2. The seven viruses analysed all have a similar pattern of amino acid composition and this differs markedly from the pattern of the eight inclusion-body proteins. Proportional to the total amino acid content, the former have a strikingly greater content of arginine and serine, and less lysine and tyrosine than the latter.

3. There is more variability among the viruses than among the inclusion-body proteins, but significant differences exist between any two members of either group.

4. The differences in amino acid composition tend to parallel the morphological classification (Bergold, 1953) rather than the groupings based on the nucleic acid analysis (Wyatt, 1952).

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Amino Acid Utilization in Bacterial Growth

2. A STUDY OF THREONINE-ISOLEUCINE RELATIONSHIPS IN MUTANTS OF *ESCHERICHIA COLI*

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The possibility that threonine is an intermediate in the synthesis of isoleucine in micro-organisms has been considered in earlier publications (Teas, 1948; Umbarger & Adelberg, 1951). Teas (1948) described growth experiments in which isoleucine-requiring mutants of *Neurospora* were able to use the keto analogue, α -oxo- β -ethylbutyric acid, as well as any of a series of four-carbon compounds; namely, α -aminobutyric acid, α -oxobutyric acid and DL-threonine. Evidence from growth studies for the relationship between threonine and isoleucine in micro-organisms has been summarized by Umbarger & Adelberg (1951). From their analysis of the data they have classified the threonine-requiring organisms into three classes: (1) those which respond to L-threonine only; (2) those which show an absolute requirement for isoleucine, D-threonine or

one of several other four-carbon compounds; (3) a group which responds as group 2 but will utilize L-threonine also.

The present paper is concerned principally with the relationship of L-threonine to isoleucine and to the four-carbon intermediates, D-threonine, α -aminobutyric acid, α -oxobutyric acid and α -oxo- β -hydroxybutyric acid. The approach has been to combine inhibition analysis (Shive, 1950) with quantitative growth measurement employing several mutants of *Escherichia coli* (Maas & Davis, 1950).

EXPERIMENTAL

Strains used. *Escherichia coli*, wild type ML3 (Monod), L-threonine requireers ML41, ML52 and ML54 obtained by Monod from the wild type ML3.

Esch. coli, wild type W (ATCC 9637) (Davis), L-threonine requirer M43-6 from wild type W, and D-threonine requirer M97-21 from wild type W, both obtained by Davis. All strains were maintained on meat-broth agar slants by weekly transfer.

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Testing methods. The testing methods, as well as calibration of the photoelectric instrument against cell count, remain as described by Hirsch & Cohen (1953) unless otherwise stated. DL-Serine, DL-threonine, DL- α -aminobutyric acid, L-valine, L-leucine, L-isoleucine, DL-methionine, L-arginine, L-lysine, L-glutamic and L-aspartic acids were Hoffmann-La Roche products. L-Serine, D-serine, L-threonine, D-threonine, L- α -aminobutyric acid and D- α -aminobutyric acid were the gift of Dr J. P. Greenstein of the U.S. Public Health Service, Bethesda, Maryland, and were more than 99.9% optically pure. Synthetic DL-homoserine was given by Dr M. D. Armstrong, University of Utah, and Dr H. J. Teas, California Institute of Technology, DL-homocysteine by Dr E. Borek, Columbia University and α -oxobutyric acid by Dr D. M. Bonner, Yale University. α -Oxo- β -hydroxybutyric acid was prepared from 2-oxo- β -bromobutyric acid according to Sprinson & Chargaff (1946). The sample of the bromo acid was a gift of Dr D. B. Sprinson, Columbia University.

We wish to thank most sincerely the above-mentioned persons for their generous gifts and for their indispensable co-operation. Our thanks are equally extended to Drs J. Monod, B. D. Davis, and A. M. Pappenheimer, jun., for providing us with the bacterial strains used in this study.

