K_2HPO_4, 3.0g of KH_2PO_4, 0.1g of $MgSO_4,7H_2O$ and 1.2g of Na_2SO_4. This was supplemented with 2g of L-threonine and 0.5g of yeast extract (Difco Laboratories, Detroit, MI, U.S.A.). Yeast extract was not required for the growth of isolates N2 and P4. Other media contained 2g of an alternative carbon source/litre, and 1g of $(NH_4)_2SO_4$/litre replaced the Na_2SO_4. Media were adjusted to pH7 with HCl or NaOH and sterilized by autoclaving. Liquid culture was usually either in 100ml of medium in 250ml conical flasks or in 1-litre batches in 2-litre conical flasks, on a rotary shaker at 30°C. Cultures were harvested in the mid-exponential phase of growth by centrifugation at 5000*g* for 20min. Bacterial pellets were suspended in 0.1M-potassium phosphate buffer, pH7, re-centrifuged and resuspended in the appropriate buffer.

Measurement of O_2 uptake by washed bacterial suspensions

O_2 uptake was measured manometrically in Warburg flasks by using conventional procedures (Umbreit *et al.*, 1964), under the conditions described previously (Higgins *et al.*, 1968).

Preparation of enzyme extracts

Gram-positive bacteria suspended in buffer were disrupted ultrasonically as previously described by Faulkner & Turner (1974). Suspensions of *Brevibacterium* and *Corynebacterium* species required ultrasonic treatment for a total of about 4min at full power in the MSE 100W ultrasonic disintegrator rather than the usual 2min. Gram-negative bacteria were disrupted in an Aminco–French pressure cell, as previously described (Turner, 1966). In all cases disrupted cells were centrifuged at 100000*g* for 90min at 0°C to remove cell debris. Supernatants were used for enzyme purification and assays.

Enzyme assays

These were done at 37°C unless otherwise stated. Protein was measured by a biuret procedure described previously (Turner, 1966) or by the more sensitive method of Lowry *et al.* (1951).

L-Threonine 3-dehydrogenase. Activity was measured either colorimetrically or spectrophotometrically depending on the circumstances.

(*a*) Colorimetric assay. This was carried out by a modification of the method described by Green & Elliott (1964). Reaction mixtures contained 250μmol of Tris/HCl buffer, pH8.6, 10μmol of NAD^+, 300μmol of KCl, 0.2ml of enzyme preparation and 120μmol of L-threonine in a total volume of 1ml. Reactions were usually started by the addition of threonine to the other components. After incubation for 15min, 0.5ml of 25% (w/v) trichloroacetic acid was added, protein was removed by centrifugation at 2000*g* for 10min and 1ml samples of supernatants were assayed colorimetrically for aminoacetone (Pickard *et al.*, 1968).

(*b*) Spectrophotometric assay. This was carried out as described above except that 5μmol of NAD^+ was used and initial rates were measured by recording the L-threonine-dependent reduction of NAD^+ at 340nm.

L-Threonine dehydratase. Activity was assayed colorimetrically by the procedure of Datta (1966), except that reaction mixtures contained 125μmol of potassium phosphate buffer, pH8, 0.1μmol of pyridoxal phosphate, 0.2ml of enzyme preparation and 30μmol of L-threonine in a total volume of 1ml.

L-Threonine aldolase. Activity was looked for by a colorimetric method for the detection and measurement of acetaldehyde (Paz *et al.*, 1965). Reaction mixtures contained 20μmol of Tris/HCl buffer (Trizma grade from Sigma Chemical Co., St. Louis, MO, U.S.A.), cell-free extract and 100μmol of L-threonine in a total volume of 1ml. Reactions were terminated by the addition of an equal volume of aldehyde reagent (0.4% *N*-methylbenzothiazolone hydrazone in 0.1M-3,3-dimethylglutaric acid/NaOH buffer, pH4) and colours developed by the procedure described elsewhere (Jones & Turner, 1973). Assays were carried out at pH8.5, at which all known bacterial L-threonine aldolases are active (Bell & Turner, 1973), and under a variety of other conditions as indicated in the text.

2-Amino-3-oxobutyrate–CoA ligase. Preliminary assays were carried out by the method of McGilvray & Morris (1969). Two other assays were used as a routine.

(*a*) Colorimetric assay depending on the formation of acetyl phosphate from acetyl-CoA produced in the ligase reaction. Reaction mixtures contained 300μmol of morpholinopropane sulphonic acid/NaOH buffer, pH8, 150μmol of L-threonine, 60μmol of NAD^+, 0.5 unit of L-threonine 3-dehydrogenase (see below), 1.5μmol of CoA, 5 units of phosphotransacetylase (Boehringer und Soehne G.m.b.H., Mannheim, Germany), 5μmol of K_2HPO_4 and approx. 1.5mg of extract protein, in a total volume of 1.5ml. The

