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Inhibition of Isoleucyl-Transfer Ribonucleic Acid Synthetase in *Escherichia coli* by Pseudomonic Acid

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The mode of action of the antibiotic pseudomonic acid has been studied in *Escherichia coli*. Pseudomonic acid strongly inhibits protein and RNA synthesis *in vivo*. The antibiotic had no effect on highly purified DNA-dependent RNA polymerase and showed only a weak inhibitory effect on a poly(U)-directed polyphenylalanine-forming ribosomal preparation. Chloramphenicol reversed inhibition of RNA synthesis *in vivo*. Pseudomonic acid had little effect on RNA synthesis in a regulatory mutant, *E. coli* B AS19 RC^{rel}, whereas protein synthesis was strongly inhibited. In pseudomonic acid-treated cells, increased concentrations of ppGpp, pppGpp and ATP were observed, but the GTP pool size decreased, suggesting that inhibition of RNA synthesis is a consequence of the stringent control mechanism imposed by pseudomonic acid-induced deprivation of an amino acid. Of the 20 common amino acids, only isoleucine reversed the inhibitory effect *in vivo*. The antibiotic was found to be a powerful inhibitor of isoleucyl-tRNA synthetase both *in vivo* and *in vitro*. Of seven other tRNA synthetases assayed, only a weak inhibitory effect on phenylalanyl-tRNA synthetase was observed; this presumably accounted for the weak effect on polyphenylalanine formation in a ribosomal preparation. Pseudomonic acid also significantly de-repressed threonine deaminase and transaminase B activity, but not dihydroxyacid dehydratase (isoleucine-biosynthetic enzymes) by decreasing the supply of aminoacylated $tRNA^{Ile}$. Pseudomonic acid is the second naturally occurring inhibitor of bacterial isoleucyl-tRNA synthetase to be discovered, furanomycin being the first.

In a previous paper (Hughes & Mellows, 1978) it was shown that in *Staphyloccocus aureus* cells, pseudomonic acid (Fig. 1) strongly inhibits both protein and RNA synthesis. DNA and cell-wall formation were affected to a lesser extent, whereas oxidative phosphorylation, as measured by ATP concentrations, was slightly enhanced by the antibiotic. It was further suggested that inhibition of RNA synthesis might be a consequence of the stringent control mechanism, caused by the pseudomonic acid-induced deprivation of one or more amino acids (Pederson *et al.*, 1973; Haseltine & Block, 1973). The mode of action studies have been continued with *Escherichia coli* MRE 600, and two mutant strains of *E. coli* B AS19 (RC^{str} and RC^{rel}). The antibiotic produces similar effects on macromolecular synthesis in *E. coli* MRE 600 to those previously observed in *S. aureus*, but higher concentrations are required. In this paper we present evidence to show that at low concentrations, inhibition of RNA synthesis is a consequence of the stringent control mechanism governed by the *rel* gene. Bacteriostasis, caused by low concentrations of pseudomonic acid, can be relieved by addition of isoleucine to the medium. In pseudomonic acid-treated cells the degree of aminoacylation of $tRNA^{Ile}$ was drastically decreased. The antibiotic was shown to be a powerful inhibitor of highly purified *E. coli* B isoleucyl-tRNA synthetase.

Materials and Methods

Materials

The purified sodium salt of pseudomonic acid was kindly donated by Beecham Pharmaceuticals, Research Division, Chemotherapeutic Research Centre, Betchworth, Surrey, U.K., and was used in this form throughout this work. Nucleotides, deoxyribonuclease I (electrophoretically pure), pyruvate kinase, salmon sperm DNA, phosphoenolpyruvate, poly(U), bulk tRNA (from *E. coli*, strain W) and amino acids were from Sigma (London) Chemical Co., Kingston upon Thames KT2 7BH, U.K. GF/C glass-fibre filter discs were from Whatman, Maidstone, Kent, U.K. Poly(ethyleneimine) (PEI)–cellulose MN300-coated t.l.c. plates were purchased from Camlab, Cambridge, U.K. $[^{32}P]P_i$ (carrier-free; original sp. radioactivity 3Ci/mmol of phosphorus) was purchased from New England Nuclear, Boston, MA, U.S.A.; [2-^{14}C]uridine (sp. radioactivity 55.0mCi/mmol), L-[U-^{14}C]phenylala-

nine (sp. radioactivity 10.0mCi/mmol), L-[U-^{14}C]isoleucine (sp. radioactivity 10.0mCi/mmol), [2-^{14}C]thymidine (sp. radioactivity 56mCi/mmol), and [5-^{3}H]UTP (sp. radioactivity 10000 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Highly purified and electrophoretically homogeneous *E. coli* B isoleucyl-tRNA synthetase and DNA-dependent RNA polymerase were kindly provided by Dr. C. J. Bruton and Dr. D. Glover respectively. Chloramphenicol, rifampicin and tetracycline were commercially available materials.

Radiochemical techniques

Radioactivity counting was performed in a Beckman LS-200B liquid-scintillation counter. Non-aqueous samples, glass-fibre filters and poly(ethyleneimine)–cellulose adsorbent were counted for radioactivity in a toluene-based scintillant (5ml) comprising butyl-PBD [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] (0.6%) and naphthalene (5%). Aqueous samples were counted for radioactivity in Bray's liquid scintillant comprising naphthalene (6%), 2,5-diphenyloxazole (0.4%), 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.02%), methanol (10%, v/v) and ethylene glycol (2%, v/v) in dioxan.

Continuous monitoring of bacterial growth

This was followed by using a Bonet Maury Biophotometer (John Yvon, Instruments Division, 91160 Longjumeau, France) with a continuous readout at 600nm.

Bacterial strains and culture conditions

E. coli MRE 600 was grown in nutrient broth at 37°C on a rotary shaker. A 0.1% inoculum of an overnight culture was dispersed into fresh prewarmed nutrient broth (50ml). Experiments were started when the A_{600} had reached approximately 0.2, indicating steady exponential growth. In nutrient broth, the minimum inhibitory concentration of pseudomonic acid against *E. coli* MRE 600 is 60μg/ml. In the study of pseudomonic acid-induced isoleucine auxotrophy, a minimal medium was used, containing (per litre) K_2HPO_4 (7g), KH_2PO_4 (3g), $(NH_4)_2SO_4$ (1g), $MgSO_4,7H_2O$ (0.1g), sodium citrate,$2H_2O$ (0.5g) and glucose (2g). In this medium the minimum inhibitory concentration of pseudomonic acid is 25μg/ml.

E. coli B, AS19 RC^{str}[*rel*$^+$ (NF541)] and *E. coli* B, AS19 RC^{rel} [*rel*$^-$ (NF542)], isogenic except for the *rel* locus and originally prepared by Fiil & Friesen (1968), were kindly provided by Dr. N. Fiil, University Institute of Microbiology, Øster Farimagsgade 2A, DK-1353, Copenhagen, Denmark. The mutants, which contain a deficient cell wall, require cytosine, thymidine, uridine and leucine for growth. The cells were grown at 37°C in Tris-buffered glucose-salts medium containing (per litre): glucose (2.0g), $(NH_4)_2SO_4$ (0.1g), casamino acids (2.0g), sodium citrate (0.5g), $MgSO_4,7H_2O$ (0.1g), $FeCl_3$ (0.00054g), Tris/HCl, pH 7.4 (6.055g), cytosine (0.05g), thymidine (0.05g), uracil (0.05g), leucine (0.1g) and KH_2PO_4 (0.272g). In this medium the minimum inhibitory concentration of pseudomonic acid against both mutant strains of *E. coli* B AS19 is 0.2μg/ml. Experiments in which [^{32}P]P_i was used were carried out in a low-phosphate medium, obtained by decreasing the concentration of KH_2PO_4 to 0.0272g/l and additionally containing KCl (0.15g/l). All experiments were carried out during steady exponential growth.