RESULTS

Nutritional properties of the L-threonine requirers. Under the conditions of growth employed, all strains requiring L-threonine were equally responsive to it. Mutants ML41, ML52 and M43-6 were found to grow only on L-threonine. The concentration of L-threonine required for half-maximal growth of mutant ML54 is approximately 4×10^{-5} M (incubation time 20 hr.). This mutant was found to grow also on D-threonine under conditions to be described later and on DL-homoserine. The optimum concentration of homoserine is 5×10^{-5} M, at which concentration growth corresponds to that obtained with 10^{-5} M L-threonine. Above 5×10^{-5} M homoserine, growth decreases. The above three compounds only were found to promote growth of ML54, even though the growth period was prolonged in the testing of other substances.

Requirements of mutant M97-21. Strain M97-21 grows on either L-isoleucine, DL- α -aminobutyric acid, α -oxobutyric acid, or D-threonine (personal communication from Dr B. D. Davis). We find that

this mutant can use D- or L- α -aminobutyric acid and α -oxo- β -hydroxybutyric acid as well. D-Threonine supports growth at high levels only (10^{-3} M). At optimum level the growth response is poorer than that obtained on the other growth factors. L-Threonine does not promote growth in concentrations as high as 10^{-2} M and inhibits partially the growth on D-threonine.

Inhibition by L-serine of the growth of the L-threonine requirers. Since the intention was to study by means of inhibition analysis the utilization of L-threonine, several amino acids were examined as potential inhibitors to the growth of the L-threonine-requiring mutants. It was found that none of the following, at the concentrations indicated in parentheses, inhibited the growth of mutant ML54 on 10^{-4} M L-threonine; DL-methionine (10^{-2} M), L-valine (10^{-2} M), L-isoleucine (10^{-2} M), glycine (10^{-2} M).

In contrast, the growth of all the L-threonine-requiring mutants was completely inhibited by the presence of DL-serine in sufficient quantity. The mutant ML54 was most thoroughly investigated in this respect. The inhibition is competitive in character (Table 1) and the inhibition index (defined as the ratio serine/threonine necessary to inhibit growth completely) is approximately 50. Experiments made with L- and D-serine showed that the inhibitory action is due to L-serine, and that D-serine is virtually inactive. It was found that at serine levels of 10^{-2} M and higher, the inhibition could not be overcome by additional threonine or other substances effective as reversal agents in the presence of lower levels of serine. It should be noted that levels of serine lower than that which inhibited growth completely at a given level of threonine markedly enhanced the growth obtained in the presence of threonine alone (Table 1).

Total reversal of the inhibition by serine could be obtained by addition of appropriate levels of D-threonine, DL- α -aminobutyric acid, L- α -aminobutyric acid and α -oxobutyric acid. Partial reversal was obtained with L-isoleucine and α -oxo- β -hydroxybutyric acid (Table 2). The competitive or non-competitive character of the reversals could not be determined because of the solubility limit

Table 1. *Competitive inhibition of the growth of the L-threonine-requiring mutant ML54 of Esch. coli by DL-serine*

Incubation: 20 hr. In this and subsequent tables, the concentrations given are the final molar concentrations. When racemic mixtures are used the concentrations given are those of the L isomer. Growth is expressed as units of the Meunier photometer.

DL-Threonine (M)	DL-Serine (M)						
	0	5×10^{-4}	1×10^{-3}	2.5×10^{-3}	5×10^{-3}	1×10^{-2}	2×10^{-2}
5×10^{-5}	75	131	99	0	0	—	—
1×10^{-4}	114	—	153	33	0	—	—
2.5×10^{-4}	127	—	—	143	107	15	0
5×10^{-4}	113	—	—	—	—	28	0

imposed by the high inhibition ratio and because of a secondary inhibitory action exerted by L-isoleucine and α -aminobutyric, α -oxobutyric and α -oxo- β -hydroxybutyric acids in the presence of serine. Some deductions can, however, be drawn from the study of the D-threonine requirer presented below.

