MODE OF ACTION OF THE ANTIBIOTIC, PSEUDOMONIC ACID

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ABSTRACT

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Pseudomonic acid is a recently discovered, narrow spectrum antibiotic produced by the bacterium, *Pseudomonas fluorescens* (NCIB 10586). Initial studies on the mode of action of the drug in bacteria, in which the effect of the substance on the cellular metabolic processes was studied, were conducted with a highly sensitive strain of *Staphylococcus aureus*. *In vivo*, the drug had a marked effect on both protein and RNA synthesis, whereas DNA and cell wall synthesis were affected considerably less. Oxidative phosphorylation was not inhibited. Studies were continued both *in vitro* and *in vivo* using a number of *Escherichia coli* strains.

The antibiotic had no effect on the function of highly purified DNA-dependent RNA polymerase from *E. coli* B and showed only a weak inhibitory effect on a poly U directed, poly Phe forming ribosomal preparation. Pseudomonic acid caused only partial inhibition of RNA synthesis, but marked inhibition of protein synthesis in a strain of *E. coli* whose RNA synthesis was not stringently controlled. In cells whose RNA synthesis was under stringent control, pseudomonic acid treatment caused a significant increase in the levels of ppGpp, pppGpp and ATP, whereas the GTP pool size decreased. These observations suggested 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 twenty common amino acids, only isoleucine reversed the *in vivo* inhibitory effect. The antibiotic was found to be a powerful inhibitor of isoleucyl-tRNA synthetase both *in vivo* and *in vitro*. A weak inhibitory effect on phenylalanyl-tRNA synthetase was also observed, which presumably accounted for the weak effect on *in vitro* poly Phe formation.

Using electrophoretically pure *E. coli* B isoleucyl-tRNA synthetase, steady state kinetic studies showed that the drug behaves as a competitive inhibitor with respect to isoleucine, inhibiting the formation of the enzyme-aminoacyl-adenylate complex, but not the subsequent transfer of isoleucine to tRNA. Using a crude preparation of aminoacyl-tRNA synthetases, prepared from rat liver, it was demonstrated that pseudomonic acid also inhibited the charging of tRNA(Ile) in eukaryotic cells, but the effect was less marked than that on the prokaryotic isoleucyl-tRNA synthetase. Equilibrium dialysis studies gave a binding ratio of isoleucyl-tRNA synthetase to pseudomonic acid of 1.0:0.85. It remains to be established whether the antibiotic binds at the isoleucine binding site or at another site on the enzyme. The significance of these observations is discussed.

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INTRODUCTION.

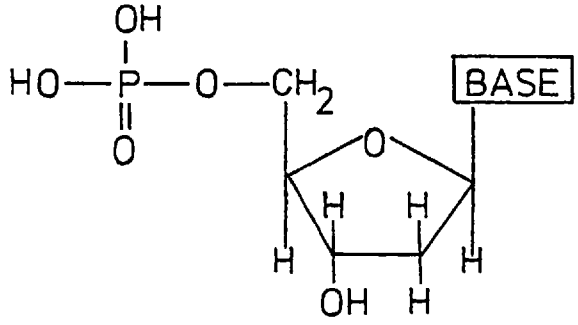
1. PROTEIN SYNTHESIS IN PROKARYOTIC ORGANISMS.

1.1. Introduction.

In recent years a much greater understanding of the function of proteins has been realised. They fulfil diverse biochemical roles and vary greatly in amino acid sequence, structure and molecular weight. The basic building blocks of proteins are the 20 common amino acids, although some proteins contain modified amino acid residues. The synthesis of these molecules is one of the major processes performed by "living" systems. An elaborate sequence of events is necessary to ensure that the correct sequence of amino acids is joined together. The sequence of amino acid residues in a given protein is all-important in determining its function. The many facets of protein synthesis make it vulnerable to the action of a wide variety of metabolic inhibitors. Differences found between the eukaryotic and prokaryotic protein synthesising machinery have been exploited very successfully in chemotherapy. Initial studies on the mode of action of pseudomonic acid indicated that it too belonged to the class of inhibitors which arrest protein synthesis in prokaryotic organisms. The following account presents an up-to-date general review of our understanding of the mechanism of protein synthesis and discusses the roles of the various ribosomal proteins and factors involved in the translation of the genetic code to a specific sequence of amino acids in a protein.

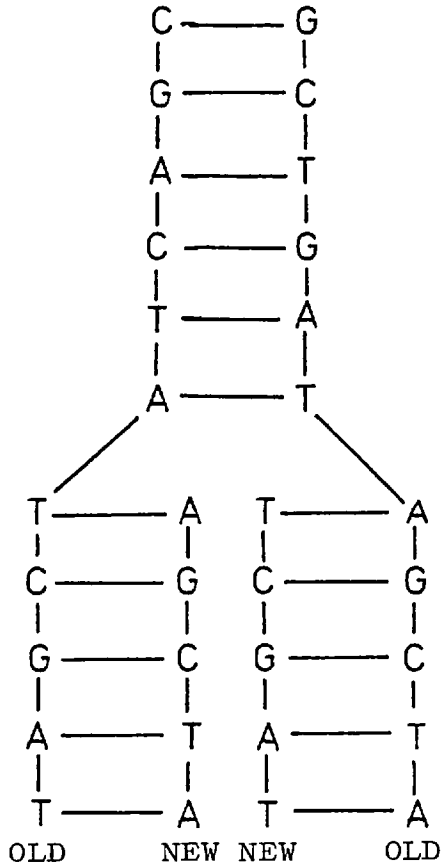
1.2. Synthesis of messenger, ribosomal and transfer RNAs.

The deoxyribonucleic acid (DNA) in cells is composed of units of deoxyribonucleotides. Each nucleotide contains three characteristic components: a) a nitrogenous heterocyclic base, which is either a pyrimidine or a purine derivative b) a pentose c) a molecule of phosphoric acid.



The two common pyrimidines found in DNA are thymidine and cytosine and the two common purine derivatives are adenine and guanine. Successive nucleotide units are covalently linked in identical fashion by phosphodiester bridges formed between the 3'-hydroxyl group of one nucleotide and the 5'-hydroxyl group on the next. Normally, DNA isolated from different organisms and viruses has two strands in a complementary double helical arrangement. The two strands are anti-parallel i.e. one has its linkages 3'→ 5' and the other 5'→ 3'. They are coiled in helical fashion around the same axis thus forming a double helix. As far as genetic function is concerned, the most important feature of DNA structure is the fact that the bases of one strand are paired in the same planes with the bases of the other strand. The pairing is specific for the base pairs Adenine (A)— Thymidine (T) and Guanine (G) — Cytosine (C). The three main stages of information transfer are a) replication of DNA in cell division b) transcription of DNA into ribonucleic acid (RNA)

FIGURE 1. A DIAGRAMMATIC REPRESENTATION OF THE PRINCIPLE OF DNA REPLICATION.



c) translation of RNA codons into protein. Replication of DNA can be considered in a simplified form to occur as shown in Figure 1. By virtue of the fidelity of base pairing, each of the two new cells formed receives an identical set of genetic information as determined by the base sequences in the DNA. Ribonucleic acid is similar in primary structure to DNA except that uridine (U) is substituted for thymidine. The sugar moiety in RNA has both a 2'- and a 3'-hydroxyl group. The enzyme responsible for synthesising RNA complementary to one strand of DNA is called DNA-directed RNA polymerase. (Ribonucleic acid itself can be the template, as for example in the replication of some viruses when RNA-dependent RNA synthesis is said to occur). The polymerase adds mono-nucleotide units to the 3'-hydroxyl end of the RNA chain and thus builds RNA in the 5'→ 3' direction, anti-parallel to the DNA strand used as template. In this way, the single-stranded RNA contains the corresponding sequence of bases and hence the same genetic message as the other DNA strand. The base sequence of RNA is read in triplets. Of the sixty four triplets (Table 1) available (provided by the four bases), each of sixty one codes for a specific amino acid, while three are common termination codons. In this way, the sequence of bases in the DNA conveys the sequence of amino acids in the proteins. Ribosomal (r) and transfer (t) RNAs are also synthesised by DNA-dependent RNA polymerase. The specific sequence of bases in these other two classes of RNA is also carried by the DNA.

TABLE 1. THE GENETIC CODE.

FIRST LETTER	SECOND LETTER: U	SECOND LETTER: C	SECOND LETTER: A	SECOND LETTER: G	THIRD LETTER
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA Term	UGA Term	A
	UUG Leu	UCG Ser	UAG Term	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

1.3. Synthesis of charged tRNA molecules.

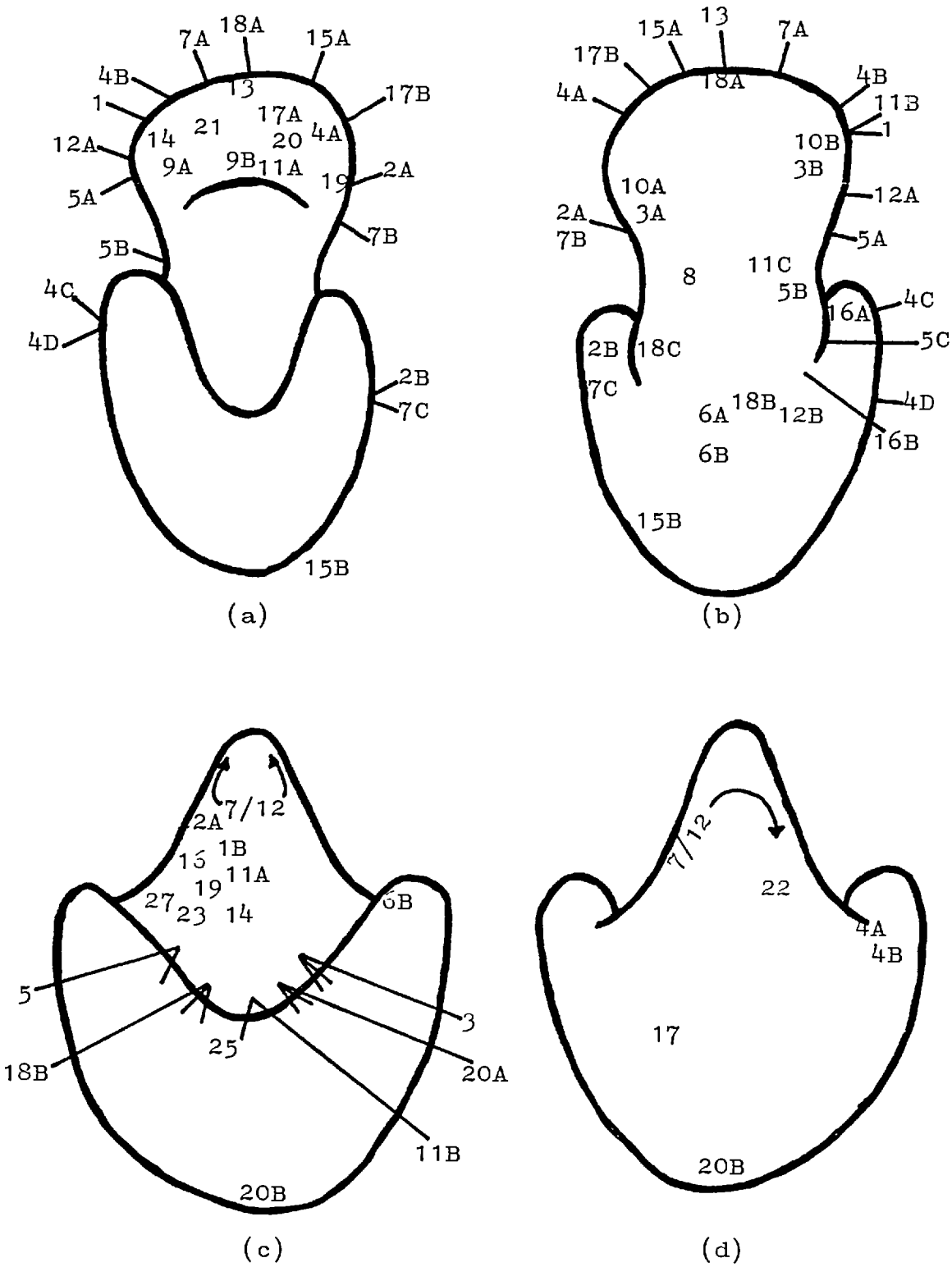
Prior to the commencement of protein synthesis, it is necessary for each of the 20 different amino acids to be esterified to their corresponding tRNAs via covalent bond formation with the 3'-hydroxyl group of the ribose residue. The formation of the aminoacylated tRNAs is catalysed by a class of enzymes called aminoacyl-tRNA synthetases. The reaction produces an aminoacylated tRNA in which the tRNA acts as a vehicle for transporting the amino acid to the ribosomal complex for inclusion in the growing peptide chain. Each amino acid is highly specific for its cognate tRNA and the aminoacylation reaction for each amino acid is catalysed by the corresponding aminoacyl-tRNA synthetase. The overall reaction can be summarised as follows:

$$\alpha\alpha + \text{tRNA} + \text{ATP} \xrightarrow{Mg^{++}} \text{aminoacyl-tRNA} + \text{AMP} + PP_i$$

This class of enzymes will be discussed more fully in the second part of this review.

The remaining sequence of events occurs on the intracellular organelles called ribosomes. In prokaryotes, these organelles have a particle weight of about 2.7 megadaltons and a sedimentation coefficient of 70. Under certain conditions they can be dissociated into 50S and 30S subunits. The 50S subunit contains two RNA components having sedimentation coefficients of 23S and 5S and 34 proteins (designated L1 - L34) (Fig. 2). Each 30S subunit contains one molecule of 16S RNA and 21 proteins (designated S1 - S21) (Fig. 2). One protein (S20) from the small subunit and

FIGURE 2. THE DISTRIBUTION OF ANTIGENIC SITES FOR THE PROTEINS OF THE 30S SUBUNIT (a,b) AND FOR THE 50S SUBUNIT (c,d).



Redrawn from Kurland (1977).

one protein (L26) from the large subunit are identical and the total number of proteins within the 70S ribosome is 54. Fifteen proteins from the 30S subunit and fifteen from the 50S subunit have been sequenced. (Stöffler & Wittmann, 1977).

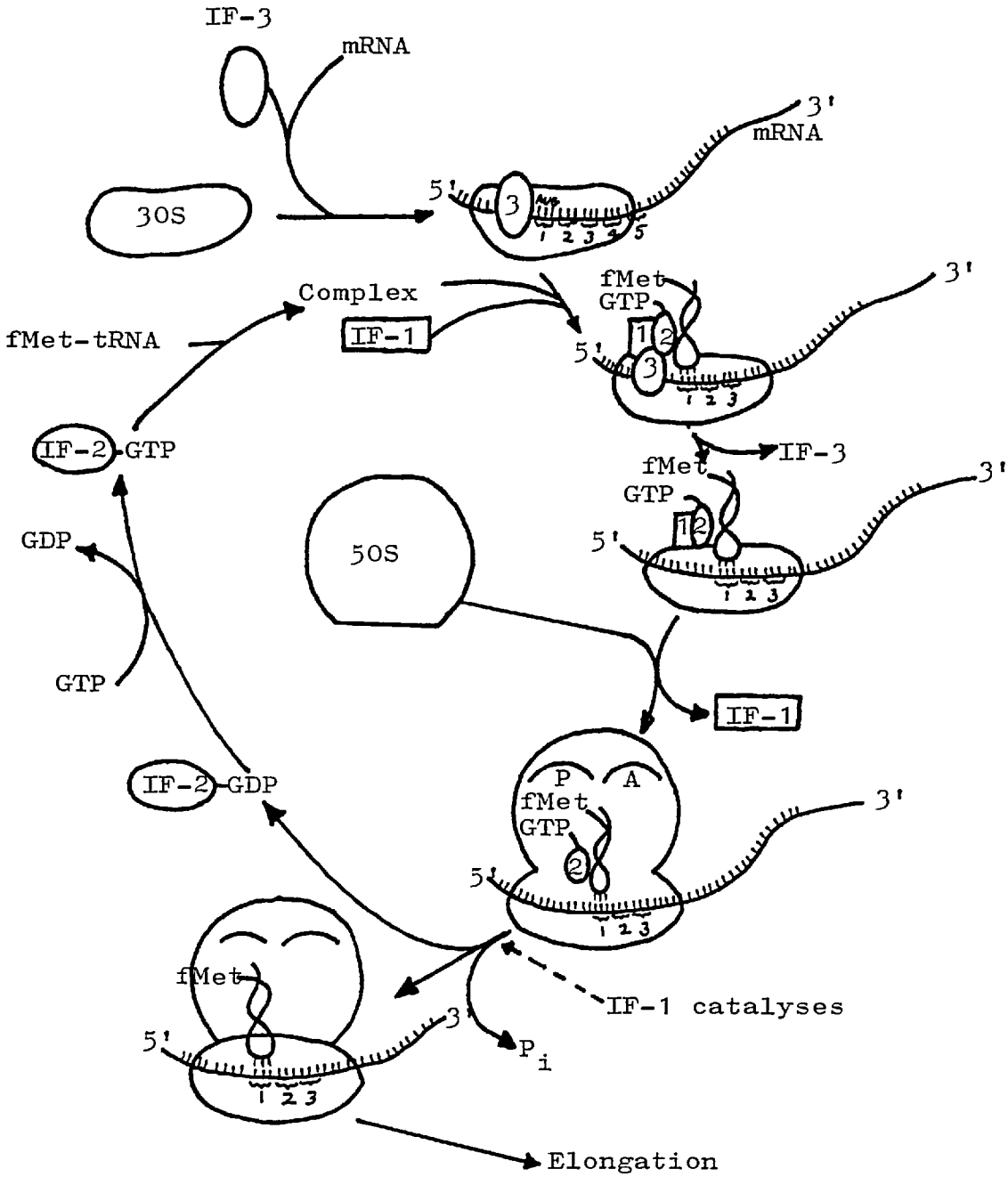
1.4. Initiation of protein synthesis.

The first codon (triplet of bases) to be translated on the mRNA is the initiation codon (AUG) which codes for methionine. E. coli contains two methionine-specific tRNAs, both of which can be charged to give methionyl-tRNA. However, the methionine residue of one can be formylated by transfer of the formyl residue from formyltetrahydrofolic acid. The methionine of the other cannot (Ochoa & Mazumder, 1974). The formylmethionyl-$tRNA_F$ (fmet-$tRNA_F$) must have some unique features which distinguish it from other tRNAs since it is not bound to the elongation factor Tu (EF-Tu) in contrast to other aminoacyl-tRNAs. It does form, however, an initiation complex, and is recognised by the initiation factors (see later). Unlike the methionyl-$tRNA_M$, it can also interact with the GUG codon.

Dissociation of 70S ribosomes into subunits is an obligatory step for initiation of translation on natural mRNA templates. With synthetic polynucleotides, however, undissociated ribosomes may be able to "initiate" protein synthesis (Kurland, 1972). During the initiation process (Fig. 3) the following events are believed to occur, although the exact sequence is not known for certain. Three protein factors (IF1-3) that can interact with ribosomes are involved in the formation of the initiation complex. Initiation

factor 3 (IF-3) (molecular weight 21,000) binds to a 30S subunit. There is much speculation as to whether IF-3 actively promotes dissociation of the 70S ribosomes or merely acts as an "anti-association" factor. The roles of the other factors in this action are not clear. Cross-linking IF-3 to ribosomal proteins shows that three of the seven with which it can interact, S11, S12 and S14, (Fig. 2) are located at the interface between the two ribosomal subunits (Traut et al., 1974). This may well be important in explaining the mechanism of IF-3 induced dissociation of the ribosome. At this stage, mRNA can interact with the 30S subunit. At this point, IF-1 (molecular weight 9,000, a small protein, 90 amino acids long) may bind to the 30S.IF-3.mRNA complex. Its exact role is not clear. It has been suggested that it may stabilise IF-2 binding (Fakunding & Hershey, 1973) and/or allow it to bind in a configuration which then allows IF-2 to trigger the GTPase site, thus favouring the recycling of IF-2 and the accommodation of fmet-$tRNA_F$ in the peptidyl (P) site on the ribosome (one of the two defined tRNA binding sites on the ribosome, the other being the aminoacyl (A) site). (Kay & Grunberg-Manago, 1972). Fmet-$tRNA_F$ and GTP bind to the 30S.(IF-3).(IF-2).(IF-1).mRNA complex. IF-2 has a free sulphydryl group that is essential for both GTP (Lelong et al., 1970) and fmet-$tRNA_F$ binding to this protein (Groner & Revel, 1973). It is believed (Vermeer et al., 1973a,b) that IF-3 dissociates from the complex before the binding of the fmet-$tRNA_F$. A 50S subunit then associates with the 30S.(IF-2).(IF-1).mRNA.fmet-tRNA.GTP complex. It has been suggested that the hydrolysis of GTP may facilitate the

FIGURE 3. THE INITIATION OF PROTEIN SYNTHESIS ON BACTERIAL RIBOSOMES.



Redrawn from Metzler (1977).

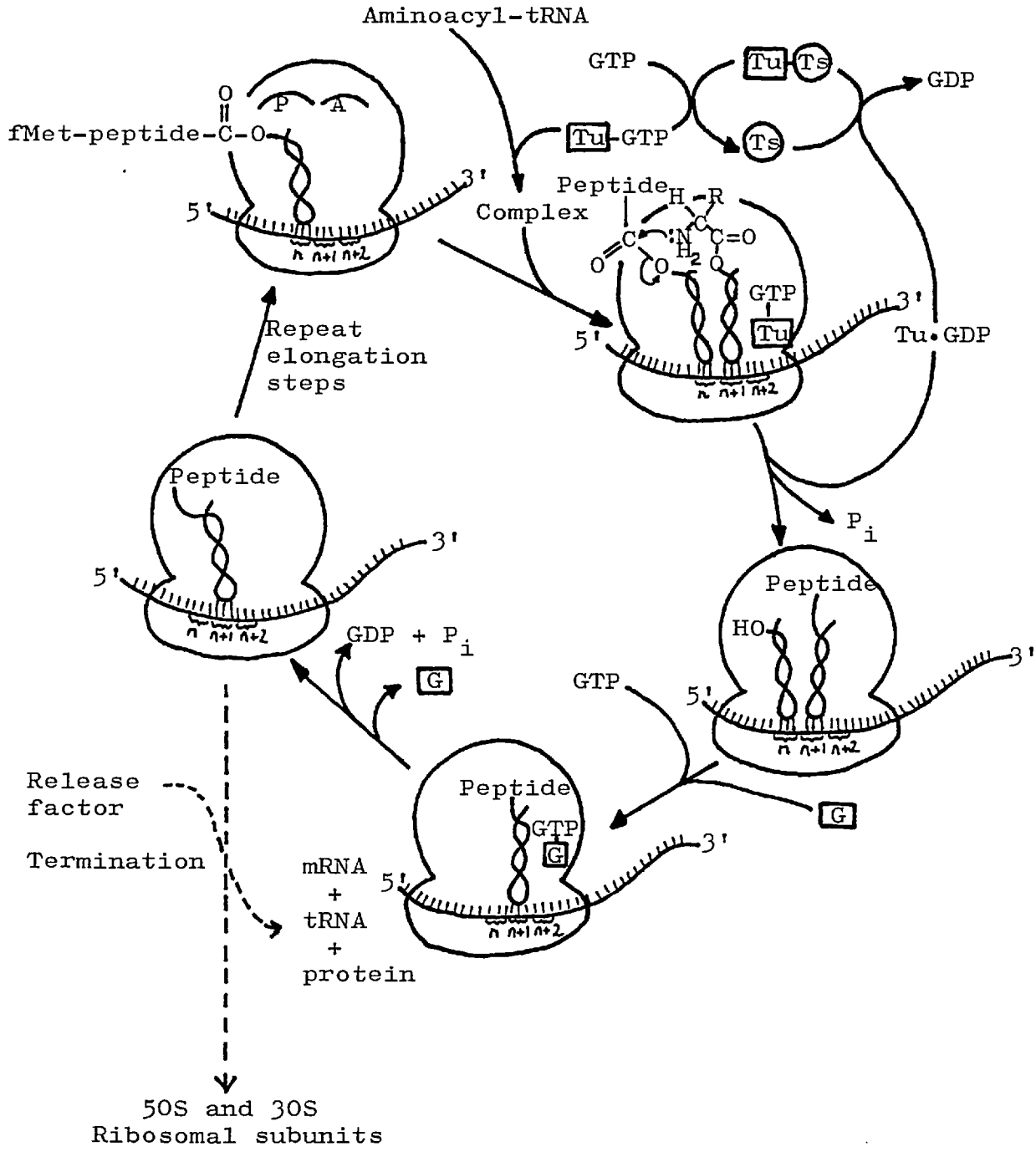
the release of IF-2, but it may also be necessary for the correct positioning of the fmet-$tRNA_F$ on the ribosome (Benne et al., 1973). Ribosomes may not be able to accommodate both IF-2 and EF-Tu (see later) at the same time (Modolell et al., 1973). Thus the incorrect positioning of the fmet-$tRNA_F$ may be a reflection of this. IF-1 may dissociate just before or after 50S binding.

1.5. Elongation.

In the next stage (Fig. 4), a second aminoacyl-tRNA binds in the A-site on the ribosome. This is mediated through base pairing of the anti-codon triplet of the second tRNA with the codon triplet of the mRNA. Before binding occurs the tRNA has in turn to bind to the protein EF-Tu (molecular weight 42,000 - 46,000) and a molecule of GTP. The most striking requirement for the interaction of aminoacyl-tRNA with EF-Tu.GTP is the aminoacyl group (Miller & Weissbach, 1977). As previously mentioned, fmet-$tRNA_F$ does not interact with EF-Tu.GTP. If the 5'-terminal cytosine is converted to uracil by a bisulphite promoted deamination, this allows the 5'-terminus to pair with an adenine in the 3'-end and produces an acceptor stem characteristic of the other aminoacyl-tRNAs (Schulman et al., 1974). Thus, modified fmet-$tRNA_F$ can interact with EF-Tu like other aminoacyl-tRNAs.

When this second aminoacyl-tRNA binds to the ribosome, GTP is hydrolysed and an EF-Tu.GDP complex is released. If a nonhydrolysable analogue of GTP is used, the aminoacyl-tRNA can still bind, but the EF-Tu and analogue remain bound

FIGURE 4. THE ELONGATION OF THE GROWING PEPTIDE CHAIN.



Redrawn from Metzler (1977).

to the ribosome and no peptidyl transfer takes place (see later) (Lucas-Lenard et al., 1969). Elongation factor Ts (EF-Ts, molecular weight 28,000) is believed to catalyse the regeneration of free EF-Tu (Miller & Weissbach, 1970). The EF-Tu dependent binding of tRNA to the A-site also requires 5S RNA, which has sequences complementary to the TψC loop (Fig. 8) of the tRNA molecule. This binding process is inhibited by the oligonucleotide TψCG. The 50S subunit proteins L5, L18 and L25 and the 5S RNA will also bind this tetranucleotide (Erdman et al., 1973, Richter et al., 1973). The 30S subunit also contributes to the A-site tRNA binding. Inactivation of certain guanine residues in the 16S RNA will inhibit tRNA, but not mRNA binding to the subunit. When tRNA is present in the A-site, the inactivation is prevented (Erdman et al., 1973). Recent experimental evidence has shown that proteins S1, S11 and S21 participate directly in the binding of tRNA to the 30S subunit (Fanning et al., 1978).

The error frequency for each amino acid inserted has been set at less than 1 in 3,000 (Loftfield & Vanderjagt, 1972). However, a cognate interaction between a pair of complementary triplets is only ten times more stable than the corresponding interaction with triplets containing a single mismatched pair of nucleotides. How, then, is the high rate of fidelity of translation achieved in vivo and in vitro? It has been suggested that another part of the tRNA molecule, other than the anti-codon triplet, interacts with the ribosome i.e. the 5S RNA interacting with the TψC loop of tRNA. In solution, in crystals and when bound to EF-Tu, tertiary interactions of the 5S RNA component with the D loop of the tRNA (Fig. 8) mask the TψCG sequence. This sequence must therefore become

unmasked when the tRNA binds to the A-site (Wagner & Garrett, 1978), thus preferentially enhancing the binding of cognate tRNAs. The codon therefore appears to function as an allosteric effector that unmasks universal sites in the tRNA that can bind to cognate structures in the ribosome. It can be shown (Kurland et al., 1975) that if such a mechanism were in play, the error frequency of translation would increase with the speed of peptide bond formation. This may explain the mode of action of the antibiotic streptomycin. In the presence of this drug, peptide elongation is more rapid than normal and therefore miscoding occurs. Recent experimental proof for this comes from the observation (Galas & Branscomb, 1976) that the rate of in vivo peptide elongation on streptomycin resistant ribosomes from E. coli is slower than on ribosomes from the wild-type organism.

1.6. Peptide bond formation.

A peptide bond is formed by the reaction of the amino group of the newly bound aminoacyl-tRNA with the esterified carboxyl group of the terminal amino acid residue of the peptidyl-tRNA by a nucleophilic displacement. The elongated peptidyl-tRNA is now bound at the A-site. Neither ATP nor GTP is required for peptide bond formation. The active site catalysing the reaction is a specific site on the 50S subunit which does not require any protein factors. The 50S subunit proteins, L16, L11 and L6, seem to be essential for peptidyl transferase activity. They are also required for chloramphenicol binding to the ribosome. This drug is a potent inhibitor of peptidyl transferase activity (Roth & Nierhaus, 1975).

Chloramphenicol binding can be inhibited by analogues of the aminoacyl-tRNA acceptor terminus (Goldberg et al., 1977) indicating that the peptidyl transferase activity is spread over parts of the A- and P-sites. Indeed, proteins L2, L15 and L27 have been shown to be involved in the P-site area of peptidyl transferase (Dietrich et al., 1974; Czernilofsky et al., 1974) while L6, L11 and L16 seem to be located at the A-site (Dietrich et al., 1974).

1.7. Translocation

Following peptide bond formation, deacylated tRNA is ejected from the P-site and the peptidyl-tRNA transferred from the A- to the P-site by the simultaneous movement of the ribosome along the mRNA, such that a new triplet codon is exposed to direct the next cycle of reactions. This translocation step (Fig. 4) involves the protein factor EF-G (molecular weight 80,000). After having bound GTP, the EF-G.GTP complex binds to the ribosome and undergoes a conformational change leading to a stronger association of the guanosine nucleotide. Recent sequencing data has shown that the GTP binding site of EF-G is located in its N-terminal domain (Girshovich et al., 1978). Hydrolysis of GTP leading to the formation of a EF-G.GDP complex would reverse this conformational change, facilitating both the release of EF-G.GDP from the ribosome and the dissociation of this complex. Fusidic acid, an antibiotic that inhibits translocation is believed to preserve the prehydrolytic conformation after GTP hydrolysis (Girbes et al., 1977). The antibiotic thiostrepton inhibits the formation of the EF-G.GTP.ribosome complex and the EF-Tu

catalysed binding of aminoacyl-tRNA and its associated GTPase activity and the interaction of IF-2 with ribosomes (Lucas-Lenard & Beres, 1974). This observation combined with results obtained with other antibiotics suggests there exists a region on the 50S subunit involved in EF-G and EF-Tu binding and GTPase activity. The sites may be identical, partially overlapping, or there may be an allosteric interaction between the factor binding sites on the 50S subunit. The two acidic proteins L7 and L12 seem to be located at this site(s) and are structurally very similar (Hamel et al., 1972). One major difference is that the N-terminal serine residue is acetylated in L7 but not in L12 (Terhorst et al., 1972). The L7 and L12 proteins are present in the 50S particle in a 1:1 ratio and there are greater than two per mole of 50S subunits (Moller et al., 1972).

In addition to similarities in amino acid composition, each has a high α-helical content (cf proteins involved in contractile processes e.g. myosin, tropomyosin and flagellin). The α-helical content of a mixture of L7 and L12 decreases significantly in the presence of large excesses of GDP, GTP or GMP-PCP, while similar concentrations of GMP or ATP have no significant effect on the degree of helicity (Brot et al., 1972). It remains to be established whether the high α-helical content has any relevance to the process of translocation.

1.8. Stringent response.

When bacterial cells are deprived of, or are unable to activate one or more essential amino acid(s), a drastic

reduction in the synthesis of stable RNA (tRNA and rRNA) occurs. The inhibition is mediated by the interaction of (p)ppGpp with RNA polymerase. pppGpp and ppGpp are synthesised on ribosomes from ATP and GTP or GDP and the process is catalysed by a ribosome-bound protein factor (stringent factor) that transfers the β,γ-diphosphate moiety as a unit from ATP to the 3'-hydroxyl group of GTP or GDP (Sy & Lipmann, 1973). The stringent factor (SF) bound to the ribosome.mRNA complex is activated when uncharged, but codon specific, tRNA is bound to the ribosomal acceptor site. During synthesis of (p)ppGpp, uncharged tRNA is cyclically bound to and released from the ribosome with each molecule of ATP hydrolysed, while the stringent factor remains bound to the 70S ribosomes. Activation of SF and hence synthesis of (p)ppGpp does not occur when aminoacyl-tRNA is bound (Richter, 1976). In vivo, (p)ppGpp is metabolically labile and is rapidly turned over. The enzyme responsible for its degradation is associated with the ribosomal fraction of cells and intact ribosomes are not required for its activity. In an attempt to find the ribosomal proteins involved in (p)ppGpp production, Christiansen and Nierhaus (1976) concluded that 26 ribosomal proteins were able to stimulate (p)ppGpp formation, but the most marked effect was demonstrated by S^4, S7, S9, S11, L15 and L20. They proposed that these proteins are involved in SF binding to the ribosome but not necessarily in actual (p)ppGpp production. It has been found (Parker et al., 1976) that an alteration of L11, caused when a mutation occurs in the Rel C gene of E. coli, results in a change from a stringent to a relaxed phenotype. Rel C^- ribosomes, however,

bind SF as efficiently as Rel C^+ ribosomes, in agreement with Christiansen and Nierhaus' theory of SF binding. As stated earlier, L11 is believed to be involved in the A-site binding of tRNA and the peptidyl transferase activity located there. A mutation in this protein might therefore mask the response of SF to uncharged, codon-specific tRNA binding in the A-site, providing the stimulus for the stringent response.

1.9. Termination.

The final stage in protein synthesis is release of the completed peptide from the last coded tRNA attached to the P-site on the ribosome (Fig. 4). Termination is signalled on the mRNA by one or more of three codons, UAA, UAG and UGA. These triplets do not code for any of the tRNA species. One of two protein releasing factors - RF-1 for UAA and UAG or RF-2 for UAA and UGA - binds to the ribosome in a codon-specific manner. Thus, suppressor tRNA molecules (tRNA molecules that can recognise and substitute amino acids at the site of a terminator codon) can compete for the binding of release factors to the ribosome(Ganoza & Tompkins, 1970). Proteins S9 and S11 appear to be needed for the codon-directed binding of RF (Tate & Caskey,1974) and, less markedly, the codon-directed hydrolysis of peptidyl-tRNA. A third factor, RF-3, is also involved in release and seems to stimulate directly the binding and dissociation of RF-1 and RF-2 from ribosomes (Goldstein & Caskey, 1970). Ribosomal proteins L7 and L12 are involved in RF binding which is reflected in the observation that release factors cannot

bind to the ribosome in the presence of EF-G (Hamel et al., 1972). GTP hydrolysis, allowing RF destabilisation and release, is believed to be required for peptide release (Tate & Caskey, 1974). The peptidyl transferase reaction involves the hydrolysis of tRNA from the peptidyl-tRNA chain in the P-site followed by peptide bond formation with the $-NH_2$ group of the aminoacyl-tRNA in the A-site. Hydrolysis of peptidyl-tRNA by itself is catalysed by ribosomes (Caskey et al., 1971). Not unexpectedly then, peptidyl transferase activity is required for peptide release. Chloramphenicol, a potent inhibitor of peptidyl transferase, also prevents codon-directed peptide release (Vogel et al., 1969). Release factor is needed for the hydrolysis since differential inactivation of RF-1 and RF-2 by N-ethyl maleimide results in partially inactivated RF-2 which can recognise codons normally but is markedly impaired in its ability to participate in peptidyl-tRNA hydrolysis (Tate & Caskey, 1974). It would appear that for termination to occur, the peptidyl-tRNA must be located at the P-site (Tompkins et al., 1970). Translocation must therefore occur subsequent to formation of the last peptide bond and prior to termination. This P-site requirement is additional evidence for participation of peptidyl transferase in hydrolysis at termination.