Measurements of protein, RNA and DNA synthesis

The incorporation of [^{14}C]isoleucine or [^{14}C]-phenylalanine, [^{14}C]uridine and [^{14}C]thymidine into acid-insoluble material was followed as previously described (Hughes & Mellows, 1978). After the incorporation of ^{14}C-labelled amino acids, culture samples (0.5ml) were added to 10% trichloroacetic acid (6ml), placed on ice for 30min and finally heated at 90–95°C for 20min. The precipitates were collected on Whatman GF/C glass-fibre filters, washed with 5% trichloroacetic acid (2×6ml) and ethanol (2×6ml), dried and counted for radioactivity. Samples (0.5ml) from [^{14}C]uridine- and [^{14}C]thymidine-incorporation experiments were added to 2% sodium dodecyl sulphate (0.5ml), and placed on ice. After 30min, 5% trichloroacetic acid (6ml) was added to each and the samples left for a further 30min before filtering etc., as described above.

Determination of nucleotide concentrations

Following the incorporation of [^{32}P]P_i, the intracellular concentrations of ATP, GTP, guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) were determined as described by Cashel (1969). Culture samples (50μl) were mixed with ice-cold 2M-formic acid (50μl), whirled and left on ice for 30min. The samples were centrifuged and samples (10μl) of the supernatants spotted on poly(ethyleneimine)–cellulose chromatography plates, which had previously been soaked in water for at least 30min and dried. The chromatograms were developed in 1.5M-KH_2PO_4 (pH 3.4); the R_F values of the various nucleotides were identical with those reported by Cashel (1969). The ^{32}P-labelled nucleotide spots were located by overnight radioautography, scraped off the chromatogram and counted for radioactivity.

A 0.3% inoculum of an overnight culture of

E. coli B, AS19 *rel*$^+$ or *E. coli* B, AS19 *rel*$^-$ grown in the high-phosphate medium was transferred to prewarmed low-phosphate medium (5 ml) and incubation continued at 37°C to an A_{576} value of approx. 0.1. [^{32}P]P_i was added to give 70 μCi/ml, the culture shaken well, and 1 ml portions transferred to each of two 5 ml conical flasks. The cultures were vigorously agitated at 37°C on a shaking water bath. One served as a control, whereas the other was treated with pseudomonic acid. Samples (50 μl) were removed at frequent intervals.

Measurement of RNA synthesis in vitro

DNA-dependent RNA polymerase, which had been isolated and highly purified from *E. coli* B by the method of Chamberlin & Berg (1962), was used. The assay contained the following ingredients in 0.2 ml: 40 mM-KCl, 15 mM-$MgCl_2$, 10 mM-2-mercaptoethanol, 5 mM-ATP, 5 mM-GTP, 5 mM-CTP, 5 mM-[^{3}H]UTP (5 μCi), 20 μg of salmon sperm DNA, 50 mM-Tris/HCl, pH 8.1, 80 mM-NaCl, glycerol (10%, v/v) and 10 units of purified enzyme. The mixture was incubated at 37°C for 1 h and the reaction terminated by the addition of 2 M-HCl (0.5 ml). After 15 min on ice the precipitate was collected on a GF/C glass-fibre filter, washed successively with 1 M-HCl (4 × 10 ml) and alcohol (2 × 10 ml), dried and counted for radioactivity as above.

Measurement of protein synthesis in vitro

A supernatant obtained by centrifugation at 30000 *g* was prepared from frozen *E. coli* MRE 600 Cells (Porton) by the method of Gould *et al.* (1973) except that deoxyribonuclease I (1 μg/ml) was added to the broken-cell suspension and stood 15 min at 0°C before centrifugation. Endogenous mRNA was destroyed by incubating the S_{30} fraction at 37°C for 40 min just before use. Each 1 ml of the incubation mixture contained 14 mM-magnesium acetate, 50 mM-KCl, 9 mM-2-mercaptoethanol, 1 mM each of a mixture of 20 amino acids, 2.5 mM-ATP, 1 mM-GTP, 2.5 mM-phosphoenolpyruvate, pyruvate kinase (20 μg) and 100 mM-Tris/HCl buffer, pH 7.8. At the end of the preincubation, sucrose (10%, w/v) was added and the mixture dialysed overnight at 4°C against 100 mM-Tris/HCl buffer, pH 7.8, containing 10% sucrose.

A sample of the above dialysate containing ribosomes (1 mg) was incubated at 37°C with the following in a final volume of 1 ml: pyruvate kinase (20 μg), 5 mM-phosphoenolpyruvate, 3 mM-GTP, 10 mM-ATP, 100 mM-Tris/HCl, pH 7.8, 14 mM-magnesium acetate, 50 mM-KCl, 14 mM-2-mercaptoethanol, [^{14}C]-phenylalanine (2 μCi; sp. radioactivity 10 mCi/mmol) and poly(U) (100 μg). After 30 min, 1 M-NaOH containing 0.25% (w/v) phenylalanine was added and the incubation continued at 37°C for 5 min and then at room temperature during 15 min. Trichloroacetic acid (10%, 4 ml) was added, whirled, left for 10 min and the mixture finally heated at 90°C for 10 min. The precipitate was collected on a Whatman GF/C glass-fibre filter, washed, dried and counted for radioactivity as described above.

Preparation of E. coli MRE 600 cell extracts and determination of threonine deaminase, transaminase B and acetohydroxyacid synthetase activities

Cells used for preparing extracts were grown in 1 litre quantities of mineral salt/glucose medium in 4 litre conical flasks that were incubated at 37°C on an orbital shaker. The cells were harvested after 6 h growth by centrifugation for 10 min at 10000 *g*. The cell pellets were suspended in one-tenth their growth volume of 0.05 M-potassium phosphate buffer, pH 8.0, containing 0.1 mM-L-isoleucine. The cells were centrifuged and rewashed. Approx. 0.5 g of cells were disrupted by sonication in 0.05 M-phosphate buffer containing 0.1 mM-isoleucine at 4°C in a MSE ultrasonicator operating at optimum frequency (3 × 30 s bursts). Isoleucine was added to the buffer to stabilize threonine deaminase activity (Freundlich & Umbarger, 1963). The sonicated suspension was centrifuged at 12000 *g* for 15 min at 0°C and the clear supernatant kept on ice. Assays for threonine deaminase and acetohydroxyacid synthetase were carried out immediately after centrifuging owing to their reported instability (Szentirmai & Umbarger, 1968). Protein was determined by the method of Lowry *et al.* (1951).

Threonine deaminase activity was determined by a modification of the procedure of Szentirmai & Umbarger (1968). A sample (100 μl) of a solution containing 0.1 M-NH_4Cl, 1 mM-pyridoxal phosphate in 1 M-Tris/HCl buffer, pH 8.0, and 0.2 M-L-threonine (100 μl) were added to cell extract (1 ml) and the mixture incubated at 37°C for 15 min. The reaction was stopped by the addition of 50% trichloroacetic acid (0.1 ml) and the mixture centrifuged at 10000 *g* for 10 min. The α-oxobutyrate formed in the supernatant was measured by the method of Friedmann & Haugen (1943). A 0.1% solution of dinitrophenylhydrazine in 2 M-HCl was added to the supernatant (1 ml). After 5 min the solution was vigorously extracted with ethyl acetate (4 ml) and the aqueous layer discarded. The ethyl acetate layer was extracted with 10% Na_2CO_3 solution (4 ml). The aqueous layer was separated and washed with ethyl acetate (3 × 2 ml). The aqueous fraction was diluted 2-fold with 1.5 M-NaOH. After 10 min the A_{520} was recorded and the amount of α-oxobutyrate present extrapolated from a calibration curve.

