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Bacterial Catabolism of Threonine

THREONINE DEGRADATION INITIATED BY L-THREONINE HYDRO-LYASE (DEAMINATING) IN A SPECIES OF *CORYNEBACTERIUM*

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1. Three bacterial isolates capable of growth on L-threonine medium only when supplemented with branched-chain amino acids, and possessing high L-threonine dehydratase activity, were examined to elucidate the catabolic route for the amino acid. 2. Growth, manometric, radiotracer and enzymic experiments indicated that L-threonine was catabolized by initial deamination to 2-oxobutyrate and thence to propionate. No evidence was obtained for the involvement of L-threonine 3-dehydrogenase or L-threonine aldolase in threonine catabolism. 3. L-Threonine dehydratase of *Corynebacterium* sp. F5 (N.C.I.B. 11102) was partially purified and its kinetic properties were examined. The enzyme exhibited a sigmoid kinetic response to substrate concentration. The concentration of substrate giving half the maximum velocity, [$S_{0.5}$], was 40mM and the Hill coefficient (*h*) was 2.0. L-Isoleucine inhibited enzyme activity markedly, causing 50% inhibition at 60μM, but did not affect the Hill constant. At the fixed L-threonine concentration of 10mM, the effect of L-valine was biphasic, progressive activation occurring at concentrations up to 2mM-L-valine, but was abolished by higher concentrations. Substrate-saturation plots for the L-valine-activated enzyme exhibited normal Michaelis–Menten kinetics with a Hill coefficient (*h*) of 1.0. The kinetic properties of the enzyme were thus similar to those of the 'biosynthetic' isoenzyme from *Rhodopseudomonas spheroides* rather than those of the enteric bacteria. 4. The synthesis of L-threonine dehydratase was constitutive and was not subject to multivalent repression by L-isoleucine or other branched-chain amino acids either singly or in combination. 5. The catabolism of L-threonine, apparently initiated by a 'biosynthetic' L-threonine dehydratase in the isolates studied, depended on the concomitant catabolism of branched-chain amino acids. The biochemical basis of this dependence appeared to lie in the further catabolism of 2-oxobutyrate by enzymes which required branched-chain 2-oxo acids for their induction.

A number of alternative routes of L-threonine catabolism have been demonstrated in bacteria. Only more recently have the biochemical and phylogenetic significance of these routes been examined (Bell *et al.*, 1972; Bell & Turner, 1976*a*).

In most bacteria capable of growth on L-threonine, the amino acid is metabolized by the initial action of L-threonine 3-dehydrogenase (EC 1.1.1.103). The product of this oxidation, 2-amino-3-oxobutyrate, is cleaved to acetyl-CoA and glycine by the action of a CoA-ligase (Bell & Turner, 1976*a*; McGilvray & Morris, 1969). L-Threonine aldolase (EC 2.1.2.1)-initiated dissimilation has been demonstrated in a limited number of bacteria (Morris, 1969; Willetts & Turner, 1971; Kumagai *et al.*, 1972).

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Studies of L-threonine catabolism initiated by 'catabolic' L-threonine dehydratase (L-threonine hydro-lyase, EC 4.2.1.16) have concentrated on two bacteria grown anaerobically on media containing high concentrations of the amino acid (Umbarger & Brown, 1957; Tokushige & Hayaishi, 1972).

A survey of isolates capable of growth on L-threonine (Bell *et al.*, 1972) revealed a group of three organisms (*Corynebacterium* sp. F5, *Brevibacterium* spp. F9 and G1) which possessed substantial L-threonine dehydratase activity to the exclusion of any other threonine-catabolizing enzyme. The properties of the dehydratase indicated that it was, in common with that of other isolates, a 'biosynthetic' isoenzyme. The route of L-threonine catabolism in these bacteria and the metabolic role of the 'biosynthetic' L-threonine dehydratases have now been examined. Evidence for metabolism via 2-oxobutyrate and propionate

and its relationship with branched-chain amino acid catabolism is presented.

Materials and Methods

Micro-organisms and media

The bacteria used in this study were isolated from soil by virtue of their ability to grow on L-threonine as the major C and N source. The bacteria were identified and given provisional genus names together with a strain designation (S. C. Bell, T. R. G. Gray & J. M. Turner, unpublished work) *Corynebacterium* sp. F5 has now been lodged with. the National Collection of Industrial Bacteria and given the accession number N.C.I.B. 11102.

Bacteria were maintained on nutrient broth and cultivated on semisynthetic media as described for similar soil isolates (Bell & Turner, 1976*a*). Threonine medium contained 2g of L-threonine/litre plus mineral salts supplemented with 0.05% yeast extract or 2mM-L-valine.

Measurement of O_2 uptake by washed bacterial suspensions

O_2 uptake was measured manometrically by using conventional equipment and procedures (Umbreit *et al.*, 1964). Incubation mixtures contained 150μmol of potassium phosphate buffer, pH7, 20μmol of substrate and approx. 1mg dry wt. of cells in a total volume of 2.8ml. Manometer readings were taken at 10min intervals over a period of not less than 90min.

2-Oxo acid utilization by washed bacterial suspensions

Incubation mixtures contained 1mmol of potassium phosphate buffer, pH7.0, 10μmol of 3-methyl-2-oxobutyrate or 50μmol of 2-oxobutyrate and approx. 25mg dry wt. of cells in a total volume of 10ml. Incubations were started by the addition of bacterial suspensions and carried out at 30°C with shaking. At timed invervals 0.2ml samples were removed, centrifuged (2000*g* for 20min) and the supernatant was assayed for 2-oxo acid. Appropriate controls were included.