A study into the assignment of the roles of the nucleic acid and protein components of the ribosome has only recently begun (Kurland, 1977). The compact nature of the ribosome predisposes towards protein-protein and protein-nucleic acid interactions making it unlikely that many proteins in isolation are responsible for catalysing individual reactions. It has been suggested that the dissociable factors, which

are not present all the time on all ribosomes, may be exceptions to this generalisation (Weissbach & Pestka, 1977). Examination of the interactions of specific inhibitors of protein synthesis with ribosomal proteins and nucleic acids has enabled more inferences to be made about structure and function relationships. The use of physical techniques, such as electron microscopy, circular dichroism and X-ray crystallography will play an important role in exploring such features of the ribosome complex and its role in protein synthesis.

The preceding review has been a very brief account of the main sequence of events in protein synthesis. The subject has been reviewed in greater detail by Brimacombe et al. (1976), Grunberg-Manago and Gros (1977), Haselkorn and Rothman-Denes (1973), Kurland (1977) and Weissbach and Pestka (1977).

2. NATURALLY OCCURRING INHIBITORS OF PROTEIN SYNTHESIS.

A large number of naturally occurring compounds which interfere with steps involved in protein synthesis have now been characterised, and these have been the subject of a recent review (Pestka, 1977). In some cases, the mode of action is known in great detail. Table 2 (Pestka, 1977) gives a comprehensive list of those which have been characterised sufficiently to enable the target sites in the protein synthesising machinery to be established. The very small group of inhibitors so far characterised that specifically inhibit the charging of tRNA are discussed in the following section.

TABLE 2 . INHIBITORS OF PROTEIN SYNTHESIS.

INHIBITOR	MW	SITE OF INHIBITION	STEP INHIBITED	REACTION INHIBITED	INHIBITION OF PRO- OR EUKARYOTES	INHIBITION IN INTACT CELLS	INHIBITION IN CELL-FREE EXTRACTS
Althiomycin	439.47	50S	E	Transpeptidation	Pro	+	+
Aminoglycosides							
Dihydrostreptomycin	583.62	30S	E(I,T)	AA-tRNA binding	Pro	+	+
Streptomycin	581.58	30S	E(I,T)	AA-tRNA binding	Pro	+	+
Bluensomycin	585.58	30S	E	AA-tRNA coding	Pro	+	+
Gentamycin C_1	477.61	30S	E	AA-tRNA coding	Pro	+	+
Kanamycin	484.50	30S (50S)	E	(AA-tRNA coding; transpeptidation	Pro	+	+
Neomycin B	614.67	30S (50S)	E	(AA-tRNA coding; transpeptidation	Pro (Eu)	+	+
Neomycin C	614.67	30S (50S)	E	(AA-tRNA coding; transpeptidation	Pro (Eu)	+	+
Paromomycin	615.65	30S	E(I,T)	AA-tRNA binding	Pro (Eu)	+	+
Spectinomycin	332.36	30S	E	Translocation	Pro	+	+
Kasugamycin	379.38	30S	I	fMet-$tRNA_F$ binding	Pro	+	+
4-Aminohexose pyrimidine nucleosides							
Amicetin	618.69	50S	E	Transpeptidation	Pro, Eu	+	+
Bamicetin	604.66	50S	E	Transpeptidation	Pro, Eu	+	+
Plicacetin	517.59	50S	E	Transpeptidation	Pro, Eu	+	+
Blasticidin S	422.45	50S	E	Transpeptidation	Pro, Eu	+	+
Gougerotin	443.42	50S	E	Transpeptidation	Pro, Eu	+	+
Hikizimycin	583.55	50S	E	Transpeptidation	Pro, Eu	+	+
Anisomycin	265.31	60S	E	Transpeptidation	Eu	+	+
Aurintricarboxylic acid	422.35	30S, 40S	I (E)	mRNA binding to ribosomes	Pro, Eu	-	+
Bottromycin A_2	823.08	50S	E	Translocation	Pro	+	+

TABLE 2 continued.

Chloramphenicol	323.13	50S	E	Transpeptidation	Pro	+	+
Cryptopleurine	377.47	40S, 60S	E	Transpeptidation	Eu	+	+
Diphtheria toxin (intact)	63,000	EF-T2	E	Translocation	Eu	+	-
Diphtheria toxin (frag. A)	24,000	EF-T2	E	Translocation	Eu	-	+
Edeine A	738.89	30S, 40S	I, E	fMet-tRNA$_F$,AA-tRNA binding	Eu	+	+
					Pro	-	+
Ediene B	780.93	30S, 40S	I, E	fMet-tRNA$_F$,AA-tRNA binding	Eu	+	+
					Pro	-	+
Emetine	480.63	60S	E	(Translocation)	Eu	+	+
Fusidic acid	515.72	EF-G, EF-T2	E	Translocation	Eu	-	+
					Pro	+	+
Glutarimides							
Acetoxy-cycloheximide	339.39	60S	E	Transpeptidation, (translocation)	Eu	+	+
Cycloheximide	281.35	60S	E(I,T)	Transpeptidation, (translocation)	Eu	+	+
Streptovitacin A	297.35	60S	E	Transpeptidation, (translocation)	Eu	+	+
Streptimidone	293.37	60S	E	Transpeptidation, (translocation)	Eu	+	+
Guanylyl-5'-methylene diphosphonate	521.22	EF-Tu, EF-G, IF-2, EF-T1, EF-T2	I, E	GTP hydrolysis	Pro, Eu	-	+
Harringtonine	531.61	40S	I	Met-tRNA$_F$ binding	Eu	+	+
Kirromycin (mocimycin)	796.98	EF-Tu	E	(EF-Tu.GDP release from ribosomes. Other EF-Tu-dependent reactions affected)	Pro	+	+
X5108	811.01	EF-Tu	E		Pro	+	+

TABLE 2 continued.

Lincosamides							
Lincomycin	406.55	50S	E(I,T)	Transpeptidation, (termination)	Pro	+	+
Clindamycin	424.99	50S	E(I,T)	Transpeptidation, (termination)	Pro	+	+
Celestosaminides							
Celesticetin	528.63	50S	E	Transpeptidation	Pro	+	+
Macrolides							
Carbomycin	842.00	50S	E	Transpeptidation, (translocation)	Pro	+	+
Erythromycin A	733.95	50S	E	Transpeptidation, (translocation)	Pro	+	+
Leucomycin A_1	785.98	50S	E	Transpeptidation, (translocation)	Pro	+	+
Niddamycin	783.96	50S	E	Transpeptidation, (translocation)	Pro	+	+
Oleandomycin	687.88	50S	E	Transpeptidation, (translocation)	Pro	+	+
Spiramycin III	899.14	50S	E(I)	Transpeptidation, (translocation)	Pro	+	+
Tylosin	904.11	50S	E	Transpeptidation, (translocation)	Pro	+	+
2-(4-Methyl-2,6-dinitroanilino)-N-methyl-propionamide (MDMP)	282.26	40S	I	40S.mRNA-Met-tRNA coupling with 60S	Eu	+	+
Micrococcin	1119	50S	E	Translocation, (AA-tRNA binding)	Pro	+	+
Negamycin	248.28	30S	I(E,T)	Initiation, AA-tRNA coding, termination	Pro (Eu)	+	+
Pactamycin	560.66	30S, 40S	I(E)	fMet- and Met-$tRNA_F$ binding	Pro, Eu	+	+
Puromycin	471.52	50S, 60S	E	Causes release of nascent peptides	Pro, Eu	+	+

TABLE 2 continued.

Sparsomycin	361.44	50S, 60S	E(T)	Transpeptidation, (termination)	Eu Pro	– +	+ +
Streptogramins A							
Griseoviridin	477.54	50S	E(I,T)	Transpeptidation, (termination)	Pro	+	+
Mikamycin A	525.61	50S	E(I,T)	Transpeptidation, (termination)	Pro	+	+
Ostreogrycin A	525.61	50S	E(I,T)	Transpeptidation, (termination)	Pro	+	+
PA114 A	525.61	50S	E(I,T)	Transpeptidation, (termination)	Pro	+	+
Staphylomycin M_1	525.61	50S	E(I,T)	Transpeptidation, (termination)	Pro	+	+
Streptogramin A	525.61	50S	E(I,T)	Transpeptidation, (termination)	Pro	+	+
Vernamycin A	525.61	50S	E(I,T)	Transpeptidation, (termination)	Pro	+	+
Virginiamycin M	525.61	50S	E(I,T)	Transpeptdation, (termination)	Pro	+	+
Streptogramins B							
Mikamycin B	866.98	50S	E	(Translocation)	Pro	+	+
Ostreogrycin B	866.98	50S	E	(Translocation)	Pro	+	+
PA 114 B-1	866.98	50S	E	(Translocation)	Pro	+	+
Staphylomycin S	823.91	50S	E	(Translocation)	Pro	+	+
Streptogramin B	866.98	50S	E	(Translocation)	Pro	+	+
Vernamycin Bα	866.98	50S	E	(Translocation)	Pro	+	+
Viridogrisein	879.03	50S	E	(Translocation)	Pro	+	+
Virginiamycin S	823.91	50S	E	(Translocation)	Pro	+	+
Tetracycline	444.43	30S, 40S, (50S)	E(T)	AA-tRNA Binding	Eu Pro	– +	+ +
Chlorotetracycline	478.88	30S, 40S, (50S)	E(T)	AA-tRNA Binding	Eu Pro	– +	+ +

TABLE 2 continued.

Tenuazonic acid	197.24	60S	E	Transpeptidation	Eu	+	+
Thiostrepton group							
Multhiomycin	1064.24	50S	E	Initiation, GTP hydrolysis, AA-tRNA binding, translocation	Pro	+	+
Siomycin A	1711.99	50S	E	Initiation, GTP hydrolysis, AA-tRNA binding, translocation	Pro	+	+
Sporangio-mycin	1850	50S	E	Initiation, GTP hydrolysis, AA-tRNA binding, translocation	Pro	+	+
Thiopeptin B	1752.01	50S	E	Initiation, GTP hydrolysis, AA-tRNA binding, translocation	Pro	+	+
Thiostrepton	1646.90	50S	E	Initiation, GTP hydrolysis, AA-tRNA binding, translocation	Pro	+	+
L-1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK)	351.85	EF-Tu	E	AA-tRNA binding	Eu Pro	+ -	- +
Trichothecanes							
Crotocin	332.40	60S	E	Transpeptidation, (termination)	Eu	+	+
Trichodermin	292.38	60S	E	Transpeptidation, (termination)	Eu	+	+
Trichodermol	250.34	60S	E	Tranpeptidation, (termination)	Eu	+	+
Trichothecin	332.40	60S	E	Transpeptidation, (termination)	Eu	+	+

TABLE 2 continued.

Crotocol	264.32	60S	I	Initiation	Eu	+	+
Diacetoxy-scirpenol	366.41	60S	I	Initiation	Eu	+	+
Nivalenol	312.32	60S	I	Initiation	Eu	+	+
T-2 Toxin	466.53	60S	I	Initiation	Eu	+	+
HT-2 Toxin	424.50	60S	I	Initiation	Eu	+	+
Verrucarin A	502.57	60S	I	Initiation	Eu	+	+

The "Site of inhibition" refers to the site of interaction of the inhibitor producing the major inhibition. These include 30 or 50S subunits and EF-Tu, EF-Ts and EF-G for prokaryotes; and correspondingly 40 and 60S subunits and EF-T1 and EF-T2 for eukaryotes.
The "Step inhibited" refers to one of the three major general stages of protein biosynthesis: initiation (I), elongation (E), and termination (T).
"Reaction inhibited" refers to the specific partial reaction inhibited or otherwise affected.
Statements in the table which are in parentheses refer to effects which occur at high concentrations of the inhibitors, may not be relevent in intact cells, or are questionable for other reasons.

3. NATURALLY OCCURRING INHIBITORS OF AMINOACYL-tRNA SYNTHETASES.

Apart from the substrate 'structural analogue' inhibitors of aminoacyl-tRNA synthetases, only a few naturally occurring compounds which inhibit these enzymes have been isolated and characterised. The target synthetase of each of the known inhibitors is shown in Table 3 . Perhaps with the exception of chalcomycin, all the inhibitors appear to be specific for one aminoacyl-tRNA synthetase. There are a few examples where slight inhibition of related-amino acid aminoacyl-tRNA synthetases also occurs (Hutter *et al.*, 1966; Tanaka *et al.*, 1969). Since the synthetases have evolved a high degree of specificity for their cognate amino acid (see section 4) many amino acid analogues are powerful inhibitors of the aminoacyl-tRNA synthetases (Szentirmai *et al.*, 1968; Anderson & Santi, 1976). Indolmycin, although a naturally produced inhibitor, is structurally very similar to tryptophan (Fig. 5). Kinetic experiments showed it to be an effective competitive inhibitor (with respect to tryptophan) of the tryptophanyl-tRNA synthetase from *E. coli*, $K_i = 8 - 9 \times 10^{-6}$M (Werner *et al.*, 1976). It appeared to be less effective on a eukaryotic tryptophanyl-tRNA synthetase that was isolated from rat liver. Ochratoxin A, although a somewhat larger molecule than indolmycin (Fig. 5), nevertheless also acts as an amino acid analogue, since it has as part of its structural framework a phenylalanyl moiety. Using phenylalanyl-tRNA synthetase isolated from *Bacillus subtilis* 168I$^-$, Konrad and Roschenthaler (1977) showed that ochratoxin A behaved as a competitive antagonist (with respect to phenylalanine) of this enzyme, $K_i = 3.0 \times 10^{-6}$M.

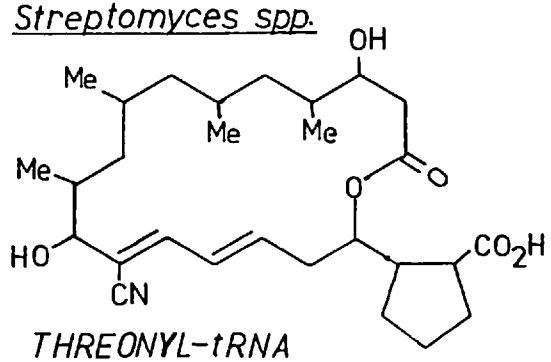
TABLE 3. INHIBITORS OF AMINOACYL-tRNA SYNTHETASES.

INHIBITOR	TARGET SYNTHETASE	REFERENCE
ASK-753	Lysyl-tRNA synthetase	Shimi & Shoukry, 1976
Borrelidin	Threonyl-tRNA synthetase	Hutter et al., 1966
Chalcomycin	Glycyl-tRNA synthetase?	Jordan, 1963
Ferramido-chloromycin	Lysyl-tRNA synthetase	Shimi & Shoukry, 1976
Furanomycin	Isoleucyl-tRNA synthetase	Tanaka et al., 1969
Granaticin	Leucyl-tRNA synthetase	Ogilvie et al., 1975a,b
Indolmycin	Tryptophanyl-tRNA synthetase	Werner et al., 1976
Ochratoxin A	Phenylalanyl-tRNA synthetase	Konrad & Roschenthaler, 1977
(Pseudomonic acid	Isoleucyl-tRNA synthetase	Hughes & Mellows, 1978b)

FIGURE 5

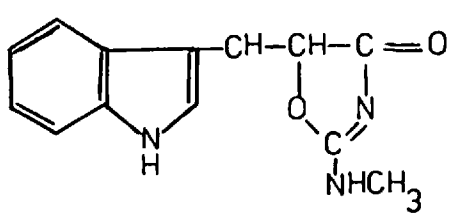
NATURALLY OCCURRING ANTIBIOTICS WHICH INHIBIT BACTERIAL AMINOACYL-tRNA SYNTHETASES

BORRELIDIN
Streptomyces spp.



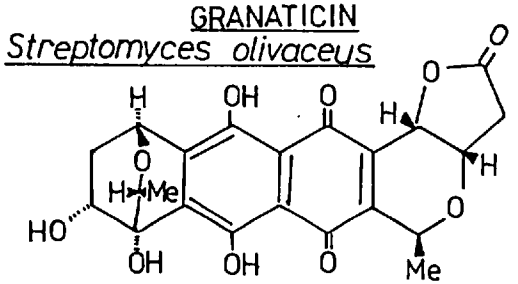
THREONYL-tRNA SYNTHETASE

INDOLMYCIN



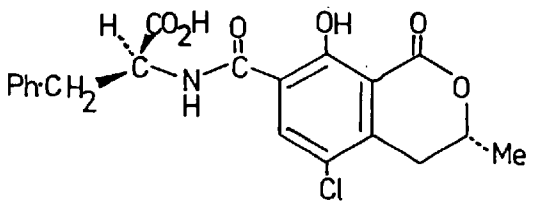
TRYPTOPHANYL-tRNA SYNTHETASE

GRANATICIN
Streptomyces olivaceus



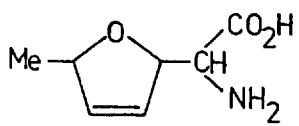
LEUCYL-tRNA SYNTHETASE

OCHRATOXIN A
Aspergillus spp.
Penicillium spp.



PHENYLALANYL-tRNA SYNTHETASE

FURANOMYCIN
Streptomyces L 803



ISOLEUCYL-tRNA SYNTHETASE

No inhibition was observed when phenylalanyl-tRNA synthetase isolated from E. coli was used. It was speculated (Konrad & Roschenthaler, 1977) that at least in E. coli, the amino group of an amino acid analogue should be unsubstituted if the compound is to be an inhibitor of the aminoacylation reaction. Furanomycin (Fig. 5) may also be a competitive inhibitor (with respect to isoleucine) of isoleucyl-tRNA synthetase, since isoleucine can overcome the inhibitory effect of the antibiotic in vivo, but conclusive experimental evidence for this is not yet available (Tanaka et al., 1969).

Preliminary experiments with borrelidin indicated that it inhibited the synthesis of threonyl-tRNA (Hutter et al., 1966). More recently, it has been shown by kinetic studies to be a non-competitive inhibitor with respect to threonine, $K_i = 3.0 \times 10^{-9}$M, inhibiting the transfer of threonine from the threonyl adenylate.enzyme complex to $tRNA^{Thr}$ (Poralla, 1975). It has been speculated (Monreal & Paules, 1970) that it acts as a threonyl-tRNA analogue (Fig. 5).

No detailed inhibitory kinetic studies have so far been conducted on the remaining inhibitors (Table 3). With the exception of borrelidin and pseudomonic acid, there is no conclusive evidence for which of the two part-reactions (section 4) catalysed by the respective synthetase is inhibited.

It is of interest to note that none of the aminoacyl-tRNA synthetase inhibitors have found use in chemotherapy, presumably because they are either too toxic for use in eukaryotes and/or they have a poor spectrum of activity against pathogenic bacteria.

Aminoacyl-tRNA synthetase inhibitors may prove very useful

probes in future investigations into the mechanism of tRNA charging in a similar manner to the way many protein synthesis inhibitors are being used in elucidating the mechanism of protein synthesis.

4. AMINOACYL-tRNA SYNTHETASES.

4.1. Introduction.

The aminoacyl-tRNA synthetases are a group of enzymes that catalyse the synthesis of charged tRNA. These molecules play a central role in protein biosynthesis as the decoders of mRNA. There is at least one specific tRNA for each amino acid. During protein synthesis, each tRNA type is esterified with its cognate amino acid by a specific aminoacyl-tRNA synthetase. The aminoacyl-tRNA is carried to the ribosome for peptide bond formation where the amino acid is placed in its correct order in the growing polypeptide chain. The anticodon nucleotide triplet of tRNA is responsible for the decoding of the codon triplet of nucleotides contained in mRNA. The activation of amino acids and their fixation to the appropriate tRNA is a crucial step in protein biosynthesis. As a group of enzymes, the synthetases have aroused interest for a number of reasons. They have the ability to recognise both nucleic acids and amino acids. The active sites recognise the three substrate molecules: tRNA, amino acid and ATP, the former having a considerably greater molecular weight (10,000) than the latter two (100-600). All the aminoacyl-tRNA synthetases catalyse the same general type of reaction and possess a degree of structural diversity. Recent experiments (Fersht, 1975; Waterson & Konigsberg, 1974) indicate, however, that more structural similarity may exist within this group of enzymes than was at first apparent. Recent techniques for improving the yield of pure enzymes from cells have enabled more detailed sequencing and X-ray

crystallographic studies to be carried out. There is still much work to be done before a detailed understanding of the chemical basis of enzyme-substrate interactions and mechanisms involved in the specific charging of tRNA is achieved.

4.2. Structure.

The majority of aminoacyl-tRNA synthetases are composed of two identical subunits (α_2): (identity of the subunits is based in most cases only on molecular weight data) (Table 4). Some contain only one polypeptide (α). Yeast phenylalanyl - tRNA synthetase (Schmidt *et al.*, 1971) and *E. coli* glycyl - tRNA synthetase (Ostrem & Berg, 1970) have been shown to contain dissimilar subunits ($\alpha_2\beta_2$). Another variant is *E. coli* glutamyl - tRNA synthetase, which is composed of two non-identical polypeptide chains ($\alpha\beta$) (Lapointe & Söll, 1972). Some sequencing data that has been obtained from the monomeric synthetases of molecular weight about 100,000, indicates that these enzymes contain homologous sequences (Koch *et al.*, 1974; Bruton *et al.*, 1974; Bruton, 1975). This suggests that these enzymes may have originated by gene duplication and fusion. More interesting, though, are the conclusions drawn by Piszkiewicz and Goitein (1974) from their experiments on the effect of limited cleavage of isoleucyl-tRNA synthetase. Similar conclusions were drawn by Rouget and Chapeville (1971) for leucyl-tRNA synthetase. The latter workers showed that two major polypeptide fragments of approximately equal size (molecular weight 55,000) and a polypeptide bridge with molecular weight 3,000 is completely excised by trypsin. With isoleucyl-

TABLE 4. MOLECULAR WEIGHT AND SUBUNIT STRUCTURE OF AA-tRNA SYNTHETASES.

ENZYME		MOLECULAR WEIGHT			NUMBER OF BINDING SITES			
AMINO ACID (SOURCE)	TYPE	α	β	NATIVE	AMINO ACID	ATP	AA-AMP	tRNA
Glu (*E. coli*)	$\alpha\beta$	56	46	102				
His (*E. coli*)	α_2	43		85	2	2		
His (*S. typhimurium*)	α_2	40		80				
Leu (yeast)	α_2	60		120				
Lys (*E. coli*)	α_2	52		104	2	2		
Lys (yeast)	α_2	69		138	2	2		
Met (*E. coli*)	α_2	86		172	2	4	2	2
Met (*B. stearothermophilus*)	α_2	66		135				
Pro (*E. coli*)	α_2	47		94				
Ser (*E. coli*)	α_2	48		95	2	2	2	2
Ser (yeast)	α_2	60		120		2		2
Ser (hen liver)	α_2	60		120				
Trp (*E. coli*)	α_2	37		74	2			2
Trp (yeast)	α_2	50		110				
Trp (beef pancreas)	α_2	54		108	2	2	2	2
Trp (human placenta)	α_2	58		118				
Trp (*B. stearothermophilus*)	α_2	35		70				
Tyr (*E. coli*)	α_2	48		97	1-2		1-2	1-2
Tyr (*B. stearothermophilus*)	α_2	44		95	1-2		1	2

TABLE 4 continued.

Arg (*E. coli*)	α			75				
Arg (*B. stearothermophilus*)	α			78	1	1		
Asp (yeast)	α			100				
Gln (*E. coli*)	α			69				1
Ile (*E. coli*)	α			112	1	1	1	1
Leu (*E. coli*)	α			105		1	1	1
Leu (*B. stearothermophilus*)	α			110				
Tyr (yeast)	α			46				
Val (*E. coli*)	α			110	1	1	1	1
Val (yeast)	α			122	1	1		1
Val (*B. stearothermophilus*)	α			110				
Gly (*E. coli*)	$\alpha_2\beta_2$	80	33	225			2	
Phe (*E. coli*)	$\alpha_2\beta_2$	94	39	267	2		2	2
Phe (*E. coli*)	$\alpha_2\beta_2$	74	63	270	2		2	2
Phe (rat liver)	$\alpha_2\beta_2$	74	69	290				

tRNA synthetase, two polypeptides of molecular weight 76,000 and 41,000 are formed. After cleavage, these two peptides tend to reassociate with each other. Piszkiewicz and Goitein suggested a possible tertiary structure for isoleucyl-tRNA synthetase, in which two polypeptides, approximately globular in shape, associate at an interface by non-covalent bonding and are connected by one relatively short segment of polypeptide. Trypsin would cleave a peptide bond within this segment resulting in a conformational change in the active site. It is possible to speculate that the monomeric synthetases may have evolved from the dimeric form, by fusion, during the course of evolution. More sequencing data is required to substantiate this.

4.3. Catalysis.

At one time, it was thought, and available experimental data seemed to indicate (Table 4), that most of the dimeric enzymes bound two amino acid and two ATP molecules, whereas the monomeric enzymes bound only one molecule of each. Recent experiments with synthetases purified from Bacillus stearothermophilus (Fersht, 1975) have shown that the monomeric valyl-tRNA synthetase indeed has two active sites. A complex (E.Val-AMP.ATP.Val) is formed which probably slowly gives an unstable $E.(Val\text{-}AMP)_2$ complex. In view of this and the fact that a few of the monomeric enzymes examined so far have repeating sequences, it has been suggested (Fersht, 1975) that all of the synthetases have at least two active sites. The second site may be difficult to detect by normal steady-state kinetic measurements and binding assays, as these enzymes exhibit negative cooperativity

of substrate binding and "half of the sites reactivity".

The overall reaction catalysed by the synthetases can be summarised as follows:

$$1)\ \alpha\alpha + tRNA + ATP \xrightarrow[Enzyme]{Mg^{++}} \underset{(\alpha\alpha\text{-}tRNA)}{\text{aminoacyl-tRNA}} + AMP + PP_i$$

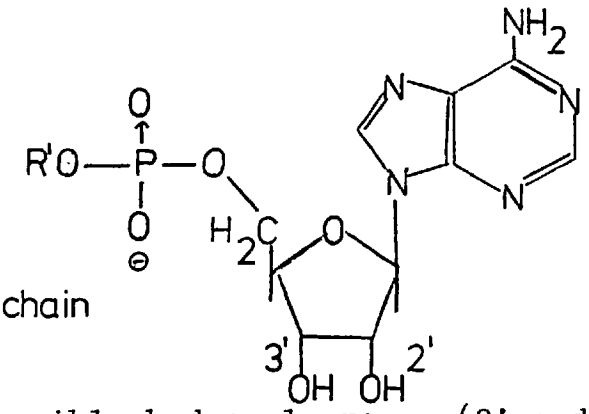
Generally, the reaction is considered to occur in two steps, A and B.

$$A)\ \alpha\alpha + ATP + Enz \rightleftharpoons Enz.\alpha\alpha\text{-}AMP + PP_i$$

$$B)\ Enz.\alpha\alpha\text{-}AMP + tRNA \longrightarrow \alpha\alpha\text{-}tRNA + Enz + AMP$$

There has been some controversy as to whether reaction (A) is an artifact and whether an adenylate intermediate is indeed a true intermediate in the overall reaction. However, the work of Eldred and Schimmel (1972a) and Fersht and Kaethner (1976) seems to prove that for the isoleucyl- and tyrosyl-tRNA synthetases of E. coli, formation of charged tRNA does proceed via an adenylate intermediate. Reaction (A) can be studied both by measuring E.αα-AMP formation, usually by isolating the complex by gel filtration, or by measuring the exchange of $[^{32}P]\,P_i$ into ATP. In the aminoacyl adenylate, the carboxyl group of the amino acid forms an anhydride linkage with the 5'-phosphoryl group of adenylic acid and the two oxygen atoms of the amino acid are retained. The overall reaction (1) and reaction (B) can both be followed by measuring the incorporation of isotopically labelled amino acid into cold acid-precipitable

material.



R'=nucleic acid side chain

There are two possible hydroxyl groups (2' and 3') available for esterification on the ribose residue of the terminal adenosine residue of all tRNAs (see above diagram). For a time it was debated as to whether the reaction was specific for the 2'- or the 3'-hydroxyl groups. It has been shown that at neutral pH, the amino acid can migrate very rapidly ($t_{\frac{1}{2}}$ in milliseconds) between the two hydroxyl groups, so that any attempt to determine this would only measure the equilibrium mixture, which is normally 70% esterification at the 3'-position and 30% at the 2'-position (Ofengand, 1977). Later work using non-isomerisable analogues of tRNA seemed to indicate that at least for some of the synthetases, there was specificity for either the 3'-OH or the 2'-OH and some exhibited no specificity (Ofengand, 1977). So far, 2'- or 3'-specificity is associated with synthetases for a given amino acid, and it remains the same regardless of the source of the synthetase. The recognition of the charged tRNA by EF-Tu.GTP (see review on protein synthesis) is probably specific for the 2'-isomer and the rapid rate of chemical migration very likely ensures that all αα-tRNAs can interact with the cognate enzyme regardless of the OH-group originally acylated.

The specificity of the charging of tRNA, on the basis of Michaelis-Menten enzyme-substrate interaction with one

recognition step, discriminates against smaller isoteric compounds to the extent of no more than 1 in 20. For example, isoleucyl-tRNA synthetase with only one proven binding site for isoleucine, cannot completely exclude the smaller valine molecule, which has a hydrogen atom in place of one of the methyl groups in isoleucine. Yamane and Hopfield (1977) proposed a kinetic proof-reading theory to account for the fidelity of the charging process (for another explanation see the review on isoleucyl-tRNA synthetase). This theory does not detail any specific mechanism of rejection, but simply states that along the reaction path, intermediates should have access to rejection paths in addition to the main pathway leading to the final product. They concluded that proof-reading of isoteric amino acids is more efficient than proof-reading of tRNAs. This is based mainly on the observation that the K_m values for cognate and non-cognate amino acids differ by a factor much greater than 10^3, whereas the K_m values of a given synthetase for isoteric amino acids usually differ only by a factor of 10^2. Under such circumstances, there would be little necessity to evolve an efficient tRNA proof-reading apparatus.

4.4. Interaction with tRNA.

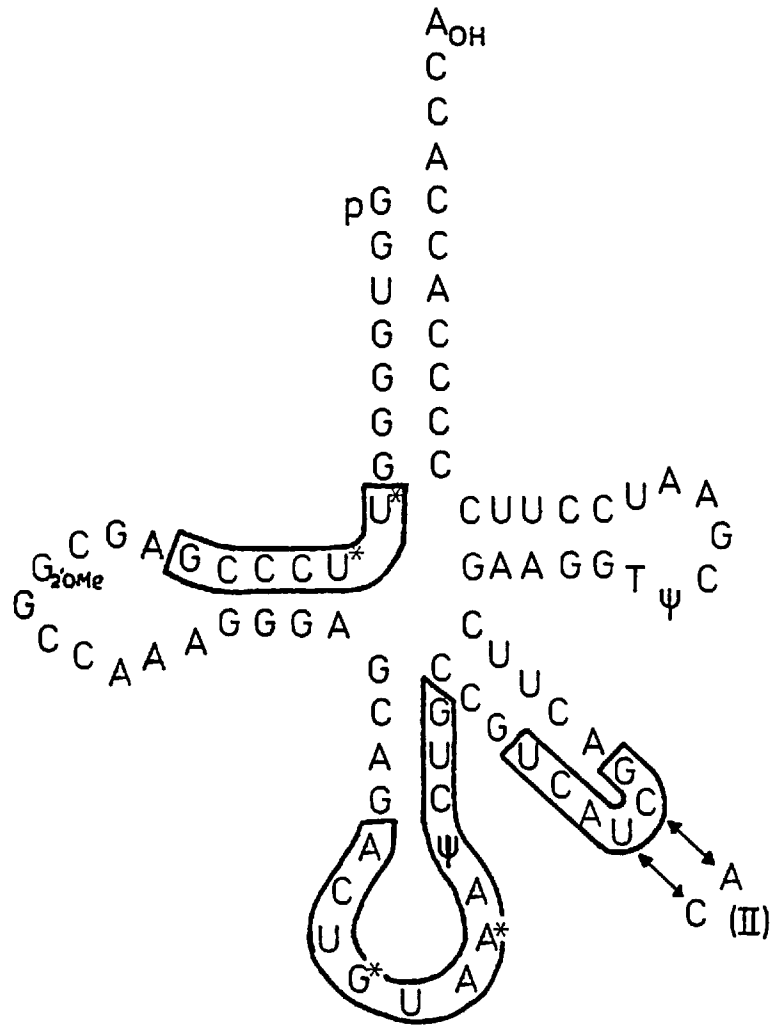
Since there is more than one anticodon for a number of amino acids, it would be expected that there would also be more than one tRNA specific for a given amino acid in the same organism. There is not, however, the full complement of sixty one species generally found, since pairing of the third base of a codon is not always strict. There is only

one synthetase in prokaryotes for each amino acid. Certain features of the tRNA structure must therefore be recognised by the synthetase to maintain cognate charging. Methanol will enable $tRNA^{Phe}$ to interact with isoleucyl-tRNA synthetase and to be mischarged at a much greater rate than normal (Yarus, 1972). The compact structure of tRNAs may be loosened by methanol and this may be required for the specificity of the nucleic acid - protein interaction. In 20% methanol, the standard free energy for the binding of $tRNA^{Phe}$ to isoleucyl-tRNA synthetase is fully two thirds that of the cognate $tRNA^{Ile}$. Since the sequences of $tRNA^{Ile}$ and $tRNA^{Phe}$ are not themselves two thirds homologous, Yarus (1972) suggested that this was evidence that a) the region of interaction is a selected group of nucleotides smaller than the whole or b) the sequence specificity (in 20% methanol at least) is not high, and alternative nucleotides are accepted by the protein.

Using purified phenylalanyl-tRNA synthetase from E. coli K10, Bartmann et al. (1975) found that the enzyme had two active sites for $tRNA^{Phe}$ binding. The sites appear not to be identical and their binding affinities differ by a factor of thirteen. These workers suggested that one site may be involved in specific recognition of $tRNA^{Phe}$ on the basis that spectral changes, due to both electrostatic interactions and intercalation of particular tryptophan moieties on the enzyme with $tRNA^{Phe}$, do not occur when a second $tRNA^{Phe}$ molecule binds. The sites may be differentiated after binding of the first substrate, since the subunit composition ($\alpha_2\beta_2$) of the phenylalanyl-tRNA synthetase would suggest that both sites are identical.

Which parts of the tRNA molecule are actually in contact with the surface of the enzyme? It might be expected that the 3'-terminus, where the amino acid is esterified, would be at the active site. Also, the anticodon loop might be expected to be in contact with the enzyme surface since this nucleotide sequence is specific for each type of tRNA. Under suitable conditions, ultra violet irradiation of synthetase-tRNA complexes can provide a covalently linked derivative. This was the approach used by Schoemaker and Schimmel (1974) who used the tyrosyl-tRNA synthetase and the two isoacceptor species of tRNA, $tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$ isolated from E. coli B. They found that pieces of the dihydrouridine arm, of the anticodon loop and of the extra loop are joined to the enzyme (Fig. 6). The 3'-terminus of the tRNA (CCA_{OH}) has also been shown to be protected by the enzyme (Schoemaker & Schimmel, 1974), even though under the conditions described above, it was not cross-linked. Caution must be exercised in interpreting the cross-linking results as they only prove that a reactive base(s) and protein residue(s) are in close proximity and not necessarily that the nucleotide regions are necessary for synthetase recognition. The cross-linked sites appear to fall into one branch of the L-shaped three dimensional structure of crystalline tRNA (Fig. 7) (Kim et al., 1973). $tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$ differ only by two bases in the extra loop, yet the extent of cross-linking of the extra loop fragment in $tRNA_1^{Tyr}$ is somewhat greater than that in $tRNA_2^{Tyr}$. Since the native tertiary structure is believed to be essentially the same in both species (Yang & Crothers, 1972), it may be that the reactivity of the two variable bases may

FIGURE 6. THE SEQUENCE AND CLOVERLEAF STRUCTURE OF E. COLI $tRNA_1^{Tyr}$.