reaction was started by the addition of L-threonine and stopped by heating at 100°C for 5min. The rate of acetyl phosphate formation, estimated by reaction with hydroxylamine and $FeCl_3$, was linear with incubation time up to 20min when extracts of *Corynebacterium* sp. P6 were used. For the assay, 1ml of sample (0–5μmol of acetyl phosphate) was added to 0.5ml of neutralized hydroxylamine reagent (4M-NH_2OH,HCl / 3.5M-NaOH, 1:1, v/v). After 10min at room temperature (about 23°C), 1.5ml of $FeCl_3$ reagent [(5% (w/v) $FeCl_3$, $6H_2O$ in 0.1M-HCl/12% (w/v) trichloroacetic acid/3M-HCl, 1:1:1, by vol. was added. Any precipitate was removed by centrifuging and the extinction of the supernatant read at 540nm. The concentration of acetyl phosphate was calculated from the molar extinction coefficient $\varepsilon = 4.17 \times 10^2 M^{-1} \cdot cm^{-1}$ determined by using acetohydroxamic acid as a reference standard. The addition of L-threonine 3-hydrogenase to reaction mixtures containing extracts of bacteria grown on threonine medium had little effect on lyase activity.

(*b*) Assay of aminoacetone formation in the presence and absence of CoA. L-Threonine 3-dehydrogenase assays were carried out, by the colorimetric assay of aminoacetone formation (see above), with and without CoA (1.5mM). Extract protein concentration and incubation time were adjusted so that about 2μmol of aminoacetone/ml of reaction mixture was formed in the absence of CoA. An amount of 2-amino-3-oxobutyrate equivalent to the amount of aminoacetone lost was assumed to be cleaved to glycine+acetyl-CoA. Where extracts exhibited low L-threonine 3-dehydrogenase activity, e.g. when bacteria were grown on succinate media, 0.5 unit of the partially purified enzyme was added to reaction mixtures.

L-*Serine hydroxymethyltransferase* (*EC* 2.1.2.1). The assay was a modification of the method of Scrimgeour & Huennekens (1962). Reaction mixtures contained 75μmol of potassium phosphate buffer, pH7.5, 50μmol of glycine, 2.5μmol of formaldehyde (freshly prepared), 0.53μmol of tetrahydrofolic acid (prepared as a solution in 50mM-2-mercaptoethanol), 0.03μmol of pyridoxal phosphate and 0.2ml of extract protein, in a total volume of 1ml. Reactions were started by the addition of glycine, and after incubation for 10–30min were terminated by the addition of 0.5ml of 15% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation and formaldehyde was assayed in 0.5ml samples by the colorimetric method of Nash (1953).

'*Glycine-cleavage system*'. The activity of this enzyme was assayed by two methods, similar to those described by McGilvray & Morris (1969). Reaction mixtures contained 250μmol of potassium phosphate buffer, pH7.5, 40μmol of glycine, 2μmol of NAD^+, 2μmol of $NADP^+$, 1μmol of tetrahydrofolic acid (as a solution in 50mM-2-mercaptoethanol), 0.2μmol of pyridoxal phosphate, 5μmol of Benzyl Viologen and 1–4mg of extract protein in a total volume of 1.5ml. For the radioactive assay of glycine cleavage, 0.2μCi of [U-^{14}C]glycine was added to incubation mixtures. The assay procedures used were those described by McGilvray & Morris (1969).

L-*Serine dehydratase* (*EC* 4.2.1.13). This enzyme was assayed colorimetrically by the procedure of McGilvray & Morris (1969). Conditions were chosen so that the amount of product formed was proportional to protein concentration and time. At low protein concentrations a lag in pyruvate formation was commonly observed.

Other enzyme assays. Standard methods were used to assay 1-aminopropan-2-ol-*O*-phosphate phospholyase (Faulkner & Turner, 1974), glycine–pyruvate aminotransferase (Rowsell *et al.*, 1969), glyoxylate carbo-ligase (Kornberg & Gotto, 1961) and *erythro*-β-hydroxyaspartate dehydratase (Kornberg & Morris, 1965).

Partial purification of L-*threonine 3-dehydrogenase and* L-*serine dehydratase from Corynebacterium sp. B6 by anion-exchange chromatography*

Crude extracts were prepared in 0.1M-Tris/HCl buffer, pH7.5. Approx. 20ml of extract, containing 400mg of protein, was applied to a column (20cm × 1.5cm diam.) of Whatman DE 52 DEAE-cellulose equilibrated with Tris/HCl buffer. Protein was eluted with an increasing concentration gradient of 0–0.5M-KCl over a total volume of 500ml, and 6ml fractions of eluate were collected. The presence of L-threonine 3-dehydrogenase and L-serine dehydratase in fractions was determined by the standard colorimetric assay methods. The results of one experiment are shown in Fig. 1. Specific-activity measurements showed approx. 17-fold and 12-fold purification of the respective enzymes. L-Threonine 3-dehydrogenase preparations were found to be free of 2-amino-3-oxobutyrate–CoA ligase activity.

Chemicals

Aminoacetone was prepared from its commercially available derivative 1-aminopropan-2-one semicarbazide hydrochloride (Eastman–Kodak Co., Rochester, NY, U.S.A.). DL-*allo*-Threonine was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K., L-*allo*-threonine from Calbiochem, Los Angeles, CA, U.S.A., D-threonine from Sigma Chemical Co., St. Louis, MO, U.S.A., and all other chemicals were from commercial sources.