Table 2. *Suppression of the inhibition by L-serine of growth of mutant ML54 by several compounds proposed as intermediates between threonine and isoleucine*

Incubation: 20 hr. DL-threonine 5×10^{-5} M in L form in all tubes. No suppression is obtained with L-valine, L-leucine, DL-methionine (or combinations of the three) L-lysine, L-arginine, L-glutamic acid, β -hydroxybutyric acid, L-aspartic acid or DL-homocysteine. The secondary inhibitory action shown by α -oxobutyric acid in the presence of serine is not included in this table, but appears at a concentration of 10^{-4} M.

Added compound	Concn. (M)	Growth (Meunier units)	
		Without DL-serine	With DL-serine (2.5×10^{-3} M)
Nil	—	82	3
DL- α -Amino-butyric acid	10^{-5}	103	13
	3.3×10^{-5}	—	85
	5×10^{-5}	116	—
	10^{-4}	119	46
α -Oxobutyric acid	10^{-6}	79	20
	5×10^{-6}	79	53
	10^{-5}	83	87
α -Oxo- β -hydroxy-butyric acid	10^{-5}	76	12
	5×10^{-5}	77	40
	10^{-4}	80	13
D-Threonine	10^{-4}	82	22
	5×10^{-4}	84	50
	10^{-3}	86	98
L-Isoleucine	5×10^{-6}	80	55
	10^{-5}	84	44
	5×10^{-5}	77	0

Sparing effect of certain compounds on L-threonine. In the presence of limiting quantities of L-threonine, extra growth was obtained by appropriate levels of D-threonine, α -aminobutyric, α -oxobutyric and α -oxo- β -hydroxybutyric acids (Table 3). No such effect was obtained with various levels of L-isoleucine, L-valine, L-leucine, DL-methionine, L-lysine, L-arginine and L-glutamic acid.

Inhibition by DL-serine of the growth of the D-threonine requirer M97-21. It was felt that a study of the effect of serine on the D-threonine requirer M97-21, which grows on any of the supposed intermediates between threonine and isoleucine, might throw some light on the site of action of serine, particularly in view of the above-cited difficulties encountered in attempting to determine the competitive or non-competitive character of the reversal of serine inhibition of the L-threonine requirers.

Table 3. *Sparing effect of several compounds on L-threonine for mutant ML54*

Incubation: 20 hr. DL-threonine (final concn. 2.5×10^{-5} M as L isomer) present in all tubes.

Sparing compound	Concn. (M)	Growth (Meunier units)
Nil	—	48
DL- α -Aminobutyric acid	10^{-5}	80
	5×10^{-5}	81
α -Oxobutyric acid	10^{-5}	46
	5×10^{-5}	67
α -Oxo- β -hydroxybutyric acid*	10^{-4}	64
	5×10^{-4}	19
D-Threonine	5×10^{-5}	53
	10^{-4}	68

* This compound becomes consistently inhibitory when its level is raised.

Table 4. *Competitive inhibition of the growth of Esch. coli mutant M97-21 on D-threonine by DL-serine*

Incubation: 20 hr.								
D-Threonine (M)	Growth in Meunier units in presence of DL-serine (M)							
	0	10^{-4}	2.5×10^{-4}	5×10^{-4}	10^{-3}	2.5×10^{-3}	5×10^{-3}	10^{-2}
5×10^{-4}	42	26	9	3	1	0	—	—
10^{-3}	61	—	32	27	21	9	0	—
5×10^{-3}	62	—	68	42	31	—	11	11
10^{-2} *	47	—	48	26	0	—	0	—

* Level of 10^{-2} M D-threonine is in itself inhibitory as evidenced by reduced growth of control with this level of threonine.