Enzyme extracts and enzyme assays

Suspensions of bacteria were disrupted ultrasonically, cell debris was removed by centrifuging and the protein content of extracts measured as previously described (Bell & Turner, 1976*a*). Enzyme assays were done at 37°C unless otherwise stated.

2-Oxo acid dehydrogenase. Activity was assayed colorimetrically by the procedure of Hayakawa *et al.* (1966). Reaction mixtures contained 150μmol of potassium phosphate buffer, pH7, 25μmol of potassium ferricyanide, 0.2μmol of thiamin pyrophosphate, 15μmol of 2-oxo acid and enzyme preparation in a total volume of 1.5ml. The ferrocyanide produced by the oxidative decarboxylation of the 2-oxo acid was determined by the method of Reed & Williams (1966).

L-Threonine aldolase. Activity was assayed by measuring acetaldehyde production enzymically by using commercial alcohol dehydrogenase (C. F. Boehringer und Soehne, Mannheim, Germany). The linked oxidation of NADH was followed spectrophotometrically at 340nm (Morris, 1969).

L-Threonine 3-dehydrogenase. Activity was looked for by the colorimetric and spectrophotometric assays previously described (Bell & Turner, 1976*a*).

Partial purification of L-threonine dehydratase

Crude extracts of *Corynebacterium* sp. F5 were prepared in 0.1M-potassium phosphate buffer, pH7. Approx. 4ml of extract, containing 44mg of protein, was added to a column of Whatman DE 52 DEAE-cellulose (25cm×1.5cm diam.) equilibrated with 0.1M-potassium phosphate buffer, pH7, containing 50μM-pyridoxal phosphate and 0.1M-KCl. Protein was eluted with a linear gradient of 0.1–0.6M-KCl in the same buffer/cofactor mixture, over a total volume of 400ml. Fractions of eluate (approx. 5ml) were collected and their L-threonine dehydratase activities determined by the colorimetric assay (see above).

Incorporation of radioactivity from L-[^{14}C]threonine

The procedures used to study the incorporation of ^{14}C from L-[U-^{14}C]threonine into constituents of the 'ethanol-soluble' fraction of bacteria were similar to those described for the metabolism of acetate by Kornberg (1958). Radioactive metabolites of L-[U-^{14}C]threonine, produced after incubation with cell-free extracts, were also obtained by extraction procedures. The extracts were chromatographed by t.l.c. on cellulose in two dimensions. The solvent systems were pyridine/water (4:1, v/v) and ethanol/water/aq. NH_3 (sp.gr. 0.880) (180:10:1, by vol.). Chromatograms were left in contact with X-ray film for radioautographic detection of labelled compounds.

The incorporation of labelled L-threonine into cells during growth (1μCi; 0.4μmol/100ml of medium) was examined by the following procedure. At timed invervals 2ml samples were removed aseptically from a 100ml culture and the cell density measured turbidimetrically as A_{540}^{1cm}. Bacteria were removed from 1ml portions of culture by filtering through Millipore filters (HAWP, 0.45μm pore size), and subsequently washed with 1ml of 0.1M-potassium phosphate buffer, pH7.0. Filters were then attached to planchets, dried and radioactivity was measured by end-window counting. Portions of the filtered medium were assayed for radioactivity by liquid-scintillation counting (Hall & Cocking, 1965).

Chemical methods

Characterization of 2,4-dinitrophenylhydrazones. Hydrazone derivatives of both authentic and

biologically formed aldehydes and oxo acids were examined by t.l.c. on cellulose with propan-2-ol/water/aq. NH_3 (sp.gr. 0.880) (20:2:1, by vol.) as the solvent system.

2-Oxo acid measurement. The 2-oxo acids were determined as their 2,4-dinitrophenylhydrazones by the colorimetric method of Ning & Gest (1966).

Aminoacetone measurement. The colorimetric assay procedure described by Higgins *et al.* (1968) was used.

Chemicals

L-[U-^{14}C]Threonine was from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were prepared or obtained from commercial sources as described previously (Bell & Turner, 1976*a*).

Results

Preliminary observations

Brevibacterium spp. F9 and G1 and *Corynebacterium* sp. F5 exhibited similar nutritional abilities. All three bacteria grew well aerobically at 30°C on the basal medium containing L-threonine as the major C and N source supplemented with yeast extract. Acetate and glycine could also be utilized as growth substrates, growth on acetate commencing after a 24h lag. Growth on glycine+acetate as the major C sources appeared to be diauxic. DL-1-Aminopropan-2-ol and propionate each supported good growth of all bacteria, a characteristic unusual for bacteria capable of growth on L-threonine (Bell & Turner, 1976*b*). It was notable that yeast extract was not required for growth on these compounds.