Results

Selection of bacteria for study

Previous results had shown that most isolates capable of growth on simple synthetic media con-

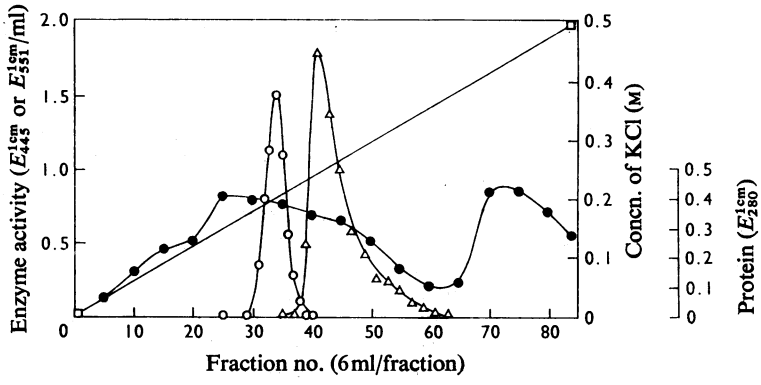

Fig. 1. *Partial purification of enzymes from Corynebacterium sp. B6*

Crude extract was applied to a column of DEAE-cellulose, protein eluted with a linear gradient of KCl and enzyme activities were assayed as described in the text. The gradient in concentration of KCl present in Tris/HCl buffer is indicated (□). Protein was detected by its E_{280} (●). L-Threonine 3-dehydrogenase E_{551} (△) and L-serine dehydratase E_{445} (○) activities were detected by colorimetric methods and are expressed in arbitrary units. Enzyme activities were found only in the regions shown; points indicating absence of either enzyme from other fractions are omitted for clarity.

Table 1. *Oxidation of possible metabolites of L-threonine by washed suspensions of selected bacteria*

The bacteria were grown on L-threonine as the major source of carbon and nitrogen as described in the Materials and Methods section. Results obtained after growth on succinate+$(NH_4)_2SO_4$ medium are shown in parentheses in the appropriate cases. O_2 uptake by washed suspensions was measured manometrically by using conventional procedures (Umbreit *et al.*, 1964). Rates of O_2 uptake are corrected for endogenous values of 0.3–0.7 μmol of O_2 absorbed/h per mg dry wt. of bacteria.

	O_2 absorbed (μmol/h per mg dry wt. of bacteria) during incubations with substrate (20mM)					
Bacterium	L-Threonine	Acetate+ glycine	Acetate	Glycine	L-Serine	Pyruvate
Achromobacter sp. F14	4.8 (0.4)	5.3 (3.5)	3.2 (3.9)	4.0 (1.3)	4.7 (1.7)	4.3 (3.3)
Alcaligenes sp. N2	4.3	5.7	3.6	3.2	5.2	4.5
Bacillus sp. N5	5.7	5.4	3.1	4.2	5.4	4.3
Brevibacterium sp. P2	3.7 (0)	4.6 (2.7)	2.7 (2.5)	2.9 (0.3)	3.9 (1.6)	4.2 (3.8)
Brevibacterium sp. B10	6.3	6.8	4.1	4.3	4.7	5.3
Corynebacterium sp. B6	6.8 (0.2)	6.3 (3.7)	2.6 (3.8)	5.2 (1.7)	5.0 (3.6)	4.7 (5.9)
Flavobacterium sp. P4	5.2	4.7	1.8	3.6	4.6	4.1
Pseudomonas sp. Y1	4.8	5.6	1.7	4.4	4.6	4.5

taining L-threonine as the major substrate possessed inducibly synthesized L-threonine 3-dehydrogenase at high activity in cell-free extracts (S. C. Bell, T. R. G. Gray & J. M. Turner, unpublished work). Bacteria representative of each of the seven genera involved were selected for further study. These are listed in Table 1. Isolate B10, a second representative of the genus *Brevibacterium*, was originally of uncertain identity but was included in the study because of its exceptionally high L-threonine 3-dehydrogenase activity.

Growth studies

Each of the bacteria selected grew well on defined basal medium containing L-threonine, acetate+ glycine, acetate, glycine, L-serine, pyruvate or succinate as carbon and energy sources (except isolate B10, which grew poorly on acetate). None of the bacteria grew on aminoacetone, DL-1-aminopropan-2-ol or glyoxylate. Propionate supported the growth of only four of the isolates (B6, B10, F14 and Y1).

Manometric experiments

O_2 uptake by washed suspensions of each isolate, after growth on L-threonine or succinate media, was stimulated by various substrates as shown in Table 1. Essentially the same pattern of oxidation was seen after growth on L-threonine. No oxidation of 2-oxobutyrate, aminoacetone, DL-1-aminopropan-2-ol,

propionate or glyoxylate was detected. A comparison of rates of O_2 uptake after growth on succinate rather than L-threonine showed that the oxidations of L-threonine, acetate+glycine, glycine and L-serine were markedly stimulated by growth on the amino acid (Table 1). In all cases O_2 uptake with glycine+acetate was greater than with glycine alone and was comparable with that for L-threonine oxidation.