Table 5. *Inhibition of the growth of Esch. coli mutant M97-21 on L- α -aminobutyric acid by DL-serine*

Incubation: 20 hr.						
L- α -Aminobutyric acid (M)	Growth in Meunier units in presence of DL-serine (M)					
	0	10^{-3}	2.5×10^{-3}	5×10^{-3}	10^{-2}	2×10^{-2}
10^{-5}	55	17	11	9	0	—
5×10^{-5}	86	37	—	6	0	—
10^{-4}	82	30	—	6	1	—
5×10^{-4}	79	70	—	3	0	0
10^{-3}	93	—	—	4	0	0

Growth of the D-threonine requirer on either D-threonine or DL- α -aminobutyric acid was inhibited by DL-serine. When D-threonine was the growth factor, both D- and L-serine were inhibitory. Growth on D-threonine was somewhat poor, as already stated, but in spite of this, it was possible to demonstrate that its inhibition by serine was competitive in nature (Table 4). On the other hand, inhibition of the growth on α -aminobutyric acid appeared to be unrelated to the amount of amino-butyric acid present (Table 5).

Relationship of L- and D-threonine in the growth of ML54. Large amounts of D-threonine (10^{-3} M) were found to satisfy the growth requirements of ML54, that is to say, final growth obtained by extending the growth period to 24 hr. was equal to the maximum growth obtainable over the same period with optimum quantities of L-threonine (10^{-4} M). This was not the case with the other L-threonine requirers, ML41, ML52 and M43-6, which grew but slightly on levels of D-threonine as high as 10^{-2} M. The slight growth obtained could be accounted for by the 0.1% of L-threonine possibly present as contaminant of the D isomer. This explanation could not account for the growth obtained with ML54.

The growth of ML54 on D- and L-threonine is shown in Fig. 1. Growth on D-threonine is very slow initially and the rate increases gradually with time. The shape of the D-threonine curve is much more in favour of a slow conversion of D-threonine into L-threonine or into a precursor of L-threonine than of a selection phenomenon or the utilization of D-threonine as such. Both the selection of a D-threonine utilizing mutant and a phenomenon of adaptation are made improbable by the finding that cells from full growth on D-threonine behave in the same fashion as the original inoculum when reseeded on D-threonine.

It should be re-emphasized at this point that mutant M97-21 gives no observable growth in the presence of L-threonine on concentrations as high as 10^{-2} M even with a period of growth extended to 48 hr.

L-Threonine deaminase. The possibility that the difference between mutants M97-21 and ML54 could be the inability of M97-21 to convert L- into D-threonine was then considered, and a search made for a racemase for threonine and for an L-threonine deaminase in both mutants. The procedure of Wood & Gunsalus (1951) with both acetone-dried and vacuum-dried cells, previously aged according to Lichstein & Christman (1948), was followed in an effort to demonstrate the presence of a racemase assaying alternatively with L- and D-threonine.

Kidney D-amino-acid oxidase and snake ophio-L-amino-acid oxidase could not be used since these enzymes are virtually inactive on the threonine

isomers (Bender & Krebs, 1950; Zeller, 1948). Consequently, an L-threonine deaminase preparation was used to detect the appearance of L-threonine (when D-threonine was the substrate) or its disappearance (when L-threonine was the substrate). This very active preparation was a cell-free extract obtained by Dr B. Nisman, of this laboratory (unpublished data) by ultrasonic disruption of cells of *Clostridium sporogenes*.

Under the conditions of these experiments, the presence of a racemase could not be demonstrated either in M97-21 or in ML54.

On the other hand, an L-threonine deaminase was readily demonstrated in both mutants tested, using vacuum-dried cells without addition of cofactors. Since the deamination product of L-threonine in

Fig. 1. Growth of mutant ML54. A, 5×10^{-5} M L-threonine; B, growth on 10^{-3} M D-threonine. The initial response seen with D-threonine can be accounted for as resulting from less than 10^{-6} M L-threonine possibly present as a contaminant. The organism grown in the presence of 10^{-6} M L-threonine will give an optical density of 0.04 which corresponds to 8-10 Meunier units.

Table 6. Inhibition of the growth of mutant M97-21 by L-threonine and DL-homoserine

Concn. of inhibitor (M)	Growth in Meunier units in presence of	
	L- α -Aminobutyric acid (10^{-5} M)	L-Isoleucine (5×10^{-5} M)
0	60	81
L-Threonine 10^{-4}	53	88
10^{-3}	17	87
2×10^{-3}	15	82*
DL-Homoserine 10^{-5}	63	78
10^{-4}	65	77
10^{-3}	0	0

* With levels tested L-threonine could not be shown to be inhibitory to the growth on L-isoleucine.