The two bases in the extra loop, which distinguish $tRNA_2^{Tyr}$ from $tRNA_1^{Tyr}$, are indicated.

Heavy lines denote fragments that are found to be associated with enzyme.

Redrawn from Schoemaker & Schimmel (1974).

be greater in $tRNA_1^{Tyr}$ than in $tRNA_2^{Tyr}$.

Locating the amino acid residues involved in the binding of tRNA to synthetases has proved more difficult. It was suggested (Bosshard et al., 1978) that terminal amino groups of lysine residues might be involved in binding tRNA by the formation of salt bridges to the phosphate backbone of the tRNA or just by steric shielding. These workers used the method of differential labelling to test this hypothesis. This technique compares the chemical reactivities of the terminal amino groups of lysine residues in the free synthetase and the synthetase-tRNA complex. The finding that no lysine groups on tyrosyl-tRNA synthetase showed increased reactivities on $tRNA^{Tyr}$ binding and that three lysine groups showed decreased activity indeed implied that certain lysine residues may be shielded by the tRNA or sterically hidden. Only by carrying out the labelling at different pH values will it be possible to distinguish between these possibilities, since if the terminal amino group has been brought near to an acidic function, e.g. a phosphate group of the tRNA backbone, its pK will have increased and hence its reactivity towards acetic anhydride (the labelling agent used) decreased. Tyrosyl-tRNA synthetase from B. stearothermophilus contains two identical subunits of molecular weight 44,000 (Koch, 1974). It might therefore be expected to have two binding sites for $tRNA^{Tyr}$. However, experimental data obtained so far is indecisive on this point. Two non-equivalent tRNA binding sites appear to be available at pH 6.5 (Bosshard et al., 1975). However, this was determined by fluorescence measurements which are prone to experimental uncertainties. Using conditions very similar

to those used in the above differential labelling study, 0.87 $tRNA^{Tyr}$ binding sites per enzyme dimer have been determined (Fersht & Jakes, 1975) by the equilibrium gel filtration method. It was concluded (Bosshard et al., 1978) that since three of the lysine amino groups showed decreased reactivity, but only one by a factor of 6.8, that only one $tRNA^{Tyr}$ binds to the enzyme on a site that may well span both subunits.

At present, the complete amino acid sequences of only a few aminoacyl-tRNA synthetases have been determined (Winter et al., 1977). As more sequences become available, this information, combined with detailed tertiary structure analysis and the type of chemical labelling studies described above, will enable more detail concerning the mechanism of action of this group of enzymes to be obtained.

4.5. Isoleucyl-tRNA synthetase.

Isoleucyl-tRNA synthetase, of all the aminoacyl-tRNA synthetases has perhaps received the most attention, by many groups of research workers. So far, all the isoleucyl-tRNA synthetases which have been purified, whatever their source, have been shown to be single polypeptides of molecular weights variously reported between 102,000 and 110,000. The amino acid composition of the enzymes from three strains of E. coli (K12, B and MRE 600) have been determined (Table 5). In addition to minor differences in amino acid composition, the three distinct enzymes appear to be structurally different as manifested by the demonstration of different N-terminal sequences for the B and MRE 600

TABLE 5.

AMINO ACID COMPOSITION OF E. COLI ISOLEUCYL-tRNA SYNTHETASE.

AMINO ACID	NUMBER OF RESIDUES PER MOLE ENZYME E. COLI		
	B*	MRE 600≠	K12**
Tryptophan	19	17	ND
Lysine	61	60	59
Histidine	26	23	31
Arginine	45	44	39
Half-cystine	14	14	14
Aspartic acid	88	90	94
Threonine	46	44	49
Serine	44	42	52
Glutamic acid	91	90	96
Proline	47	43	44
Glycine	72	75	81
Alanine	93	91	93
Valine	59	70	79
Methionine	23	22	23
Isoleucine	48	44	47
Leucine	83	73	81
Tyrosine	32	30	39
Phenylalanine	31	29	36

Taken from:

*Durekovic, A., Flossdorf, J. & Kula, M.R. (1973) Eur. J. Biochem. 36, 528-533

≠Baldwin, A.N. & Berg, P. (1966) J. Biol. Chem. 241, 831-838

**Fersht, A.R. & Kaethner, M.M. (1976) Biochemistry 15, 818-823

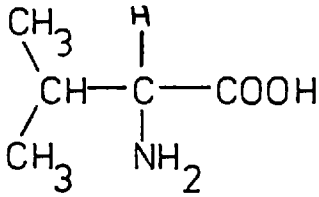
enzymes (Durekovic et al., 1973). It has been suggested (Fersht & Kaethner, 1976) that the diverse regions do not involve the catalytic sites of the enzymes.

Like most of the other synthetases so far studied, sulphydryl groups play an important part in catalysis (Stern et al., 1966). Isoleucine binding and the catalysis of the pyrophosphate exchange reaction were thought to be dependent in particular on the activity of one reduced sulphydryl group (Iaccarino & Berg, 1969). N-Ethyl maleimide inactivated the sulphydryl groups on the enzyme isolated from E. coli B. The one very rapidly reacting group could be protected by prior binding with isoleucine and ATP. Using the E. coli MRE 600 enzyme, a specific cysteinyl residue at or close to the catalytic site of isoleucine has also been found (Rainey et al., 1976; Kula, 1974). The protein was modified by using N-ethyl maleimide in one experiment and L-isoleucyl - bromomethyl ketone in the other. Initial sequencing studies showed that the same cysteine residue was labelled in each case. More detailed investigation of the catalytic and substrate binding properties of the modified protein, however, ruled out the essential participation of the rapidly alkylated cysteine sulphydryl group during catalysis (Rainey et al., 1977). It is believed (Rainey et al., 1977) that the previous results are ascribed to reduced binding of L-isoleucine, so that there exists a reduced level of enzyme.amino acid complex, but which can proceed further to product formation. The finding that alkylation of the particular fast-reacting sulphydryl group does not eliminate catalysis may not, however, rule out the essential function of another, yet unidentified, cysteine sulphydryl group. The integrity of this particular

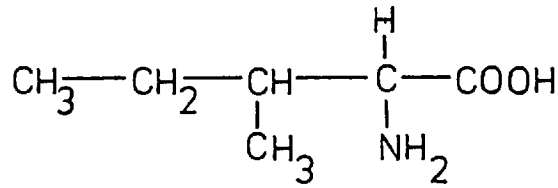
sulphydryl group is required for optimal binding of L-isoleucine and maximum synergistic complexing with ATP. It has been proposed (Rainey et al., 1977) that an unfavourable, yet minute rearrangement of the active site, affecting the position and ionisation of amino acid side chains occurs. The L-isoleucyl methyl moiety must be removed from the catalytic site subsequent to alkylation most probably by a rearrangement involving the sulphydryl groups. It may be that an arm-like structure carrying this moiety transiently approaches the amino acid binding site and thereby elicits an as yet unknown inactivating effect on the amino acid transfer step. It has also been proposed (Iaccarino & Berg, 1969) that a linkage between the L-isoleucine catalytic site and the tRNA binding site would be disrupted.

The interaction of isoleucine with its synthetase and the nature of the active site has also aroused interest, since valine differs from isoleucine only by having a hydrogen atom in place of the methyl group of isoleucine.

VALINE



ISOLEUCINE



On the basis of physico-chemical interactions, discrimination against valine can be no more than 1 in 20 (Yamane & Hopfield, 1977). However, in protein synthesis in vivo, the error of misplacing a valine for an isoleucine is less than 1 in 3,000 (Loftfield & Vanderjagt, 1972). In vivo there is no more mischarging of $tRNA^{Ile}$ with valine than with other amino acids. Isoleucyl-tRNA synthetase is, however, capable of activating valine to form an enzyme-bound valyl-adenylate

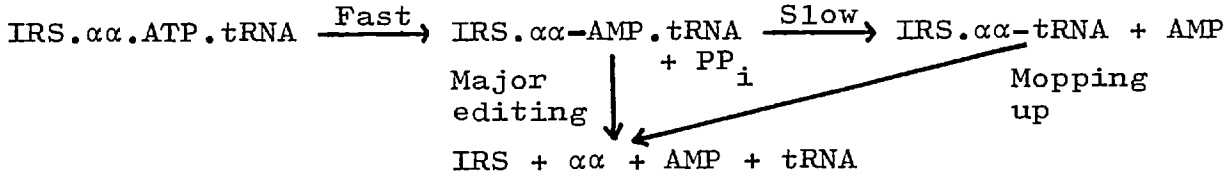
complex (Baldwin & Berg, 1966). However, when the enzyme.Val-AMP complex is presented with $tRNA^{Ile}$, valine is not transferred to $tRNA^{Ile}$, but the Val-AMP complex is hydrolysed to valine and AMP. Any alteration of the tRNA which destroys the isoleucine acceptor activity also destroys the ability of the tRNA to induce hydrolysis of the enzyme-bound valyl-adenylate (Baldwin & Berg, 1966). Eldred and Schimmel (1972b) provided evidence that Val-$tRNA^{Ile}$ is an intermediate in the $tRNA^{Ile}$-induced hydrolysis of the enzyme-bound Val-AMP complex. Ile-$tRNA^{Ile}$ itself can be slowly deacylated by isoleucyl-tRNA synthetase in the absence of AMP and PP_i. However, the controversy continues over the exact mechanism for preventing the mischarging of $tRNA^{Ile}$. In a recent paper, Fersht (1977) presents evidence that a two-stage editing mechanism exists, in which the majority of the Val-AMP is destroyed before the transfer to tRNA. This would be the major editing step while the hydrolytic activity of isoleucyl-tRNA synthetase towards Val-$tRNA^{Ile}$ would be a second editing step to mop up any mischarged tRNA formed by the Val-AMP escaping the first editing step. The editing procedure is necessary because, as stated before, there are insufficient structural differences between some pairs of amino acids to discriminate between them by a simple mechanism. The enzyme may be in a different, hydrolytically active, conformation during the transfer of the amino acid from the adenylate to the tRNA, viz:

$$\text{IRS} \longrightarrow \text{IRS*.Val-AMP.tRNA} \xrightarrow{1.2\,\text{sec}^{-1}}$$

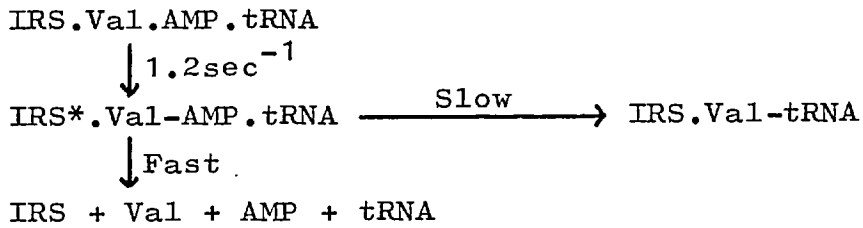
$$\text{IRS*.Val-tRNA} \xrightarrow{150\,\text{sec}^{-1}} \text{Val} + \text{tRNA} + \text{IRS}$$

where IRS = isoleucyl-tRNA synthetase.

Alternatively, there may be a different specificity mechanism i.e. a hydrolytic site, in which Val-AMP is hydrolysed before the transfer of the valyl residue to the tRNA. The hydrolytic activity of the isoleucyl-tRNA synthetase towards valyl-tRNA^{Ile} would then be just a double check to mop up any mis-acylated tRNA that leaks through the first editing step. It is proposed that the hydrolytic site is just large enough to accept valine, but it can exclude isoleucine (Fersht, 1977a).



It is still possible, however, for a rate determining change in enzyme conformation to give an activated intermediate.



For isoleucine, the transfer step would be much faster than the hydrolytic step. As stated earlier, although isoleucyl-tRNA synthetase is a monomeric enzyme, there is a degree of sequence homology similar to other monomeric synthetases (Kula, 1973). It is possible that two binding sites, not necessarily identical, for isoleucine may exist on each molecule. However, so far there is no definite evidence for this. The active site of isoleucyl-tRNA synthetase, purified from E. coli B was probed using structural analogues of

isoleucine and ATP by Holler *et al.* (1973). It was concluded that one of the structural components of the active site is an ion pair. Binding of L-isoleucine is associated with an opening of the ion pair and formation of two new ion pairs through the α-ammonium group and the carboxylate group of isoleucine respectively. The positive charge paired with the substrate carboxylate group may simultaneously interact with the oligophosphate group of ATP. Since the binding of L-isoleucine and ATP is independent (Holler *et al.*, 1971) this positively charged group must have a similar position in both enzyme.ligand complexes. Since the ion pair can be opened when ATP binds to its specific site, once this open conformation has been stabilised, binding of isoleucine-like compounds is made much easier.

Structural analogues of isoleucine have also been used recently to investigate the isoleucyl-tRNA synthetase purified from *E. coli* MRE 600 (Flossdorf *et al.*, 1976a,b). The binding properties were examined using an analytical centrifugation technique. It was found that a primary amino group, together with a not too small side chain were the pre-requisites for ligand recognition by the enzyme. Specificity for the L-isomers seemed to be absolute. But the carboxyl group could be replaced by substituents that were more or less hydrophobic. Side chain branching at the β-carbon atom was also important.

Although for many years the charging of tRNA was considered to occur in two stages,

$$\alpha\alpha + ATP + Enzyme \rightleftharpoons \alpha\alpha\text{-}AMP.Enzyme + PP_i$$

$$\alpha\alpha\text{-}AMP.Enzyme + tRNA \longrightarrow \alpha\alpha\text{-}tRNA + AMP + Enzyme$$

the mechanism has only recently been shown (Fersht & Kaethner, 1976) to be correct in the case of isoleucyl-tRNA synthetase from E. coli K12. Although there seems to be no doubt that the formation of the aminoacyl-adenylate.enzyme complex, is the fastest stage, it was shown that the transfer of the isoleucyl moiety to $tRNA^{Ile}$ is the rate determining step. This contrasts with the earlier observations of Yarus and Berg (1969), and Eldred and Schimmel (1972a) who found that the rate determining step for the E. coli B enzyme was release of isoleucyl-$tRNA^{Ile}$ from the enzyme. Fersht and Kaethner (1976) proposed the following reaction scheme for the enzyme at high substrate concentrations:

$$IRS.tRNA.Ile.ATP \longrightarrow IRS.tRNA.Ile\text{-}AMP + PP_i \xrightarrow{Ile}$$

$$IRS'.tRNA.Ile\text{-}AMP.Ile \longrightarrow IRS + Ile\text{-}tRNA + Ile + AMP$$

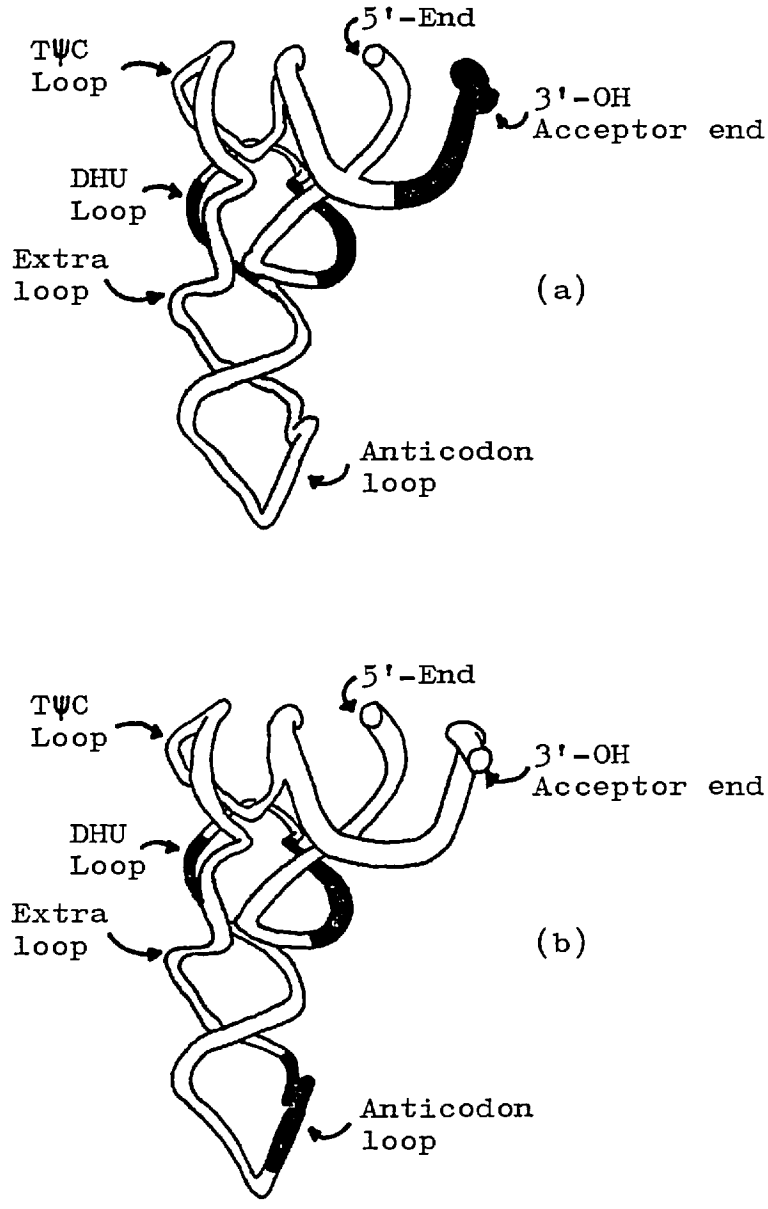
In contrast, with phenylalanyl-tRNA synthetase from E. coli K10, it was shown (Holler, 1976) that a slow rearrangement of the Phe-$tRNA^{Phe}$, before dissociation from the enzyme was the rate limiting step.

The binding site for $tRNA^{Ile}$ on the isoleucyl-tRNA synthetase enzyme isolated from E. coli B has been examined by photochemically cross-linking the tRNA in situ to the enzyme (Budzik et al., 1975). In addition, $tRNA^{Ile}$ was

cross-linked to valyl-tRNA synthetase from yeast. This enzyme is known to mischarge $tRNA^{Ile}$ with valine. In each case, three distinct parts of the nucleic acid were found to be cross-linked. Two of these are the same in both complexes and involve the dihydrouridine stem and loop regions (Fig. 8). The third linkage is different in each case, involving the 3'-terminus in the cognate case and the 3'-side of the anticodon in the non-cognate case. It would appear logical that one site of interaction would be the anticodon loop. In fact, it has been shown in other studies that this area is shielded by the enzyme (Schimmel et al., 1972). Also, the 3'-terminus of the $tRNA^{Ile}$ must make contact with the valyl-tRNA synthetase since it attaches valine at this point. Although these studies are not conclusive, they do, however, give a useful indication of areas of contact between $tRNA^{Ile}$ and isoleucyl-tRNA synthetase. Later work, using ^{3}H-labelling of purine units in the tRNA (Schoemaker & Schimmel, 1976) showed that four purine residues in the $tRNA^{Ile}$ were substantially perturbed (showed retarded labelling rates) when bound to isoleucyl-tRNA synthetase. They were located at or near the 3'-terminus at the interface of the dihydrouridine stem and loop (Fig. 7). This substantiates the previous photochemical cross-linking studies. In addition, two other sites, one in the anticodon and one in the amino acid acceptor - TψC helix appear to be perturbed by the enzyme (Figs. 7 and 8).

As yet, no complete sequence has been reported for isoleucyl-tRNA synthetase. At the present time, the enzyme from E. coli MRE 600 is being sequenced by Kula's group at the Universität Regensburg and Gesellschaft für Biotechnologische

FIGURE 7. THE CONFORMATION OF $tRNA^{Ile}$ FROM E. COLI B SHOWING REGIONS CROSS-LINKED (a) TO ITS COGNATE AMINOACYL-tRNA SYNTHETASE & (b) TO YEAST VALYL-tRNA SYNTHETASE.



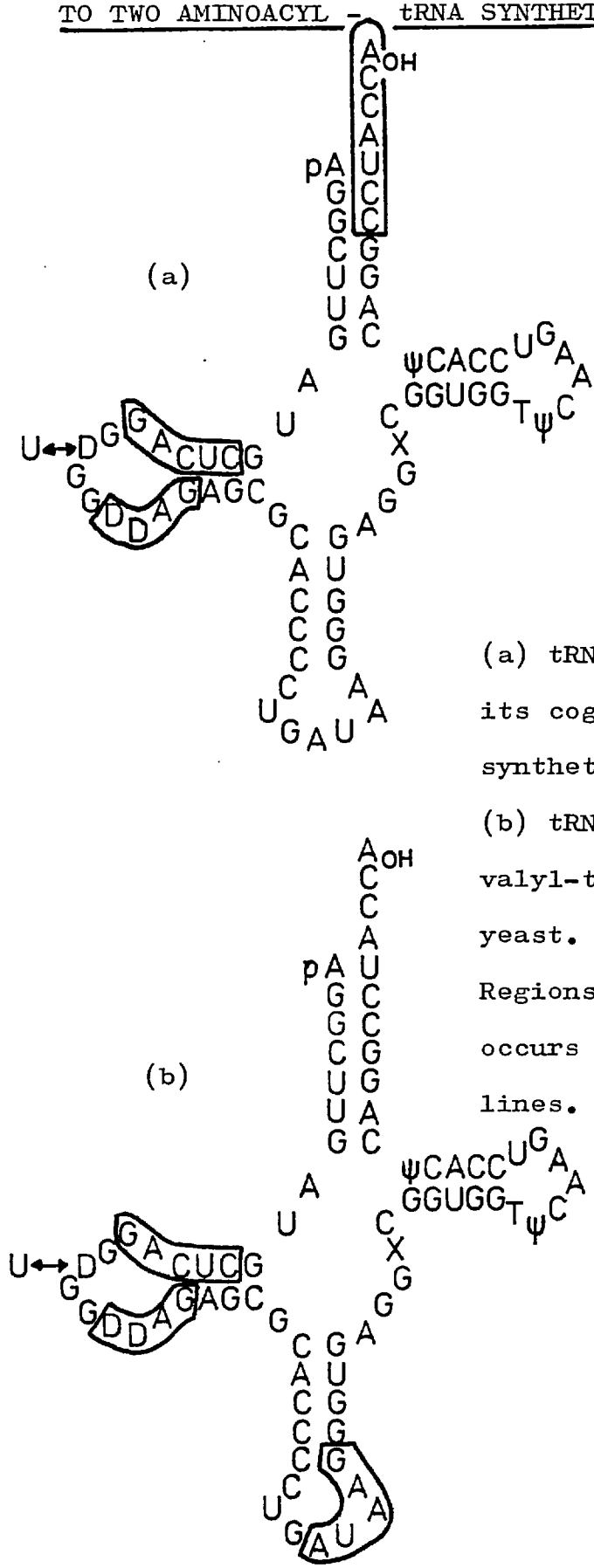
(a) $tRNA^{Ile}$ cross-linked to its cognate aminoacyl-tRNA synthetase.

(b) $tRNA^{Ile}$ cross-linked to valyl-tRNA synthetase from yeast.

Regions where cross-linking occurs are shown by heavy shading.

Redrawn from Budzik *et al.* (1975).

FIGURE 8. THE SEQUENCE & CLOVERLEAF STRUCTURE OF $tRNA^{Ile}$ FROM E. COLI B SHOWING REGIONS CROSS-LINKED TO TWO AMINOACYL - tRNA SYNTHETASES.



(a) $tRNA^{Ile}$ cross-linked to its cognate aminoacyl-tRNA synthetase.

(b) $tRNA^{Ile}$ cross-linked to valyl-tRNA synthetase from yeast.

Regions where cross-linking occurs are shown by heavy lines.

Redrawn from Budzik et al. (1975).

Forschung mbH, Braunschweig-Stöckheim, West Germany. Once this information, together with a detailed X-ray crystallographic study is available, it should be possible to answer more detailed questions about the catalytic mechanism of this enzyme.

The foregoing review is a brief resume of the salient features of aminoacyl-tRNA synthetases. The subject has been reviewed more extensively by Ofengand (1977), Kisselev and Favorova (1974) and Soffer (1974).

MATERIALS AND METHODS.

MATERIALS.

a) Bacterial strains used.

Staphylococcus aureus (Oxford) N.C.T.C. 6571

Pseudomonas fluorescens NC1B 10586

Escherichia coli MRE 600

Escherichia coli B AS19 NF 541 Rel A^{+**}

Escherichia coli B AS19 NF 542 Rel A^{-**}

** Kindly supplied by Dr. N.Fiil, Universitets Mikrobiologiske Institut, Øster Farimagsgade 2A, DK-1353, Copenhagen K, Denmark.

b) Culture maintenance.

Cultures were stored in 10% glycerol in sealed ampules under liquid nitrogen at -196°C. For routine use, cultures were maintained on nutrient agar slopes in McCartney bottles at 20°C. Strains were sub-cultured every month to check for contamination.

c) Media.

(i) Nutrient broth, brain heart infusion broth and nutrient agar were obtained from Oxoid Limited, London, S.E.1.

(ii) Synthetic minimal medium (Cruikshank, 1969): 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); distilled water to 1 litre; pH 7.4 with sodium hydroxide.

(iii) Synthetic minimal medium for culturing E. coli B AS19 NF541 and NF542:
Tris-HCl pH7.4 (6.055g), $(NH_4)_2SO_4$ (0.1g), casamino acids (2g), sodium citrate (0.5g), KH_2PO_4 (0.272g), $MgSO_4.7H_2O$ (0.1g), $FeCl_3$ (0.00054g), cytosine (0.05g), thymidine (0.05g), uracil (0.05g) and glucose (2g); made up to 1 litre with

distilled water.

(iv) Low phosphate minimal medium:

Experiments in which [^{32}P]-orthophosphate was used were carried out in a medium in which the level of KH_2PO_4 was 0.0272g/litre and additionally contained KCl (0.15g/litre).

d) Isotopically labelled compounds.

The following isotopically labelled compounds were obtained from the Radiochemical Centre, Amersham, Bucks.

D-[1-^{14}C]-glucosamine	3.4mCi/mmole
[U-^{14}C]-glycine	50.0mCi/mmole
L-[U-^{14}C]-histidine	150.0mCi/mmole
L-[U-^{14}C]-isoleucine	10.0mCi/mmole
L-[U-^{14}C]-isoleucine	342.0mCi/mmole
[U-^{14}C]-labelled protein hydrolysate	54.0mCi/matom
L-[U-^{14}C]-leucine	150.0mCi/mmole
L-[methyl-^{14}C]-methionine	52.0mCi/mmole
L-[U-^{14}C]-phenylalanine	10.0mCi/mmole
Tetrasodium [^{32}P]-pyrophosphate	100.0mCi/mmole
[2-^{14}C]-thymidine	56.0mCi/mmole
DL-[methylene-^{14}C]-tryptophan	50.0mCi/mmole
L-[U-^{14}C]-tyrosine	10.0mCi/mmole
[2-^{14}C]-uridine	55.0mCi/mmole
[5-^{3}H]-uridine triphosphate	10,000mCi/mmole
L-[U-^{14}C]-valine	125.0mCi/mmole

[^{32}P]-Orthophosphate (carrier-free): original specific activity 3Ci/mmole of phosphorus was purchased from New England Nuclear, D-6072 Dreieich, West Germany.

e) Scintillation fluids.

(i) Liquid scintillation fluid had the following composition: 2-(4'-tert-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (30g), naphthalene (250g) made up to 5 litres with toluene. In experiments where small aqueous samples were radiocounted (20-50µl), the sample was dissolved in 2-methoxyethanol (5ml) prior to addition of the scintillant (10ml) of the above composition.

(ii) For larger aqueous samples (50-500µl) Bray's scintillation fluid was used, comprising: naphthalene (60g), 2,5-diphenyloxazole (4g), 1,4-bis-(5-phenyloxazol-2-yl) benzene (0.2g), methanol (100ml) and ethylene glycol (20ml) made up to 1 litre with 1,4-dioxan.

Naphthalene (Scintillation grade) was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex.

2-(4'-Tert-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole was obtained from CIBA (A.R.L.) Ltd., Duxford, Cambridge.

Toluene (Analar grade) was obtained from Fisons Scientific Apparatus, Loughborough, Leicestershire.

2,5-Diphenyloxazole (Scintillation grade) was obtained from Packard Instrument Company Inc., 220, Warenville Road, Downers Grove, Illinois 60515, U.S.A.

1,4-Bis-(5-phenyloxazol-2-yl) benzene (Scintillation grade) was obtained from Hopkins and Williams Ltd.

Methanol (Analar grade) was obtained from James Burrough Ltd.,Fine Alcohols Division, 60, Montford Place, London, S.E.11.

Ethylene glycol was obtained from Hopkin and Williams Ltd.

1,4-Dioxan (Scintillation grade was obtained from

Fisons Scientific Apparatus.

(iii) Radiocounting efficiency for ^{3}H and ^{14}C in the different scintillant cocktails was determined with ^{14}C or ^{3}H labelled standards.

(iv) Scintillation counting was performed using a Beckman LS-200B Liquid Scintillation Spectrometer.

f) Antibiotics.

(i) Pseudomonic acid was used as the sodium salt in experiments unless stated otherwise. Its purification from fermentations is described in the Methods section. In later experiments, sodium pseudomonate (92% purity, by high pressure liquid chromatography) was supplied by Beecham Pharmaceuticals Research Division, Chemotherapeutic Research Centre, Brockham Park, Betchworth, Surrey.

(ii) Chloramphenicol, nalidixic acid, penicillin G, rifampicin and tetracycline was also supplied by Beecham Pharmaceuticals Research Division.

(iii) 2,4-Dinitrophenol was obtained from BDH Chemicals Ltd., Poole, Dorset.

g) Miscellaneous.

(i) GF/C and GF/F glass fibre filter papers were obtained from Whatman Ltd., Maidstone, Kent.

(ii) Polyethyleneimine (P.E.I.)-cellulose coated thin layer chromatography plates were obtained from Camlab Ltd., Cambridge.

(iii) Silica gel (0.25mm) thin layer chromatography plates containing fluorescent indicator UV_{254} were obtained from Camlab Ltd.

(iv) Adenosine triphosphate, cytidine triphosphate, guanosine triphosphate, uridine triphosphate, deoxyribonuclease I (electrophoretically pure), amino acids, poly U, phospho-enol pyruvate, pyruvate kinase, salmon sperm DNA, bulk tRNA (from E. coli strain W) and pyrophosphatase were obtained from Sigma (U.K.) Ltd., Fancy Road, Poole, Dorset.

(v) Autoradiography film, Kodirex KD 54T, was obtained from Kodak Ltd., Victoria Road, Ruislip, Middlesex.

(vi) Sephadex G-25 (coarse) and G-50 (fine) were obtained from Pharmacia (G.B.) Ltd., Paramount House, 75, Uxbridge Road, London, W.5.

METHODS.

1. Purification of Sodium Pseudomonate.

A crude mixture of sodium salts (10g) obtained from a large-scale fermentation (Fuller et al., 1971) was extracted three times into isobutyl methyl ketone at pH 4.0-4.5 from an aqueous solution. After the volume of the extract had been reduced to ca. 4ml by evaporation under reduced pressure, distilled water (200ml) was added and the pH adjusted to 7.8 with ammonia solution (0.1M). The solution was adsorbed onto a prewashed (distilled water) and aqueous ammonia-equilibrated amberlite XAD2 resin column. The resin (1200g) was contained in a column made of precision bore borosilicate glass (125 x 5.5cm). The crude salts were purified by chromatography on the resin with a linear gradient produced by adding 0.01N methanolic ammonia to 0.01N aqueous ammonia. Fractions (95 x 100ml) were collected. To locate the fractions containing ammonium pseudomonate, a sample (5ml) of every fifth fraction was evaporated to dryness and methylated using ethereal diazomethane. Methyl pseudomonate formed a discrete spot when run on pre-coated silica gel GF_{254} plates in an isopropyl alcohol:chloroform::1:9 system and was located under ultra violet light (254nm). Fraction 65-82 were found to be rich in ammonium pseudomonate. They were combined and the volume of extracts reduced to 100ml. After pH adjustment to 4.4, the free acid was re-extracted into isobutyl methyl ketone. The sodium salt was formed by repartition into water, the pH of the aqueous layer being adjusted to 7.0-7.5 by the addition of sodium hydroxide solution. This aqueous phase

was freeze dried to obtain purified sodium pseudomonate (2.89g) as a flocculent white solid, which was stored dessicated at 0-4°C.

2. Determination of Minimum Inhibitory Concentrations (M.I.C).

A standard solution of each antibiotic was prepared (usually 1mg/ml) in double-strength nutrient broth (i.e. broth made up at twice the strength suggested by the manufacturers). Doubling dilutions of each were made into capped test tubes containing sterile double strength nutrient broth (2ml) to give final antibiotic concentrations in the range 0.03-500µg/ml. Each dilution was inoculated with 10^3-10^4 cells/ml of the organism to be tested. The M.I.C. was defined as the least antibiotic concentration at which no visible growth occurred, after sixteen hours static incubation at 37°C.

3. Agar Plate Assay for Sodium Pseudomonate.

The assay plate was prepared by pouring molten penassay base agar (250ml) at 45°-50°C that had been seeded with 10^5-10^6 cells/ml S. aureus (Oxford) into a sterile, level assay dish. After the agar had set, the surface was dried by inverting the plate in a 37°C incubator, with the lid removed, for about 30 minutes. Wells, 8mm in diameter and approximately 3cm apart were cut into the agar, using a cork borer that had been sterilised by immersion in alcohol and flamed. Suitable dilutions of the test sample and standard sodium pseudomonate solutions (0.1-100µg/ml) were prepared using 0.01M phosphate buffer, pH 7.0, as the diluent. 0.05ml of diluted solution was added to each well; at least three wells of each dilution were prepared, a record being kept of the contents of each well. After the bioassay plate

had been incubated at 37°C for 18 hours, the diameters of the inhibition zones were measured accurately by means of vernier calipers. The average zone diameter for each dilution of sample and standard antibiotic solution was determined, and a standard curve was prepared by plotting zone diameter against logarithm of antibiotic concentration. There is a linear relationship between zone diameter and the logarithm of the antibiotic concentration. Antibiotic concentrations in the test samples were determined by obtaining average zone diameters using the standard curve.

4. Determination of the Effect of Sodium Pseudomonate on a Growing Culture of S. aureus.

A 0.01% inoculum of an overnight culture of S. aureus was made into double-strength nutrient broth (200ml). Every half hour the E_{540} of the culture was determined. A viable count of the culture was determined every hour, by plating out samples (0.1ml) in five-fold replicates, of culture samples suitably diluted, onto agar plates. After three hours the culture was split and sodium pseudomonate added to one to a final concentration of 0.5μg/ml. Sampling was continued for a further four hours. The experiment was repeated using sodium pseudomonate at concentrations of 0.1μg/ml and 0.05μg/ml.

5. Measurement of Protein Synthesis in vivo.

The method used was similar to that of Uchida & Zahner (1975). A 0.1% inoculum of an overnight culture of the bacterium to be used was dispensed into double-strength nutrient broth (100ml). After about two hours of shaking incubation at 37°C, or when the E_{620} of the culture reached about 0.2, tracer [^{14}C]-isoleucine or [^{14}C]-phenylalanine

(0.2μCi/ml of the culture) was added. Samples (0.5ml) were removed and added to trichloracetic acid (TCA) (5ml), mixed well, placed on ice for 30 minutes and finally heated at 90-95°C for 20 minutes. At various time intervals, as indicated by the arrows in Figs.10-16, the culture was split and sodium pseudomonate added to give the final concentrations shown. Sampling was continued in the same way for all cultures. On cooling, the precipitate from each sample was collected by filtration on to a Whatman GF/C glass fibre filter. Each filter was washed with 5% TCA (2x6ml) and absolute alcohol (2x6ml), then dried and the radioactivity determined by liquid scintillation counting in toluene-based scintillation fluid (5ml).

For measuring the in vivo protein, RNA and DNA synthesis using E. coli strains AS19 NF541 and NF542, brain heart infusion broth was used in place of double-strength nutrient broth.