Enzymes initiating threonine metabolism

It had been established previously that when the activity of enzymes known to initiate L-threonine catabolism were assayed under standard conditions, L-threonine 3-dehydrogenase was the most active enzyme in the isolates selected for the present study (S. C. Bell, T. R. G. Gray & J. M. Turner, unpublished work). As it was unlikely that the standard conditions were optimum for enzyme assay in all cases, it was necessary to check the properties of the enzymes in crude cell-free extracts before their relative importance in threonine degradation could be assessed.

L-Threonine 3-dehydrogenase. The optimum pH for activity of each of the enzyme preparations in Tris/HCl and diethanolamine/HCl buffers (Table 2) was distinct, being within the range pH 8.5–10.5. Potassium phosphate buffer (0.1 M) inhibited activity in 0.25M-Tris/HCl buffer by 25–87% at pH 7.5. Plots of reaction velocity versus substrate concentration exhibited a sigmoid response for all extracts. The sigmoid response was also apparent with purified preparations of the enzyme (see Fig. 2). Results plotted according to the Hill (1913) equation indicated coefficients (h) of 1.25–3.20. Values of $[S]_{0.5}$ (Koshland *et al.*, 1966), the half-saturating concentration of substrate equivalent to the K_m, were in the range 13.6–20.0mM. When the potential effectors of catabolic enzymes, AMP, ADP, ATP, phosphoenolpyruvate and acetyl-CoA, were tested at 1 and 5mM, with saturating and non-saturating concentrations of L-threonine, no effect was apparent. Plots of reaction velocity versus NAD^+ concentration yielded 'normal' hyperbolic curves in all cases, double-reciprocal plots (Lineweaver & Burk, 1934) indicating apparent K_m values of 0.45–1.00mM. The addition of EDTA (5mM) or KCl (10mM) did not affect enzyme activity in any case. Concentrations of KCl up to 500mM did not affect the activity of the enzyme purified from *Corynebacterium* B6. The only stereoisomer of L-threonine active as a substrate was D-*allo*-threonine, which had 7–31% of the activity of

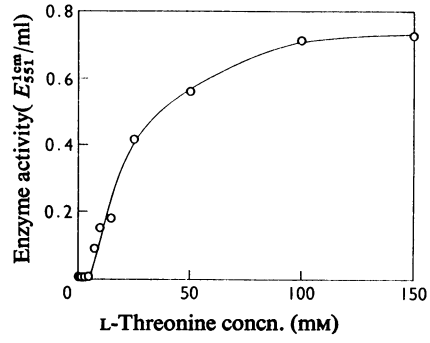

Fig. 2. *Effect of substrate concentration on the activity of L-threonine 3-dehydrogenase*

The enzyme was purified from *Corynebacterium* sp. B6 as described in the Materials and Methods section and had a specific activity of 27 μmol of product formed/min per mg of protein. Initial rates of L-threonine 3-dehydrogenase activity (○) were measured spectrophotometrically.

Table 2. *Properties of L-threonine 3-dehydrogenase from bacteria grown on L-threonine medium*

Enzyme activity in extracts was assayed as described in the Materials and Methods section, except that substrate concentrations, pH etc., were varied as appropriate.

Bacterium	pH optimum	$V_{max.}$ for L-threonine (nmol of product formed/min per mg of protein)	$[S]_{0.5}$ for L-threonine (mM)	K_m for NAD^+ (mM)	Hill coefficient (h)	Relative activity with DL-*allo*-threonine (% of activity towards L-threonine)
Achromobacter sp. F14	8.5–10.0	830	18.2	1.0	1.7	29
Alcaligenes sp. N2	9.0–10.0	577	17.2	1.0	2.9	29
Bacillus sp. N5	8.5–9.5	935	16.5	0.5	3.2	7
Brevibacterium sp. P2	9.0–10.0	970	13.6	0.5	1.8	15
Brevibacterium sp. B10	9.5–10.5	1731	14.3	0.7	2.0	31
Corynebacterium sp. B6	9.0–10.0	2040	20.0	0.8	1.5	14
Flavobacterium sp. P4	9.5–10.5	1490	15.4	0.5	1.9	0
Pseudomonas sp. Y1	9.5–10.5	730	15.2	0.6	1.3	26

the natural isomer in all extracts (except those of P4). D-Threonine and L-*allo*-threonine were inactive as substrates.

The properties of L-threonine 3-dehydrogenase in extracts of individual bacteria are summarized in Table 2. (Activities of the enzyme in various extracts are shown in Table 4.)

L-Threonine dehydratase and L-threonine aldolase. Preliminary studies indicated that the L-threonine dehydratases in crude extracts, optimally active at pH 9–10, were biosynthetic in function. In each case activity was strongly inhibited by L-isoleucine (67–97% inhibition at 1 mM) and activated by L-valine (1.4–3.2-fold at 1 mM). In the presence of 1 mM-L-valine, the K_m values for L-threonine ranged from 4.0 to 11.0 mM. Enzyme activity was not markedly affected by growth on different media.