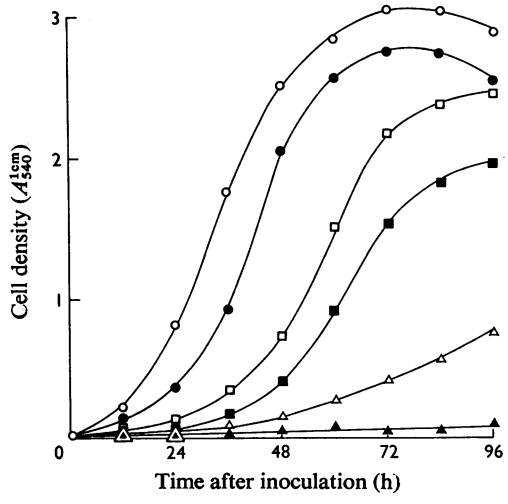


Fig. 1. *Effect of* L-*valine concentration of the growth of Corynebacterium sp. F5 on* L-*threonine medium*
The bacterium was grown on L-threonine (17mM) +salts medium as described in the Materials and Methods section, supplemented with L-valine: 0mM (▲); 0.1mM (△); 0.5mM (■); 1mM (□); 3mM (●); 5mM (○). Samples were removed aseptically at timed intervals and growth was measured spectrophotometrically.

As found during a survey of isolates grown on L-threonine (S. C. Bell, T. R. G. Gray & J. M. Turner, unpublished work), extracts of *Brevibacterium* spp. F9 and G1 and *Corynebacterium* sp. F5 exhibited substantial L-threonine dehydratase activity, i.e. 85, 202 and 154nmol of 2-oxobutyrate formed/min per mg of protein respectively.

The bacteria appeared to be biochemically similar and detailed studies were pursued on *Corynebacterium* sp. F5 except where stated.

Factors required for growth on threonine medium

As yeast extract was required for growth of *Corynebacterium* sp. F5 on L-threonine, but not on succinate or propionate, as C sources, the requirement was not auxotrophic. Supplements of vitamin and amino acid mixtures were tested to determine their ability to promote growth on L-threonine. Only a mixture of the branched-chain acids L-valine, L-isoleucine and L-leucine (each at 2mM) promoted rapid and good growth on L-threonine. Individually, L-valine was the most efficient growth promoter, markedly decreasing the lag phase before rapid growth (Fig. 1). Additional studies showed that the amino acid did not affect the final growth yield when used at concentrations below 1mM. L-Leucine also promoted growth on L-threonine, but was less effective than L-valine; growth with even 5mM-L-isoleucine was slow. The bacterium grew well on each of the branched-chain amino acids as sole sources of C and N.

A similar requirement for branched-chain amino acids during growth on L-threonine was found with isolates F9 and G1.

Manometric experiments

Washed suspensions of *Corynebacterium* sp. F5 readily oxidized acetate, glycine, L-serine and pyruvate, although this ability appeared to be constitutive (Table 1). Aminoacetone and DL-1-aminopropan-2-ol, proposed as products of L-threonine catabolism in a strain of *Bacillus subtilis* (Willetts & Turner, 1970), and also glyoxylate, were oxidized only slowly. 2-Oxobutyrate and propionate were oxidized at high rates only after growth on L-threonine+L-valine medium. The pattern of substrate oxidation was consistent with L-threonine catabolism initiated by deamination to 2-oxobutyrate.

Enzymes acting on threonine in extracts

Extracts of *Corynebacterium* sp. F5 grown on L-threonine+L-valine were examined for enzymes

Table 1. *Oxidation of possible* L-*threonine metabolites by washed suspensions of Corynebacterium sp. F5*

Corynebacterium sp. F5 was grown on the compounds shown as sole sources of C and N. O_2 uptake by washed suspensions was measured manometrically (see the Materials and Methods section). Rates of O_2 uptake were corrected for endogenous rates.

Substrate oxidized	O_2 absorbed (μmol/h per mg dry wt. of bacteria grown on medium indicated) L-Threonine (17mM) +L-valine (2mM)	Succinate+ $(NH_4)_2SO_4$
L-Threonine	4.91	0.76
2-Oxobutyrate	4.23	0.57
Propionate	3.93	0.95
Pyruvate	2.75	3.05
Acetate+glycine (each 7mM)	2.72	2.95
Acetate	2.60	2.82
L-Serine	1.95	2.10
Glycine	0.95	0.83
Glyoxylate	0.51	0.52
DL-1-Aminopropan-2-ol	0.42	0.47
Aminoacetone	0.15	0.19

known to degrade L-threonine. No L-threonine 3-dehydrogenase activity could be detected when sought under a variety of conditions, e.g. pH, substrate concentration and in the presence of thiols, chelating agents and possible regulatory effectors. Low constitutively formed L-threonine aldolase activity was found. Under optimum conditions, i.e. pH8.6 in 0.25M-Tris/HCl buffer, with L-threonine at 0.2M (K_m 22mM), activities of only 3–6nmol of acetaldehyde formed/min per mg of protein were found.

L-Threonine dehydratase. The high dehydratase activity present in crude extracts of *Corynebacterium* sp. F5 was examined to determine whether it was due to the 'catabolic' or 'biosynthetic' form of the enzyme or perhaps both. The optimum pH for activity was pH8–9 in 0.125M-Tris/HCl and -diethanolamine/HCl buffers. At corresponding pH values in 0.125M-potassium phosphate buffers activities were 50–60% greater. Plots of reaction velocity versus substrate concentration exhibited normal Michaelis–Menten kinetics, with an apparent K_m of 16mM at pH8.5. L-Valine exerted no dramatic effect, but lowered the K_m for L-threonine to approx. 13mM and the $V_{max.}$ from 170 to 140nmol of 2-oxobutyrate formed/min per mg of protein. L-Isoleucine tested at 1mM was markedly inhibitory, causing 76, 90 and 100% inhibition at L-threonine concentrations of 100, 50 and 10mM respectively. Adenine nucleotides had no apparent effect on activity at saturating and non-saturating substrate concentrations.