6. Measurement of DNA and RNA Synthesis in vivo.

Cultures were inoculated and incubated as described for protein synthesis. To follow RNA synthesis, the cultures were labelled with [^{14}C]-uridine (0.2μCi/ml) and for DNA synthesis, with [^{14}C]-thymidine. Samples of culture (0.5ml) were then added to 2% sodium lauryl sulphate (0.5ml), mixed well and then placed on ice. After 30 minutes, 5% TCA (5ml) was added to each and after mixing well, the samples were left for a further 15 minutes before filtering, washing, drying and radiocounting, as described above.

7. Measurement of Peptidoglycan Synthesis in vivo.

(a) The first method used was identical to that described above for protein synthesis, using D-[^{14}C]-glucosamine (0.2μCi/ml)as tracer.

(b) Method (a) is not specific for peptidoglycan since glucosamine can serve as a metabolic intermediate in the formation of several macromolecules in the bacterial cell. A second method was also used (Park & Hancock, 1960) in which peptidoglycan was separated from the remainder of the cellular material.

A 0.1% inoculum of an overnight culture of S. aureus was dispensed into double-strength nutrient broth (200ml). This was incubated with shaking at 37°C for 2 hours. D-[1-^{14}C]-Glucosamine was added, the culture was split (equal portions) into two 500ml flasks, and to one of the cultures antibiotic was added to give the concentration as shown in Table 6. Incubation of both cultures was continued for the various time intervals, as indicated in Table 6. The treated and untreated cells were harvested separately by centrifuging at 6,000g for 15 minutes. Both pellets were washed in distilled water, then each was resuspended in cold distilled water (2ml). A 25% TCA solution (0.5ml) was added to each. The extracts were mixed well and then left at 0°C for 10 minutes, followed by centrifugation at 4,000g for 10 minutes. The supernatants were discarded and each residue suspended in 75% ethanol (2-5ml). After 10 minutes at room temperature, both samples were again centrifuged at 4,000g for 10 minutes and the supernatants were discarded. The residues were resuspended in 5% TCA (2.5ml) and heated for 6 minutes at 90°C. After cooling, the suspensions were centrifuged at 4,000g for 10 minutes. Again the supernatants were discarded and the residues resuspended in 0.05M NH_4HCO_3 (0.95ml) containing 0.005M ammonium hydroxide. A solution (0.05ml) of trypsin (1mg/ml)

was added to each followed by incubation for 2 hours at 37°C. The suspensions were centrifuged at 4,000g for 10 minutes and the supernatant discarded. The residue was freeze dried, resuspended in distilled water (1ml) and 0.1ml aliquots of each were radiocounted in Bray's scintillation fluid (10ml).

8. Measurement of Cellular ATP Levels.

The [^{32}P]-orthophosphate exchange method of Cashel (1969), as described by Ogilvie et al., (1975b), was used. A 0.1% inoculum of an overnight culture of S. aureus was dispensed into double-strength nutrient broth (100ml) and incubated with shaking at 37°C for 3 hours. Aliquots (0.5ml) of culture were transferred to two test tubes. A solution of sodium pseudomonate was added to one of the tubes to give a final concentration of 0.5µg/ml. Sterile distilled water was added to the second tube to maintain equalvolumes in each. Both cultures were labelled with [^{32}P]orthophosphate (50µCi/ml). The cultures were incubated statically at 37°C and equally aerated using a split capillary tube. At various time intervals, as indicated in Table 9 , aliquots (0.1ml) of culture were removed and added to 2M formic acid (0.1ml). After mixing well, the samples were left on ice for 20 minutes, followed by centrifugation at 18,000g for 5 minutes. Aliquots (20µl) from each of the supernatants were spotted onto polyethyleneimine-cellulose thin layer chromatography plates which had previously been soaked in deionised water for 30 minutes and then dried. The labelled components were separated by ascending development in 1.5M KH_2PO_4 (pH 3.4) at room temperature in a closed chromatography tank. The ^{32}P - labelled nucleotides were located by overnight autoradiography using Kodak X-ray film. The R_f value of ATP (0.8)

was identical to that quoted by Cashel (1969). The spots corresponding to the ^{32}P labelled ATP were scraped off the chromatogram and radiocounted.

For measurement of ATP, GTP, ppGpp and pppGpp levels in E. coli B AS19 NF541 and NF542, the same method was also used. A 0.3% inoculum of an overnight culture of E. coli B AS19 NF541 or E. coli B AS19 NF542 grown in the high phosphate medium was transferred to prewarmed low phosphate medium (5ml) and incubation continued at 37°C to an absorbance value of approximately 0.1 at 578nm. [^{32}P]-Orthophosphate was added to give 70μCi/ml, the culture shaken well, and 1ml portions transferred to each of two 5ml conical flasks. The cultures were vigorously agitated at 37°C on a shaking water bath. One served as a control whilst the other was treated with pseudomonic acid. Samples (50μl) were removed at frequent intervals (Fig. 18).

9a. Measurement of Protein Synthesis in vitro.

The method used was that described by Lacey and Chopra (1972). A 4% inoculum of an overnight culture of S. aureus was dispersed into double-strength nutrient broth (100ml) and incubated with shaking at 37°C for $2\frac{1}{2}$ hours. The cells were harvested by centrifugation at 4°C and then washed twice in 0.05M Tris-HCl buffer pH7.6 containing ammonium acetate (50mM), magnesium chloride (20mM), potassium chloride (50mM) and dithiothreitol (0.2mM) (Buffer A). The washed cells were resuspended in buffer A (1ml), lysostophan added (50μg/ml) and the solution incubated for 10 minutes at 37°C. Deoxyribonuclease I (5μg/ml) was added and incubation continued for 30 seconds. The resultant lysates were cooled to 4°C and then centrifuged at 10,000g for 30 minutes to remove

residual whole cells. The supernatant was used in the following cell-free protein synthesising system. Cell-free extract (0.1ml) was added to 0.1ml of a solution containing adenosine triphosphate (7.5mM), guanosine triphosphate (1mM) and ^{14}C labelled amino acids (10μCi/ml) in buffer A. This mixture was incubated at 37°C for 40 minutes and then precipitated by the addition of TCA (final concentration 5%) followed by heating at 90°C for 20 minutes. Precipitates were collected, washed and dried on GF/C filters and radioactivity estimated as for protein synthesis *in vivo*.

No conclusive results were obtained using this method. After consulting with Dr. Chopra (Department of Bacteriology, Bristol University) this method was abandoned. It appears that he has experienced difficulties in obtaining consistent results with strains of *S. aureus* other than those reported in his original paper.

9b. Protein Synthesis in *E. coli* MRE 600 *in vitro*.

The method used was similar to that of Gould *et al.* (1973). Frozen washed cells (600g) of *E. coli* MRE 600 were suspended in buffer (20mM Tris-HCl, pH7.4, 10mM magnesium acetate and 10mM 2-mercaptoethanol) to a final volume of 900ml. To disrupt the cells, this suspension was passed through a Manton Gaulin continuous flow French press at 7,500psi. Deoxyribonuclease I was added to a concentration of 1μg/ml and left for 15 minutes at 0°C. The cell extracts were centrifuged for 30 minutes at 12,000g to remove any remaining whole cells and larger cell debris. The supernatant was centrifuged at 30,000g for one hour and this was used as the S30 preparation in the protein synthesis assay. Endogenous mRNA was destroyed by incubation at 37°C

for 40 minutes prior to use. The composition of the incubation mixture was as follows:- 8mM magnesium acetate, 50mM potassium chloride, 9mM 2-mercaptoethanol, 0.075mM each of twenty amino acids, 2.5mM adenosine triphosphate, 2.5mM guanosine triphosphate, 2.5mM phosphoenol pyruvate and pyruvate kinase (15µg/ml) in 80mM Tris-HCl buffer, pH7.8. The concentration of ribosomes in the S30 fraction was determined from the optical density, after dilution, at 260nm and 280nm (Warburg & Christian, 1942).

Sucrose was added to the supernatant to a concentration of 10% w/v, which was then dialysed overnight at 4°C against the same buffer that was used in the preincubation plus 10% sucrose, but without the amino acids, ATP, GTP, phosphoenol pyruvate and pyruvate kinase.

Protein Synthesis Assay.

An aliquot of the above dialysate was incubated at 37°C with the following cocktail in a final volume of 1ml:- pyruvate kinase (20µg), 5mM phosphoenol pyruvat, 3mM GTP, 10mM ATP, 14mM magnesium acetate, 50mM potassium chloride, 14mM 2-mercaptoethanol, [^{14}C]-phenylalanine (2µCi; 10mCi/mmole) and poly U (100µg). After 30 minutes, 1M sodium hydroxide containing 0.25% w/v phenylalanine was added and the incubation continued at 37°C for 5 minutes and then at room temperature for 15 minutes. Trichloracetic acid (10%, 4ml) was added, whirled, left for 10 minutes and the mixture finally heated at 90°C for 10 minutes. The precipitate was collected on a Whatman GF/C glass fibre filter, washed, dried and radio-counted as described for protein synthesis *in vivo*.

10. Measurement of RNA-Dependent RNA Synthesis Using Purified Virus Particles from Aspergillus foetidus.

The purified virus preparation was kindly provided by Dr. K. Buck, Department of Biochemistry, Imperial College, London. Each virus particle contains an enzyme which can transcribe the viral genome of double stranded RNA.

In a total assay volume of 0.2ml, purified virus (E_{260}=1.0) suspension (100µl) was incubated overnight at 30°C with Tris-HCl pH 7.9 (80mM), sodium chloride (150mM), ethylene diamine tetraacetic acid (0.1mM), magnesium chloride (3mM), ATP (3mM), GTP (3mM), CTP (3mM), UTP (3mM), [^{3}H]-UTP (17µCi/µmole), water and/or antibiotic solution. After incubation, the assay mixtures were cooled to 0°C, and TCA (10%, 3ml), also at a temperature of 0°C, containing sodium pyrophosphate (10mM) was added to each. After 15 minutes, the insoluble precipitate from each assay was collected on a GF/C filter, presoaked in TCA (2%) aontaining sodium pyrophosphate (10mM). Each filter was then washed eight times with aliquots (10ml) of TCA (2%) containing sodium pyrophosphate (10mM) and three aliquots (10ml) of absolute alcohol. The filters were then dried and radioactivity was determined as for RNA synthesis *in vivo*.

11. Measurement of DNA-Dependent RNA Polymerase Activity.

The RNA polymerase had been purified from *E. coli* B and was kindly provided by Dr. D. Glover, Department of Biochemistry, Imperial College, London. The purification procedure used (Chamberlin & Berg, 1966) was as follows:- *E. coli* cells were disintegrated by mixing with glass beads in a Warburg blender. Differential precipitation with streptomycin (0.5%), protamine (0.05%) and finally ammonium sulphate (50%) followed. The final purification step involved

DEAE cellulose chromatography. RNA synthesis was measured using the following assay system:- Tris-HCl pH 8.1 (50mM), sodium chloride (80mM), potassium chloride (40mM), magnesium chloride (15mM), 2-mercaptoethanol (10mM), glycerol (10%), ATP (5mM), CTP (5mM), GTP (5mM), UTP (5mM), [^{3}H]-UTP (5μCi), salmon sperm DNA (20μg) and 10 units of purified enzyme, plus or minus water and antibiotic solutions, in a single assay volume of 0.2ml. After static incubation at 37°C for one hour, the reaction was terminated by the addition of 2M HCl (0.5ml) to each assay. After 15 minutes on ice, the precipitate was collected on a GF/C filter, washed successively with 1M HCl (4x10ml) and absolute alcohol (2x10ml), dried and radiocounted as for RNA synthesis *in vivo*.

12. Measurement of the Growth of *E. coli* MRE 600 in the Presence of Pseudomonic Acid and One or More Amino Acids.

A biophotometer, with continuous readout at 600nm, was used to measure the effect of adding amino acid solutions to a culture of *E. coli* MRE 600 growing in minimal medium that had been treated with pseudomonic acid.

13. Measurement of Amino Acid Levels in *E. coli* MRE 600.

A 1% inoculum of an overnight culture of *E. coli* MRE 600 was dispersed into minimal medium (200ml). After shaking incubation at 37°C for 2½ hours, the culture was equally divided and pseudomonic acid (10μg/ml) added to one portion. Both cultures were incubated for a further 15 minutes and then the cells were harvested by centrifugation. The cell pellets were weighed, then acetone (2ml) was added to each. After standing at room temperature for one hour, the samples were evaporated to dryness, then petroleum ether (40-60°C boiling fraction) (2ml) was added. After 15 minutes at room

temperature, the petroleum ether was removed by centrifugation at 10,000g for 5 minutes. The pellets were then extracted again with petroleum ether (2ml). After extraction with methanol (2x2ml), both samples were evaporated to dryness and each dissolved in 0.2M HCl (5ml). The amino acids were separated using a Beckman amino acid analyser.

14. Measurement of Aminoacylation in vitro.

A modification of the procedure of Werner et al. (1976) was used. The S30 supernatant derived from E. coli MRE 600 cells (see 9b) was centrifuged at 150,000g for 5 hours. The S150 supernatant was dialysed overnight against buffer A containing sucrose (20%) and was used as a crude source of tRNA synthetases. Aminoacylation in vitro was measured by the addition of an aliquot (20µl) of the dialysed S150 supernatant to the following reagents, in a final volume of 100µl:- Tris-HCl, pH 7.5 (25mM), ATP (5mM), potassium chloride (70mM), magnesium chloride (10mM), EDTA (0.5mM) bulk tRNA (isolated from E. coli W) (250µg), radiolabelled amino acid (0.1mM, specific activity 10-150mCi/mmole) plus or minus pseudomonic acid (10µg). After the incubation at 37°C for 20 minutes, the reaction was stopped by the addition of TCA (7%, 2ml) and left on ice for 30 minutes. The samples were filtered through Whatman GF/C glass fibre filters, washed and radiocounted as for protein synthesis in vitro.

15. Measurement of the Degree of Aminoacylation of tRNA in vivo.

The procedure of Folk & Berg (1970) was employed, 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 minimal salts medium (1 litre)

pre-warmed to 37°C. This was incubated, with shaking, at 37°C for 1 hour 50 minutes. The culture was equally divided and pseudomonic acid (25μg/ml) was added to one half. Both cultures were incubated for a further 10 minutes, followed by the addition of 55% TCA (50ml) pre-warmed to 37°C. After 1 minute, 1% SDS (5ml) was added and the cultures rapidly cooled to 2-5°C by swirling in a solid CO_2-acetone bath. After 15-20 minutes, the precipitate was collected by centrifugation at 12,000g for 20 minutes and resuspended in sodium acetate (0.25M, pH 6.5) (8ml) containing 0.05% SDS and 1mM EDTA. An equal volume of phenol (saturated with 0.25M sodium acetate, pH 5.0, containing 1mM EDTA) was added. The mixture was sonicated for 1 minute (2x30 sec., with 2 minutes cooling between each) with a Branson sonicator. After centrifugation at 26,000g for 20 minutes, the aqueous layer was withdrawn and added to 4 volumes of ethanol and 0.2 volumes of 4M NaCl. The phenol layer was washed with an equal volume of sodium acetate- EDTA buffer. After centrifugation, the aqueous layer was withdrawn and added to 4 volumes of ethanol and 0.2 volumes 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 salt-ethanol mixture and left at -18°C overnight. Nucleic acids were collected by centrifugation at 26,000g for 20 minutes at 4°C. The pellet was suspended in sodium acetate buffer (0.1M, pH 4.6) (3ml). To the suspension (1.5ml) was added 0.01M sodium periodate in 0.1M sodium acetate, pH 4.6 (0.5ml). The remaining nucleic acid solution (1.5ml) was treated with 0.1M sodium acetate, pH 4.6 (0.5ml). After incubation in the dark at 37°C for 30 minutes, the nucleic

acid was reprecipitated by the addition of ethanol (4 volumes) and 5M sodium chloride (0.2 volumes) then centrifuged at 26,000g for 10 minutes. The precipitates were dissolved in 0.1M sodium acetate, pH 4.6 (2ml), containing 0.1M ethylene glycol, and incubated in the dark at 37°C for a further 10 minutes. Nucleic acid was precipitated as before with ethanol and salt. Both pellets were each dissolved in 3.6M Tris-acetate buffer, pH 8.2 (1ml) and incubated at 37°C for 2 hours. Nucleic acid was again precipitated with ethanol (4 volumes). After centrifugation, each pellet was dissolved in water (1ml).

The tRNA acceptor ability of aliquots (20µl) of each sample was assayed with several [^{14}C]-amino acids as described in the *in vitro* aminoacylation method.

16. Assay of Isoleucyl tRNA Synthetase Activity.

(a). Overall aminoacylation reaction.

Isoleucyl tRNA synthetase, purified from *E. coli* B, was very generously provided by Dr. C. Bruton, Department of Biochemistry, Imperial College, London.

This assay measured the rate of formation of [^{14}C]-isoleucyl-tRNA^{Ile} (Durekovic *et al.*, 1973). The reaction mixture (0.1ml) contained Tris-HCl buffer (100mM, pH 7.4), magnesium chloride (10mM), potassium chloride (10mM), dithiothreitol (2mM), L-[^{14}C]-isoleucine (0.1-0.01mM), 12 A_{260} units bulk tRNA, ATP (1mM) plus or minus pseudomonic acid (10-50nM) and 0.1µg of purified enzyme. Before addition of the enzyme, the reaction mixture was pre-equilibrated at 37°C. The reaction was timed from the addition of the enzyme. 5% Trichloroacetic acid was added to terminate the reaction. After being left at 0°C for 30 minutes, the precipitate from

each of the samples was collected on a GF/C glass fibre filter. These were washed with 5% TCA (2x15ml), then with absolute alcohol (2x10ml) and after drying, counted for radioactivity.

The aminoacylation assay was used to determine the effect of a number of pseudomonic acid derivatives, which had been prepared in the industrial laboratories, on the enzyme.

(b). Pyrophosphate exchange reaction.

Enzyme activity was measured by following the exchange of[^{32}P] label from pyrophosphate into adenosine triphosphate (Bergmann, 1969). The reaction volume of 1.0ml contained Tris-HCL buffer (100mM, pH 7.4), magnesium chloride (5mM), potassium fluoride (10mM), ATP (2mM),[^{32}P] -pyrophosphate (2mM, 10^4-10^5cpm/μmole), dithiothreitol (10mM), isoleucine (2-20μM) plus or minus pseudomonic acid (10-50nM) and purified isoleucyl-tRNA synthetase (250μg). The reaction mixture minus enzyme was pre-equilibrated at 37°C. The reaction was timed from the addition of enzyme and stopped by the addition of 7% perchloric acid (0.5ml) and then 10% Norit A solution (0.2ml). After 20 minutes at room temperature, the Norit A was collected on a GF/C glass fibre filter. The filter was thoroughly washed with distilled water and then glued to a planchette and radiocounted in a gas flow counter.

(c). Transfer step in isoleucyl-tRNA synthesis. Isolation of enzyme.[^{14}C]-Ile.AMP complex.

The method used was that of Rainey et al., 1977. Purified enzyme (500μg) was added to a reaction mixture of 200μl (final

volume) containing ATP (2mM), magnesium chloride (5mM), inorganic pyrophosphatease (1μg, 0.2unit), EDTA (0.1mM), L-[^{14}C]-isoleucine (20μM, 100mCi/mmole) and dithiothreitol (10mM) in potassium phosphate buffer (20mM, pH 7.5). After 2 minutes at 30°C , blue dextran was added. The mixture was placed on top of a Sephadex G-50 (coarse) column (28x0.5cm), previously equilibrated with 0.1M sodium citrate buffer (pH 6.1), 0.05M potassium chloride and 5mM dithiothreitol and eluted with the same buffer by gravity within a few minutes. The enzyme.[^{14}C]-Ile.AMP complex was located by radiocounting aliquots (20μl) from alternate fractions (160μl) that were collected from the column. The peak of activity found within the void volume corresponded to the complex and this was used for the transfer assay. In a final volume of 100μl, an aliquot (20μl) of the combined fractions containing the enzyme.[^{14}C]-Ile.AMP complex was incubated with 20 A_{260} units of bulk tRNA from E. coli W, 10mM dithiothreitol, 20mM Tris-HCl, pH 7.4, 5mM potassium chloride plus or minus pseudomonic acid. After incubation at 37°C for fifteen minutes, the reaction was terminated by the addition of 10% TCA (1.5ml). The mixture was left on ice for at least 15 minutes before being filtered onto a GF/C filter, washed with 5% TCA (20ml) and then absolute alcohol (10ml). Activity was measured by liquid scintillation counting of the dried filters.

17. Isolation of a Crude Aminoacyl-tRNA Synthetase Preparation from Rat Liver.

The method used was mainly as described by Lanks et al. (1971) and Santi and Webster (1976). Two rat livers (21gm) were homogenised in a blender at 0°C in 1.5 volumes of buffer (10mM potassium phosphate, pH 7.4, 10mM magnesium acetate,

5mM mercaptoethanol and 10% glycerol). The homogenate was centrifuged at 10,000g for 30 minutes at 5°C, then the resulting supernatant was centrifuged at 200,000g at 4°C for 2 hours. The post-ribosomal supernatant was dialysed and concentrated overnight at 4°C against the same buffer plus 25% glycerol. A slight precipitate formed in the dialysate and this was removed by centrifugation at 10,000g.

Aminoacylation Assay.

In a final volume of 100µl, 10µl of the above supernatant were incubated with 100mM Tris-HCl (pH 7.4), 10mM magnesium acetate, 10mM potassium chloride, 2mM ATP, 10nmoles L-[U-^{14}C]-isoleucine (10mCi/mmole), 5mM mercaptoethanol and 500µg of unfractionated yeast tRNA. After incubation at 37°C for fifteen minutes, the reaction was stopped by the addition of 5% TCA (1.5ml). The samples were left on ice for at least 20 minutes and then filtered onto GF/C filters. The filters were washed with 5% TCA (2x15ml) and then absolute alcohol (10ml). After drying, activity on the filters was determined by liquid scintillation counting.

18. Separation of the Enzyme.Pseudomonic Acid Complex on Sephadex G-50.

Sodium [9'-^{3}H]-pseudomonate (specific activity of 101.6µCi/mg) was prepared by Dr. G. Mellows.

Sodium [9'-^{3}H]-pseudomonate (20nmole) was incubated with purified isoleucyl-tRNA synthetase (250pmole) in Tris-HCl buffer, pH 7.4 (20mM) containing 10mM dithiothreitol for 15 minutes at 37°C. To separate off unbound pseudomonic acid, the incubation mixture was placed on top of a Sephadex G-50 (fine) column (0.8x8cm) and eluted under gravity in the same buffer. Fractions (90µl) were collected and 25µl

of each was radiocounted, using the methoxyethanol-butyl PBD-toluene scintillant. Alternate fractions (50µl) were qualitatively tested for protein using the procedure of McKnight (1977). The procedure was repeated using an equivalent amount of bovine serum albumin in place of the enzyme. The pseudomonic acid-enzyme complex was also treated with 0.1% sodium dodecyl sulphate at room temperature and at 100°C. The complex was also formed in the presence of magnesium chloride (10mM) and ATP (5mM, pH 7.4). Treatment of this enzyme.sodium pseudomonate complex with 0.1% sodium dodecyl sulphate at room temperature and at 100°C was also carried out. To achieve true equilibrium, the column was washed with cold pseudomonic acid at the same concentration as was used in the incubation mixture. The column was eluted with pseudomonic acid-buffer mixture.

19.Gel Electrophoresis of Enzyme. Pseudomonic Acid Complexes.

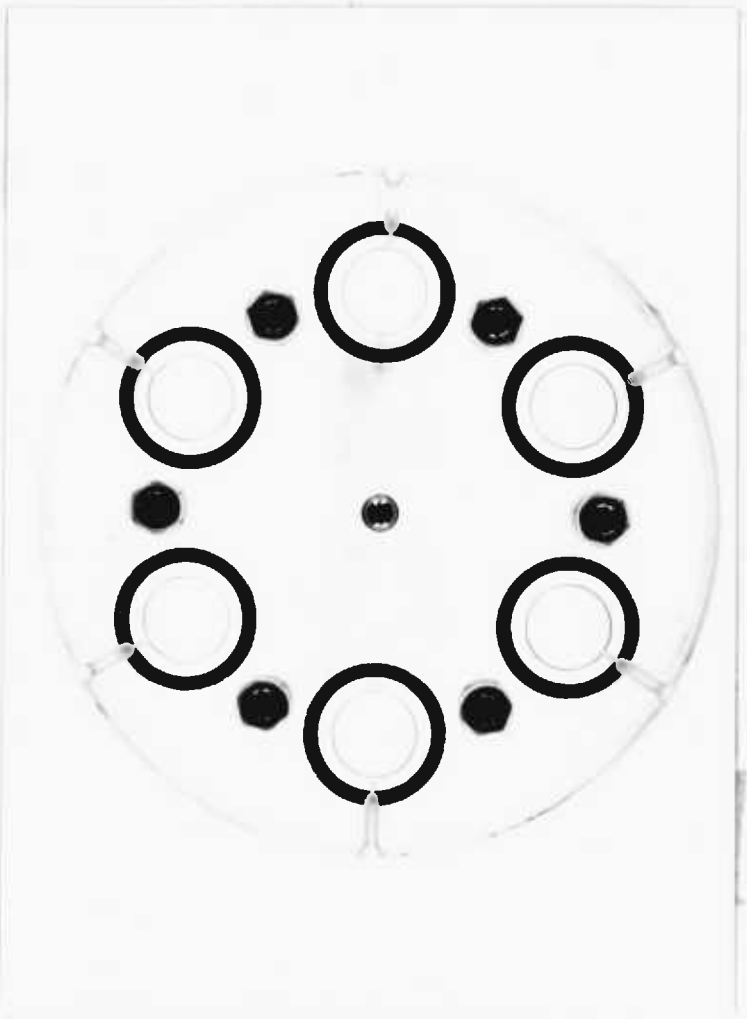
The gels were prepared by mixing 40% acrylamide solution (containing 1% methylene bis acrylamide) (4ml) with phosphate buffer, pH 8.0 or 7.4 (10ml), 1% N,N,N',N'-tetramethylene-diamine (2ml) and water (2ml) or 1% SDS (2ml) depending on the type of gel being made. After de-aeration, freshly made 1% ammonium persulphate was added (Weber & Osborn, 1969). The gel tubes (8.5x0.6cm) were filled to leave a gap of about 0.5cm at the top of each tube. Onto this surface, water was carefully layered before the gels hardened. 0.1% Bromo-phenol blue was used as a tracking dye. The samples electro-phoresed on these gels are described in the results section. Running buffer was 0.1M phosphate (pH 8.0 or 7.4) plus or minus 0.1% SDS. For urea gels, urea was added to the gel phosphate buffer to a final concentration of 6M.

Elctrophoresis was carried out at a constant current of 6mA per gel. Staining was carried overnight in small tubes containing the staining solution, composition as follows: 0.1% Coomassie brilliant blue, 10% 5-sulphosalicylic acid and 10% TCA. Destain was 5% acetic acid. Gels for slicing were rapidly frozen in powdered solid carbon dioxide. The slices (1mm thick) were each placed in a scintillation vial. After dehydration, achieved by leaving the gels at 37°C overnight, the gel matrix was broken down by adding 100 volume hydrogen peroxide and 880 ammonia (99:1) (0.25ml). The vials were tightly capped then left overnight at 37°C for 24 hours. Methoxyethanol (5ml) and butyl-PBD scintillant (10ml) were added to each and the radioactivity of each determined by liquid scintillation counting.

20. Equilibrium Dialysis of Isoleucyl-tRNA Synthetase.Pseudomonic Acid Complex.

The small scale equilibrium dialysis apparatus, as shown in Fig.17 was used. The volume available in each compartment was approximately 0.8ml. Dialysis membranes were cut from Visking tubing, boiled in five changes of EDTA, then rinsed and boiled again in ten changes of distilled water, before use. Filling and emptying the chambers was carried out using 1ml disposable syringes and needles. Each compartment contained in a final volume of 0.5ml: 100mM Tris-HCl, pH 7.4, 10mM magnesium chloride, 10mM potassium chloride, 10mM dithiothreitol and sodium [9-^{3}H]-pseudomonate (50nM-500nM). Isoleucyl-tRNA synthetase (414nM) was placed on one side of each compartment. The apparatus was rotated continually at 4°C for 18 hours. Experiments in the absence of enzyme showed that, with sodium [9-^{3}H]-pseudomonate, equilibrium was

FIGURE 17. SMALL SCALE EQUILIBRIUM DIALYSIS APPARATUS.



always reached after 12 hours. Three samples (100µl) were removed from each compartment and counted for radioactivity in methoxyethanol, naphthalene, butyl-PBD and toluene scintillant.

21. Preparation of Isotopically Labelled Pseudomonic Acid.

Two methods were used, the first involved feeding L-[^{14}C]-methyl -methionine to a growing culture of Pseudomonas fluorescens (NC1B 10856) and extracting the [^{14}C]-pseudomonic acid. In the second, the pseudomonic acid was labelled with ^{3}H in the 9' position by Dr. G. Mellows, using a chemical method, to give sodium [9'-^{3}H]-pseudomonate of specific activity 101.6µCi/mg.

For the preparation of ^{14}C labelled sodium pseudomonate, seed stage medium (4x100ml), (comprising nutrient broth (13g), peptone (5g), disodium hydrogen phosphate (2.6g), potassium dihydrogen phosphate (2.4g), glucose (1.1g) made up to 1l with distilled water) was inoculated with Pseudomonas fluorescens (NC1B 10856) and incubated at 30°C for 24 hours. An aliquot (7ml) of this culture was transferred into each of 10x100ml of production medium comprising: potassium dihydrogen phosphate (3.3g), disodium hydrogen phosphate (0.65g), potassium chloride (0.5g), manganese chloride (0.003g), magnesium sulphate heptahydrate (0.375g), ground nut meal (21g) and made up to 1l with distilled water. (The pH was adjusted to pH 7.05 with sodium hydroxide solution). The cultures were incubated on a rotary shaker at 24°C. After 8 hours, L-[^{14}C]-methyl -methionine (250µCi) was equally distributed between the 10 flasks and incubation continued for a further 16 hours. The cells were removed from the culture by centrifugation at 15,000g for 30 minutes. The pellets were washed

once with distilled water. The combined supernatants were extracted three times with isobutyl methyl ketone at pH 4.5. The isobutyl methyl ketone extracts were combined and the solvent removed by rotary evaporation. 0.01M Ammonia solution (10ml) was added and the pH adjusted to 0.01M ammonia solution. To ensure that conversion to the ammonium salt was complete, mixing was continued overnight. Ammonium [^{14}C]-pseudomonate was then purified as described in Method (1). The purity of the resulting [^{14}C]-pseudomonic acid was determined by autoradiography of a thin layer chromatogram (silica gel GF_{254}) of the sample that had been developed in isopropyl alcohol-chloroform (1:9 v/v) and by high pressure liquid chromatography. The specific activity was found to be 1.1μCi/mg.

RESULTS.

RESULTS.

(a). Bacteriostasis and Reversibility.

The primary effect of pseudomonic acid against S. aureus at concentrations (0.05-0.5μg/ml) close to the minimum inhibitory concentration (M.I.C.) of 0.05μg/ml (Table 7) is bacteriostasis (Fig. 9) (see also Sutherland et al., 1976). Cells treated with pseudomonic acid within these concentration limits, followed by transfer to fresh medium, recover spontaneously after several hours. At higher concentrations, the effect of the antibiotic becomes increasingly bacteriocidal.

(b). Effect of Pseudomonic Acid on Macromolecular Synthesis in S. aureus.

The synthesis of protein, RNA and DNA was measured in the presence of increasing concentrations of pseudomonic acid, by following the incorporation of [U-^{14}C]-phenylalanine, [2-^{14}C]-uridine and [2-^{14}C]-thymidine respectively into TCA precipitable material. The incorporation of all three labelled compounds was always inhibited simultaneously but protein and RNA synthesis appeared to be more sensitive than DNA synthesis (Fig. 10, Table 8).

Pseudomonic acid also inhibited the formation of cell wall peptidoglycan (Fig. 13). However, in the short term, the inhibition of peptidoglycan synthesis was less pronounced than the inhibition of RNA, DNA and protein synthesis (Table 8). The more marked effect of penicillin G (Table 6), which inhibits the cross-linking process in peptidoglycan synthesis (Gale et al., 1972) suggested that cell wall formation is a less likely target site. This is supported by the observation that pseudomonic acid treated cells do not lyse as do penicillin

TABLE 7.

MINIMUM INHIBITORY CONCENTRATION (M.I.C.) VALUES.

Bacterium	Inhibitor	M.I.C. (μg/ml)	Medium
S. aureus (Oxford)	Sodium pseudomonate	0.05	Double strength nutrient broth
"	Chloramphenicol	3.0	"
"	Nalidixic acid	25.0	"
"	Tetracycline	0.3	"
"	2,4-Dinitrophenol	1.0	"
"	Penicillin G	0.03	"
"	Rifampicin	0.02	"
E. coli MRE 600	Sodium pseudomonate	50.0	"
"	Tetracycline	1.0	"
"	Rifampicin	10.0	"
"	Chloramphenicol	6.0	"
"	Sodium pseudomonate	25.0	Minimal salts medium
E. coli AS19 NF541	Sodium pseudomonate	0.3	Brain heart infusion broth
E. coli AS19 NF542	Sodium pseudomonate	0.3	"
E. coli AS19 NF541	Sodium pseudomonate	0.1	Minimal salts medium
E. coli AS19 NF542	Sodium pseudomonate	0.1	"

FIGURE 9. THE EFFECT OF DIFFERENT CONCENTRATIONS OF PSEUDOMONIC ACID ON A GROWING CULTURE OF S. AUREUS.

An exponentially growing culture of S. aureus was divided into two equal portions. One culture served as the control, while pseudomonic acid (0.05μg/ml) was added to the other cultures. The experiment was repeated using different pseudomonic acid concentrations as indicated in the Figure. ●, Control; o, Pseudomonic acid (0.05μg/ml) ▲, Pseudomonic acid (0.1μg/ml); ■,Pseudomonic acid (0.5μg/ml).

Samples were withdrawn from both cultures and the viable count of each determined (see Materials and Methods section).