Transaminase B activity was determined by the method of Szentirmai & Umbarger (1968). The procedure measures the pyridoxal phosphate-

dependent conversion of [^{14}C]isoleucine into 3-methyl-2-oxo[^{14}C]pentanoate. At the end of the incubation the residual [^{14}C]isoleucine was removed by passing the mixture down a small Dowex-50 (H^+ form) ion-exchange column. A portion (1 ml) of the effluent plus washings containing the ^{14}C-labelled α-oxoacid was counted for radioactivity in Bray's scintillation fluid.

Acetohydroxyacid synthetase activity was measured by an adaptation of the method of Bauerk *et al.* (1964), which follows acetolactate formation from pyruvate and acetaldehyde. A sample (200μl) of a solution containing sodium pyruvate (0.2M), $MgCl_2$ (0.05M), thiamine pyrophosphate (0.4mg/ml) in 0.5M-potassium phosphate buffer (pH 8.0) was added to 1 ml of cell extract and the mixture incubated at 37°C for 30min. The reaction was quenched by the addition of 50% (v/v) H_2SO_4 (0.1 ml) and the mixture kept at 37°C for 30min to allow complete conversion of acetolactate into acetoin to take place. After centrifugation acetoin in the supernatant was measured colorimetrically by the method of Westerfield (1945).

Measurement of aminoacylation in vitro

This was carried out by a modification of the procedure of Werner *et al.* (1976). The S_{30} supernatant derived from *E. coli* MRE 600 cells (see earlier) was centrifuged at 150000*g* for 5h. The S_{150} supernatant was dialysed overnight and was used as a crude source of tRNA synthetases. Aminoacylation *in vitro* was measured by the addition of a sample (20μl) of the dialysed S_{150} supernatant to the following reagents in a final volume of 100μl: 25mM-Tris/HCl, pH7.5, 5mM-ATP, 70mM-KCl, 10mM-magnesium acetate, 0.5mM-EDTA, 250μg of bulk tRNA, 1nM-amino acid (sp. radioactivity 10–150mCi/mmol) with or without 10μg of pseudomonic acid. After the incubation at 37°C for 20min the reaction was stopped by the addition of 7% trichloroacetic acid (2ml) and left on ice for 30min. The samples were filtered through Whatman GF/C glass-fibre filters, washed and counted for radioactivity.

Determination of the degree of aminoacylation of tRNA in vivo

The procedure of Folk & Berg (1970*b*) was used incorporating the modifications of Lewis & Ames (1972), but using a crude enzyme preparation as a source of aminoacyl-tRNA synthetase. A 1% inoculum of an overnight culture of *E. coli* MRE 600 was made into 1 litre of minimal salts medium (prewarmed to 37°C). This was incubated with shaking at 37°C for 1h 50min. The culture was equally divided and pseudomonic acid (25μg/ml) was added to one portion. Both cultures were incubated for a further 10min followed by the addition of 55% trichloroacetic acid (50ml) prewarmed to 37°C. After 1 min 1% sodium dodecyl sulphate (5ml) was added and the cultures rapidly cooled to 2–5°C by swirling in a solid CO_2/acetone bath. After 15–20min the precipitate was collected by centrifugation at 12000*g* for 20min and resuspended in sodium acetate (0.25M, pH6.5) (8ml) containing 0.05% sodium dodecyl sulphate and 0.001M-EDTA. An equal volume of phenol (saturated with 0.25M-sodium acetate, pH5.0, containing 0.001M-EDTA) was added. The mixture was sonicated for 1min (2 × 30s bursts with 2min cooling between each) with a Branson sonicator. After centrifugation at 26000*g* for 20min the aqueous layer was withdrawn and added to 4vol. of ethanol and 0.2vol. of 4M-NaCl. The phenol layer was washed with an equal volume of sodium acetate/EDTA buffer. After centrifugation the aqueous layer was removed, added to the NaCl/ethanol mixture and left at −18°C overnight. Nucleic acids were collected by centrifugation at 26000*g* for 20min at 4°C. The pellet was resuspended in sodium acetate buffer (0.1M, pH4.6) (3ml). To 1.5ml of this suspension was added 0.01M-sodium periodate in 0.1M-sodium acetate, pH4.6 (0.5ml). The remaining 1.5ml of ribonucleic acid was treated with 0.1M-sodium acetate, pH4.6 (0.5ml). After incubation in the dark at 37°C for 30min the nucleic acid was reprecipitated by the addition of ethanol (4vol.) and 5M-NaCl (0.2vol.) then centrifuged at 26000*g* for 10min. The precipitates were dissolved in 0.1M-sodium acetate, pH4.6 (2ml), containing 0.1M-ethylene glycol, and incubated in the dark at 37°C for a further 10min. Nucleic acid was precipitated as before with ethanol and NaCl. Both pellets were each dissolved in 3.6M-Tris/acetate buffer, pH8.2 (1ml), and incubated at 37°C for 2h. Nucleic acid was again precipitated with ethanol (4vol.). After centrifugation each pellet was dissolved in water (1 ml).

The tRNA-acceptor ability of each sample was assayed with several ^{14}C-labelled amino acids as described for the aminoacylation method *in vitro*.

Assay of isoleucyl-tRNA synthetase

The procedure of Durekovic *et al.* (1973) was used. The reaction mixture consisted of 100μl of a solution of 100mM-Tris/HCl, pH7.0, 10mM-KCl, 1mM-ATP, 10mM-$MgCl_2$, 2mM-dithiothreitol, 12A_{260} units of bulk tRNA, [^{14}C]isoleucine (sp. radioactivity 10mCi/mmol, 10nmol per assay), 0.1μg of enzyme, with or without 10μg of pseudomonic acid. After incubation at 37°C for 20min the reaction was stopped by the addition of 7% trichloroacetic acid (2ml) and the mixture left on ice for 30min. The precipitate was collected on a Whatman GF/C

glass-fibre filter, washed successively with 5% trichloroacetic acid (2 × 10ml) and alcohol (2 × 10ml), dried and counted for radioactivity.

Results

Inhibition of the synthesis of macromolecules

To check whether pseudomonic acid (Fig. 1) produces a similar effect on macromolecular synthesis in *E. coli*, as in *S. aureus*, the rate of synthesis of protein, RNA and DNA was measured in the presence of increasing concentrations of the antibiotic. Inhibition of the assimilation of labelled phenylalanine, uridine and thymidine (e.g. 93%, 80% and 41% respectively at a concentration of 50μg of pseudomonic acid/ml) occurred simultaneously, but protein and RNA synthesis were affected predominantly, as was also the case in *S. aureus* (Hughes & Mellows, 1978). In an attempt to distinguish between primary inhibitory effects on both processes and an inhibitory effect on one process leading to rapid inhibition of the other, protein and RNA synthesis were measured *in vitro*. The activity of highly purified *E. coli* B RNA polymerase was not affected by pseudomonic acid, whereas rifampicin, a powerful inhibitor of the polymerase, almost totally inhibited the enzyme at one-tenth the concentration of pseudomonic acid tested (Table 1). In a ribosomal preparation obtained from *E. coli* MRE 600, the same concentration of pseudomonic acid resulted in only a slight depression of poly (U)-directed formation of polyphenylalanine (Table 2), whereas tetracycline and chloramphenicol, at the same concentration, exerted a much stronger inhibitory effect.

These observations, although indicating that pseudomonic acid preferentially inhibits protein synthesis *in vitro*, do not account for the marked inhibitory effects on protein (and RNA) synthesis *in vivo*.