L-Threonine aldolase activity could not be detected in extracts of any of the bacteria under any of a variety of assay conditions. A range of pH values, thiol-protecting compounds, chelating agents and potential effectors was tested.

Other key enzymes of threonine metabolism

Evidence suggesting a key role for L-threonine 3-dehydrogenase prompted a search for enzymes involved in the further metabolism of its immediate product 2-amino-3-oxobutyrate. The route involving glycine and serine as intermediates (McGilvray & Morris, 1969) was of particular interest.

2-Amino-3-oxobutyrate–CoA ligase. Preliminary assays were carried out with glycine and acetyl-CoA as substrates, by measuring the glycine-dependent formation of CoA spectrophometrically (McGilvray & Morris, 1969). This assay was unsatisfactory for the enzyme in crude extracts on two counts: all extracts examined exhibited significant acylase activity (20–84 nmol of acetyl-CoA cleaved/min per mg of protein) and the rates of reaction were measured in the non-physiological direction. An assay system was therefore devised, which used partially purified L-threonine 3-dehydrogenase as a 2-amino-3-oxobutyrate-generating system, sufficient exogenous phosphotransacetylase to convert rapidly the acetyl-CoA formed into acetyl phosphate (overcoming the acylase reaction) and a colorimetric method to measure this product (see the Materials and Methods section). Aminoacetone, formed by the spontaneous decarboxylation of 2-amino-3-oxobutyrate, did not interfere with this assay. A third assay, with similar advantages, depended on the effect of CoA on the yield of aminoacetone from L-threonine (see the Materials and Methods section). The decrease in aminoacetone production was attributable to CoA-dependent ligase activity, and this was verified by the detection of [^{14}C]glycine on chromatographs of incubation mixtures containing CoA with L-[U-^{14}C]-threonine as substrate. CoA had no effect on NAD^+-dependent L-threonine 3-dehydrogenase activity measured spectrophotometrically (McGilvray & Morris, 1969). Both newly devised assay methods gave results in good agreement and the latter was used as a routine because of its relative simplicity.

Studies of 2-amino-3-oxobutyrate cleavage in extracts of *Corynebacterium* sp. B6, by using the colorimetric assay for acetyl phosphate (see the Materials and Methods section), indicated an apparent K_m for CoA of 0.14 mM at pH 8. The addition of various metal ions, AMP, ADP or ATP, at 1 mM, had no effect on acetyl-CoA formation, i.e. the linkage between L-threonine 3-dehydrogenase and the ligase responsible for 2-amino-3-oxobutyrate cleavage.

(The activities of the ligase in extracts are shown in Table 4).

L-Serine hydroxymethyltransferase. This enzyme was assayed in the direction of serine formation, by following the glycine-dependent disappearance of formaldehyde (Scrimgeour & Huennekens, 1962). The reaction was also dependent on tetrahydrofolic acid and was enhanced by pyridoxal phosphate. A broad pH optimum, between pH 7 and 8 in phosphate buffer, was found for the enzyme in extracts of all the bacteria. Significant activity was found in all extracts examined (see Table 4.)

'Glycine-cleavage system' (glycine synthase, EC 2.1.2.10). Two assays were used for the enzyme cleaving glycine to yield CO_2 plus 5,10-methylene-tetrahydrofolic acid. The latter product is the normal source of the C_1 unit combining with glycine in the L-serine hydroxymethyltransferase reaction.

A spectrophotometric assay of 5,10-methylene-tetrahydrofolic acid formation (McGilvray & Morris, 1969), depending on endogenous $NADP^+$-dependent 5,10-methylenetetrahydrofolic acid dehydrogenase activity in extracts, showed that in dialysed extracts of *Corynebacterium* sp. B6, maximum activity depended on the presence of extract (0), tetrahydrofolic acid (0), glycine (15), $NADP^+$ (17), NAD^+ (57), pyridoxal phosphate (67) and Benzyl Viologen (87). The numbers in parentheses show the percentage of activity remaining when each component was omitted from otherwise complete reaction mixtures (see the Materials and Methods section). This assay indicated rates of only about 10 nmol of 5,10-methylenetetrahydrofolic acid formed/min per mg of protein in crude extracts, probably owing to its rapid removal by reaction with glycine to yield L-serine.

A radioactive assay of glycine cleavage, measuring the formation of $^{14}CO_2$ (McGilvray & Morris, 1969), gave activities of about 50 nmol of glycine cleaved/min per mg of protein in extracts of *Corynebacterium* sp. B6. The overall reaction was dependent for full activity on the presence of $NADP^+$ (23), tetrahydrofolic acid (33), Benzyl Viologen (48), NAD^+ (55) and

Table 3. *Properties of* L-*serine dehydratases of bacteria grown on* L-*threonine medium*

Bacteria were grown, and extracts prepared, as described in the Materials and Methods section. Enzyme activity was assayed by the method given except that pH, substrate concentration etc. were varied as appropriate.