FIGURE 9

THE EFFECT OF DIFFERENT CONCENTRATIONS OF PSEUDOMONIC ACID ON A GROWING CULTURE OF S.AUREUS OXFORD

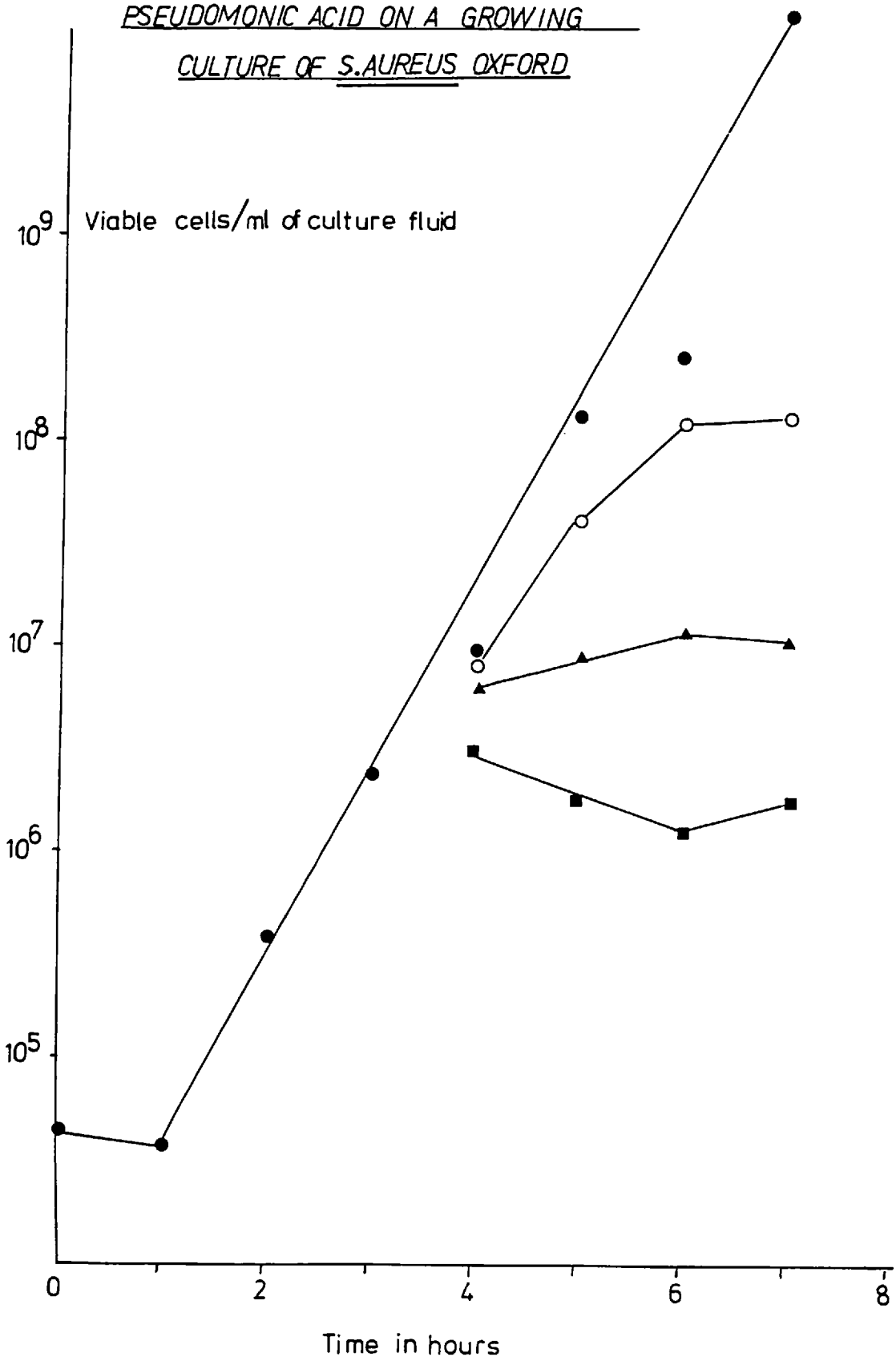


FIGURE 10.THE EFFECT OF PSEUDOMONIC ACID ON a) PROTEIN, b) RNA and c) DNA SYNTHESIS IN S. AUREUS.

An exponentially growing culture (40ml, $E_{600} \simeq 0.2$) was labelled with either [^{14}C]-phenylalanine, [^{14}C]-uridine, or [^{14}C]-thymidine (each at 0.4μCi/ml). After 4 min. the culture was split into 10ml aliquots. One subculture was used as a control, whilst the others were treated with different concentrations of pseudomonic acid (as indicated by the arrows). Samples (0.5ml) were withdrawn and the acid-precipitable radioactivity determined (Materials and Methods).

●, Control; o, 0.05μg/ml Pseudomonic acid; ▲, 0.25μg/ml Pseudomonic acid; △, 0.5μg/ml Pseudomonic acid.

FIGURE 10

EFFECT OF PSEUDOMONIC ACID ON PROTEIN, RNA & DNA SYNTHESIS IN STAPHYLOCOCCUS AUREUS

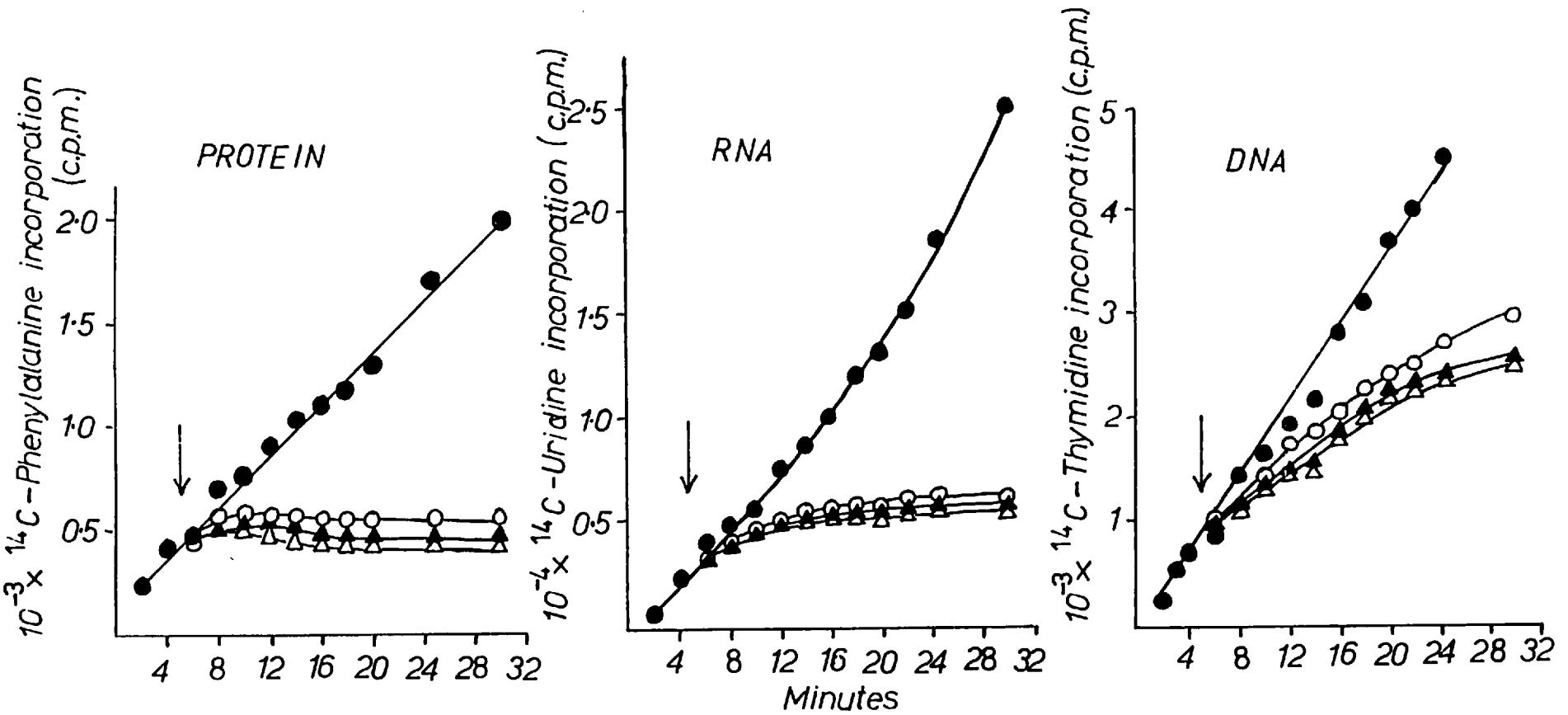


TABLE 8.

INHIBITION OF PROTEIN, RNA AND DNA SYNTHESIS BY PSEUDOMONIC ACID, IN STAPHYLOCOCCUS AUREUS.

(20 MIN AFTER ANTIBIOTIC ADDITION)

PSEUDOMONIC ACID (μg/ml)	% INHIBITION		
	PROTEIN	RNA	DNA
0.05	88	78	55
0.10	92	80	60
0.25	96	81	63
0.50	100	86	65

To a split culture was added either [^{14}C]-phenylalanine, [^{14}C]-uridine or [^{14}C]-thymidine as described in the legend to Fig. 10. The degree of inhibition was calculated from the kinetics of incorporation in pseudomonic acid treated cultures relative to the untreated controls, 20 min after the addition of the antibiotic.

FIGURE 13. THE EFFECT OF PSEUDOMONIC ACID ON PEPTIDOGLYCAN SYNTHESIS IN S. AUREUS.

D-[^{14}C]-Glucosamine (0.2μCi/ml) was added to an exponentially growing culture (50ml). The culture was split into four equal portions. One culture served as the control, and different concentrations of pseudomonic acid were added to the remaining three cultures as indicated by the arrow. Samples (0.5ml) were withdrawn and the acid-precipitable radioactivity determined (Materials and Methods).

FIGURE 13

EFFECT OF PSEUDOMONIC ACID ON CELL-WALL PEPTIDOGLYCAN SYNTHESIS IN STAPHYLOCOCCUS AUREUS

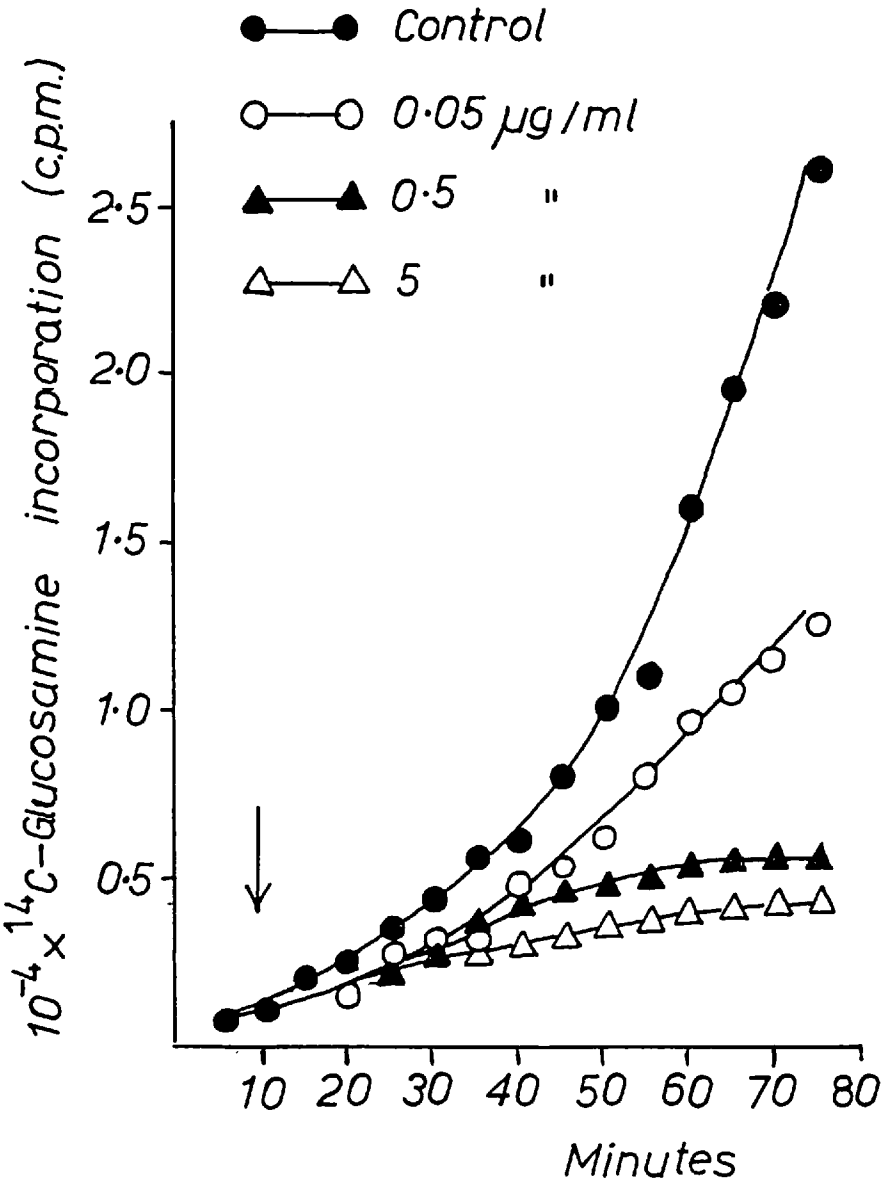


TABLE 6.

EFFECT OF PSEUDOMONIC ACID ON [^{14}C]-GLUCOSAMINE INCORPORATION INTO CELL-WALL PEPTIDOGLYCAN IN STAPHYLOCOCCUS AUREUS.

TIME (MIN)	% INHIBITION OF PEPTIDOGLYCAN SYNTHESIS
30	24
60	46
90	75
30 (Penicillin G,) (0.03μg/ml)	81

Pseudomonic acid : 0.05μg/ml

[^{14}C]-Glucosamine was added to a split culture of S. aureus. Antibiotic, as indicated above, was added to one of the cultures. After a certain time (as indicated in the Table) peptidoglycan was extracted according to the method of Park & Hancock, (1960). The degree of inhibition was calculated from the levels of [^{14}C] incorporated into peptidoglycan extracted from antibiotic treated cells compared to untreated controls.

treated cells.

At a concentration of 0.5μg/ml, pseudomonic acid significantly enhanced (37%) the cellular ATP level after 30 minutes. Under identical conditions, 2,4-dinitrophenol, a known uncoupler of oxidative phosphorylation (Gale et al., 1972) at 1mg/ml (M.I.C.) led to a 45% reduction in the ATP content of the cell (Table 9). It is therfore concluded that at low concentrations, sodium pseudomonate does not interfere with oxidative phosphorylation and energy processes dependent on it. A similar enhancement of the cellular ATP pool size has been observed in Bacillus subtilis (Ogilvie et al., 1975b) grown in the presence of granaticin, an antibiotic which specifically inhibits the aminoacylation of leucyl-tRNA (Ogilvie et al., 1975b). Later experiments using E. coli B showed that sodium pseudomonate inhibits the aminoacylation of isoleucyl-tRNA. Thus enhancement of ATP levels in S. aureus suggested that pseudomonic acid may have a mode of action similar to that of granaticin.

(c). Effect of Chloramphenicol on Pseudomonic Acid Treated S. aureus Cells.

Certain antibiotics, e.g. chloramphenicol, which inhibit protein synthesis at the ribosomal level abolish the stringent control mechanism of RNA synthesis (Cashel, 1969; Edlin & Broda, 1968; Lund & Kjeldgaard, 1972). When chloramphenicol and pseudomonic acid were simultaneously added to S. aureus cells, the incorporation of [^{14}C]-uridine was not inhibited when compared with [^{14}C]-uridine uptake in cells treated with pseudomonic acid alone (Fig. 12). This chloramphenicol breaks the stringent regulation of RNA synthesis imposed by pseudomonic acid.

TABLE 9.

EFFECT OF PSEUDOMONIC ACID ON CELLULAR ATP LEVELS IN STAPHYLOCOCCUS AUREUS.

ATP was determined by the [^{32}P]-orthophosphate exchange method.

TIME (MIN)	[^{32}P]-ATP (cpm)	
	PSEUDOMONIC ACID TREATED CELLS	CONTROL
0	322	330
10	684	645
20	1174	772
30	1466	1072
	2,4-DINITROPHENOL TREATED CELLS	
0	213	262
10	527	695
20	457	1118
30	746	1347

Pseudomonic acid : 0.5μg/ml

2,4-Dinitrophenol : 1.0mg/ml

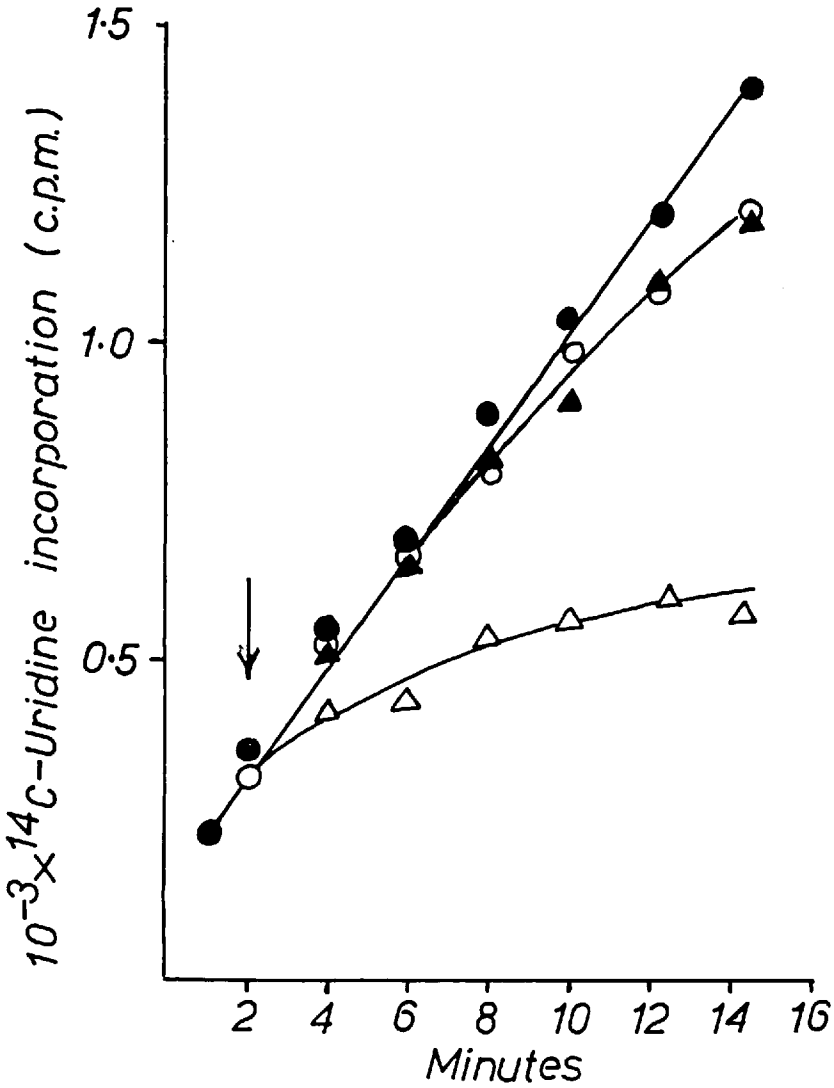
Two aliquots (0.5ml) of an exponentially growing culture ($E_{600} \simeq 0.2$) were transferred to two tubes. Pseudomonic acid (0.5μg/ml) or 2,4-dinitrophenol (1mg/ml) was added to one tube, whilst the other served as the control. [^{32}P]-orthophosphate (0.1ml, 50μCi) was added to each and the cultures incubated at 37°C, with aeration from a split capillary tube. Samples (0.1ml) were pipetted into 2M formic acid (0.1ml) and left on ice for 30 min. Aliquots (50μl) were eluted on pre-washed PEI-cellulose coated t.l.c. plates using 1.5M KH_2PO_4. The [^{32}P]-ATP spot was located by autoradiography, removed from the chromatogram and radiocounted.

FIGURE 12. THE EFFECT OF CHLORAMPHENICOL ON RNA SYNTHESIS IN A PSEUDOMONIC ACID-TREATED CULTURE OF S. AUREUS.

An exponentially growing culture was treated with [^{14}C]-uridine (0.4μCi/ml). The culture was split into four equal portions. Chloramphenicol, chloramphenicol plus pseudomonic acid and pseudomonic acid were added to each of three cultures as indicated by the arrow, whilst the other culture served as a control. Samples (0.5ml) were withdrawn and the acid-precipitable radioactivity determined (Materials and Methods).

FIGURE 12

EFFECT OF CHLORAMPHENICOL ON RNA SYNTHESIS IN PSEUDOMONIC ACID TREATED STAPHYLOCOCCUS AUREUS



control

500µg/ml chloramphenicol

500µg/ml chloramphenicol
50µg/ml pseudomonic acid

50µg/ml pseudomonic acid

(d). Effect of Pseudomonic Acid on Macromolecular Synthesis in E. coli MRE 600, E. coli AS19 NF541 and E. coli AS19 NF542.

(i) In vivo RNA, DNA and Protein Synthesis.

As was previously demonstrated for S. aureus, pseudomonic acid has a bacteriostatic effect also on E. coli MRE 600 at the minimum inhibitory concentration (M.I.C.=50μg/ml) and concentrations slightly in excess of the M.I.C. The higher M.I.C. required for E. coli compared with that for S. aureus (0.05μg/ml) is almost certainly a consequence of the reduced permeability of the outer cell wall to the antibiotic. For example, treatment of E. coli MRE 600 with metal chelating agents, e.g. EDTA, increases the permeability of the cell wall to pseudomonic acid, thus lowering the M.I.C. (R. Sutherland & B. Slocombe, persomal communication). The M.I.C. of E. coli B AS19, a mutant strain possessing a deficient cell wall is even less; 0.3μg/ml.

In the presence of increasing concentrations of pseudomonic acid, inhibition of the assimilation of [^{14}C]-labelled phenylalanine, uridine and thymidine occurred simultaneously. Protein and RNA synthesis were affected predominantly (Fig. 11) as was also the case in S. aureus.

(ii) In vitro RNA and Protein Synthesis.

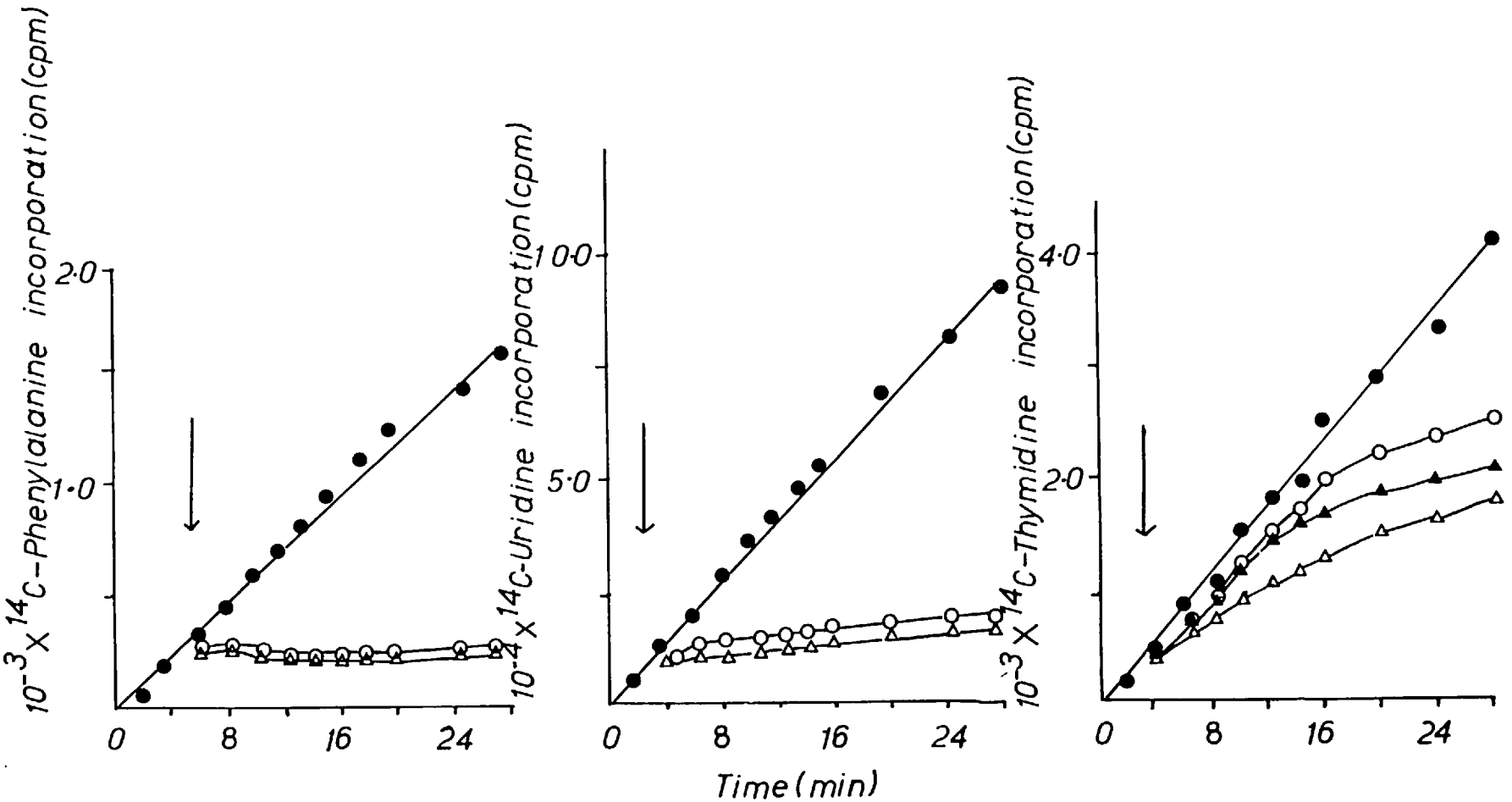
In an attempt to determine whether both RNA and protein synthesis were the targets of inhibtion by pseudomonic acid, or whether one was the primary target and inhibition of the other a secondary effect, protein and RNA synthesis were followed in vitro. The activity of highly purified E. coli B RNA polymerase was not affected by pseudomonic acid, whereas rifampicin, a powerful inhibitor of the polymerase (Sung, 1972), almost totally inhibited the enzyme at one-tenth the

FIGURE 11. THE EFFECT OF PSEUDOMONIC ACID ON a) PROTEIN, b) RNA, AND c) DNA SYNTHESIS IN E. COLI MRE 600

To an exponentially growing culture ($E_{600} \simeq 0.2$) was added either [^{14}C]-phenylalanine (0.4μCi/ml), [^{14}C]-uridine (0.4μCi/ml), or [^{14}C]-thymidine (0.4μCi/ml). The culture was divided into several equal portions. One sub-culture served as the control and different concentrations of pseudomonic acid were added to the other sub-cultures, as indicated by the arrows. Samples were withdrawn and the incorporation of ^{14}C into acid-precipitable material was determined (Materials and Methods). ●, Control; O, Pseudomonic acid (50μg/ml) treated; ▲, Pseudomonic acid (75μg/ml) treated; △, Pseudomonic acid (100μg/ml) treated

FIGURE 11

EFFECT OF PSEUDOMONIC ACID ON PROTEIN, RNA AND DNA SYNTHESIS IN E.COLI MRE 600



concentration of pseudomonic acid tested (Table 10). Pseudomonic acid also had no effect on the activity of the RNA-dependent RNA polymerase from the mycovirus isolated from Aspergillus foetidus (Table 11).

In a ribosomal preparation obtained from E. coli MRE 600, the same concentration of pseudomonic acid resulted in only a slight depression of polyuridylic acid (poly U)-directed poly phe formation (Table 12), whereas tetracycline and chloramphenicol exerted a much stronger inhibitory effect.

Whilst indicating that pseudomonic acid preferentially inhibits in vitro protein synthesis, these observations do not account for the marked in vivo inhibitory effects on protein and RNA synthesis.

(iii) In vivo RNA, DNA and Protein Synthesis in E. coli AS19 NF541 and NF542.

Using a pair of E. coli AS19 mutants NF541 and NF542, the primary target of pseudomonic acid was shown to be protein synthesis. In bacteria deprived of an amino acid, stable RNA synthesis is stringently controlled, since this situation leads to the presence of 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 & Borak, 1971) and mutations in this gene can give strains that have relaxed control of stable RNA synthesis. In the relaxed mutant strain, RNA synthesis proceeds regardless of amino acid starvation, whereas in the stringently controlled strain, RNA synthesis is strongly inhibited. E. coli AS19 NF541 and NF 542 are isogenic except for the rel gene, strain NF541 having RNA synthesis under stringent control whereas

TABLE 10.

EFFECT OF PSEUDOMONIC ACID ON RNA SYNTHESIS IN VITRO.

(Using DNA-dependent RNA polymerase from E. coli B)

ANTIBIOTIC	CONCENTRATION (μg/ml)	% INHIBITION
PSEUDOMONIC ACID	100	0
RIFAMPICIN	10	90

The incorporation of ^{3}H, from [^{3}H]-UTP, into RNA 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 levels of ^{3}H incorporation into RNA in the two experiments.

TABLE 11.

EFFECT OF PSEUDOMONIC ACID ON RNA-DEPENDENT RNA SYNTHESIS USING PURIFIED VIRUS PARTICLES FROM ASPERGILLUS FOETIDUS.

Antibiotic	% Inhibition after 16 hours incubation
Rifampicin (10μg/ml)	0
Pseudomonic acid (100μg/ml)	0

The incorporation of ^{3}H, from ^{3}H -UTP, into RNA using purified virus particles from Aspergillus foetidus as the source of RNA polymerase and 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 levels of ^{3}H incorporation into RNA in the two experiments.

TABLE 12.

EFFECT OF PSEUDOMONIC ACID ON POLY·PHE FORMATION IN VITRO.

(Using a ribosomal preparation from E. coli MRE 600)

ANTIBIOTIC	CONCENTRATION (μg/ml)	% INHIBITION
PSEUDOMONIC ACID	100	8
CHLORAMPHENICOL	100	22
TETRACYCLINE	100	58

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 (2μCi) 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.

that in strain NF542 is relaxed. On treatment with pseudomonic acid (0.3μg/ml), RNA synthesis proceeded in the relaxed mutant (Fig. 14) but immediately stopped in the stringent mutant (Fig. 15). 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., 1975b). Thus, pseudomonic acid inhibits RNA formation only in cells in which RNA synthesis is under optimum control.

(iv) Effect of Chloramphenicol on RNA Synthesis in Pseudomonic Acid Treated E. coli MRE 600 Cells.

As was observed with S. aureus, a combination of pseudomonic acid plus chloramphenicol allowed RNA synthesis to continue unabated for sometime before onset of inhibition (Fig. 16). Since the stringent control of RNA synthesis in cells is abolished by chloramphenicol (Edlin & Broda, 1968; Cashel, 1969; Lund & Kjelgaard, 1972).

(v) Purine Nucleotide Levels in E. coli AS19 NF541 and NF542 Cells that have been Treated with Pseudomonic Acid.

It is well documented that in stringent cells deprived of an amino acid, guanosine-3'-diphosphate-5'-diphosphate (ppGpp) and guanosine-3'-diphosphate-5'-triphosphate (pppGpp) accumulate, whereas in relaxed mutants, no enhancement of these nucleotide levels in observed (Cashel & Gallsnt, 1969; Cashel, 1969; Cashel & Kalbacher, 1970; Haseltine et al., 1972). 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 reduced to a

FIGURE 14. THE EFFECT OF PSEUDOMONIC ACID ON a) PROTEIN, AND b) RNA ACCUMULATION IN E. COLI B, AS19 542.

Exponentially growing cultures of E. coli B, AS19 542 to which had been added either [^{14}C]-phenylalanine (0.4μCi/ml) or [^{14}C]-uridine (0.4μCi/ml) were treated as described in the legend to Fig. 10. •, Control; o, Pseudomonic acid (0.2μg/ml) treated; ▲, Pseudomonic acid (0.3μg/ml) treated; Δ, Pseudomonic acid (0.5μg/ml) treated.

Figures in Table 19 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 the antibiotic.

FIGURE 14

EFFECT OF PSEUDOMONIC ACID ON PROTEIN AND RNA SYNTHESIS IN E.COLI AS19 542

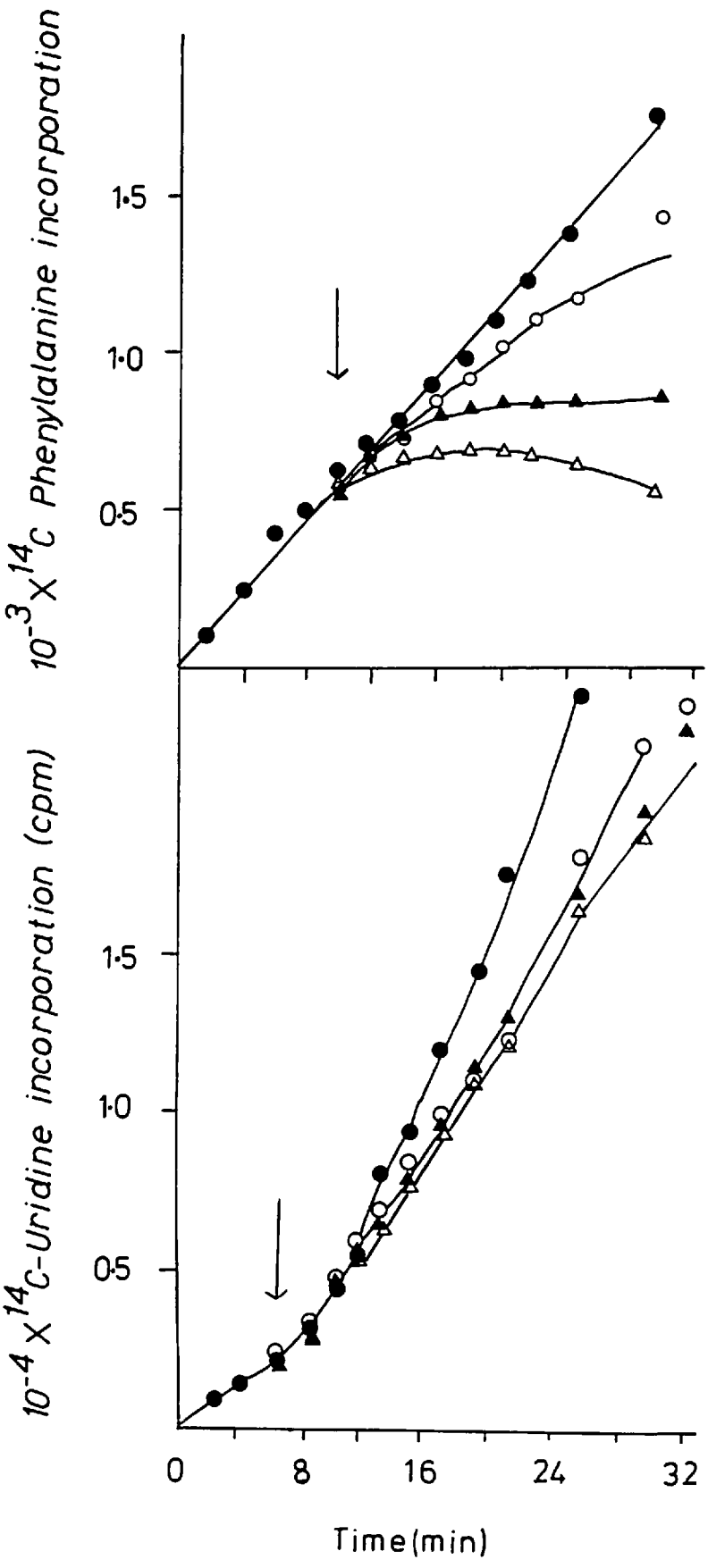


FIGURE 15. THE EFFECT OF PSEUDOMONIC ACID ON a) PROTEIN, AND b) RNA ACCUMULATION IN E. COLI B, AS19 541.

Exponentially growing cultures of E. coli B, AS19 541, to which had been added either [^{14}C]-phenylalanine (0.4μCi/ml) or [^{14}C]-uridine (0.4μCi/ml) were treated as described in the legend to Fig. 10. •, Control; o, Pseudomonic acid (0.2μg/ml) treated; ▲, Pseudomonic acid (0.3μg/ml) treated; Δ, Pseudomonic acid (0.5μg/ml) treated.

Figures in Table 19 refer to the degree of inhibition (%), calculated from the kinetics of incorporation in pseudomonic acid-treated cultures relative to the untreated control, 20 min after the addition of the antibiotic.