Effect of pseudomonic acid on protein and RNA synthesis in E. coli B, AS19 RC^{str} and RC^{rel}

In bacteria deprived of an amino acid, stable RNA synthesis is stringently controlled, since this situation leads to the presence of the uncharged cognate tRNA in the acceptor site of the ribosome (Pederson *et al.*, 1973; Haseltine & Block, 1973). The stringent regulatory mechanism is controlled by the *rel* gene (Stent & Brenner, 1961; Ryan & Borek, 1971) and mutation at this locus produces 'relaxedness'. In the relaxed (*rel*$^-$) mutant strain, RNA synthesis proceeds regardless of amino acid starvation, whereas in the stringently controlled strain (*rel*$^+$) RNA synthesis is strongly inhibited. The effect of pseudomonic acid on a pair of *E. coli* B mutant strains (AS19, RC^{str}=*rel*$^+$; AS19, RC^{rel}=*rel*$^-$), isogenic except for the *rel* gene, was examined. On treatment with pseudomonic acid (0.3μg/ml), RNA synthesis proceeds in the relaxed mutant (Fig. 2), but immediately stops in the stringent mutant (Fig. 3). In both strains, protein accumulation was equally inhibited by pseudomonic acid, but the relaxed strain responded after a short delay. This delayed response has previously been observed in relaxed strains starved of an amino acid (Sokawa *et al.*, 1971; Ogilvie *et al.*, 1975*b*). Thus pseudomonic acid inhibits RNA formation only in cells in which RNA synthesis is under optimum control.

Effect of chloramphenicol on pseudomonic acid-treated cells

Certain antibiotics that inhibit protein synthesis at the ribosomal level, e.g. chloramphenicol, allow RNA synthesis to continue unabated (Edlin & Broda, 1968; Cashel, 1969). The stringent control of RNA synthesis in cells deprived of an amino acid is also abolished by chloramphenicol (Edlin & Broda, 1968; Cashel, 1969; Lund & Kjelgaard, 1972). On addition of chloramphenicol simultaneously with pseudomonic acid to *E. coli* MRE 600 cells, RNA synthesis continued unabated for some time before the onset of inhibition (Fig. 4). Thus chloramphenicol removed the pseudomonic acid-induced stringent control mechanism.

Purine nucleotide concentrations

It is well documented that in stringent cells deprived of an amino acid, guanosine 3'-diphosphate 5'-diphosphate (ppGpp) and guanosine 3'-diphos-

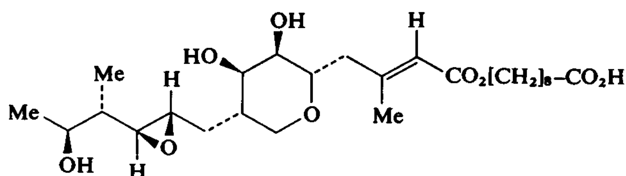


Fig. 1. *Structure of pseudomonic acid*

phate 5′-triphosphate (pppGpp) accumulate, whereas in relaxed mutants no enhancement of these nucleotide concentrations is observed (Cashel & Gallant, 1969; Cashel, 1969; Cashel & Kalbacher, 1970; Haseltine *et al.*, 1972; Ogilvie *et al.*, 1975*b*). In stringent cells the GTP pool size decreases as a consequence of the formation of ppGpp and pppGpp (Edlin & Broda, 1968; Cashel, 1969), but the ATP pool size is decreased to a lesser extent, if at all (Edlin & Broda, 1968). Changes in the pool sizes of these purine nucleotides, measured by [^{32}P]P$_i$ exchange, was compared in the two *E. coli* B strains (AS19 RC^{str} and AS19 RC^{rel}) after the addition of pseudomonic acid. In the stringent strain both ppGpp and pppGpp increased soon after the addition of pseudomonic acid (0.5 μg/ml), and reached maximum values within minutes after addition of the antibiotic (Fig. 5). Decreasing the antibiotic

Table 1. *Effect of pseudomonic acid on RNA synthesis in vitro*

The incorporation of ^{3}H, from [^{3}H]UTP, into RNA by using highly purified *E. coli* B DNA-dependent RNA polymerase and salmon sperm DNA as template, was determined in the presence and absence of the antibiotics as described in the Materials and Methods section. The degree of inhibition was calculated from the ^{3}H incorporation into RNA in the two experiments.

Antibiotic	Amount (μg/ml)	Inhibition (%)
Pseudomonic acid	100	0
Rifampicin	10	97

Table 2. *Effect of pseudomonic acid on polyphenylalanine formation in vitro*

A ribosomal fraction obtained from *E. coli* MRE 600, and from which endogenous mRNA had been destroyed, was incubated as described in the Materials and Methods section, with [^{14}C]phenylalanine (2 μCi) and poly(U). The incorporation of ^{14}C into acid-precipitable material was determined after 30 min and the degree of inhibition calculated from the kinetics of incorporation in antibiotic-treated assays, relative to the untreated controls.

Antibiotic	Amount (μg/ml)	Inhibition (%)
Pseudomonic acid	100	8
Chloramphenicol	100	22
Tetracycline	100	58

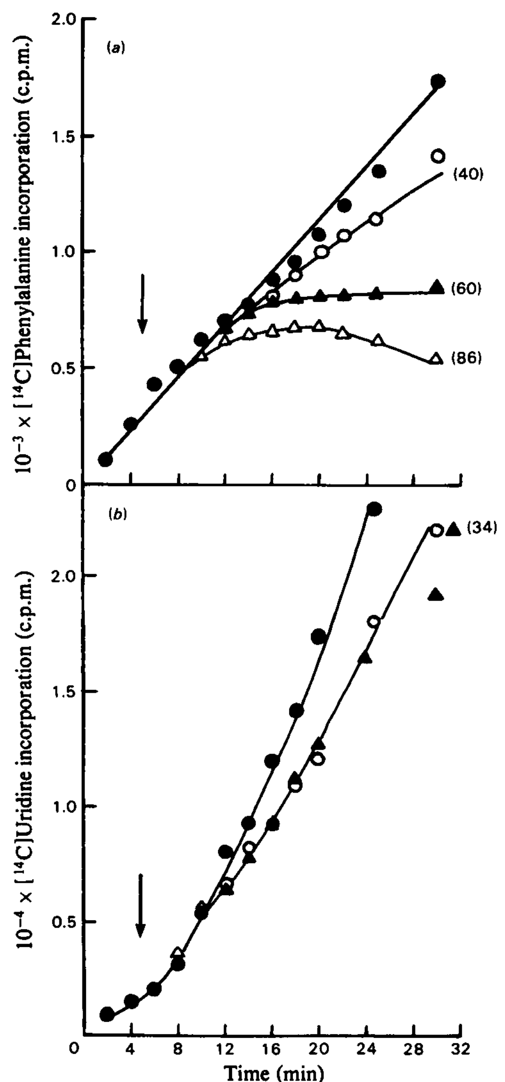


Fig. 2. *Effect of pseudomonic acid on protein (a) and RNA (b) synthesis in E. coli B, AS19 RC^{rel}*

To an exponentially growing culture (A_{600} approx. 0.2) of *E. coli* B, AS19 RC^{rel} was added either [^{14}C]-phenylalanine or [^{14}C]uridine (each at 0.4 μCi/ml). The culture was divided into several equal portions. One subculture served as the control and different concentrations of pseudomonic acid were added to the other subcultures as indicated by the arrows. Samples were withdrawn and the incorporation of ^{14}C into acid-precipitable material was determined as described in the Materials and Methods section. Symbols: ●, control; ○, pseudomonic acid (0.2 μg/ml)-treated; ▲, pseudomonic acid (0.3 μg/ml)-treated; △, pseudomonic acid (0.5 μg/ml)-treated. Values in parentheses refer to the degree of inhibition (%), calculated from the kinetics of incorporation in pseudomonic acid-treated cultures relative to the untreated controls, 20 min after the addition of antibiotic.