The properties of the L-threonine dehydratase found in crude extracts suggested that it was the 'biosynthetic' type physiologically concerned with the biosynthesis of L-isoleucine (Umbarger & Brown, 1957; Umbarger, 1973). Other evidence suggested that this enzyme, or perhaps an undetected isoenzyme form, was playing a catabolic role during the growth of *Corynebacterium* sp. F5 on L-threonine.

Properties of partially purified L-*threonine dehydratase.* Fractionation of crude extracts by ion-exchange chromatography on DEAE-cellulose revealed a single peak of L-threonine dehydratase which was eluted at 0.15M-Cl^-. No activity was found in other fractions even in the presence of AMP or ADP at 1mM. Between 4- and 5-fold purification was achieved, with approx. 45% recovery, yielding preparations of specific activity 0.35–0.65μmol of 2-oxobutyrate formed/min per mg of protein.

The enzyme was specific for L-threonine (100%), only low activities being found with L-serine (6%), L-allothreonine (5%), DL-allothreonine (3%) and D-threonine (0%). The effects of branched-chain amino acids were tested at 1mM with 10mM-L-threonine. L-Valine activated 3.1-fold, whereas L-isoleucine inhibited activity completely. L-Leucine, D-valine, D-isoleucine and adenine nucleotides had no effect.

More detailed kinetic studies showed that in the

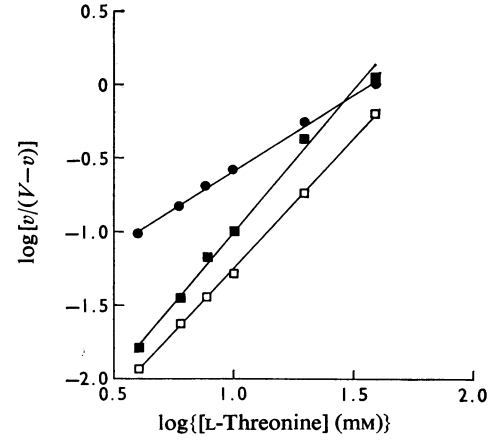


Fig. 2. *Effect of substrate concentration on the activity of* L-*threonine dehydratase in presence and absence of* L-*isoleucine and* L-*valine*

Partially purified L-threonine dehydratase was assayed at pH8.0 as outlined in the Materials and Methods section, except that the L-threonine concentration was varied from 4 to 40mM. Activity was assayed in the absence (■) and presence of 2mM-L-valine (●) or 50μM-L-isoleucine (□). The results are presented as a Hill (1913) plot. *v*, Velocity; *V*, maximum velocity (nmol of product/min per mg of protein).

absence of effectors the plot of reaction velocity versus L-threonine concentration exhibited a sigmoid response. This was accentuated by L-isoleucine even at 60 μM, but was converted into a 'normal' hyperbolic curve by 2mM-L-valine. L-Valine almost completely abolished the inhibitory effect of L-isoleucine at these concentrations (Fig. 2). Double-reciprocal plots (Lineweaver & Burk, 1934) indicated a K_m of 40mM for L-threonine in the presence of L-valine. The $V_{max.}$ value appeared to be the same in the presence or absence of effectors. Results plotted according to the Hill equation (Hill, 1913) had coefficients (h) of 1.0, 1.8 and 2.0 in the presence of 2mM-L-valine, the absence of effectors and the presence of 60 μM-L-isoleucine respectively. Whereas increasing concentrations of L-isoleucine progressively inhibited dehydratase activity at fixed substrate concentrations, the effect of L-valine was biphasic. Activity was stimulated by L-valine up to approx. 2mM, but higher concentrations activated progressively less. At 40mM-L-threonine, activation by L-valine was less marked than at 10mM-L-threonine (Fig. 3). Activation by L-valine was significantly dependent on pH, increasing from pH8.5 to 7.0. Inhibition by L-isoleucine was relatively constant within this pH range.

Partially purified L-threonine dehydratase was highly susceptible to inactivation by 1M-urea. Whereas L-isoleucine was unable to protect the enzyme against inactivation, low concentrations of L-valine (2-10mM) had a marked effect. Moreover, concentrations of L-valine higher than 2mM did not activate less in the presence of urea (Fig. 4).

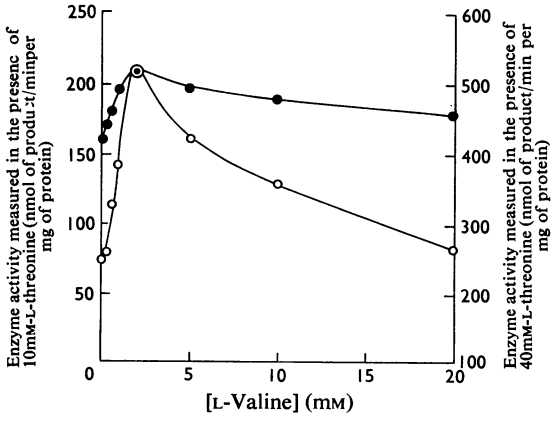


Fig. 3. *Effect of* L-*valine on* L-*threonine dehydratase activity*
Partially purified enzyme was used and assayed as described for Fig. 2 except that L-threonine was used at 10mM (○) or 40mM (●).