FIGURE 15

EFFECT OF PSEUDOMONIC ACID ON PROTEIN AND RNA SYNTHESIS IN E.COLI AS19 541

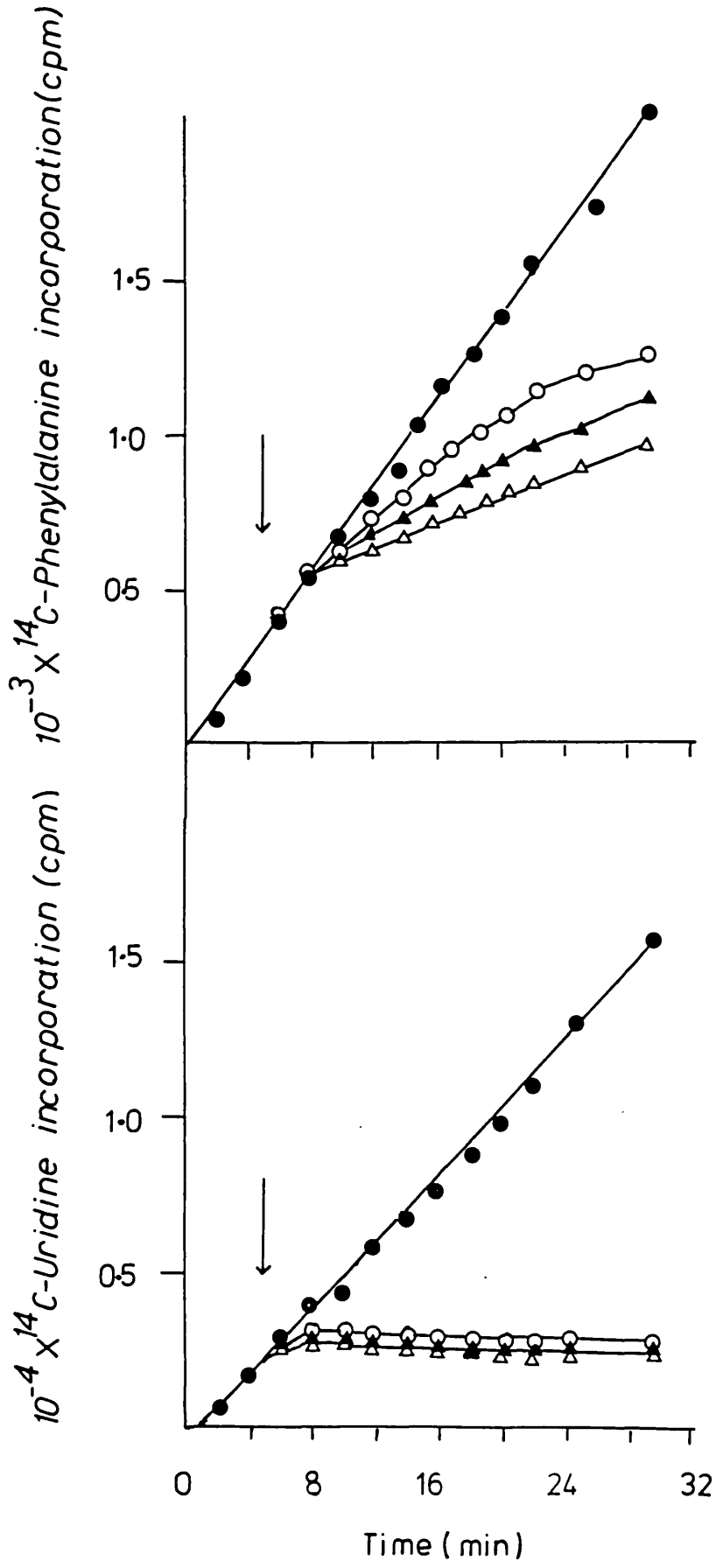


FIGURE 16. THE EFFECT OF PSEUDOMONIC ACID AND CHLORAMPHENICOL ON RNA SYNTHESIS IN E. COLI MRE 600.

[^{14}C]-Uridine (0.4μCi/ml), was added to an exponentially growing culture. The culture was divided into four equal portions. Chloramphenicol (500μg/ml), chloramphenicol (500μg/ml) plus pseudomonic acid (50μg/ml) and pseudomonic acid (50μg/ml) were added to each of three cultures as indicated by the arrow. The fourth culture served as a control. Samples (0.5ml) were withdrawn and the acid-precipitable material was determined (Materials and Methods).

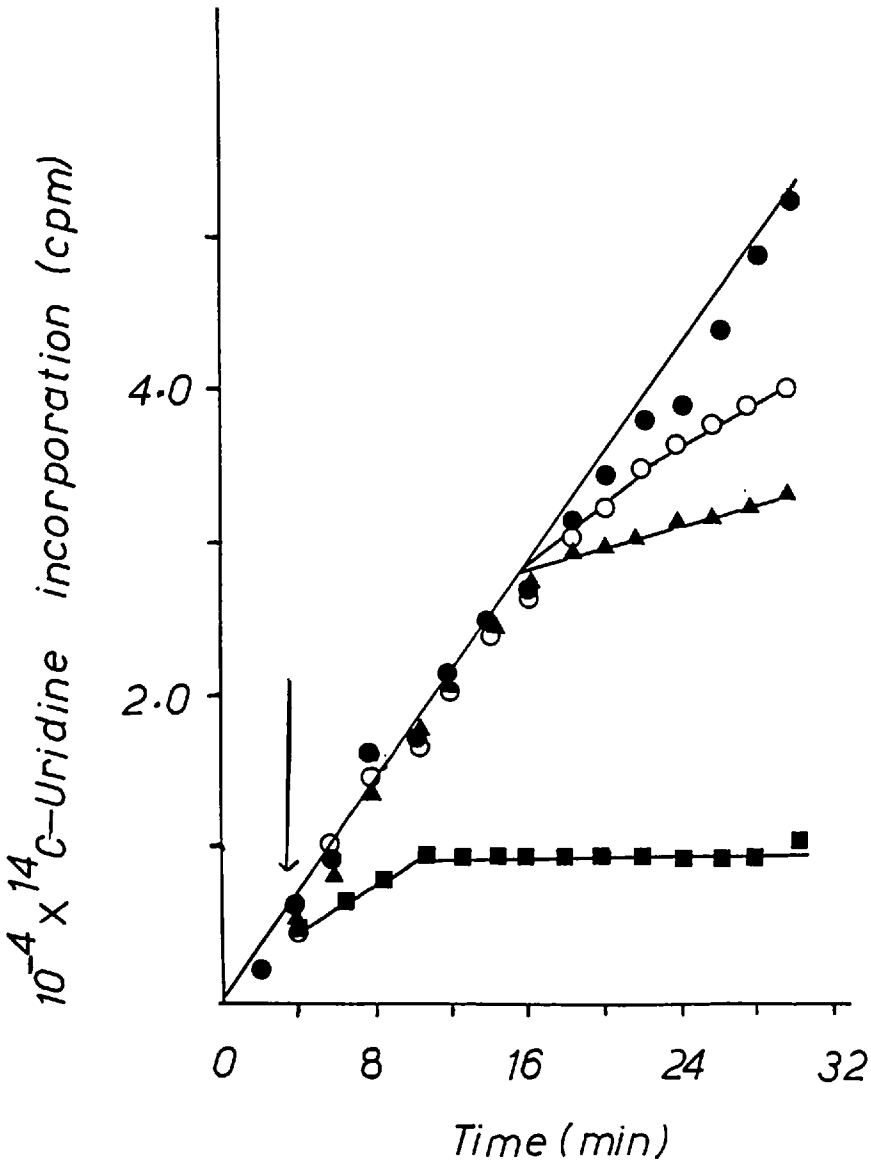
●, Control; o, Chloramphenicol treated;

▲, Chloramphenicol plus pseudomonic acid treated;

■, Pseudomonic acid treated.

FIGURE 16

EFFECT OF CHLORAMPHENICOL ON RNA SYNTHESIS IN PSEUDOMONIC ACID TREATED E.COLI MRE 600



lesser extent, if at all (Edlin & Broda, 1968). Changes in the pool sizes of these purine nucleotides, measured by [^{32}P]-orthophosphate exchange was compared in the two E. coli B strains (AS19 NF541 and NF542) after the addition of pseudomonic acid. In the stringent strain (NF541), both ppGpp and pppGpp increased soon after the addition of pseudomonic acid (0.5μg/ml), and reached maximum levels within minutes after addition of the antibiotic (Fig. 18). Reducing the antibiotic concentration prolonged the onset of the increase in ppGpp and pppGpp levels, but the effect was equally marked down to a concentration of 0.1μg/ml of pseudomonic acid (Mellows & Slaney, unpublished work). The ATP pool size also continued to increase after the addition of pseudomonic acid, confirming the absence of a primary effect of pseudomonic acid on oxidative phosphorylation. 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 (strain NF542), at a pseudomonic acid concentration of 0.5μg/ml, both GTP and ATP levels continued to increase after antibiotic addition, whereas ppGpp maintained a low basal level, showing no increase after addition of antibiotic (no pppGpp was observed). The levels of ATP, GTP, ppGpp and pppGpp after pseudomonic acid treatment therefore follow those observed during deprivation of an amino acid.

(e). 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-salts medium can be

FIGURE 18. ppGpp AND pppGpp NUCLEOTIDE ACCUMULATION IN E. COLI B AS19 541 TREATED WITH PSEUDOMONIC ACID.

[^{32}P] - Orthophosphate (65μCi/ml) was added to an exponentially growing culture ($E_{600} \simeq 0.2$) in Tris-buffered glucose-salt medium, containing phophate at a concentration of 0.2mM. Two aliquots (1ml) were removed and incubated separately. One served as a control, whilst pseudomonic acid was added to the other at the time indicated by the arrows. Samples (50μl) were removed and the ppGpp, pppGpp, ATP and GTP nucleotide levels determined (Materials and Methods).

a) ^{32}P radioactivity appearing in ppGpp and pppGpp

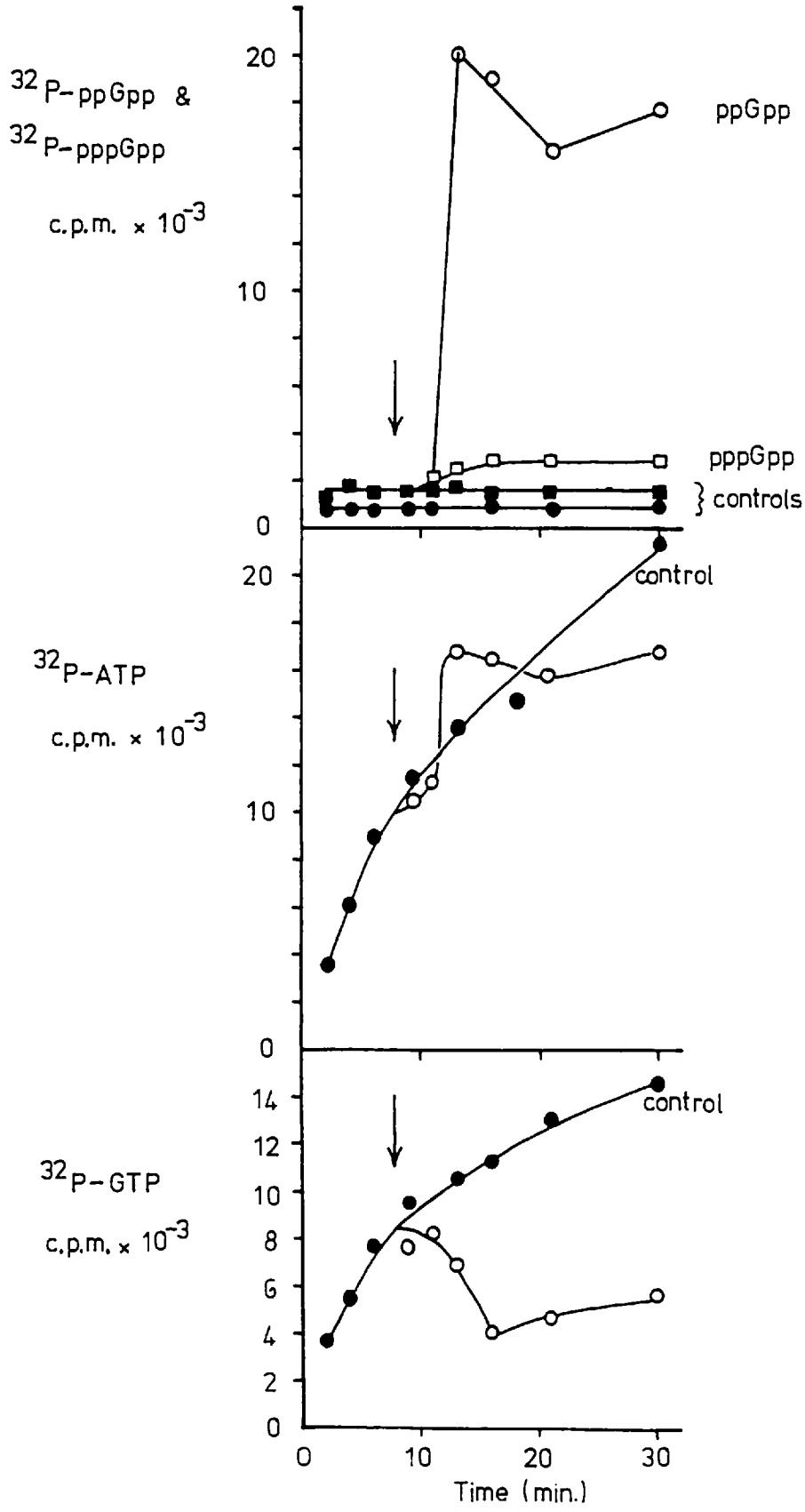
b) ^{32}P radioactivity appearing in ATP

c) the labelling of GTP

●,■ Control; o,□ Nucleotide levels after onset of pseudomonic acid inhibition.

FIGURE 18

ppGpp & pppGpp NUCLEOTIDE ACCUMULATION IN E.COLI B, AS19 54-1 TREATED WITH PSEUDOMONIC ACID (0·5µg/ml)



alleviated by the addition of a mixture of twenty amino acids to the medium. In order to ascertain whether a single or more than one amino acid produced the relieving effect, various combinations of amino acids, from which a single amino acid was eliminated from the complete mixture of twenty 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. 19). At a concentration of 25μg/ml of pseudomonic acid, isoleucine alone, at a concentration of 0.1mM and above, was able to fully reverse the effects (Fig. 20). The effect of pseudomonic acid (25μg/ml) could be reversed by isoleucine (1mM) even up to six hours after antibiotic addition. However, the cells showed a lag period of between 4-5 hours before normal growth resumed. At the same concentration, isoleucine provides almost total relief of pseudomonic acid induced inhibition of protein synthesis (Fig. 21). Inhibition of RNA synthesis, however, is only partly relieved by 1mM isoleucine, but the onset of inhibition occurs after a lag period following antibiotic addition (Fig. 21).

(f). Level of Free Amino Acids Found in Pseudomonic Acid Treated Cells.

Analysis of the intracellular pool of amino acids extracted from E. coli MRE 600 growing in the presence and in the absence of pseudomonic acid, revealed no reduction in the level of isoleucine in antibiotic treated cells (Table 13). Generally, the levels of amino acids were increased in the pseudomonic acid treated cells, probably as a consequence of of the cessation of protein synthesis.

FIGURE 19. ISOLEUCINE AUXOTROPHY OF E. COLI MRE 600 TREATED WITH PSEUDOMONIC ACID.

A freshly inoculated culture growing in minimal medium was equally divided into four portions. After the following additions, the growth of the cultures was monitored continually at 600nm using a Biophotometer.

a) pseudomonic acid (25μg/ml)

b) pseudomonic acid (25μg/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, cysteine; each at a final concentration of 1mM).

c) pseudomonic acid (25μg/ml) plus isoleucine (final concentration of 1mM)

d) control (no additions)

FIGURE 19

ISOLEUCINE AUXOTROPHY OF E. COLI MRE 600 TREATED WITH SODIUM PSEUDOMONATE

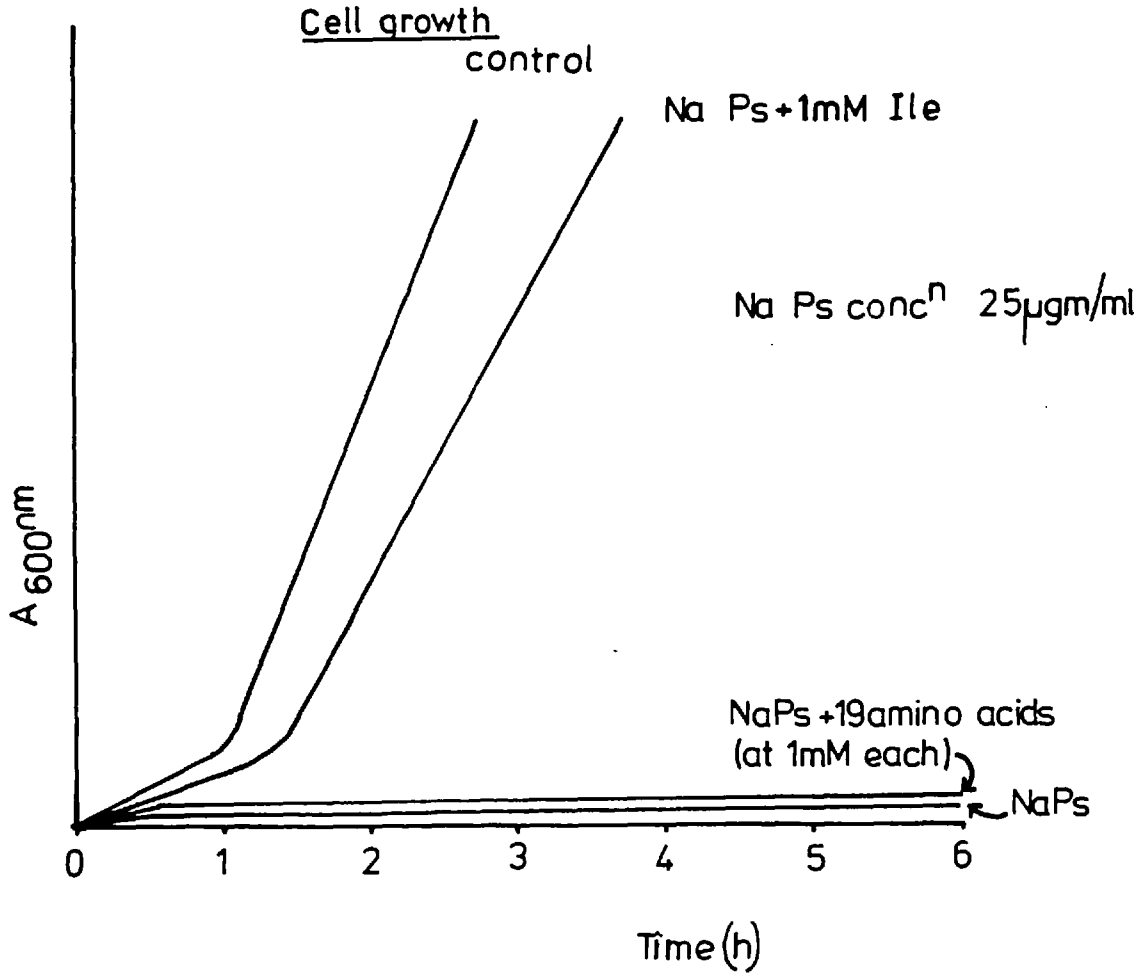


FIGURE 20. THE REVERSAL OF PSEUDOMONIC ACID INDUCED ISOLEUCINE AUXOTROPHY BY ISOLEUCINE IN E. COLI MRE 600.

Freshly inoculated cultures, growing in minimal media, were treated with:

a) pseudomonic acid (25μg/ml)

and pseudomonic acid (25μg/ml) plus increasing concentrations of isoleucine:

b) 0.01mM

c) 0.1mM

d) 0.5mM

e) 1.0mM

f) control (no additions)

Growth was monitored continuously at 600nm using a Biophotometer.

FIGURE 20

ISOLEUCINE AUXOTROPHY OF E.COLI MRE 600 TREATED WITH SODIUM PSEUDOMONATE

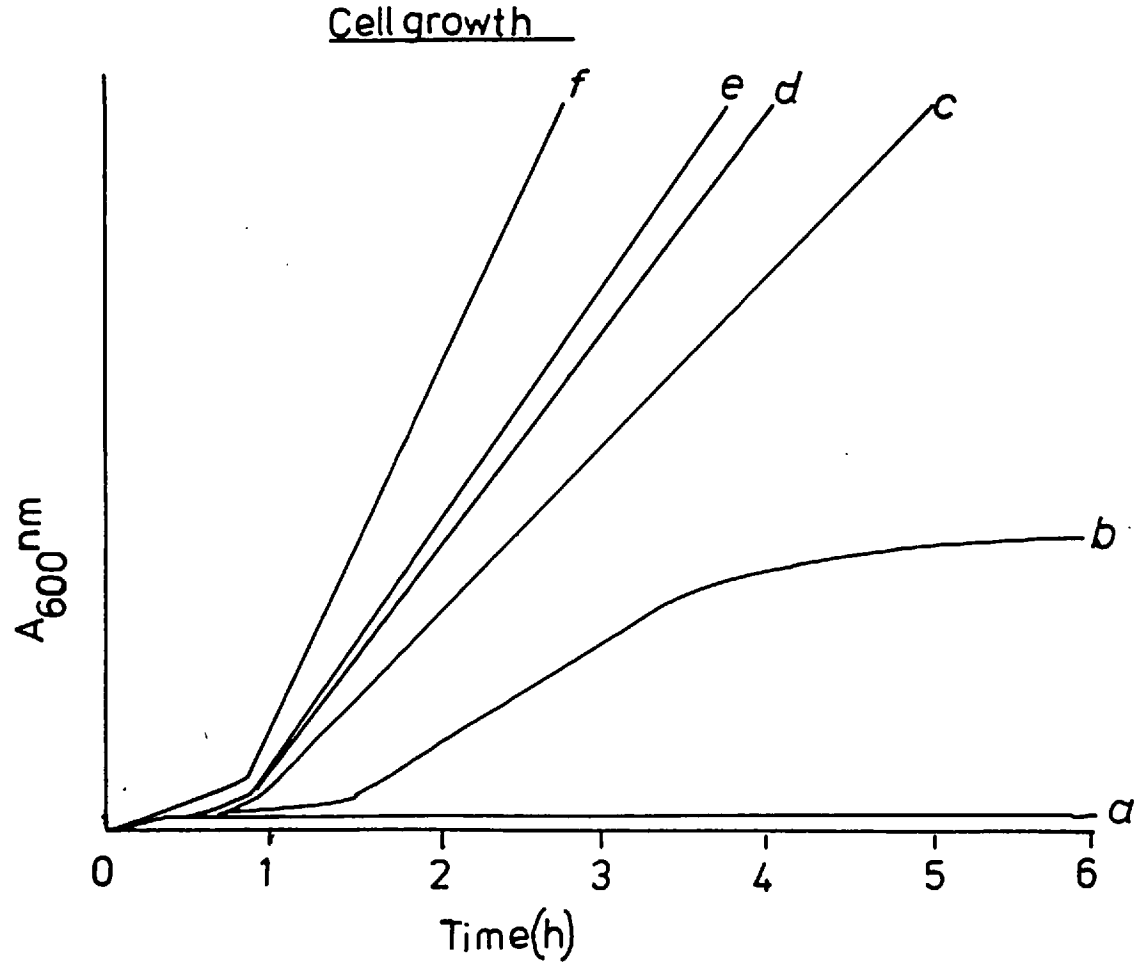


FIGURE 21. THE EFFECT OF ISOLEUCINE ON a) PROTEIN AND b) RNA SYNTHESIS IN E. COLI MRE 600 TREATED WITH PSEUDOMONIC ACID.

An exponentially growing culture in minimal medium was divided into several portions and either [^{14}C]-phenylalanine (0.4μCi/ml) or [^{14}C]-uridine (0.4μCi/ml) was added. Isoleucine (1mM), pseudomonic acid (25μg/ml) and isoleucine (1mMO plus pseudomonic acid (25μg/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 (Materials and Methods).

●, Control; ▲, Pseudomonic acid treated;

o, Pseudomonic acid plus isoleucine.

FIGURE 21

IN VIVO REVERSAL OF PSEUDOMONIC ACID INDUCED INHIBITION OF PROTEIN & RNA SYNTHESIS IN E.COLI MRE 600 BY ISOLEUCINE

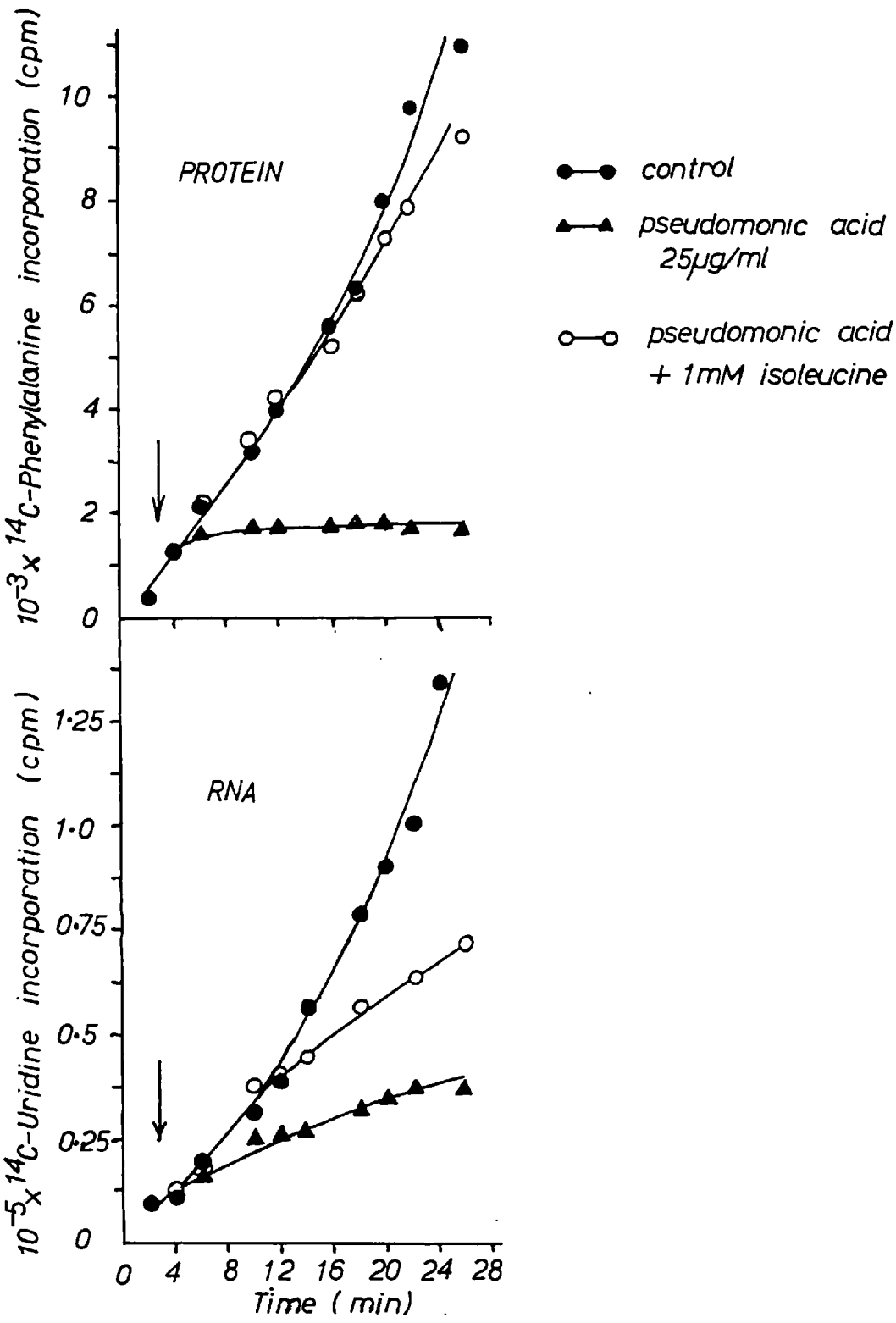


TABLE 13.

EFFECT OF PSEUDOMONIC ACID ON THE AMINO ACID POOL OF E. COLI MRE 600.

Amino acid	Control	Pseudomonic acid (20µg/ml, 10min.)
Asparagine	1.3*	0.7*
Threonine	0.3	1.0
Serine	0.2	1.1
Glutamic acid	5.3	21.0
Glycine	0.7	2.0
Alanine	3.7	10.0
Valine	4.8	24.0
Methionine	0.2	0.33
Isoleucine	0.7	1.7
Leucine	0.3	1.7
Tyrosine	0.3	0.7
Phenylalanine	0.1	0.6

* nmole/20mg wet weight of cells.

Extraction and determination of amino acids were as described in the Materials and Methods section.

(g). Degree of Aminoacylation of $tRNA^{Ile}$ in Pseudomonic Acid Treated Cells.

Since pseudomonic acid did not appear to interfere with the intracellular supply of isoleucine, the only remaining explanation for its effect on protein synthesis was that it was inhibiting tha aminoacylation of $tRNA^{Ile}$ catalysed by isoleucyl-tRNA synthetase.

The degree of aminoacylation of several tRNAs, including $tRNA^{Ile}$, in normally growing and pseudomonic acid treated cells was determined. Aminoacylation of $tRNA^{Ile}$, and several other tRNA species was examined using a cell-free system from E. coli MRE 600. The aminoacylation of $tRNA^{Ile}$ was specifically and drastically inhibited in pseudomonic acid treated cells of E. coli MRE 600 (Table 14). 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.

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 remains fully charged (Table 15). The three other tRNAs assayed remained fully charged in both antibiotic treated and untreated cells.

(h). Type of Inhibition Shown by Pseudomonic Acid on Purified Isoleucyl-tRNA Synthetase from E. coli B.

Steady state kinetic studies of the overall amino-acylation reaction using purified isoleucyl-tRNA synthetase from E. coli B showed that pseudomonic acid behaved as a competitive inhibitor with respect to isoleucine (Fig. 22). (Assays were quenched after one minute, since the aminoacylation

TABLE 14.

EFFECT OF PSEUDOMONIC ACID ON IN VITRO AMINOACYLATION OF DIFFERENT tRNA SPECIES.

AMINO ACID	% INHIBITION OF AMINOACYLATION OF tRNA
Glycine	0
Leucine	0
Valine	0
Histidine	0
Phenylalanine	10
Tyrosine	0
Tryptophan	0
Isoleucine	90

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

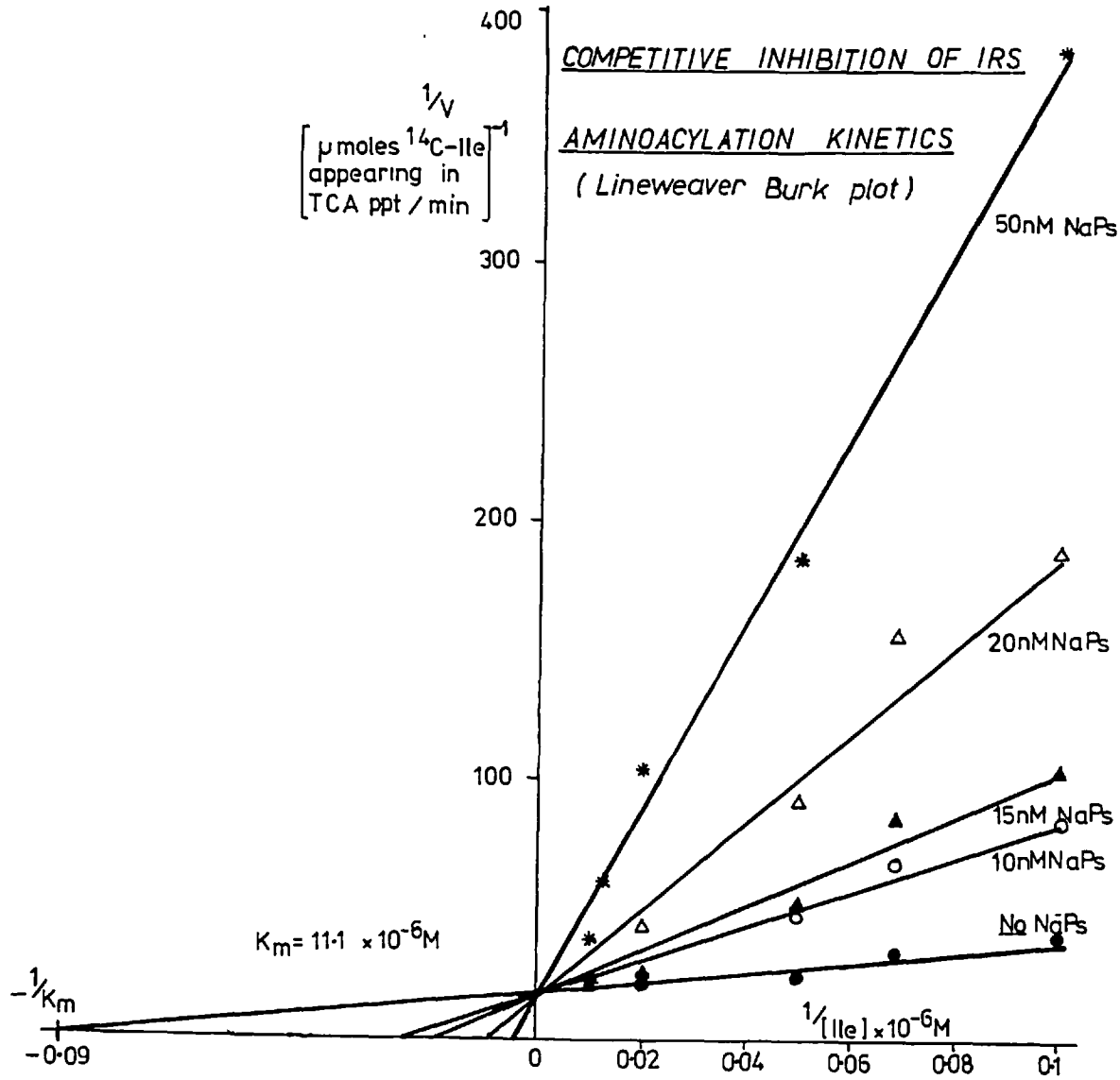
TABLE 15.

EFFECT OF PSEUDOMONIC ACID ON THE IN VIVO AMINOACYLATION OF tRNA IN E. COLI MRE 600.

AMINO ACID	CHARGED tRNA (%)	
	CONTROL	PSEUDOMONIC ACID TREATED
Isoleucine	100	60
Leucine	100	100
Histidine	100	100
Phenylalanine	100	100

An exponentially growing culture, in minimal medium, (11, $E_{620} \simeq 0.5$) was equally divided into two portions. One culture was treated with pseudomonic acid (25μg/ml) and the other served as a control. After 10 min, growth was stopped by the addition, to each culture, of 55% TCA (50ml), pre-warmed 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]-amino acid. The degree of aminoacylation *in vivo* was calculated from the tRNA acceptor activity resistant to periodate oxidation.

FIGURE 22


COMPETITIVE INHIBITION OF IRS
AMINOACYLATION KINETICS
(Lineweaver Burk plot)
1/V
[μ moles 14C-Ile appearing in TCA ppt / min]-1
400
300
200
100
50nM NaPs
20nMNaPs
15nM NaPs
10nMNaPs
No NaPs
Km= 11·1 x 10-6M
-1/Km
-0·09
0
0·02
0·04
0·06
0·08
0·1
1/[Ile] x 10-6M

of $tRNA^{Ile}$ using this system was only linear for 3 minutes). The K_i (2.5×10^{-9}M) obtained from a Dixon plot (Fig. 23) is several orders of magnitude lower than the K_m (11.1×10^{-6}; pH 7.4; 37°C, with respect to isoleucine). The charging of $tRNA^{Ile}$ by the synthetase enzyme from E. coli K12 has been shown (Fersht & Kaethner, 1976) to occur in two discrete stages:

1. $\alpha\alpha + ATP + Enz \longrightarrow Enz.\alpha\alpha\text{-}AMP + PP_i$

2. $Enz.\alpha\alpha\text{-}AMP + tRNA \longrightarrow Enz + AMP + \alpha\alpha\text{-}tRNA$

overall

$$\alpha\alpha + ATP + tRNA \xrightarrow{Enz} \alpha\alpha\text{-}tRNA + AMP + PP_i$$

Stage one can be examined separately by following the exchange of label from [^{32}P] pyrophosphate into adenosine triphosphate. Pseudomonic acid inhibited this reaction in a competitive manner with respect to isoleucine (Fig. 24). Using a Dixon plot (Fig. 25), the K_i was shown to be 6.0×10^{-9}M, in close agreement with the K_i for the inhibition of the overall aminoacylation reaction.

To examine the effect of pseudomonic acid on the transfer of isoleucine from the Enz.isoleucine-adenylate complex to $tRNA^{Ile}$, the Enz.[^{14}C]-Ile-AMP was isolated by Sephadex chromatography following incubation of the enzyme with [^{14}C]-isoleucine and ATP in the presence of pyrophosphatase. The complex was incubated with tRNA under suitable conditions (see Methods section). The results (Table 16) show that this step is insensitive to pseudomonic acid.

(i). Effect of Pseudomonic Acid on Aminoacyl-tRNA Synthetases from Rat Liver.