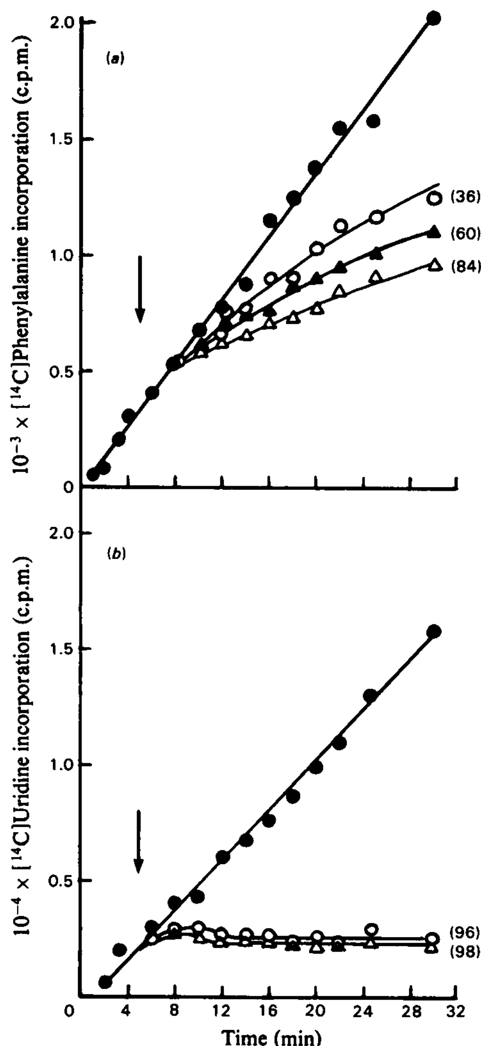


Fig. 3. *Effect of pseudomonic acid on protein (a) and RNA (b) accumulation in E. coli B, AS19 RC^{str}*

Exponentially growing cultures of *E. coli* B, AS19 RC^{str} to which had been added either [^{14}C]phenylalanine (0.4 μCi/ml) or [^{14}C]uridine (0.4 μCi/ml) were treated as described in the legend to Fig. 2. Symbols: ●, control; ○, pseudomonic acid (0.2 μg/ml)-treated; ▲, pseudomonic acid (0.3 μg/ml)-treated; △, pseudomonic acid (0.5 μg/ml)-treated. Values in parentheses refer to the degree of inhibition (%), calculated from the kinetics of incorporation in pseudomonic acid-treated cultures to the untreated controls, 20 min after the addition of the antibiotic.

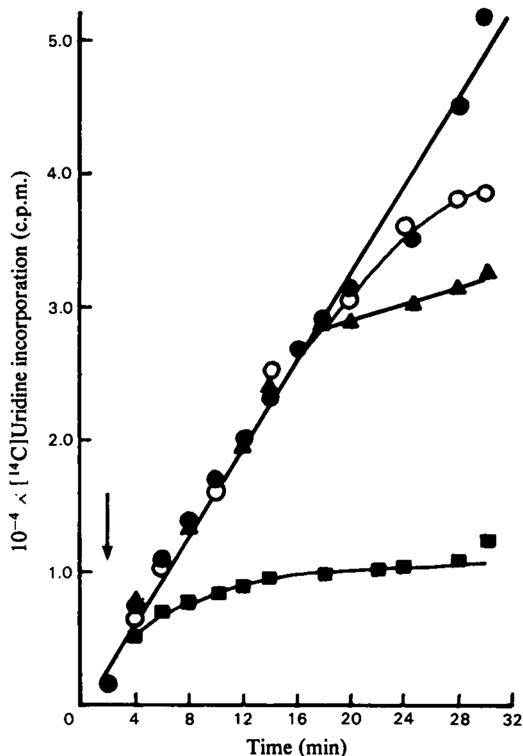
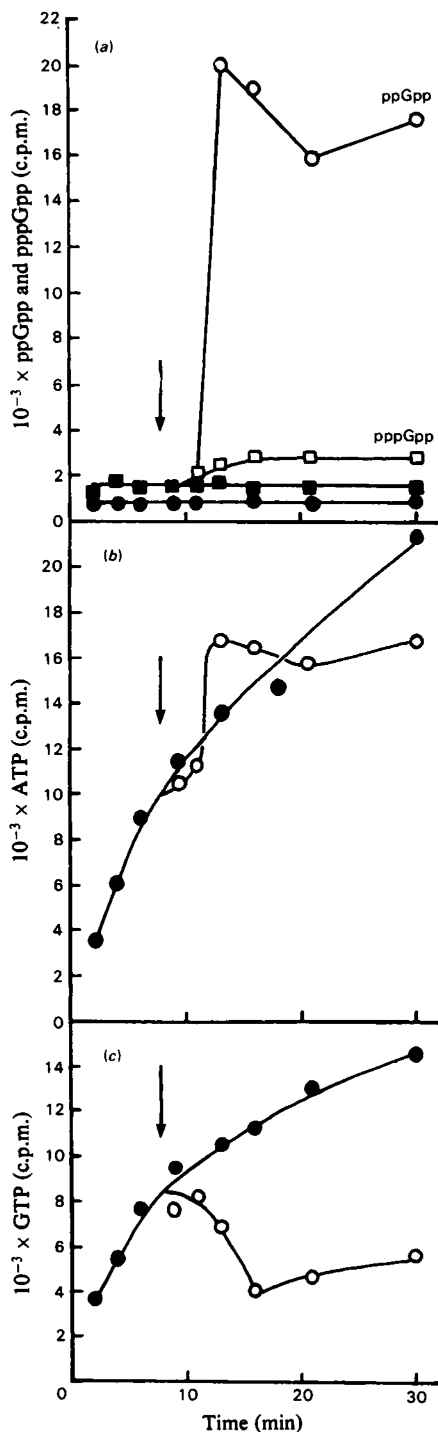


Fig. 4. *Effect of pseudomonic acid and chloramphenicol on RNA synthesis in E. coli MRE 600*

[^{14}C]Uridine (0.4 μCi/ml) was added to an exponentially growing culture. The culture was divided into four equal portions and chloramphenicol (500 μg/ml), chloramphenicol (500 μg/ml) plus pseudomonic acid (50 μg/ml) and pseudomonic acid (50 μg/ml) were added to each of three cultures as indicated by the arrow. The fourth culture served as a control. Samples (0.5 ml) were withdrawn and the acid-precipitated radioactivity determined as described in the Materials and Methods section. Symbols: ●, control; ○, chloramphenicol-treated; ▲, chloramphenicol-plus-pseudomonic acid-treated; ■, pseudomonic acid-treated.

concentration prolonged the onset of the increase in ppGpp and pppGpp concentrations, but the effect was equally marked down to a concentration of 0.1 μg of pseudomonic acid/ml (G. Mellows & A. Slaney, unpublished work). The ATP pool size also continued to increase after addition of pseudomonic acid, confirming the absence of a primary effect of pseudomonic acid on oxidative phosphorylation (Hughes & Mellows, 1978). The GTP pool size showed a rapid decrease. The ratio of ppGpp/pppGpp (about 14:1) is somewhat higher than that (3:1) observed in *E. coli* during amino acid starvation (Cashel & Gallant, 1969). In the relaxed mutant of *E. coli* B, at a pseudomonic acid concentration of 0.5 μg/ml, both GTP and ATP concentrations continued to increase after antibiotic addition, whereas



ppGpp maintained a low basal value, showing no increase after addition of antibiotic (no pppGpp was observed). The concentrations of ATP, GTP, ppGpp and pppGpp, after pseudomonic acid treatment, therefore follow those observed during deprivation of an amino acid. A similar effect of nucleotide concentrations was observed in *B. subtilis* treated with granaticin, an antibiotic that inhibits leucyl-tRNA synthetase and also causes leucine auxotrophy (Ogilvie *et al.*, 1975*a*,*b*).