Effect of growth conditions on L-*threonine dehydratase formation.* High L-threonine dehydratase activities were found in extracts of *Corynebacterium* sp. F5 grown on a variety of substrates (Table 2). It was notable that the addition of branched-chain amino acids, singly or in combination, did not repress enzyme synthesis during growth on L-threonine.

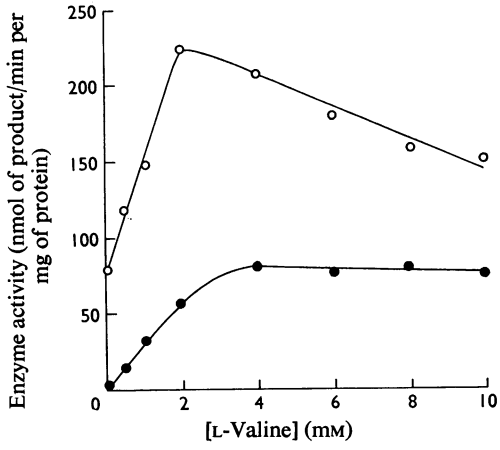


Fig. 4. *Effect of* L-*valine concentration on urea inactivation of* L-*threonine dehydratase*
Partially purified L-threonine dehydratase was assayed at pH8.0 as outlined in the Materials and Methods section except that 10mM-L-threonine was used. L-Valine was added at the appropriate concentration in the presence (●) and absence (○) of 1M-urea. Specific activity is expressed as nmol of 2-oxobutyrate formed/min per mg of protein.

Table 2. *Effect of growth substrate on* L-*threonine dehydratase activity of Corynebacterium sp F5*
The bacterium was grown on the compounds shown as sole C sources (4g/litre). $(NH_4)_2SO_4$ was added as a N source when necessary at 1g/litre. Branched-chain amino acids, when used as supplements, were present at 0.5g/litre. L-Threonine dehydratase was assayed in extracts as outlined in the Materials and Methods section.

Growth substrate	L-Threonine dehydratase activity (nmol of 2-oxobutyrate produced/min per mg of protein)
Succinate+NH_4^+	111
Propionate+NH_4^+	128
Nutrient broth	145
L-Valine	159
L-Leucine	157
L-Isoleucine	155
L-Valine (+L-leucine +L-isoleucine)	149
L-Threonine (+L-valine)	147
L-Threonine (+L-valine +L-leucine+L-isoleucine)	145

2-Oxobutyrate as a catabolite of threonine

No aminoacetone was detected in culture supernatants during growth of L-threonine+L-valine medium, but another compound which reacted with 2,4-dinitrophenylhydrazine was found. It was identified (see the Materials and Methods section) as the 2,4-dinitrophenylhydrazone of 2-oxobutyrate. Excretion of the oxo acid during growth of *Corynebacterium* sp. F5 was transient, its accumulation being greatest when growth was about one-quarter complete, and representing approx. 3% of the L-threonine originally present in the medium (see Fig. 5). A similar relationship between oxo acid excretion and growth was seen with isolates F9 and G1.

Washed suspensions of *Corynebacterium* sp. F5 grown on L-threonine+L-valine utilized 2-oxobutyrate (see the Materials and Methods section) more rapidly than did cells grown on L-valine and much more rapidly than did cells grown on succinate $+(NH_4)_2SO_4$ (Table 3). The addition of L-threonine inhibited oxo acid utilization in all cases, as also did L-valine, except with cells grown on succinate+ $(NH_4)_2SO_4$. These results suggested that 2-oxobutyrate was involved not only in the catabolism of L-threonine, but also the catabolism of L-valine in this bacterium.

Metabolism of [^{14}C]threonine by bacteria and bacterial extracts

Attempts were made to detect and identify L-threonine catabolites by adding 2μCi of L-[U-^{14}C]-threonine to 5ml of an aerated suspension of *Corynebacterium* sp. F5 grown on L-threonine+ L-valine, incubated at 30°C. Rapid sampling and chromatographic analysis (see the Materials and Methods section) revealed three metabolites after 2.5min incubation, the major one being 2-oxobutyrate. The remaining two compounds were not identified. No radioactive aminoacetone, 1-aminopropan-2-ol, glycine, glyoxylate, serine or pyruvate were detected in these experiments.

Radioactive L-threonine (2μCi; 75μmol) was also incubated with cell-free extracts (10mg of protein) of the bacterium, in a system including 150μmol of potassium phosphate buffer, pH7.5, in a total volume of 1.5ml. Samples were removed, deproteinized and analysed by t.l.c. (see the Materials and Methods section), at different time-intervals. Boiled-enzyme controls were found to contain radioactive L-threonine only. Labelled 2-oxobutyrate was identified as an early-formed product, its formation being inhibited by 90% by 10mM-isoleucine. A product tentatively identified as propionyl-CoA was found when the reaction mixtures were supplemented with 5mM-NAD^+, 1mM-CoA and 1mM-thyamin pyrophosphate, its formation also being inhibited by isoleucine.