Of four aminoacyl-tRNA synthetases assayed (Table 17), isoleucyl-tRNA synthetase in this system proved most sensitive

FIGURE 23

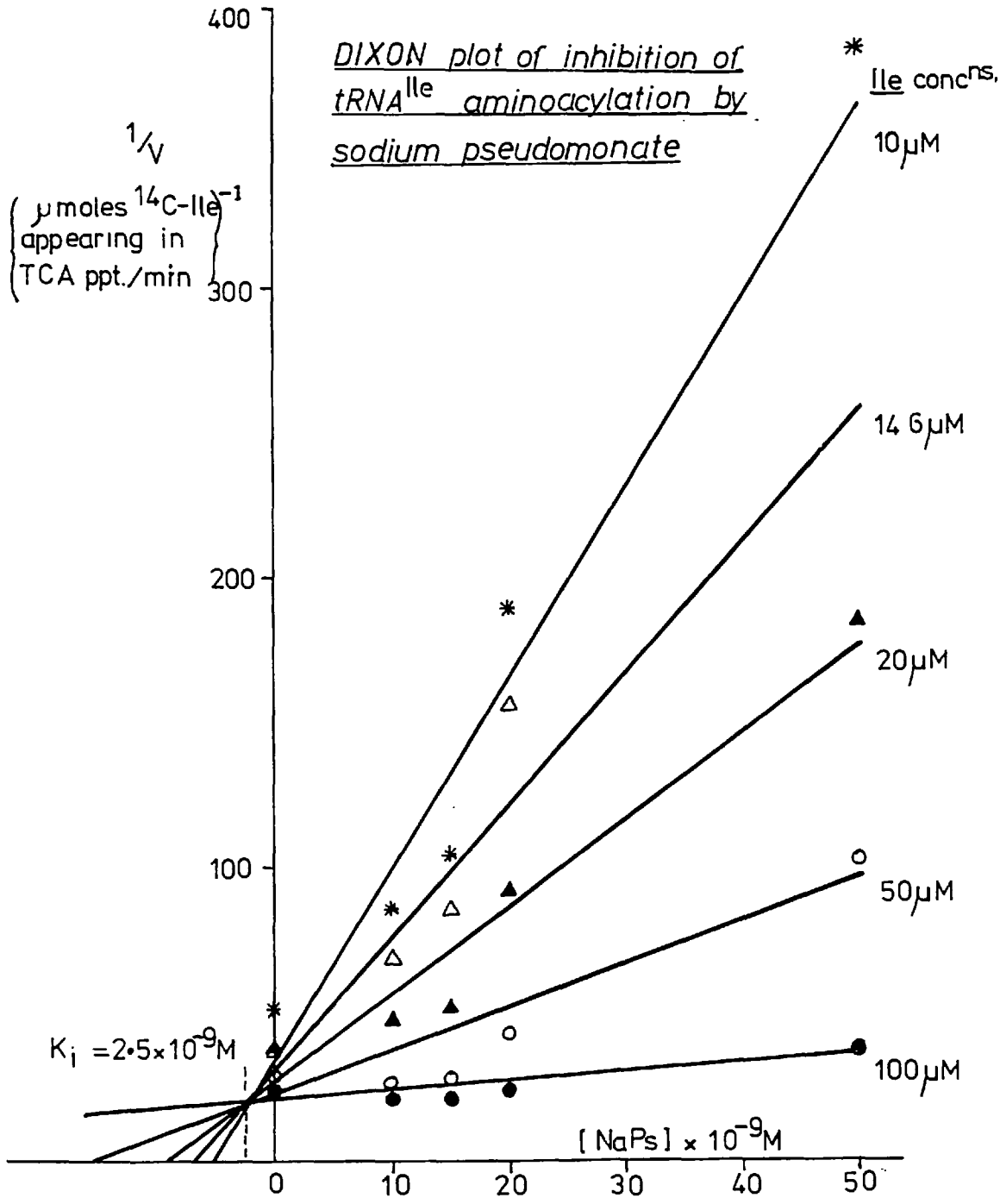

DIXON plot of inhibition of tRNA^Ile aminoacylation by sodium pseudomonate
1/V
(μmoles 14C-Ile appearing in TCA ppt./min)^-1
Ile concns.
10μM
14.6μM
20μM
50μM
100μM
Ki = 2·5×10^-9M
[NaPs] × 10^-9M
400
300
200
100
0
10
20
30
40
50

FIGURE 24

LINEWEAVER BURK PLOT OF INHIBITION OF PYROPHOSPHATE EXCHANGE REACTION BY SODIUM PSEUDOMONATE

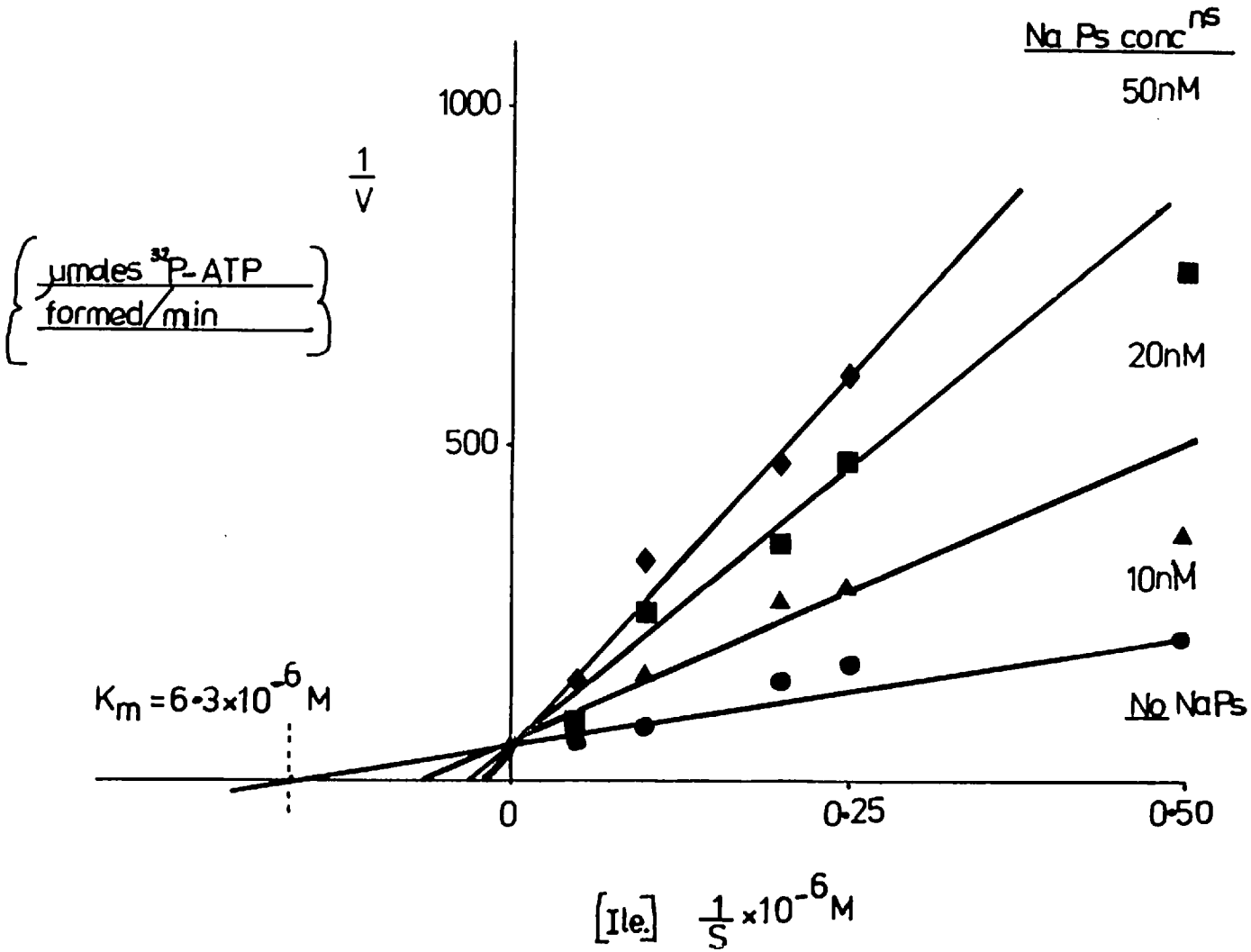


FIGURE 25

DIXON PLOT OF INHIBITION OF PYROPHOSPHATE EXCHANGE REACTION BY SODIUM PSEUDOMONATE

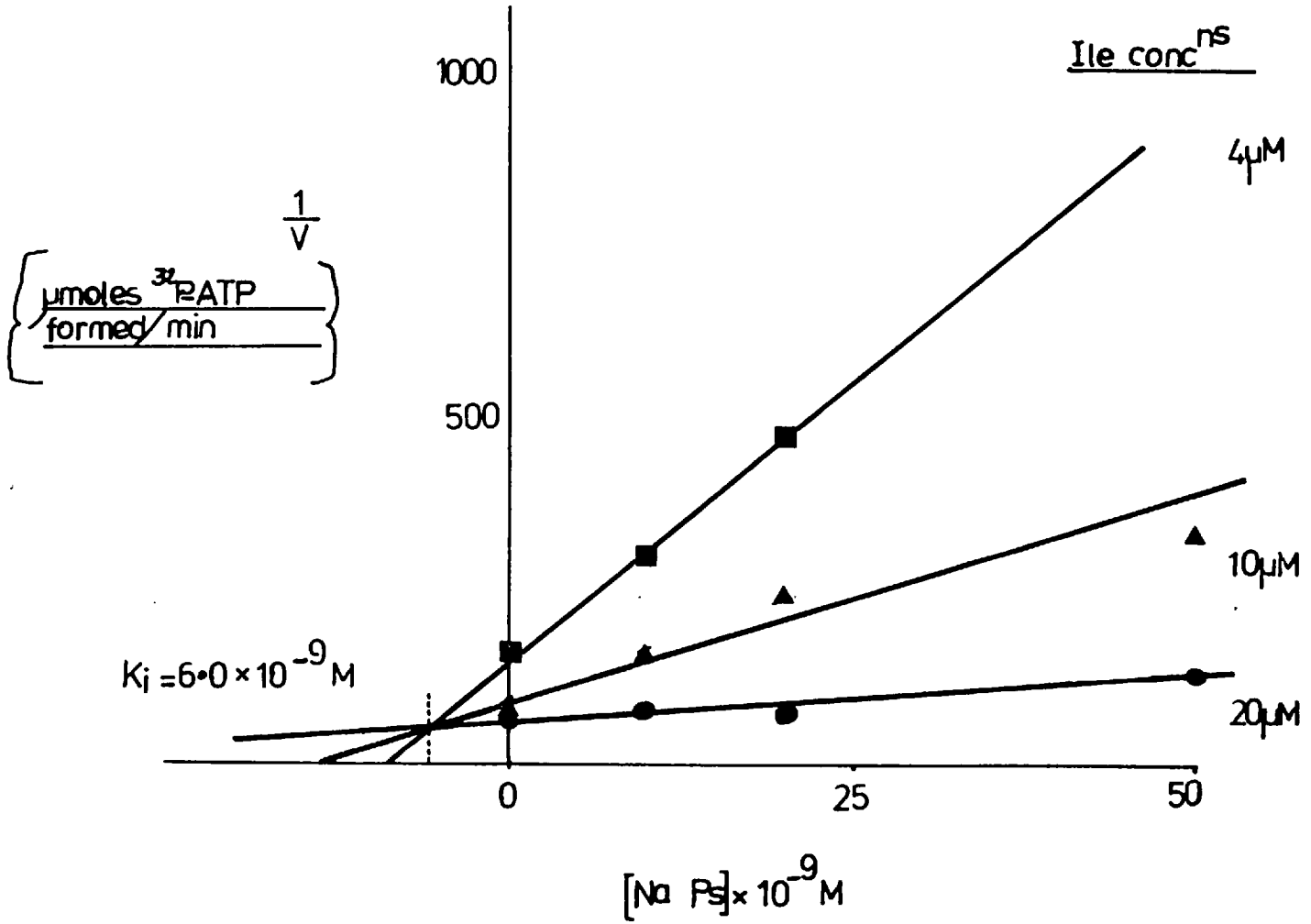


TABLE 16.

EFFECT OF PSEUDOMONIC ACID ON THE FORMATION OF [^{14}C]-ISOLEUCYL-tRNA^{Ile} FROM tRNA^{Ile} AND ^{14}C -ISOLEUCYL-AMP.ENZYME COMPLEX.

counts/min		
Control	tRNA Absent	Pseudomonic acid treated
479	0	524

Isoleucyl-tRNA synthetase.[^{14}C]-Ile-AMP complex was incubated with tRNA for 15 minutes at 37°C as described in the Materials and Methods section. The reaction was quenched with 10% TCA and then the precipitate collected on a glass fibre filter.

TABLE 17.

EFFECT OF PSEUDOMONIC ACID ON AMINOACYLATION OF tRNAs IN A RAT LIVER PREPARATION.

Amino Acid	% Inhibition of Charging at 100μg/ml Pseudomonic Acid Concentration
Isoleucine	60
Valine	0
Leucine	0
Phenylalanine	10

Using a 200,000g supernatant from rat liver as a source of tRNA synthetases, the aminoacylation of different tRNA species using the corresponding [U-^{14}C]-amino acid, was determined in the presence and absence of pseudomonic acid (100μg/ml), as described in the Materials and Methods section. The degree of inhibition of aminoacylation was calculated from the incorporation of ^{14}C into TCA precipitable material in the presence and absence of pseudomonic acid.

to pseudomonic acid, but the inhibitory effect was weak compared to the effect on the prokaryotic enzyme. Kinetic studies showed that the rat liver isoleucyl-tRNA synthetase had a K_m of 5.4×10^{-6}M (pH 7.4; 37°C, with respect to isoleucine) (Fig. 26) and a K_i of 20×10^{-6}M for pseudomonic acid (Fig. 27). The K_m for isoleucine from both eukaryotic and prokaryotic organisms is similar: 5.4×10^{-6}M and 11.1×10^{-6}M respectively. Interestingly, phenylalanyl-tRNA synthetase also appeared slightly sensitive in the rat liver system, as was demonstrated for the E. coli enzyme.

(j). Sephadex Chromatography of Pseudomonic Acid.Isoleucyl-tRNA Synthetase Complex.

The antibiotic.enzyme complex appears to be stable towards Sephadex chromatography, and could be conveniently isolated by this method (Fig. 28). A binding ratio of [9'-^{3}H]-pseudomonic acid to isoleucyl-tRNA synthetase of 0.73 to 1.0 was obtained from column chromatography on Sephadex G-50 under equilibrium conditions (Fig. 28). Inclusion of Mg^{2+} and ATP in the incubation mixture did not increase the binding ratio. Pseudomonic acid can only be separated from the isoleucyl-tRNA synthetase by using severe protein denaturing conditions e.g. boiling in 0.1% sodium dodecyl sulphate (SDS). This non-covalent, yet very tight association, between pseudomonic acid and isoleucyl-tRNA synthetase was predictable from the kinetic experiments. To test the specificity of the interaction between pseudomonic acid and isoleucyl-tRNA synthetase, [9'-^{3}H]-pseudomonic acid was incubated with bovine serum albumin and the complex subjected to column chromatography as described above. No label was found associated with the bovine serum albumin fractions

FIGURE 26

Competitive inhibition of rat liver IRS aminoacylation kinetics (Linweaver-Burk plot)

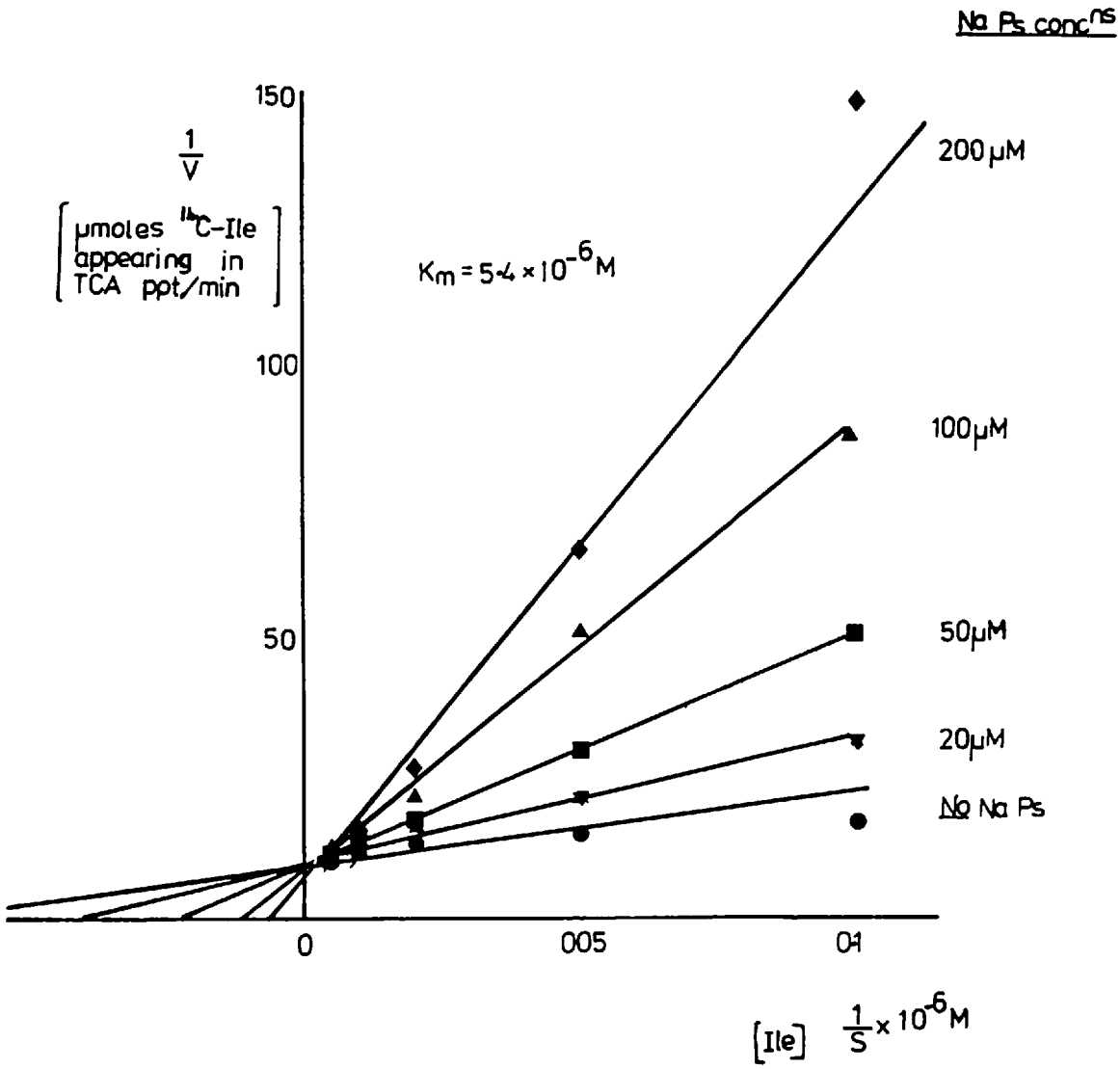


FIGURE 27

Dixon plot of inhibition of $tRNA^{Ile}$ aminoacylation by sodium pseudomonate

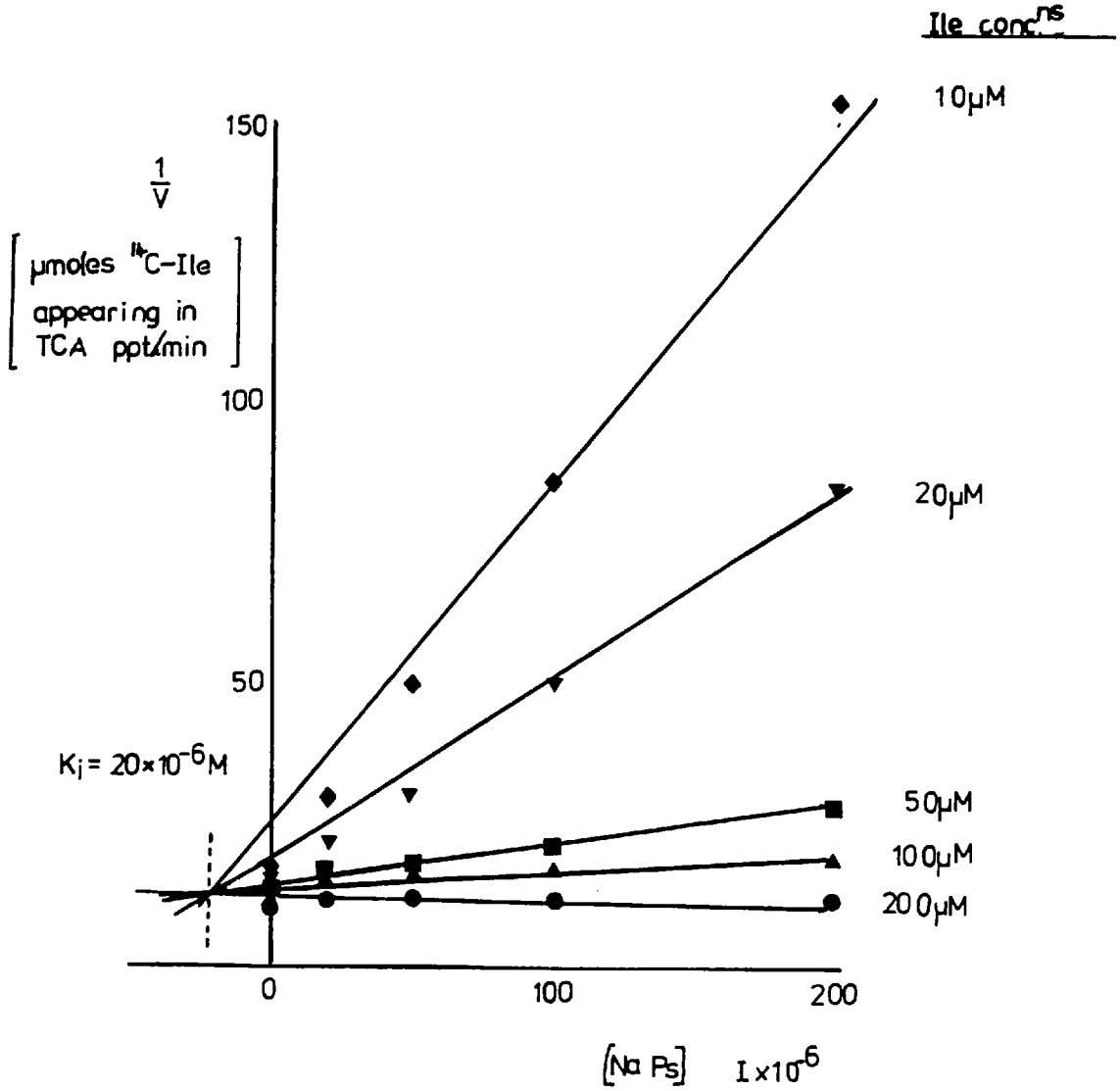


FIGURE 28. G-50 SEPHADEX GEL FILTRATION STUDIES ON SODIUM [9'-^{3}H]-PSEUDOMONATE.ENZYME COMPLEX.

Isoleucyl-tRNA synthetase (211pmole) was incubated at 37°C with sodium [9'-^{3}H]-pseudomonate (20nmole; 50.8μCi/μmole) in 20mM Tris-HCl buffer pH 7.2 and 10mM dithiothreitol plus or minus 10mM magnesium chloride and 5mM ATP for 15 minutes. The mixture was filtered on Sephadex G-50 (fine) (0.8x8cm) with the same buffer. Fractions (90μl) were collected, 25μl Of each was radiocounted and 50μl tested for protein according to the procedure of McKnight, (1977).

← - - - → Protein positive fractions.

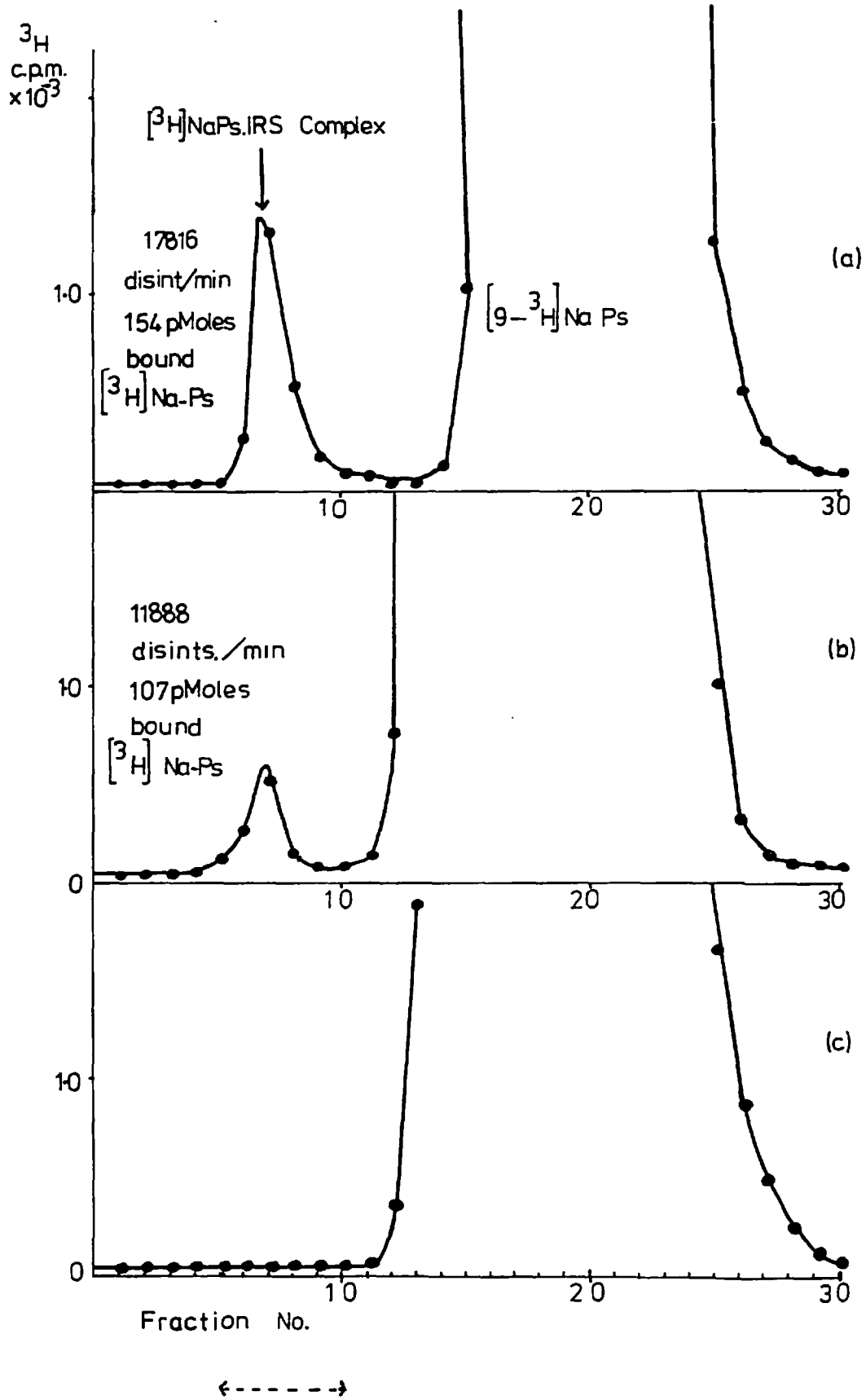
(a) Reaction mixture applied directly to column
Binding ratio IRS:Pseudomonic acid::1:0.73

(b) Reaction mixture treated with 0.1% SDS for 10 minutes at 24°C prior to gel filtration.
Binding ratio IRS:Pseudomonic acid::1:0.51

(c) Reaction mixture treated with 0.1% SDS for 10 minutes at 100°C prior to gel filtration.

FIGURE 28

G-50 SEPHADEX GEL FILTRATION STUDIES ON [^{3}H]NaPs.IRS COMPLEX



from the column.

(k). Gel Electrophoresis of Pseudomonic Acid.Isoleucyl-tRNA Complex.

When an excess of pseudomonic acid is incubated with the enzyme and the resulting mixture run on 0.1% SDS polyacrylamide gels, two bands can be clearly resolved (Fig. 29), one of which has the same electrophoretic mobility as native isoleucyl-tRNA synthetase. However long the pseudomonic acid and enzyme are incubated, two bands always result. If the complex is boiled in 0.1% SDS prior to electrophoresis or if the gels contain 6M urea, only one band appears with the same mobility as the enzyme alone. This infers that the complex is only partially slable under conditions of 0.1% SDS at room temperature prior to electrophoresis.

An attempt to separate the [9'-^{3}H]-pseudomonic acid. enzyme complex from free pseudomonic acid using native and 0.1% SDS polyacrylamide gel electrophoresis, by monitoring the radioactivity in 1mm gel slices proved unsuccessful. The ^{3}H -antibiotic tended to smear too much, preventing the identification of clear peaks.

(l). Equilibrium Dialysis of Pseudomonic Acid.Isoleucyl-tRNA Synthetase Complex.

A Scatchard plot of the equilibrium dialysis results confirmed a binding ratio of pseudomonic acid to isoleucyl-tRNA synthetase of 0.85 to 1.0 (Fig. 30). The dissociation constant for the complex was found to be approximately 75×10^{-9}M.

FIGURE 29. GEL ELECTROPHORESIS OF SODIUM PSEUDOMONATE.ENZYME COMPLEX.

0.1% SDS Acrylamide Gels.

a) 10μg Isoleucyl-tRNA Synthetase.

b) 10μg Isoleucyl-tRNA Synthetase plus 100μg Sodium Pseudomonate.

c) 10μg Isoleucyl-tRNA Synthetase plus 10μg Sodium Pseudomonate.

6M Urea Gels.

d) 10μg Isoleucyl-tRNA Synthetase.

e) 10μg Isoleucyl-tRNA Synthetase plus 100μg Sodium Pseudomonate.

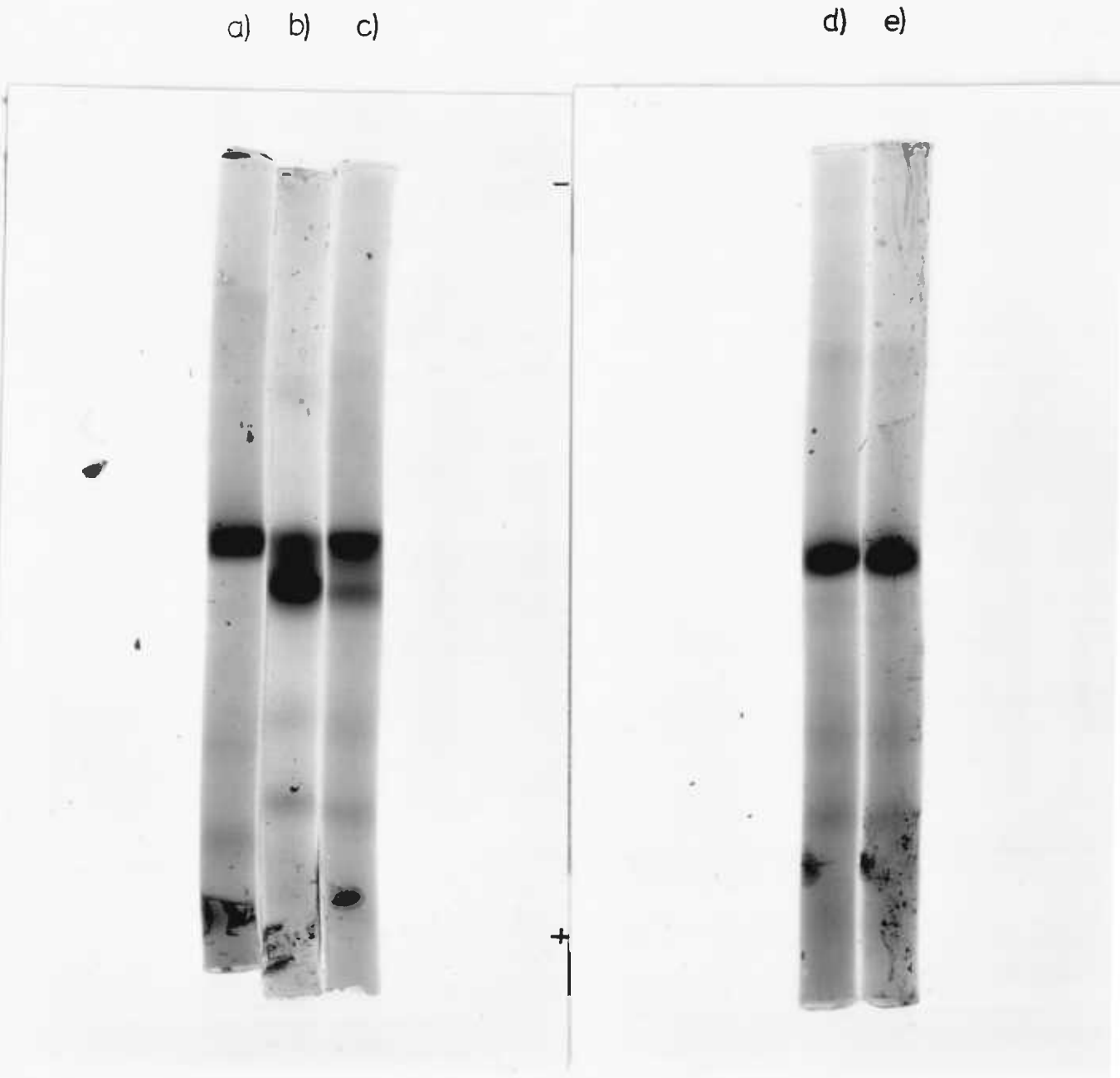
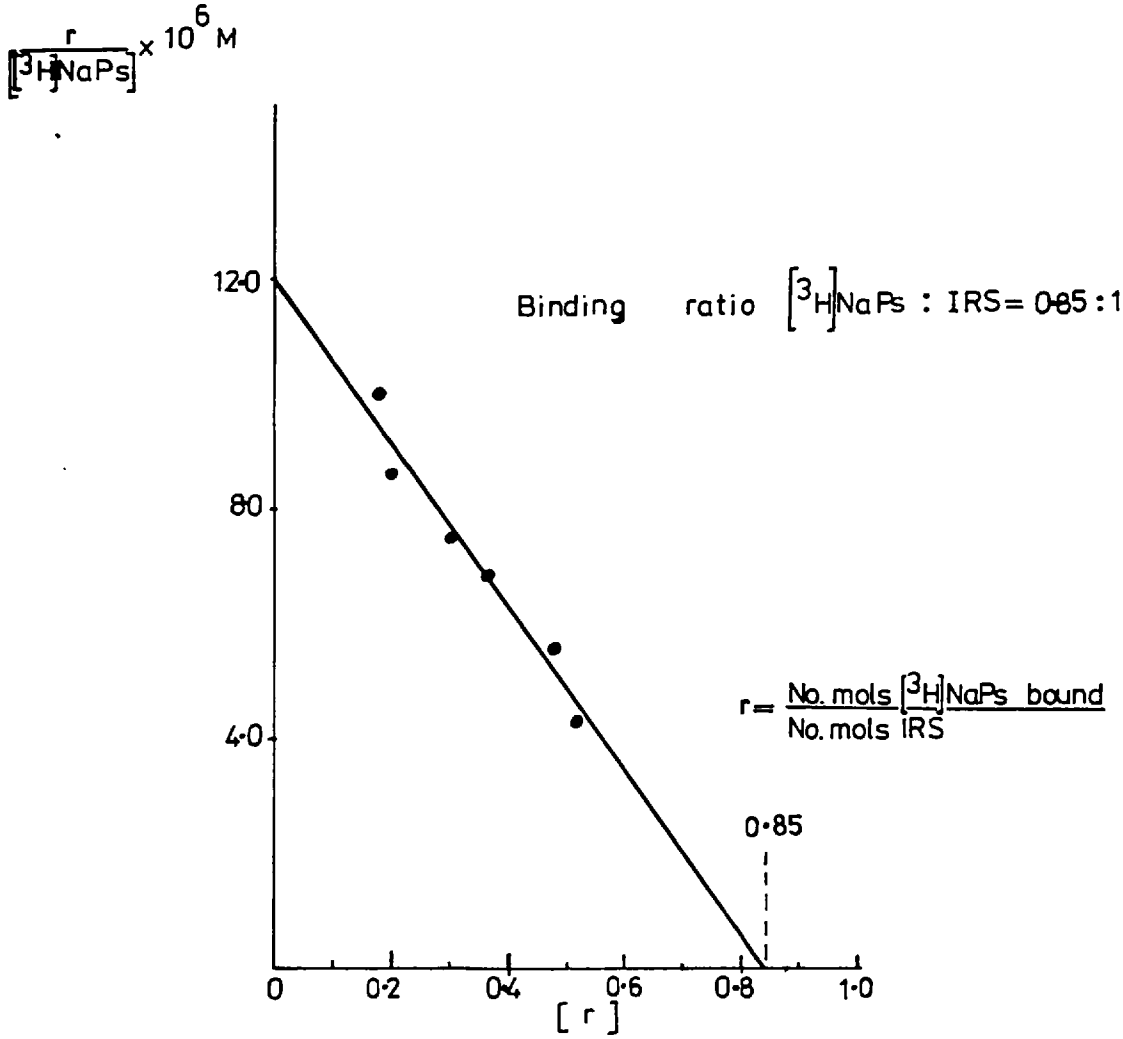


FIGURE 30

SCATCHARD PLOT OF [^{3}H]NaPs BINDING TO IRS



For reasons of confidentiality, at this point in time, the effect of a number of pseudomonic acid derivatives on the isoleucylation of $tRNA^{Ile}$ catalysed by *E.coli* B isoleucyl-tRNA synthetase, cannot be included in this thesis.