Pseudomonic acid-induced isoleucine auxotrophy of E. coli cells

A preliminary study showed that inhibition of *E. coli* MRE 600 cultures growing in a glucose/salt medium can be alleviated by the addition of a mixture of 20 amino acids to the medium. To ascertain whether a single or more than one amino acid produced the relieving effect, various mixtures of amino acids, from which a single amino acid was eliminated from the complete mixture of 20 essential amino acids, were added to the growth medium. Isoleucine was found to be obligatory and specific for counteracting the inhibitory effect of pseudomonic acid (Fig. 6). At a concentration of 25 μg of pseudomonic acid/ml, isoleucine alone (1 mM) was able to fully reverse the effects even up to 7 h after addition of the antibiotic. At the same concentration, isoleucine provides almost total relief of pseudomonic acid-induced inhibition of protein synthesis (Fig. 7). Inhibition of RNA synthesis, however, is only partly relieved by 1 mM-isoleucine, but the onset of inhibition occurs after a lag period after antibiotic addition.

Derepression of isoleucine biosynthetic enzymes

The terminal steps of isoleucine, valine and leucine biosynthesis share common enzymes (Fig. 8), which

Fig. 5. *ppGpp and pppGpp nucleotide accumulation in E. coli B, AS19 RC^{str} treated with pseudomonic acid*
[^{32}P]P$_i$ (65 μCi/ml) was added to an exponentially growing culture (A_{600} approx. 0.2) in Tris-buffered glucose/salt medium, containing phosphate at a concentration of 0.2 mM. Two samples (1 ml) were removed and incubated separately. One served as a control, and pseudomonic acid (0.5 μg/ml) was added to the other at the time indicated by the arrows. Samples (50 μl) were removed and the ppGpp, pppGpp, ATP and GTP nucleotide concentrations determined as described in the Materials and Methods section. (*a*) ^{32}P radioactivity appearing in ppGpp and pppGpp; (*b*) ^{32}P radioactivity appearing in ATP; (*c*) the labelling of GTP. ●, ■, Control; ○, □, nucleotide concentrations after onset of pseudomonic acid inhibition.

are regulated by the cognate aminoacylated tRNA species through a complex multivalent repression mechanism (Freundlich *et al.*, 1962; Jackson *et al.*, 1974; Calhoun & Hatfield, 1975; Umbarger, 1975; Calhoun, 1976). The *ilv* ABCDE gene cluster is specific for the terminal steps in isoleucine and valine synthesis and has three different recognition sites for regulation. Only the '*ilv* ADE' gene cluster, respectively controlling the synthesis of threonine deaminase, dihydroxyacid dehydratase and transaminase B, responds to excess isoleucine, leucine and valine (Dwyer & Umbarger, 1968). The '*ilv* B' gene, controlling acetohydroxyacid synthetase, responds only to a multivalent repression signal from valine and leucine (Dwyer & Umbarger, 1968; Blatt *et al.*,

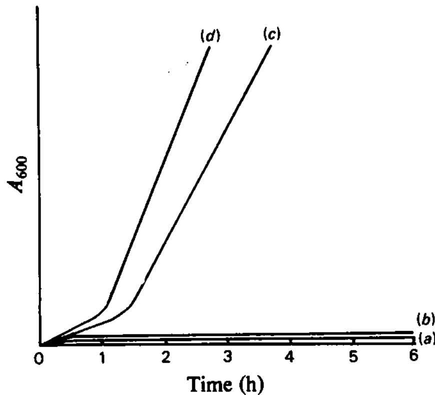


Fig. 6. *Isoleucine auxotrophy of E. coli MRE 600 treated with pseudomonic acid*

A freshly inoculated culture growing in minimal media was equally divided into four portions. After the following additions, the growth of the cultures was continually monitored at 600nm by using a biophotometer. (*a*) pseudomonic acid (25 μg/ml)-treated; (*b*) pseudomonic acid (25 μg/ml) plus a mixture of 19 amino acids (alanine, arginine, asparagine, aspartate, glutamate, glutamine, glycine, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine; each at a final concentration of 1 mM); (*c*) pseudomonic acid (25 μg/ml) plus isoleucine (final concentration of 1 mM); (*d*) control (no additions).

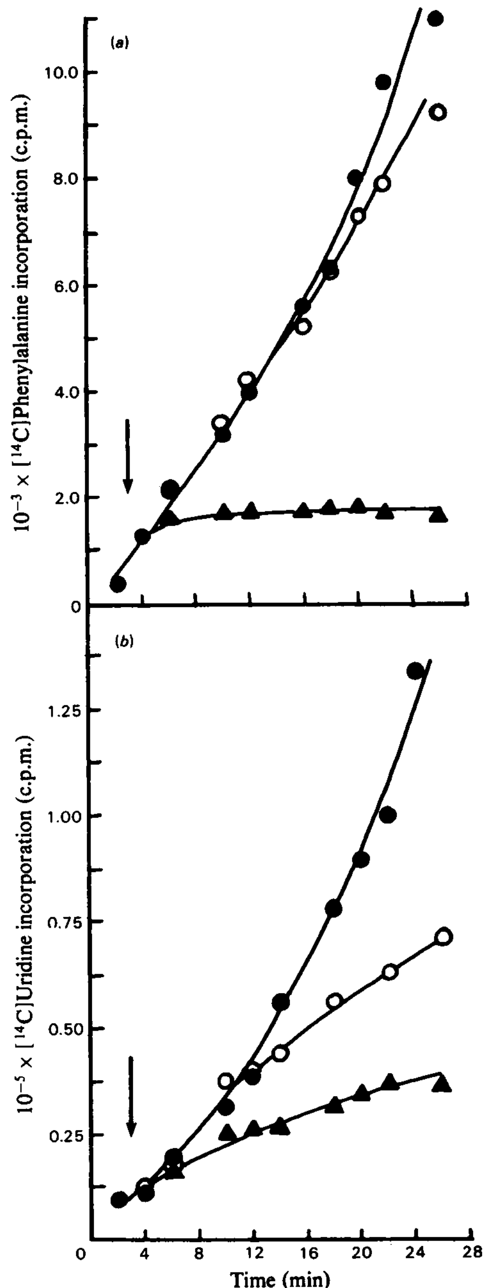


Fig. 7. *Effect of isoleucine on (a) protein and (b) RNA synthesis in E. coli MRE 600 treated with pseudomonic acid*

An exponentially growing culture in minimal media was divided into several equal portions and either [^{14}C]phenylalanine (0.4 μCi/ml) or [^{14}C]uridine (0.4 μCi/ml) was added. Isoleucine (1 mM), pseudomonic acid (25 μg/ml) and isoleucine (1 mM) plus pseudomonic acid (25 μg/ml) were added simultaneously at the time indicated by the arrows. Samples were withdrawn and the incorporation of ^{14}C into acid-precipitable material was determined as described in the Materials and Methods section. Symbols: ●, control; ▲, pseudomonic acid-treated; ○, pseudomonic acid plus isoleucine.