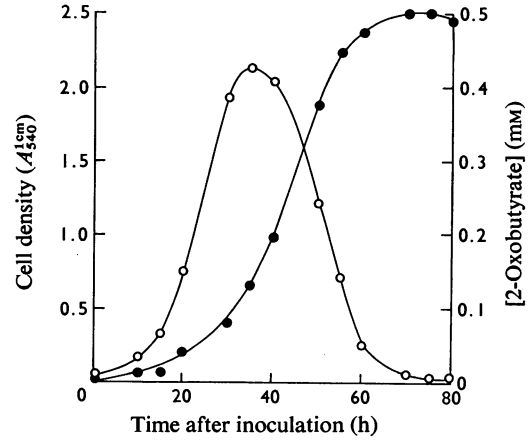


Fig. 5. *2-Oxobutyrate formation and re-utilization during the growth of Corynebacterium sp. F5 on L-threonine +L-valine*

The bacterium was grown on 17mM-L-threonine+ 2mM-L-valine+salts medium as described in the Materials and Methods section. At timed intervals 2ml samples were removed aseptically and the cell density (●) and 2-oxobutyrate concentration (○) measured.

Table 3. *Utilization of 2-oxo acids by washed suspensions of Corynebacterium sp. F5*

The bacterium was grown in media containing the compounds shown as sole sources of C and N. After harvesting and washing, suspensions were incubated with the 2-oxo acid, and the rate of its removal was followed as described in the Materials and Methods section. Rates of utilization are expressed as nmol of 2-oxo acid removed/h per mg dry wt. of cells.

Addition (10mM)	Oxo acid	Utilization of 2-oxo acid (nmol/h per mg dry wt. cells) after growth on the medium shown: Succinate+$(NH_4)_2SO_4$	L-Threonine+L-valine	L-Valine
None	2-Oxobutyrate (5mM)	30	230	113
+L-Threonine		5	127	15
+L-Valine		32	145	15
None	3-Methyl-2-oxobutyrate (1mM)	10	89	48

Possible links between L-*threonine catabolism and the requirement of branched-chain amino acids for growth*

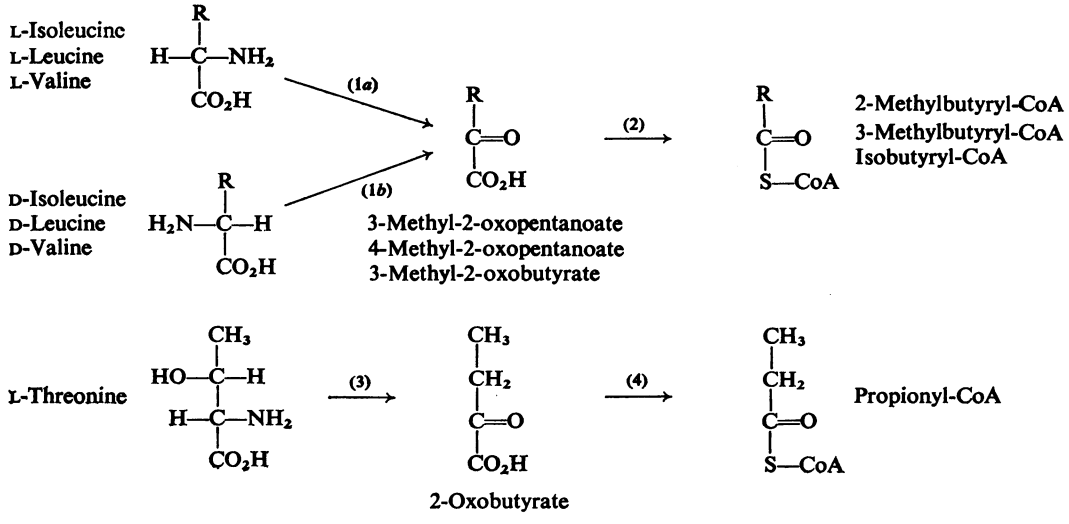
The results of growth, manometric, enzymic and radiotracer studies suggested that growth on L-threonine involved 2-oxobutyrate and 'propionate' as catabolites. Enzymic evidence suggested that a 'biosynthetic' L-threonine dehydratase was responsible for the initial catabolic step. Two possible explanations of the requirement for L-valine for growth were considered. Firstly, L-valine was needed to active the dehydratase or antagonize feedback inhibition of the enzyme by L-isoleucine *in vivo*. Secondly, branched-chain amino acids were necessary to induce enzymes needed for the metabolism of 2-oxobutyrate or some product during growth on L-threonine.

Growth studies. D-Valine was found to promote the growth of *Corynebacterium* sp. F5 on L-threonine, although the concentrations required were higher than those of L-valine. Good growth was promoted by 5mM-D-valine. L-Isoleucine did not inhibit growth on L-threonine+L-valine medium, and at concentrations above 5mM increased the growth yield. It therefore seemed unlikely that branched-chain amino acids promoted growth on L-threonine as a direct result of their modulation of L-threonine dehydratase activity. This was confirmed by the finding that although the bacterium could not grow on 2-oxobutyrate+$(NH_4)_2SO_4$ medium, the addition of branched-chain amino acids promoted rapid growth. As no supplement was needed for good growth on propionate+$(NH_4)_2SO_4$ medium, it seemed likely that the locus of action of L-valine was the metabolism of 2-oxobutyrate to propionate or its CoA-derivative.