Bacterium	pH optimum	$V_{max.}$ for L-serine (nmol of product formed/min per mg of protein)	$[S]_{0.5}$ for serine (mM)	Hill coefficient (*h*)	Activity with 10mM-Mg^{2+} (% of control lacking Mg^{2+})	Activity with 10mM-Ca^{2+} (% of control lacking Ca^{2+})
Achromobacter sp. F14	7.5–8.5	1280	12.5	2.6	290	17
Alcaligenes sp. N2	7.0–9.0	259	41.0	1.0, 3.2	413	180
Bacillus sp. N5	8.0–8.5	436	22.5	2.6	147	34
Brevibacterium sp. P2	7.5–8.6	215	16.0	2.2	250	47
Brevibacterium sp. B10	7.5–9.0	1032	13.3	1.9	150	4
Corynebacterium sp. B6	7.5–8.5	528	19.6	2.2	188	64
Flavobacterium sp. P4	8.5–9.5	370	33.0	2.7	218	130
Pseudomonas sp. Y1	8.0–9.0	1186	26.3	1.9	195	72

pyridoxal phosphate (58). Again, the numbers in parentheses indicate the percentage of activity remaining when the component was omitted. Activity was undetectable when boiled extract was used. Chromatographic procedures demonstrated that reaction mixtures contained [^{14}C]serine as well as unused [^{14}C]glycine, confirming that the 'glycine-cleavage system' acted in concert with L-serine hydroxymethyltransferase. The radioactive assay of activities in extracts for all the bacteria showed broad pH optima in the range pH 6.5–8.0. Enzyme activity in extracts varied markedly according to growth conditions (see Table 4).

L-Serine dehydratase. Extracts of all the threonine-grown bacteria had high activity. The pH optima were in the range pH 7.0–9.0 depending on the source (see Table 3). Pyridoxal phosphate (0.1mM) did not stimulate activity in extracts, and of a number of cations tested, only Mg^{2+} stimulated activity to a marked degree, up to 4-fold. The addition of Ca^{2+} caused inhibition in most but not all cases (Table 3). The relation between L-serine concentration and reaction velocity was represented by a markedly sigmoid Michaelis–Menten plot in all cases. Similar results were obtained with the enzyme purified from *Corynebacterium* sp. B6. Values of $[S]_{0.5}$ between 13 and 41mM were found, and Hill coefficients (*h*) of 1.85–2.70 calculated, for crude extracts. Values of 20.0mM and only 1.4 respectively were found for the purified enzyme.

Although relatively low L-threonine dehydratase activity was detectable in most bacterial extracts, the findings that L-serine dehydratase was not inhibited by L-isoleucine (1mM) and that the ratio of activities towards L-serine and L-threonine varied markedly according to growth conditions (Table 4) showed that the two activities were due to different enzymes. Only very low D-serine dehydratase activities, less than 6nmol of pyruvate formed/min per mg of protein, were detectable in extracts.

Of a variety of possible effectors tested, ADP stimulated L-serine dehydratase activity in extracts of *Flavobacterium* sp. P4, to a variable extent, the Michaelis–Menten plot remaining sigmoid. The effect was not observed with the enzyme from other bacteria, and AMP, ATP, phosphoenolpyruvate and acetyl-CoA, each tested at 1 and 5mM, had no effect. The optimum pH for activity in extracts of *Flavobacterium* sp. P4 was higher than for other isolates.

Additional enzymes of glycine catabolism. No significant *erythro*-β-hydroxyaspartate dehydratase or glyoxylate carbo-ligase activities, characteristic of the β-hydroxyaspartate and glycerate pathways of glycine metabolism (Kornberg & Morris, 1965; Kornberg & Gotto, 1961), were detected in extracts assayed under a wide variety of conditions. Similarly, no glycine–pyruvate aminotransferase activity, indicating metabolism via glyoxylate, could be found.

Aminoacetone as a catabolite of L-*threonine*

The elucidation of catabolic pathway for aminoacetone, involving L-1-aminopropan-2-ol and propionate as intermediates (Faulkner & Turner, 1974), led to a search for this pathway for L-threonine metabolism via 2-amino-3-oxobutyrate. In addition to the results of growth and manometric experiments (Table 1), assays for the key enzyme 1-aminopropan-2-ol *O*-phosphate phospholyase showed that the enzyme was absent from extracts of threonine-grown bacteria.

Effect of growth conditions on metabolic activity

In addition to the effect of growth conditions on oxidative ability (Table 1), the activities of enzymes

Table 4. *Enzyme activities in extracts of bacteria grown on different media*

The bacteria were grown on the media shown, cell-free extracts were prepared and enzyme activities measured as described in the Materials and Methods section. L-Threonine 3-dehydrogenase activity was assayed at pH 9.5 with L-threonine at 200mM and NAD^+ at 10mM. L-Serine dehydratase activity was measured at pH 8 with L-serine at 300mM.