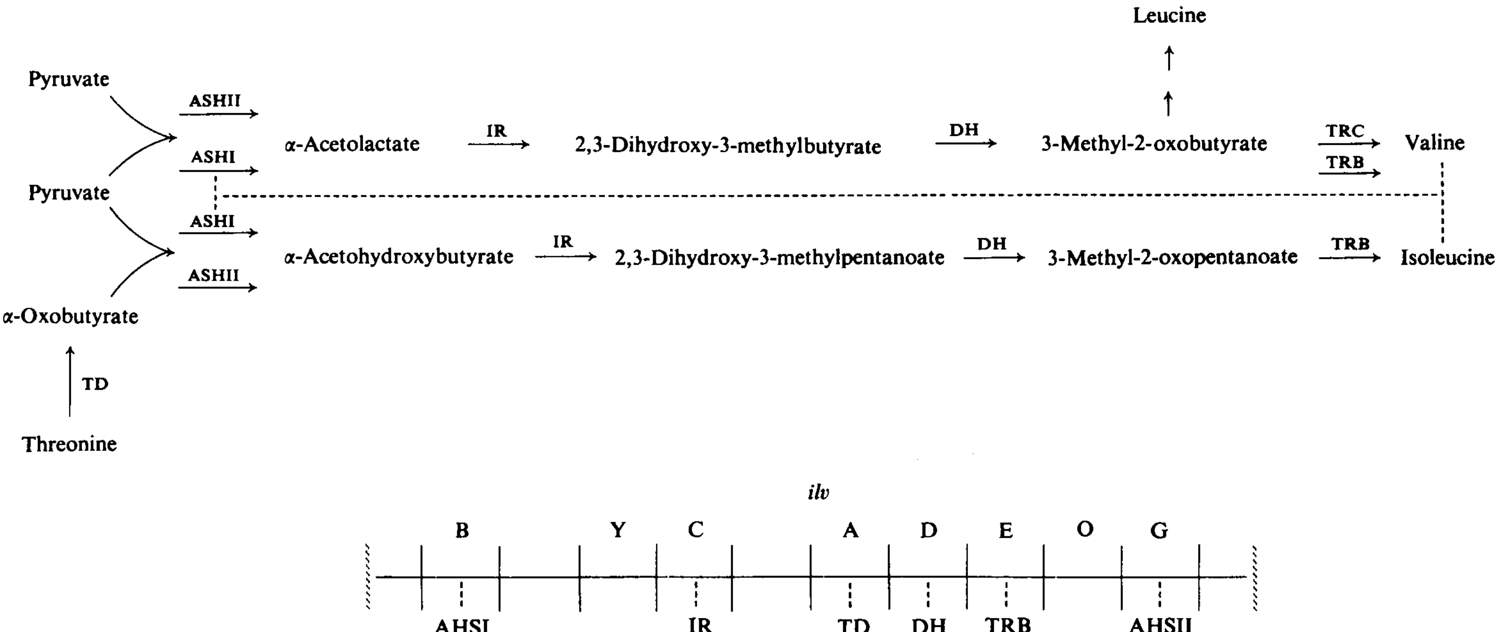


Fig. 8. *Biosynthesis of isoleucine, valine and leucine*

Enzymes catalysing transformations are abbreviated: TD, threonine deaminase; ASHI, end-product-inhibited acetohydroxyacid synthetase; ASHII, acetohydroxyacid synthetase (not end-product-inhibited); IR, acetohydroxyacid isomeroreductase; DH, dihydroxyacid dehydratase; TRB, transaminase B; TRC, transaminase C. Gene *ilvY* specifies an element required for induction of gene *ilvC* by acetohydroxyacids. Gene *ilvO* is a locus that, after mutation from the wild-type state, produces a regulatory element required for gene-*ilvG* expression and enhanced gene-*ilvEDA* expression (Smith & Umbarger, 1977).

Table 3. *Derepression of threonine deaminase, transaminase B and acetohydroxyacid synthetase in E. coli MRE 600 treated with pseudomonic acid*

Two 1 litre cultures, growing in mineral salt/glucose medium, were treated with pseudomonic acid (25 μg/ml), 1 h post inoculation (A_{578} 0.03), and another pair of 1 litre cultures served as a control. After a further 5 h the cells were harvested from each pair of cultures by centrifugation and the respective concentrations of threonine deaminase, transaminase B and acetohydroxyacid synthetase determined as described in the Materials and Methods section.

Enzyme	Specific activity: Control	Specific activity: Pseudomonic acid-treated	Enzyme activity enhancement
Threonine deaminase (μmol of oxobutyrate formed/min per mg of protein)	0.0053	0.0165	3.1
Transaminase B (μmol of [^{14}C]isoleucine disappearing/min per mg of protein)	0.030	0.101	3.4
Acetohydroxyacid synthetase (μmol of acetolactate formed/min per mg of protein)	0.083	0.120	1.4

1972). Consequently, if pseudomonic acid interferes with the supply of isoleucine leading to decreased concentrations of charged tRNA^{Ile}, derepression of the former three enzymes, but not acetohydroxyacid synthetase, nor acetohydroxyacid isomeroreductase, which is controlled by substrate induction rather than end-product repression (Arfin *et al.*, 1969), would be expected. Of three of the isoleucine biosynthetic enzymes assayed in pseudomonic acid-treated *E. coli* MRE 600 cells both threonine deaminase and transaminase B were significantly derepressed, whereas acetohydroxyacid synthetase activity was only slightly enhanced (Table 3). The above observations confirm that pseudomonic acid inhibits protein synthesis by causing intracellular starvation of isoleucine.

Degree of aminoacylation of tRNA^{Ile} in pseudomonic acid-treated cells

At this stage two possible causes of pseudomonic acid-induced inhibition of protein synthesis were considered: (1) inhibition of aminoacylation of tRNA^{Ile} by isoleucyl-tRNA synthetase; (2) interference with the intracellular supply of isoleucine. The degree of aminoacylation of several tRNA species, including tRNA^{Ile}, in normally growing and pseudomonic acid-treated cells was determined. The aminoacylation of tRNA^{Ile} was specifically and drastically inhibited in pseudomonic acid-treated *E. coli* MRE 600 cells (Table 4). Of the other amino acids assayed, the antibiotic had no effect on the respective aminoacylation processes with the exception of the activation of phenylalanine, which was inhibited only slightly.

Table 4. *Effect of pseudomonic acid on aminoacylation of different tRNA species in vitro*

By using a 150000 *g* supernatant from *E. coli* MRE 600 as a source of tRNA synthetases, the aminoacylation of different tRNA species with the corresponding U-^{14}C-labelled amino acid was determined in the presence and absence of pseudomonic acid (100 μg/ml), as described in the Materials and Methods section. The degree of inhibition of aminoacylation was calculated from the incorporation of ^{14}C into trichloroacetic acid-precipitable material in the presence and absence of pseudomonic acid.

Amino acid	Inhibition of aminoacylation of tRNA (%)
Glycine	0
Leucine	0
Valine	0
Histidine	0
Phenylalanine	10
Tyrosine	0
Tryptophan	0
Isoleucine	90

This result was further substantiated by the observation that tRNA^{Ile}, isolated from pseudomonic acid-treated cells, was only partly charged, whereas tRNA^{Ile} in untreated cells remained fully charged (Table 5). The three other tRNA species assayed remained fully charged in both antibiotic-treated and untreated cells.

Inhibition of isoleucyl-tRNA synthetase in vivo

In the presence of excess of isoleucine and tRNA^{Ile} (as bulk tRNA from *E. coli* strain W), 100 nM-pseudomonic acid inhibits the aminoacylation of electrophoretically pure *E. coli* B isoleucyl-tRNA synthetase at a concentration of 1 nM by 81 %.

Discussion

Pseudomonic acid causes reversible suppression of growth and protein and RNA synthesis in *E. coli* MRE 600. The antibiotic had no effect on purified DNA-dependent RNA polymerase from *E. coli* B, and only a slight inhibitory effect on the poly (U)-directed formation of polyphenylalanine in a ribosomal preparation from *E. coli* MRE 600 *in vitro*.

Table 5. *Effect of pseudomonic acid on the aminoacylation of tRNA in E. coli MRE 600 in vivo*

An exponentially growing culture in minimal media (1000 ml, A_{260} approx. 0.5) was equally divided into two portions. One culture was treated with pseudomonic acid (25 µg/ml) and the other used as a control. After 10 min, growth was stopped by the addition, to each culture, of 55% trichloroacetic acid (50 ml) prewarmed to 37°C. tRNA was separately extracted from each culture, and equally divided into two portions. One portion was treated with periodate. The aminoacyl-tRNA in both extracts was then hydrolysed. Finally, the intact tRNA in both extracts was charged with the respective ^{14}C-labelled amino acid. The degree of aminoacylation *in vivo* was calculated from the tRNA-acceptor activity resistant to periodate oxidation.