L-*Valine effect and* [^{14}*C*]*threonine metabolism.* An inoculum of L-valine-grown bacteria gave rise to rapid growth on, and L-[U-^{14}C]threonine incorporation from, L-threonine+L-valine medium. Succinate +$(NH_4)_2SO_4$-grown cells grew only after a lag of 8–9h. Bacteria growing exponentially on L-valine incorporated radioactivity from [^{14}C]threonine immediately and 11.5 times as rapidly as similar cultures growing on succinate+$(NH_4)_2SO_4$. These results were consistent with the hypothesis that L-valine facilitated the metabolism of L-threonine or probably its catabolite 2-oxobutyrate. Together with other results they suggested that branched-chain amino acids, e.g. L-valine, promoted the formation of enzymes necessary for the complete metabolism of L-threonine.

Branched-chain amino acid catabolism

In bacteria a branched-chain 2-oxo acid dehydrogenase catalyses one of the reactions constituting a common pathway for the catabolism of L- (and D-) branched-chain amino acids (Massey *et al.*, 1974, 1976). The enzyme is broadly specific and in *Pseudomonas putida* the inducers of the dehydrogenase are the branched-chain oxo acids rather than the amino acids (Marshall & Sokatch, 1973). The possible relationship between L-threonine and branched-chain amino acid catabolism is indicated



Scheme 1. *Microbial catabolism of branched-chain amino acids and* L-*threonine via 2-oxo acids*
The enzymes are: (1*a*) branched-chain amino acid transaminase; (1*b*) D-amino acid dehydrogenase; (2) branched-chain 2-oxo acid dehydrogenase; (3) L-threonine dehydratase; (4) '2-oxobutyrate dehydrogenase'.

in Scheme 1. The effect of growth conditions on the metabolism of 3-methyl-2-oxobutyrate and 2-oxobutyrate (Table 3) indicated that the utilization of both of these compounds was induced by growth of *Corynebacterium* sp. F5 on L-valine or L-valine-supplemented media. The growth of the bacterium on L-threonine medium was promoted by the addition of 2mM-2-oxoisovalerate, although the lag before rapid growth was longer than when L-valine was added. The same growth yield was obtained in both cases.

2-Oxo acid dehydrogenase activity was detected in extracts of *Corynebacterium* sp. F5 by the method of Hayakawa *et al.* (1966) as outlined in the Materials and Methods section. The optimum pH for activity was pH7.5 and the reaction was completely dependent on the addition of thyamin pyrophosphate. The enzyme was found to be extremely labile, however. High blank values could be decreased by dialysis against 0.1M-potassium phosphate buffer, pH7.5, but this treatment resulted in the loss of considerable activity. Specific activities for 2-oxobutyrate, 3-methyl-2-oxobutyrate, 3-methyl-2-oxopentanoate and 4-methyl-2-oxopentanoate were 10.2, 9.8, 9.5 and 9.4nmol oxidized/min per mg of protein.

Discussion

The results of the experiments described here are consistent with the catabolism of L-threonine by a *Corynebacterium* sp. by initial deamination to 2-oxobutyrate, its oxidative decarboxylation to propionyl-CoA and thence common intermediary metabolites. It seems highly likely that threonine deamination is catalysed by a 'biosynthetic' dehydratase which normally supplies 2-oxobutyrate required for isoleucine biosynthesis. It also seems likely that 2-oxobutyrate is catabolized by a broadly specific branched-chain oxo acid dehydrogenase, the formation of which is induced by 2-oxo acids derived from valine, isoleucine or leucine, but not by 2-oxobutyrate itself. This provides an example of the way in which a catabolic pathway may evolve by using elements normally associated with other areas of metabolism.

The threonine dehydratase, found at high activity in cell-free extracts of the *Corynebacterium*, was of the 'biosynthetic' type on the criteria that its formation was constitutive, it was susceptible to inhibition by isoleucine, valine protected against denaturation, valine was also a positive effector and antagonist of isoleucine and neither AMP nor ADP acted as effectors (Umbarger, 1973). Atypically, formation of the enzyme was not subject to multivalent repression by isoleucine plus the other branched-chain amino acids (Freundlich *et al.*, 1962). This would be a prerequisite for the catabolism of threonine via 2-oxobutyrate by the bacterium. The properties of the dehydratase from the *Corynebacterium* were similar to those of the enzyme in *Rhodopseudomonas spheroides* (Datta, 1966; Barritt, 1971), a yeast (Katsunuma *et al.*, 1971) and a species of *Brevibacterium* (Miyajima & Shiio, 1972) rather than in the enteric bacteria. In the latter case, valine only normalizes the substrate–response curve in the presence of isoleucine (Umbarger, 1973). The enzyme from *Corynebacterium* sp. F5 is also atypical in that it exhibited a co-operative response to threonine even in the absence of isoleucine. The normal Michaelis–Menten kinetics observed for the enzyme in crude extracts may have been due to the carry-over of valine from the growth medium. This would also account for the fact that additional valine did not activate the non-purified dehydratase. The reason why the K_m for L-threonine was lower in extracts than in purified preparations is uncertain.

The evidence that L-threonine was metabolized via 2-oxobutyrate *in vivo* seems clear. The oxo acid accumulated transiently during the growth of the *Corynebacterium* on threonine+valine and washed cell suspensions metabolized [^{14}C]threonine to radioactive 2-oxobutyrate. Similarly the oxo acid was rapidly metabolized by the bacterium after growth on valine-supplemented medium. Similar evidence suggested that propionate, or its 'active' form, was an intermediate of threonine catabolism.