		Enzyme activities (nmol/min per mg of protein) in crude extracts					
Bacterium	Growth substrates	L-Threonine 3-dehydrogenase	2-Amino-3-oxobutyrate–CoA ligase	L-Threonine dehydratase	'Glycine-cleavage system'	L-Serine hydroxymethyl-transferase	L-Serine dehydratase
Achromobacter sp. F14	L-Threonine	590	212	20	21	31	308
	Glycine	21	2	19	14	47	163
	Succinate	39	3	27	0	38	25
Alcaligenes sp. N2	L-Threonine	725	279	0	13	28	219
	Glycine	35	17	9	8	72	216
	Succinate	52	19	6	2	38	48
Bacillus sp. N5	L-Threonine	472	179	7	17	39	386
	Glycine	23	12	10	22	122	402
	Succinate	38	17	14	0	38	29
Brevibacterium sp. P2	L-Threonine	870	65	15	38	26	190
	Glycine	16	1	9	80	32	230
	Succinate	24	2	14	5	24	18
Brevibacterium sp. B10	L-Threonine	968	242	5	27	57	418
	Glycine	28	9	11	41	48	475
	Succinate	40	14	15	5	39	300
Corynebacterium sp. B6	L-Threonine	1040	364	11	56	77	315
	Glycine	42	14	17	29	93	226
	Succinate	75	25	22	1	67	240
Flavobacterium sp. P4	L-Threonine	815	250	4	12	17	270
	Glycine	14	4	9	6	64	243
	Succinate	32	7	15	0	35	0
Pseudomonas sp. Y1	L-Threonine	865	308	5	15	65	1090
	Glycine	58	17	15	8	74	692
	Succinate	158	32	11	1	82	259

in extracts of bacteria grown on three different media were studied. Assays were carried out under the conditions found to be appropriate from detailed studies of their properties. The results are shown in Table 4. Of the enzymes capable of acting on L-threonine, only the dehydrogenase had its formation increased dramatically by growth on the amino acid. This was so in all cases. The synthesis of 2-amino-3-oxobutyrate–CoA ligase was similarly induced by growth of the bacteria on threonine but not glycine or succinate media. Whereas L-serine hydroxymethyltransferase was found in extracts after growth on succinate, as well as on threonine or glycine media, L-serine dehydratase was found at high activity only after growth on threonine or glycine in all but two cases (isolate B6 and B10), where enzyme synthesis appeared to be constitutive. The 'glycine-cleavage system' was found only after growth on threonine or glycine, although activities were rather low in many cases, possibly owing to enzyme lability or underestimation by the assay procedure.

Discussion

The results of growth, manometric and enzymic experiments were consistent with the catabolism of L-threonine by cleavage to acetate plus glycine, the glycine being further metabolized via L-serine. Similar results were obtained with representatives of the genera *Achromobacter*, *Alcaligenes*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Flavobacterium* and *Pseudomonas*. The same route has been shown to operate in a species of *Arthrobacter* (McGilvray & Morris, 1969) and in a species of the fungus *Fusarium* (Willetts, 1972*a*). It appears from these detailed studies alone that the use of the catabolic route is widespread in the microbial kingdom.

The newly studied bacterial L-threonine 3-dehydrogenases were synthesized during growth on threonine and were highly active. The induced formation of a potent NAD^+-linked threonine dehydrogenase was first reported by McGilvray & Morris (1969), who found activity of 153 nmol/min per mg of protein in crude extracts of an *Arthrobacter* species and purified the enzyme about 40-fold (McGilvray & Morris, 1971). The activity of the inducibly synthesized L-threonine 3-dehydrogenase of *Pseudomonas oxalaticus* was found to be 720 nmol/min per mg of protein in extracts (Blackmore & Turner, 1971), but the best known source of the enzyme is now *Corynebacterium* sp. B6 (Table 2). This enzyme was purified about 17-fold to a specific activity of over 30 μmol/min per mg of protein (Fig. 1). The inducibly formed biodegradative enzymes described here differ in a number of respects from those investigated earlier, and from L-threonine 3-dehydrogenases formed constitutively by *Rhodopseudomonas spheroides* (Neuberger & Tait, 1960), *Staphylococcus aureus* (Green, 1964), *Escherichia coli* (Turner, 1967) and *Bacillus subtilis* (Willetts & Turner, 1970). Perhaps the most interesting difference is their response to increasing concentrations of substrate. Sigmoid kinetics, consistent with homotropic co-operative effects, have not been reported for the enzyme previously and were confirmed for the enzyme purified from *Corynebacterium* sp. B6. Similar kinetic behaviour of the enzyme *in vivo* may ensure that sufficient L-threonine is available for biosynthetic purposes at low concentrations, and that degradation does not occur at high rates until high intracellular concentrations are reached. Similar reasoning can be applied to the behaviour of the biodegradative L-serine dehydratases found.

The synthesis of 2-amino-3-oxobutyrate–CoA ligase, assayed by newly devised procedures, was induced by growth on threonine but not on glycine medium, confirming the enzyme's role in the CoA-dependent cleavage of the 3-oxo acid (McGilvray & Morris, 1969) rather than as an 'aminoacetone synthetase' (Elliott, 1958). An improved assay method, measuring rates of reaction in the physiological direction, enabled the K_m for CoA (0.14 mM) to be determined for the first time by using a high-activity preparation (350 nmol/min per mg of protein) from *Corynebacterium* sp. B6.

The finding that CoA markedly decreased the yield of aminoacetone from L-threonine in extracts indicated the efficient linkage of the dehydrogenase and ligase enzymes. The linkage was confirmed by experiments using L-[^{14}C]threonine in incubation mixtures.