Esch. coli is α -oxobutyric acid (Chargaff & Sprinson, 1943) on which M97-21 grows readily, it is difficult to account for the failure of this organism to grow on L-threonine.

Mutant M97-21 grows better on D-threonine than on the racemic mixture; we therefore looked for a possible inhibition by L-threonine of the growth of M97-21 on factors other than D-threonine. It was found that L-threonine is inhibitory to the growth of M97-21 on L- α -aminobutyric acid but not on L-isoleucine at the levels tested (Table 6). This raised the possibility that the failure of M97-21 to grow on L-threonine was due to an inhibitory action of the L-threonine on the utilization of its dissimilation products, as precursors of L-isoleucine.

DL-Homoserine as inhibitor. DL-Homoserine was inhibitory to all the L- and D-threonine requirers, even to ML54 for which it is a growth factor within a narrow range of concentrations. The inhibitory levels of homoserine vary for the different strains of the L-threonine requirers. With the D-threonine requirer M97-21, growth on L- α -aminobutyric acid and L-isoleucine were both inhibited, while L-threonine inhibited growth on L- α -aminobutyric acid only (Table 6).

The growth of the wild type W, from which M97-21 is derived, was completely inhibited by 10^{-3} M DL-homoserine (Table 7). This inhibition could be overcome by adding to the medium either iso-

leucine or valine. Similarly, the growth of the mutant M97-21 on isoleucine, when totally inhibited by homoserine, could be restored by the addition of valine (Table 8).

DISCUSSION

The competitive nature of the inhibition by L-serine of growth on L-threonine indicates that the utilization of L-threonine is involved, whether it be its incorporation as such into protein, or its utilization for the synthesis of some other metabolite. That the serine inhibition is overcome only by compounds previously listed as possible intermediates in the synthesis of L-isoleucine, and that these same compounds show a sparing effect over L-threonine, can be taken as evidence that it is rather utilization in the direction of isoleucine that is interfered with by serine. However, such facts as can be gained from growth experiments cannot furnish conclusive evidence of the degree to which the carbon chain of threonine is involved in the synthesis of isoleucine. Ehrensward (1952), from studies with acetate doubly labelled with ^{13}C and ^{14}C suggests that the C-4 chain of threonine appears not to be incorporated as a block into the isoleucine molecule. More evidence of this type must be accumulated before this biosynthetic pathway can be clarified.

The mutant M97-21 was found to possess an active L-threonine deaminase which should, in the absence of some inhibitory influence of L-threonine, permit this mutant to grow on L-threonine. The deamination product of L-threonine is α -oxobutyric acid, which, when supplied in the medium, supports growth. The failure, then, of M97-21 to grow on L-threonine may be due to its inability to deaminate L-threonine at the pH prevailing under conditions which support growth, or to an inhibitory influence of L-threonine. The inhibitory effect of both L-threonine and DL-homoserine has been demonstrated. This recalls the condition described by Davis (1952) where dehydroshikimic acid inhibits the utilization of its own derivative, shikimic acid, for the synthesis of phenylalanine and tyrosine. In mutant M97-21 the inhibition by homoserine is overcome by isoleucine or valine. The wild type, from which M97-21 is derived, is also inhibited by DL-homoserine. This inhibition is suppressed as well by isoleucine or valine.