	Charged tRNA (%)	
Amino acid	Control	Pseudomonic acid-treated
Isoleucine	100	60
Leucine	100	100
Histidine	100	100
Phenylalanine	100	100

Although these results indicate that the primary target of pseudomonic acid is one (or more) of the steps involved in protein synthesis, the latter observation suggests that inhibition is not a consequence of a direct effect on ribosomal function. These observations also suggest that the simultaneous inhibition of both protein and RNA accumulation *in vivo* must therefore be a consequence of the pseudomonic acid-induced stringent control of RNA synthesis, resulting from amino acid starvation (Neidhardt, 1966; Edlin & Broda, 1968; Cashel, 1969). This is confirmed by results obtained with a stringent and relaxed pair of *E. coli* B mutants (isogenic except for the *rel* gene), which rules out a direct effect *in vivo* of pseudomonic acid on RNA synthesis. In the same experiments it was shown that the ATP pool size increases in pseudomonic acid-treated cells, confirming the previous observation in *S. aureus* (Hughes & Mellows, 1978) that oxidative phosphorylation and energy processes dependent on it are not affected.

Addition of excess isoleucine reverses the inhibition caused by low concentrations of the antibiotic. Pseudomonic acid caused a significant derepression of threonine deaminase and transaminase B, but not dihydroxyacid dehydratase (isoleucine biosynthetic enzymes) confirming the auxotrophy for isoleucine, since the former two enzymes are regulated by fully aminoacylated-tRNA^{Ile} (Szentirmai & Umbarger, 1968; Iaccarino & Berg, 1971; Blatt & Umbarger, 1972) in addition to leucyl-tRNA^{Leu} (Alexander *et al.*, 1971; Umbarger, 1975) and valyl-tRNA^{Val} (Eidlic & Neidhardt, 1965). A similar derepression of the three specific leucine enzymes by defective aminoacyl-tRNA synthetases in bacterial mutants has previously been observed (Alexander *et al.*, 1971; Umbarger, 1975). It was further demonstrated that the antibiotic markedly decreases the aminoacylation of tRNA^{Ile} in pseudomonic acid-treated cells and also inactivates purified *E. coli* B isoleucyl-tRNA synthetase. That a decrease in aminoacylation of tRNA^{Ile} could be a consequence of the inhibition of protein synthesis is ruled out by the specificity of the antibiotic for isoleucyl-tRNA synthetase and the observations of Cassio & Mathien (1974) and Glazier & Schlessinger (1974), who found that aminoacylation increases during inhibition of protein synthesis.

Of the other tRNA synthetases assayed in a crude cell-free preparation from *E. coli* MRE 600, pseudomonic acid exerted only a slight inhibitory effect on the function of phenylalanyl-tRNA synthetase. Inhibition of poly(U)-directed synthesis of polyphenylalanine is presumably a consequence of this. Thus the primary action of pseudomonic acid, leading to bacteriostasis in *E. coli* cells is the inhibition of isoleucyl-tRNA synthetase. More recently we have shown that pseudomonic acid is a competitive inhibitor, with respect to isoleucine, of isoleucyl-tRNA synthetase. The antibiotic specifically inhibits the formation of the enzyme–aminoacyladenylate complex and not the subsequent transfer step of the isoleucyl moiety to tRNA^{Ile} (J. Hughes & G. Mellows, unpublished work). On structural grounds it therefore seems possible that the terminal residue of the epoxide chain, having the same carbon skeleton as L-isoleucine, competes with the amino acid for the L-isoleucine-binding site on isoleucyl-tRNA synthetase, which is known to contain two hydrophobic domains respectively accomodating the methyl and ethyl groups of the amino acid (Fig. 9) (Holler *et al.*, 1973; Flossdorf *et al.*, 1976).

Furanomycin, a structural analogue of isoleucine, from *Streptomyces* L-803, has also been shown to be a competitive antagonist of isoleucyl-tRNA synthetase in *E. coli* and *Bacillus subtilis*, as well as in a rat liver homogenate (Tanaka *et al.*, 1969). Pseudomonic acid also competitively inhibits the aminoacylation of tRNA^{Ile} in a rat liver preparation, but the inhibitory effect is weak compared with that observed with the bacterial enzyme (J. Hughes & G. Mellows, unpublished work). Five other antibiotics, borrelidin (Hunter *et al.*, 1966; Nass & Hasenback, 1970), granaticin (Ogilvie *et al.*, 1975*a*,*b*), ferramidochloromycin (Shimi *et al.*, 1975) and ASK-753 (Shimi & Shoukry, 1976), and ochratoxin A (Konrad & Roschenthaler, 1977) have also recently been shown to specifically inhibit threonyl-tRNA synthetase, leucyl-tRNA synthetase, lysyl-tRNA synthetase and

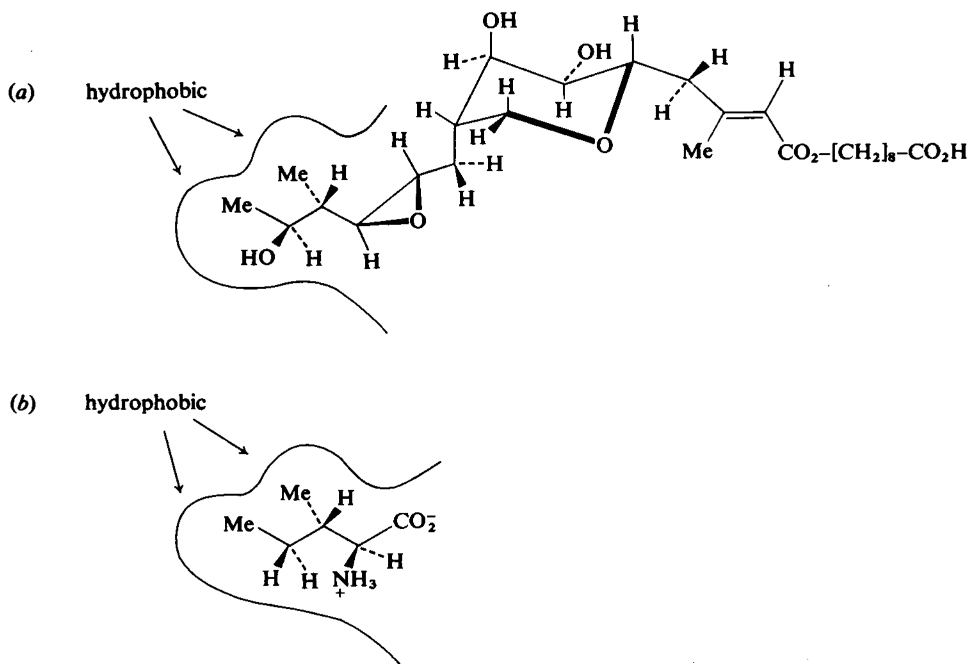


Fig. 9. *Illustration of hydrophobic-binding domains on isoleucyl-tRNA synthetase accommodating (a) terminus of epoxide side chain of pseudomonic acid and (b) aliphatic side chain of* L-*isoleucine*

phenylalanyl-tRNA synthetase respectively. Several amino acid analogues have also been shown to inhibit the corresponding tRNA synthetases; these are thiaisoleucine (Szentirmai *et al.*, 1968; isoleucine analogue), indolmycin (Werner *et al.*, 1976; tryptophan analogue) and *N*-benzyl-2-phenylethylamine derivatives, e.g. *N*-benzyl-D-amphetamine (Anderson & Santi, 1976; phenylalanine analogues). Also several mutants with altered tRNA synthetases, which are auxotrophic for their cognate amino acids, have been described (Folk & Berg, 1970*a*,*b*; Iaccarino & Berg, 1971; Straus & Ames, 1973).

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