The further metabolism of glycine was shown to operate by the L-serine pathway in all the bacteria studied. There was no evidence for metabolism by the glycerate pathway occurring in *Ps. oxalaticus* (Blackmore & Turner, 1971) and two species of *Penicillium* (Willetts, 1972*b*). Both L-serine hydroxymethyltransferase and the 'glycine-cleavage system' were found. The synthesis of the 'glycine-cleavage system', needed for 5,10-methylenetetrahydrofolic acid formation, was clearly induced by growth on threonine or glycine media. Although the average activity of 25 nmol/min per mg of protein in bacterial extracts was relatively low, it was significantly higher than the value of 0.5 nmol/min per mg of protein calculated to be the enzyme activity in extracts of the *Arthrobacter* species studied by McGilvray & Morris (1969). The inducibly formed L-serine dehydratases, shown to differ from the biosynthetic L-threonine dehydratases also present in extracts, exhibited kinetic behaviour consistent with a biodegradative role.

No evidence for the operation of an aminoacetone pathway of L-threonine catabolism was obtained. The inability of any of the bacteria to grow on aminoacetone, 1-aminopropan-2-ol or propionate, their

inability to oxidize these compounds after growth on threonine medium and the absence of the key phospholyase enzyme showed that aminoacetone is not an intermediate of L-threonine metabolism in the isolates studied. The same conclusion has been drawn from a study of isolates which are capable of growth on L-threonine, 1-aminopropan-2-ol and propionate media (Bell & Turner, 1976). It seems unlikely that any commonly occurring bacterium degrades L-threonine by a route involving aminoacetone as an obligatory intermediate.

This work was carried out with the aid of a grant from the Medical Research Council. We thank Miss E. Duggan for expert technical assistance. The interest and support of Professor T. W. Goodwin, F.R.S., is gratefully acknowledged.

References

Bell, S. C. & Turner, J. M. (1973) *Biochem. Soc. Trans.* **1**, 678–681
Bell, S. C. & Turner, J. M. (1976) *Biochem. Soc. Trans.* **4**, 497–500
Blackmore, M. A. B. & Turner, J. M. (1971) *J. Gen. Microbiol.* **67**, 243–246
Datta, P. (1966) *J. Biol. Chem.* **241**, 5836–5844
Elliott, W. H. (1958) *Biochim. Biophys. Acta* **29**, 446–447
Faulkner, A. & Turner, J. M. (1974) *Biochem. J.* **138**, 263–276
Green, M. L. (1964) *Biochem. J.* **92**, 550–555
Green, M. L. & Elliott, W. H. (1964) *Biochem. J.* **92**, 537–549
Higgins, I. J., Pickard, M. A. & Turner, J. M. (1968) *J. Gen. Microbiol.* **54**, 105–114
Hill, A. V. (1913) *Biochem. J.* **7**, 471–480
Jones, A. & Turner, J. M. (1973) *Biochem. J.* **134**, 167–182
Kornberg, H. L. & Gotto, A. M. (1961) *Biochem. J.* **78**, 69–82
Kornberg, H. L. & Morris, J. G. (1965) *Biochem. J.* **95**, 577–586
Koshland, D. E., Nemethy, G. & Filmer, D. (1966) *Biochemistry* **5**, 365–392
Lessie, T. G. & Whiteley, H. R. (1969) *J. Bacteriol.* **100**, 878–889
Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–663
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
McGilvray, D. & Morris, J. G. (1969) *Biochem. J.* **112**, 657–671
McGilvray, D. & Morris, J. G. (1971) *Methods Enzymol.* **17B**, 580–584
Morris, J. G. (1969) *Biochem. J.* **115**, 603–605
Nash, T. (1953) *Biochem. J.* **55**, 416–421
Neuberger, A. & Tait, G. H. (1960) *Biochim. Biophys. Acta* **41**, 164–165
Paz, M. A., Blumenfeld, O. O., Rojkind, M., Henson, E., Furfine, C. & Gallop, P. M. (1965) *Arch. Biochem. Biophys.* **109**, 548–559
Pickard, M. A., Higgins, I. J. & Turner, J. M. (1968) *J. Gen. Microbiol.* **54**, 115–126
Rowsell, E. V., Snell, K., Carnie, J. A. & Al-Tai, A. H. (1969) *Biochem. J.* **115**, 1071–1073
Scrimgeour, K. G. & Huennekens, F. M. (1962) *Methods Enzymol.* **5**, 838–843
Turner, J. M. (1966) *Biochem. J.* **99**, 427–433
Turner, J. M. (1967) *Biochem. J.* **104**, 112–121
Umbarger, H. E. & Brown, B. (1957) *J. Bacteriol.* **73**, 105–114
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1964) *Manometric Techniques*, 4th edn., pp. 1–17, Burgess Publishing Co., Minneapolis
Willetts, A. J. (1972*a*) *Antonie van Leeuwenhoek* **38**, 591–603
Willetts, A. J. (1972*b*) *J. Gen. Microbiol.* **73**, 71–83
Willetts, A. J. & Turner, J. M. (1970) *Biochem. J.* **117**, 27P–28P