Table 7. *Inhibition of the growth of the wild type Esch. coli (W) by DL-homoserine and its suppression by L-isoleucine or L-valine*

Incubation: 18 hr.			
DL-Homoserine (M)	L-Isoleucine (M)	L-Valine (M)	Growth (Meunier units)
0	0	0	93
10^{-5}	0	0	94
10^{-4}	0	0	79
10^{-3}	0	0	0
10^{-3}	10^{-5}	0	23
10^{-3}	10^{-4}	0	46
10^{-3}	10^{-3}	0	85
10^{-3}	0	10^{-5}	36
10^{-3}	0	10^{-4}	85
10^{-3}	0	10^{-3}	82
10^{-3}	10^{-5}	10^{-5}	38
10^{-3}	10^{-4}	10^{-4}	89
10^{-3}	10^{-3}	10^{-3}	85

Table 8. *Suppression of the homoserine inhibition on the growth of Esch. coli mutant M97-21 by L-valine*

Incubation: 22 hr. L-Isoleucine concn. 10^{-4} M throughout.						
DL-Homoserine (M)	Growth in Meunier units in presence of L-valine (M)					
	0	10^{-5}	10^{-4}	10^{-3}	5×10^{-3}	10^{-2}
0	99	—	—	77	66	15*
10^{-3}	30	—	—	—	—	—
2×10^{-3}	0	0	2	26	71	11*

* L-Valine inhibits competitively the growth of M97-21 on L-isoleucine.

Further experiments are in progress on the nature of the block in M97-21, i.e. whether it is lacking an enzyme or is inhibited by a metabolite which it accumulates and which is involved in the valine-isoleucine equilibrium.

SUMMARY

1. The growth of four mutants of *Escherichia coli* requiring L-threonine is inhibited by the presence of L-serine in the culture medium.

2. The inhibition by DL-serine is competitive and is suppressed by D-threonine, α -aminobutyric acid, α -oxobutyric acid, α -oxo- β -hydroxybutyric acid and L-isoleucine.

3. All the above-listed compounds, except L-isoleucine, exert a sparing effect in the utilization of L-threonine.

4. A mutant requiring either D-threonine, α -aminobutyric acid or L-isoleucine is also inhibited by DL-serine, when grown on D-threonine or α -aminobutyric acid.

5. These results lead to the hypothesis that L-isoleucine synthesis depends on L-threonine utilization in these mutants.

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Stoichiometry of the Citrulline Phosphorylase Reaction of *Streptococcus lactis*

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It has recently been shown that the anaerobic degradation of the carbamido group of L-citrulline by *Streptococcus faecalis* (Knivett, 1953; Slade, 1953) and *Strep. lactis* (Korzenovsky & Werkman, 1953*a*, *b*) is coupled to the esterification of orthophosphate. Adenosine diphosphate (ADP), Mg^{++} and orthophosphate are the necessary components for the formation of ornithine, carbon dioxide and ammonia, as well as for the synthesis of acid-labile phosphate bonds (Knivett, 1953; Slade, 1953; Korzenovsky & Werkman, 1953*a*, *b*). Incorporation of radioactive phosphorus into adenosine triphosphate (ATP) (Knivett, 1953) or phosphorylation of glucose in the presence of iodoacetate (Korzenovsky & Werkman, 1953*a*, *b*) indicates that ADP functions as a phosphate acceptor resulting in the synthesis of ATP. Although adenosine 5-phosphate (AMP) can replace ADP (Korzenovsky & Werkman, 1953*b*) its role as a phosphate acceptor is not clear because the absence of enzymes capable of catalysing the reaction $2\text{ADP} \rightleftharpoons \text{AMP} + \text{ATP}$ has not been shown.

The purpose of the present paper is to establish the stoichiometry of the citrulline phosphorylase reaction according to the following equations:

METHODS

Conditions for the culture of *Strep. lactis* and preparation of cell-free extracts have been described (Korzenovsky & Werkman, 1953*b*). Extracts were dialysed against distilled water at 1° for 8 hr. and then diluted with 0.5M potassium phosphate buffer, pH 6.2-6.3, to contain about 5 mg. of protein per 0.1 ml. The diluted extracts contained approximately 5 μ moles of orthophosphate per 0.1 ml.

The reaction was conducted in 25 ml. Erlenmeyer flasks. Enzyme, orthophosphate (pH 6.2-6.3), maleate buffer (maleic acid brought to pH 6.2-6.3 with NaOH solution), $MgCl_2$ and iodoacetate were incubated for 10 min. Purified hexokinase and glucose, when used, were then added and the