Less direct evidence pointed to a branched-chain oxo acid dehydrogenase as the enzyme responsible for the oxidative decarboxylation of 2-oxobutyrate to propionyl-CoA. 3-Methyl-2-oxobutyrate, known to induce formation of the enzyme in *Pseudomonas putida* (Massey *et al.*, 1974, 1976), promoted growth of *Corynebacterium* sp. F5 on threonine almost as effectively as did its precursor valine. Similarly growth on 2-oxobutyrate+$(NH_4)_2SO_4$ was promoted by valine. Bacteria grown on threonine+valine were then capable of utilizing 3-methyl-2-oxobutyrate as well as 2-oxobutyrate. Enzymic evidence for the branched-chain oxo acid dehydrogenase in the bacterium was obtained, but it was extremely labile and only low specific activities were measured. After growth on valine-supplemented media, its activity towards 2-oxobutyrate although low was similar to that towards 3-methyl-2-oxobutyrate and related branched-chain oxo acids.

The requirement by *Corynebacterium* sp. F5 for valine for growth on threonine or 2-oxobutyrate, but not propionate or succinate, is explicable in terms of the induction of the branched-chain oxo acid dehydrogenase. L-Valine may additionally antagonize isoleucine inhibition of L-threonine dehydratase, although D-valine did not act as an antagonist yet promoted growth on threonine. D-Valine, in common with its L-stereoisomer, is known to be degraded via 2-methyl-2-oxobutyrate,

and thus would induce branched-chain oxo acid dehydrogenase formation.

A catabolic role for a 'biosynthetic' threonine dehydratase has not been reported previously. Specific 'catabolic' enzymes are known in anaerobically grown *Escherichia coli* and *Clostridium tetanomorphum*, and these enzymes have been studied extensively (see Umbarger, 1973). An AMP-activated enzyme has also been demonstrated in aerobically grown *Azotobacter chroococcum* (Gupta, 1971). In contrast with *Corynebacterium* sp. F5 and isolates F9 and G1, none of these bacteria is capable of growth on threonine as the major source of carbon and energy. The recruitment of a 'biosynthetic' enzyme for threonine catabolism and the co-metabolism of 2-oxobutyrate and oxo acids by a route for the degradation of branched-chain amino acids suggest mechanisms for the evolution of new catabolic pathways.

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References

Barritt, G. J. (1971) *J. Bacteriol.* **105**, 718–721
Bell, S. C. & Turner, J. M. (1976*a*) *Biochem. J.* **156**, 449–458
Bell, S. C. & Turner, J. M. (1976*b*) *Biochem. Soc. Trans.* **4**, 497–500
Bell, S. C., Turner, J. M., Collins, J. & Gray, T. R. G. (1972) *Biochem. J.* **127**, 77P
Datta, P. (1966) *J. Biol. Chem.* **241**, 5836–5844
Freundlich, M., Burns, O. R. & Umbarger, H. E. (1962) *Proc. Natl. Acad. Sci. U.S.A.* **48**, 1804–1808
Gupta, Y. P. (1971) *Enzymologia* **41**, 91–98
Hall, T. C. & Cocking, E. C. (1965) *Biochem. J.* **96**, 626–633
Hayakawa, T., Hirashima, M., Ide, S., Hamada, M., Okabe, K. & Koike, M. (1966) *J. Biol. Chem.* **241**, 4694–4699
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
Katsunuma, T., Elsässer, S. & Holzer, H. (1971) *Eur. J. Biochem.* **24**, 83–87
Kornberg, H. L. (1958) *Biochem. J.* **68**, 535–542
Kumagai, H., Nagate, T., Yoshida, H. & Yamada, H. (1972) *Biochim. Biophys. Acta* **258**, 779–790
Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–663
Marshall, V. P. & Sokatch, J. R. (1973) *J. Bacteriol.* **110**, 1073–1081
Massey, L. K., Conrad, R. S. & Sokatch, J. R. (1974) *J. Bacteriol.* **118**, 112–120
Massey, L. K., Sokatch, J. R. & Conrad, R. S. (1976) *Bacteriol. Rev.* **40**, 42–54
McGilvray, D. & Morris, J. G. (1969) *Biochem. J.* **112**, 657–671
Miyajima, R. & Shiio, I. (1972) *J. Biochem.* (*Tokyo*) **71**, 951–960
Morris, J. G. (1969) *Biochem. J.* **115**, 603–605
Ning, C. & Gest, H. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1823–1827
Reed, L. J. & Williams, C. R. (1966) *Methods Enzymol.* **9**, 247–265
Tokushige, M. & Hayaishi, O. (1972) *J. Biochem.* **72**, 469–477
Umbarger, H. E. (1973) *Adv. Enzymol.* **37**. 349–395
Umbarger, H. E. & Brown, B. (1957) *J. Bacteriol.* **72**, 105–112
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1964) *Manometric Techniques*, 4th edn., pp. 1–17, Burgess Publishing Co., Minneapolis
Willetts, A. J. & Turner, J. M. (1970) *Biochem. J.* **117**, 27P–28P
Willetts, A. J. & Turner, J. M. (1971) *Biochim. Biophys. Acta* **252**, 105–110