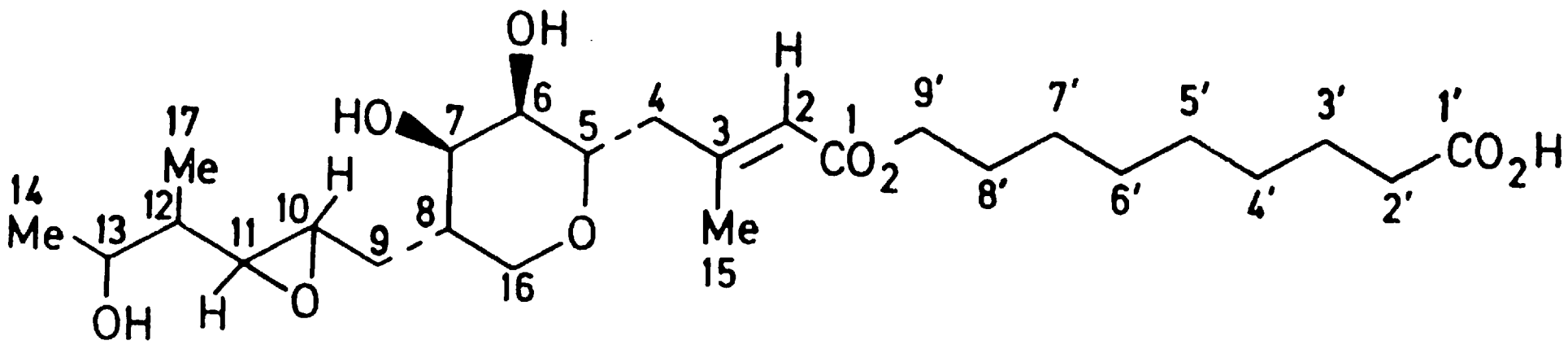
DISCUSSION.

DISCUSSION.

Pseudomonic acid is a recently discovered antibiotic, produced by the bacterium Pseudomonas fluorescens NC1B 10586. Conditions have been developed for its large scale production (Fuller et al., 1971). The gross structure of pseudomonic acid (Fig. 31) was determined by Chain & Mellows (1977a) from chemical and spectroscopic studies and an X-ray crystallographic study of a derivative has additionally provided the absolute configuration of the eight assymetric centres in the molecule (Alexander et al., 1978). The antibiotic has a fairly narrow spectrum of antibacterial activity (B. Slocombe & R. Sutherland, unpublished results), being mainly an inhibitor of Gram-positive bacteria, but in addition showing good activity against Neisseria species and Haemophilus influenzae. E. coli strains can be made more susceptible to pseudomonic acid by prior treatment with EDTA, an ion chelating agent (B. Slocombe & R. Sutherland, personal communication), indicating that lack of penetration may well explain why many Gram-negative organisms are resistant to the drug. The novel structure of the antibiotic together with its lack of cross-resistance with a wide range of common antibiotics prompted a study into its mode of action in bacteria. It was considered that if a detailed understanding of the mode of action of pseudomonic acid could be determined at the molecular level, then this information could be useful in directing chemical modification of the drug to increase its efficacy as an antibiotic. Additionally, the solution to the problem of permeability, especially in Gram-negative organisms might be more easily overcome,

FIGURE 31

PSEUDOMONIC ACID



if an understanding on which part(s) of the pseudomonic acid molecule was important for its mode of action could be found.

Initial studies aimed at delineating which major cellular process was affected most by pseudomonic acid, were carried out using Staphylococcus aureus (Oxford), as this Gram-positive organism was very susceptible to the antibiotic at low concentrations and had been used in routine assays for the antibiotic. Pseudomonic acid causes reversible suppression of the growth of S. aureus at concentrations around its minimum inhibitory concentration (M.I.C.). Much higher levels are required before the effect of the drug becomes bacteriocidal (B. Slocombe & R. Sutherland, personal communication). Of the major biosynthetic pathways examined in vivo, pseudomonic acid was found to markedly inhibit protein and RNA synthesis, measured by [^{14}C]-phenylalanine and [^{14}C]-uridine incorporation into trichloroacetic acid precipitable material. DNA and cell wall formation, as measured by [^{14}C]-thymidine and [^{14}C]-glucosamine assimilation, were affected to a lesser extent, and interference with these processes was therefore considered to be secondary. Finally, the antibiotic did not interfere with oxidative phosphorylation, since the cellular pool of ATP increased slightly rather than decreased in cells treated with pseudomonic acid. Controls were carried out in all these experiments, by using substances that were known inhibitors of each of these five cellular processes (Tables 6, 9 and 18). A comparison of the affect of pseudomonic acid at its M.I.C. with that of the respective inhibitor at its M.I.C. level could be made. With respect to the former observation, it is clear

TABLE 18.

INHIBITION OF PROTEIN, RNA AND DNA SYNTHESIS IN VIVO IN S. AUREUS BY CHLORAMPHENICOL, RIFAMPICIN AND NALIDIXIC ACID.

Antibiotic	% Inhibition after 20 minutes		
	Protein	RNA	DNA
Chloramphenicol (5µg/ml)	97	6	N.D.
Rifampicin (0.03µg/ml)	65	79	N.D.
Nalidixic acid (50µg/ml)	N.D.	N.D.	80

N.D. = Not Determined

To a split culture was added either [^{14}C]-phenylalanine, [^{14}C]-uridine or [^{14}C]-thymidine as described in the legend to Fig. 10. The degree of inhibition was calculated from the kinetics of incorporation in pseudomonic acid treated cultures relative to the untreated controls, 20 minutes after the addition of the antibiotic.

that a primary affect on either RNA or protein synthesis could lead to a rapid inhibition of the other pathway as a result of *in vivo* regulation of both. Rifampicin, a potent inhibitor of RNA polymerase (Sung, 1972), rapidly inhibits both RNA and protein synthesis *in vivo* due to the marked instability of bacterial messenger RNA. As soon as RNA synthesis is inhibited the supply of mRNA will cease, resulting in the arrest of protein synthesis. Conversely, if protein synthesis in inhibited as a consequence of lack of an amino acid(s) or a species of charged tRNA, both events give rise to the presence of uncharged tRNA in the A-site of functional ribosomes (Ogilvie *et al.*, 1975b). This provides the stimulus for the stringent response (see Introduction), the production of guanosine 5'-diphosphate-3'-diphosphate (ppGpp) and guanosine 5'-triphosphate-3'-diphosphate (pppGpp), which in turn inhibit the synthesis of stable RNA (tRNA and rRNA), by inhibiting the formation of polymerase.stable RNA.promoter complexes (Travers, 1976). Certain antibiotic inhibitors of ribosomal function, e.g. chloramphenicol, tetracycline, fusidic acid will remove the stringent response (Lund & Kjeldgaard, 1972). It was found in the preliminary experiments with *S. aureus* that the *in vivo* inhibition of RNA synthesis was relieved by the addition of chloramphenicol to pseudomonic acid treated cells. This result suggested, at this point, that the antibiotic-induced inhibition of RNA synthesis was probably a secondary effect and might be caused by the pseudomonic acid-induced stimulation of the stringent response. To clarify this point, an attempt was made to study the *in vitro* effect of pseudomonic acid on protein synthesis, using a cell-free system from *S. aureus* cells, prepared according to the method of Lacey &

Chopra (1972). Although some time was spent developing and modifying these preparations, consistent control results could not be achieved. For this reason, and, more especially, since the methods for obtaining purified enzyme preparations for protein and RNA synthesis were available for *E. coli*, all subsequent work was carried out with strains of this Gram-negative bacterium. In *in vivo* studies using *E. coli* MRE 600, more of the drug was necessary to bring about bacteriostasis (M.I.C. 50μg/ml) than with *S. aureus* due to poor penetration of the antibiotic through the *E. coli* cell wall. The effect of pseudomonic acid on *in vivo* RNA, protein and DNA formation was repeated in *E. coli* MRE 600. Findings similar to those observed in *S. aureus* were noted.

Interestingly, pseudomonic acid had no effect on the *in vitro* incorporation of [^{3}H]-UTP into RNA by highly purified *E. coli* B DNA-dependent RNA polymerase. Using a cell-free protein synthesising system from *E. coli* MRE 600 prepared essentially as described by Gould *et al.* (1973), pseudomonic acid (10μg/ml) had only a slight inhibitory effect on the *in vitro* poly U directed formation of poly-[^{14}C]-phe, as measured by [^{14}C]-phenylalanine incorporation into acid-precipitable material. Whilst these results indicated that the primary target of pseudomonic acid was one (or more) of the steps involved in protein synthesis, the latter observations combined with the *in vivo* results from the *S. aureus* work, suggested that inhibition was not a consequence of a direct effect of the antibiotic on ribosomal function. These observations also suggested that the simultaneous *in vivo* inhibition of both protein and RNA accumulation in *S. aureus* must therefore be a consequence of the pseudomonic acid-induced

stringent control of RNA synthesis, resulting from amino acid starvation (Edlin & Broda, 1968; Cashel, 1969). This was confirmed by results obtained with a stringent and relaxed pair of *E. coli* B cell wall mutants (AS19 Rel A^+ and Rel A^-), isogenic apart from a mutation in the Rel gene. The lesion in the cell wall reduced the M.I.C. for pseudomonic acid for both mutants to 0.3µg/ml. It was shown that *in vivo* RNA synthesis continued in the relaxed strain treated with the antibiotic at concentrations around the M.I.C., but was markedly inhibited in the stringently controlled mutant (Table 19). These experiments ruled out a direct *in vivo* effect of pseudomonic acid on RNA synthesis, in agreement with the earlier finding that the drug had no effect on DNA-dependent RNA polymerase. Using the same pair of *E. coli* B AS19 mutants, it was also shown that pseudomonic acid, at a concentration of 0.5µg/ml, stimulated the *in vivo* production of pppGpp and ppGpp in the stringently controlled mutant, but had no effect on these nucleotide levels in the relaxed mutant. This confirmed that the primary target of pseudomonic acid was protein synthesis. Inhibition of stable RNA synthesis would thus be due to a subsequent stimulation of the stringent response. In the same experiments, it was also shown that the ATP pool size increased in pseudomonic acid treated cells, confirming the previous observation in *S. aureus* (Hughes & Mellows, 1978a) that oxidative phosphorylation and energy processes dependent on it were not affected.

A preliminary study showed that inhibition of *E. coli* MRE 600 cultures growing in glucose salt medium, could be alleviated by the addition of all 20 common amino acids. In order to determine whether a single or more than one amino

TABLE 19.

EFFECT OF PSEUDOMONIC ACID ON PROTEIN & RNA SYNTHESIS IN VIVO IN E. COLI B AS19 541 & AS19 542.

PSEUDOMONIC ACID (μg/ml)	% INHIBITION OF ^{14}C - PHENYLALANINE UPTAKE		% INHIBITION OF ^{14}C - URIDINE UPTAKE	
	AS19 541	AS19 542	AS19 541	AS19 542
0.1	33	24	98	34
0.2	36	40	98	34
0.3	60	60	97	34
0.5	84	86	98	36

% Inhibition calculated 20 minutes after antibiotic addition.

produced the relieving effect, various mixtures of amino acids from which a single amino acid was eliminated from the complete mixture on 20 amino acids were added to the growth medium. It was found that when isoleucine and only this amino acid was left out of the mixture of 20 amino acids, cell growth stopped. Thus pseudomonic acid caused isoleucine auxotrophy in E. coli MRE 600. At this point it was confirmed that the antibiotic could cause isoleucine auxotrophy, either by restricting the formation of isoleucine, by interfering with some facet of its biosynthesis, or by interfering with the assimilation of isoleucine into protein. The former possibility was investigated by examining the cellular amino acid pool. If the antibiotic interfered with the biosynthesis of isoleucine, then the cellular concentration of this amino acid would decrease. The amino acid pools from control and pseudomonic acid treated cells were extracted, analysed and quantitated. Although amino acid analyses varied somewhat between repeated experiments, it was apparent that there was no evidence of a decrease in the level of isoleucine in pseudomonic acid treated cells. Generally, the levels of all amino acids, including isoleucine, tended to be elevated, compared to levels in control cells. Amino acid pools tended to be enlarged in cells that have been treated with inhibitors of protein synthesis, as one might expect (Ogilvie et al., 1975a). It is now known that the formation of isoleucine is regulated by a complex mechanism, involving isoleucyl-tRNA as effector rather than the amino acid (Figs. 32 and 33). In preliminary experiments conducted by Mellows and Shand (Hughes & Mellows, 1978b), in which cellular levels of isoleucyl-tRNA were assessed indirectly, three of the enzymes

FIGURES 32 and 33. THE BIOSYNTHESIS OF ISOLEUCINE, LEUCINE AND VALINE.

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

FIGURE 32

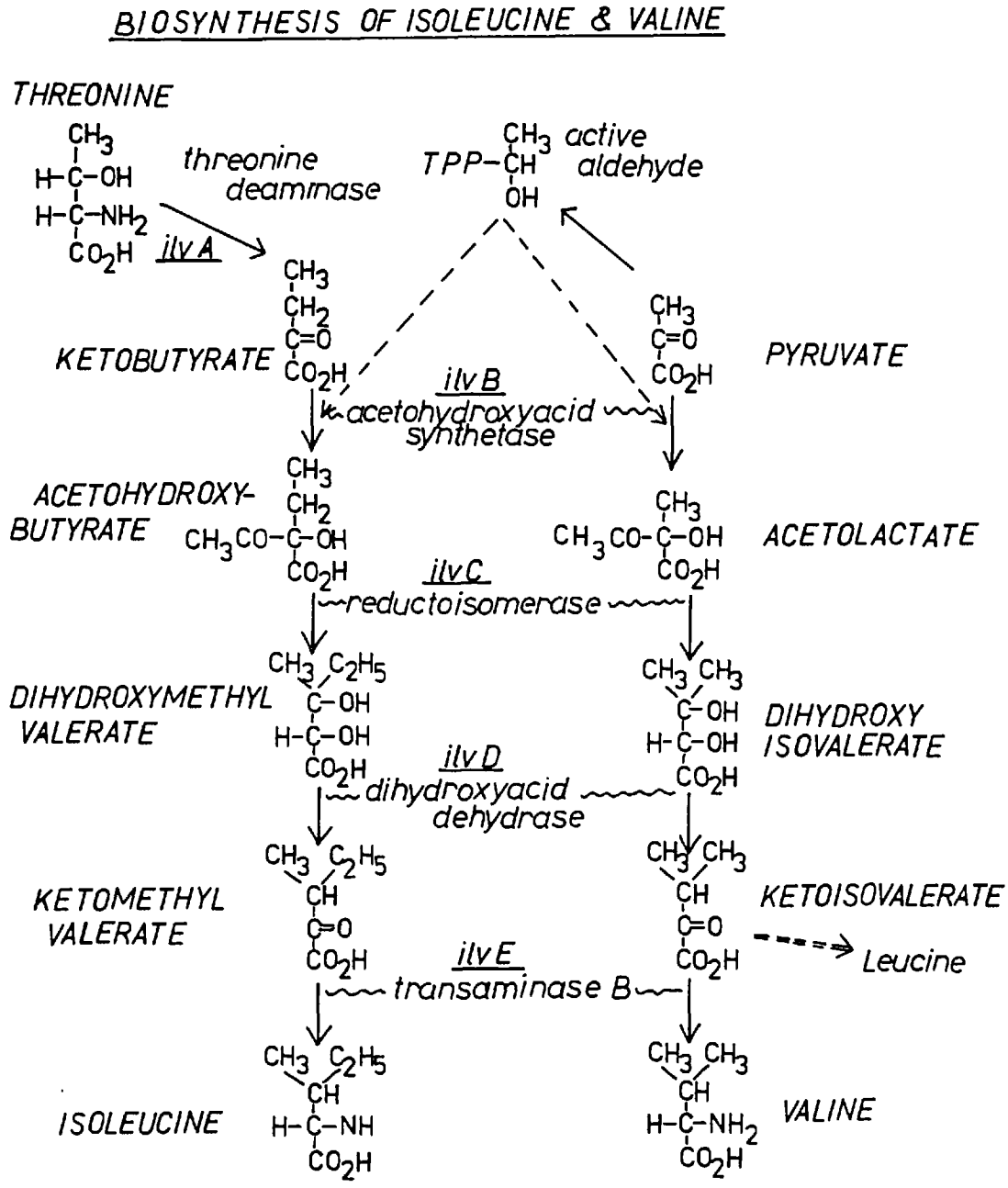
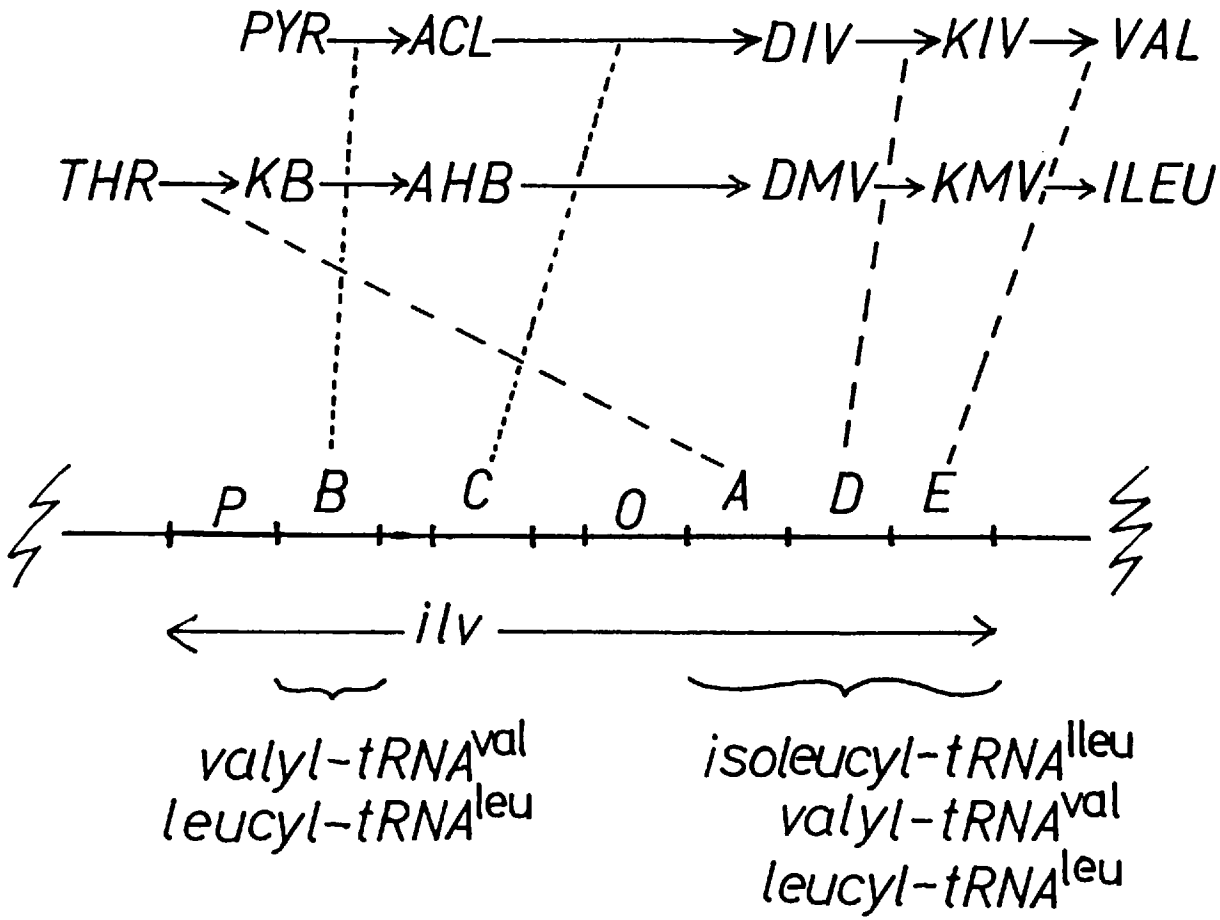

BIOSYNTHESIS OF ISOLEUCINE & VALINE
THREONINE
threonine deamminase
ilvA
active aldehyde
TPP
KETOBUTYRATE
PYRUVATE
ilvB
acetohydroxyacid synthetase
ACETOHYDROXY-BUTYRATE
ACETOLACTATE
ilvC
reductoisomerase
DIHYDROXYMETHYL VALERATE
DIHYDROXY ISOVALERATE
ilvD
dihydroxyacid dehydrase
KETOMETHYL VALERATE
KETOISOVALERATE
Leucine
ilvE
transaminase B
ISOLEUCINE
VALINE

FIGURE 33

ARRANGEMENT OF 'ILV' GENOME ON E.COLI CHROMOSOME



involved in isoleucine biosynthesis were assayed in extracts from pseudomonic acid treated and control cell cultures of E. coli MRE 600. Pseudomonic acid caused a significant derepression of threonine deaminase and transaminase B, but not of dihydroxyacid dehydratase (Table 20). These results confirmed the apparent auxotrophy for isoleucine that appeared to be induced by pseudomonic acid, since the former two enzymes are regulated by fully aminoacylated $tRNA^{Ile}$ (Szentirmai et al., 1968; Iaccarino & Berg, 1971; Blatt & Umbarger, 1975) and valyl-$tRNA^{Val}$ (Eidlic & Neidhardt, 1965). The gene controlling acetohydroxyacid synthetase has been shown to respond only to a multivalent repression signal from valine and leucine (Dwyer & Umbarger, 1968; Blatt et al., 1972), and would not therefore be derepressed under conditions where isoleucyl-$tRNA^{Ile}$ was present at much reduced levels**.

The experimental results obtained at this point strongly indicated that pseudomonic acid inhibited the charging of $tRNA^{Ile}$. This was proved conclusively using a cell-free aminoacyl-tRNA synthetase preparation from E. coli MRE 600 with which the effect of pseudomonic acid on the charging of several species of tRNA was examined. It was found that pseudomonic acid specifically inhibited isoleucyl-$tRNA^{Ile}$ formation. Of the seven other tRNA synthetases assayed using this system, pseudomonic acid exerted only a slight inhibitory effect on the function of phenylalanyl-tRNA synthetase. The inhibition

** Umbarger (personal communication) has some evidence to suggest that the above three enzymes are in the same unit of transcription and has further suggested that the polarity of translation is probably the explanation for the lower derepression of acetohydroxyacid synthetase that was observed.

TABLE 20.

DEREPRESSION OF THREONINE DEAMINASE & TRANSAMINASE B IN E.COLI MRE 600 TREATED WITH PSEUDOMONIC ACID (25µg/ml).

ENZYME	SPECIFIC ACTIVITY		ENZYME ACTIVITY ENHANCEMENT
	CONTROL	PSEUDOMONIC ACID TREATED	
THREONINE DEAMINASE	0.0053*	0.0165*	3.1
TRANSAMINASE B	0.030+	0.101+	3.4
ACETOHYDROXYACID SYNTHETASE	0.083≠	0.120≠	1.4

Enzyme activities expressed as :

* µmoles ketobutyrate formed / mg protein / min.

+ µmoles ^{14}C-isoleucine disappearing / mg protein / min.

≠ µmoles acetolactate formed / mg protein / min.

Two 1l cultures, growing in mineral salt-glucose medium, were treated with pseudomonic acid (25µg/ml), 1 hour post-inoculation ($E_{578} \simeq 0.03$), whilst another pair of 1l cultures served as a control. After a further five hours, the cells were harvested from each pair of cultures by centrifugation and the respective levels of threonine deaminase, transaminase B and acetohydroxy-acid synthetase determined as described in the Materials and Methods section.

by pseudomonic acid, of poly U directed synthesis of poly phe, which was discussed earlier, is presumably a consequence of this. It is of interest that phenylalanine, like isoleucine, has a hydrophobic side chain, but at the same pseudomonic acid concentration, there was no inhibition of the charging of the other similar hydrophobic amino acids (valine and leucine) (Table 14). The significance of this observation is currently unknown. It has been shown also that pseudomonic acid has no effect on the activation of the two acidic amino acids, glutamic acid and aspartic acid (Mellows & Soughton, unpublished work). However, the same workers demonstrated a 70% stimulation by pseudomonic acid of the aminoacylation of $tRNA^{Lys}$, but the molecular basis for this is again not known.

When tRNA was extracted and purified from E. coli MRE 600 cells which had been treated with pseudomonic acid, it was found that only 60% of the $tRNA^{Ile}$ was charged, compared with that in untreated cells where the $tRNA^{Ile}$ was effectively fully charged. Three other tRNA species extracted and assayed, $tRNA^{Phe}$, $tRNA^{Leu}$ and $tRNA^{His}$, remained fully charged in both pseudomonic acid treated and untreated cells. 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 (IRS) and the observations of Cassio & Mathien (1974) and Glazier & Schlessinger (1974), who found that aminoacylation increases during inhibition of protein synthesis.

Using electrophoretically homogeneous isoleucyl-tRNA synthetase which had been isolated from E. coli B, it was

shown by a steady state kinetic study that pseudomonic acid behaved as a very effective competitive inhibitor (with respect to isoleucine) (Figure 22). The K_i (2.5×10^{-9}M) obtained from a Dixon plot (Dixon, 1953) was several orders of magnitude lower than the K_m (11.1×10^{-6}M; pH 7.4; 37°C, with respect to isoleucine). The determined Michaelis-Menten constant, K_m, was slightly higher than that reported for the E. coli K12 IRS ($K_m = 5.7 \times 10^{-6}$M; pH 7.8; 25°C) (Fersht & Kaethner, 1976) and E. coli MRE 600 IRS ($K_m = 4 \times 10^{-6}$M; pH 8.0; 25°C) (Rainey et al., 1977). The charging of $tRNA^{Ile}$ has been shown (Fersht & Kaethner, 1976) to occur in two stages, although the full mechanism of the overall reaction is very complex:

1. $Ile + ATP + Enz \xrightarrow{Mg^{2+}} Enz.Ile\text{-}AMP + PP_i$

2. $Enz.Ile\text{-}AMP + tRNA^{Ile} \longrightarrow Ile\text{-}tRNA^{Ile} + Enz + AMP$

overall

$$Ile + ATP + tRNA^{Ile} \xrightarrow{Mg^{2+}} Ile\text{-}tRNA^{Ile} + AMP + PP_i$$

The rate determining step for the E. coli K12 enzyme has recently been shown (Fersht & Kaethner, 1976) to be the transfer step (equation 2) whereas that reported for the E. coli B enzyme is the diffusion of the charged tRNA from the enzyme (Yarus & Berg, 1969). Further steady state kinetic experiments showed that pseudomonic acid competitively inhibited the formation of the Enzyme.isoleucyl-AMP complex, as measured by the pyrophosphate exchange reaction (Bergmann, 1962) ($K_i = 6.0 \times 10^{-9}$M). The determined K_m (6.3×10^{-6}M; pH 7.4; 37°C) agreed well with the literature value (4.0×10^{-6}M; pH 8.0; 25°C) (Rainey et al., 1977). The IRS.[^{14}C]-Ile-AMP complex was isolated and purified by gel filtration on Sephadex G-25 following incubation of [^{14}C]-isoleucine, ATP,

magnesium chloride and pyrophosphatease with IRS. Using the IRS.[^{14}C]-Isoleucine-AMP complex, it was shown that pseudomonic acid had no effect on the transfer of [^{14}C]-isoleucine from the complex to tRNA^{Ile}. There are two possible explanations for the kinetic results. Either part of the pseudomonic acid molecule behaves as a structural analogue of isoleucine, and directly competes with isoleucine for the isoleucine binding site, or the antibiotic binds to a different part of the isoleucyl-tRNA synthetase, in such a way that the active site for isoleucine would be no longer available for isoleucine binding (cf some regulatory allosteric enzymes e.g. aspartate transcarbamoylase of E. coli (Ferdinand, 1976)). It has not been possible so far to distinguish between these two possibilities.

The kinetic experiments indicated that pseudomonic acid binds very tightly, but non-covalently to IRS. In support of this, very severe denaturing conditions, for example, urea treatment or boiling in sodium dodecyl sulphate, are required for the complete removal of the bound pseudomonic acid from the enzyme, as shown by gel electrophoresis on polyacrylamide gels (Fig. 29) and Sephadex chromatographic studies (Fig. 28). The antibiotic.enzyme complex appears to be stable towards Sephadex chromatography. A binding ratio of [9'-^{3}H]-pseudomonic acid to enzyme of 0.73:1.0 was obtained from column chromatography on Sephadex G-50 under equilibrium conditions (Fig. 28). When an excess of pseudomonic acid was incubated with the enzyme and the resulting mixture run on 0.1% SDS-polyacrylamide gels, two bands were clearly resolved (Fig. 29). One of the bands had the same electrophoretic mobility as the native enzyme. However long the

pseudomonic acid and enzyme were incubated, two bands always resulted. Addition of isoleucine, ATP and a protease inhibitor to the incubation mixture prior to electrophoresis had no effect on the appearance of the bands. This inferred that the complex was only partially stable under conditions of 0.1% SDS treatment, since boiling the complex in 0.1% SDS, prior to electrophoresis gave only one band of the same mobility as the native enzyme. Similarly, only one band was observed in 6M urea gels. The appearance of the second band was very strange, since in SDS electrophoresis, proteins have been demonstrated to have relative mobilities proportional to their molecular weights (Fig. 34). On this basis, the bands would differ by about 2,000 in molecular weight. (If the second band was indeed the pseudomonic acid.enzyme complex, the difference should only be 500). Although SDS electrophoresis has been shown (Weber & Osborn, 1969) to be reliable means of determining the molecular weights of proteins, the exact basis for its reliability is not fully understood. It is known that SDS binds to proteins in a constant weight ratio. The pre-binding of pseudomonic acid to the enzyme may prevent SDS from binding, thereby producing a second band of lower molecular weight. An attempt to separate the [9'-^{3}H]-pseudomonic acid.enzyme complex from free pseudomonic acid using native and 0.1% SDS polyacrylamide gel electrophoresis, by monitoring the radioactivity in 1mm gel slices, proved unsuccessful. The ^{3}H -antibiotic surprisingly tended to smear too much, preventing the identification of clear peaks. The binding ratio of [9'-^{3}H]-pseudomonic acid to IRS (0.85:1.0) was also determined by equilibrium dialysis. This was in good agreement with the ratio (0.73:1.0) obtained by

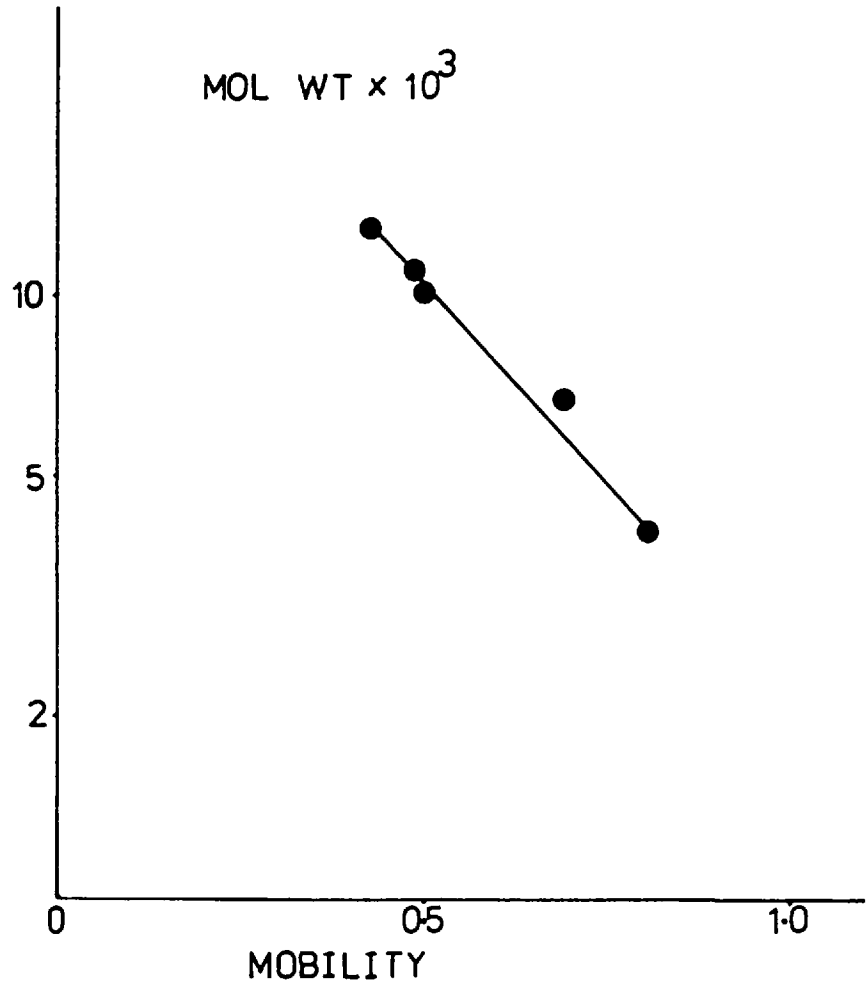
FIGURE 34. ELECTROPHORETIC MOBILITY OF PROTEINS AS A FUNCTION OF THEIR MOLECULAR WEIGHTS.

Several different proteins of known molecular wieght were subjected to gel electrophoresis in 0.1% SDS polyacrylamide gels as described in the Materials and Methods section. The relative mobility of each protein was determined by use of the following formula, devised by Weber & Osborn, (1969):

$$\text{Relative mobility} = \frac{(\text{Distance of protein migration})(\text{Length before staining})}{(\text{Length after destaining})(\text{Distance of dye migration})}$$

FIGURE 34

Electrophoretic mobility of proteins as a function of their molecular weight



Sephadex chromatography. The dissociation constant for the antibiotic.IRS complex was also determined from a Scatchard plot (Fig. 30). The K_{diss} obtained, $75x10^{-9}M$, is somewhat larger than the K_i ($5x10^{-9}M$), but the reason for the discrepancy is not known.

On structural grounds, it would seem possible that the terminal residue of the epoxide chain at C-8 of pseudomonic acid, which has the same carbon skeleton as L-isoleucine, could compete with the amino acid for its binding site on the enzyme (Fig. 35). The site is known to contain two hydrophobic domains, respectively accomodating the methyl and ethyl groups of the amino acid (Holler et al., 1973; Flossdorf et al., 1976b). It is also known that the synthetases have evolved a high degree of specificity. For example, IRS can activate valine to form IRS.val-AMP complex. However, in the presence of $tRNA^{Ile}$, the valyl-AMP is not transferred to the $tRNA^{Ile}$, but is hydrolysed by an inbuilt editing mechanism of the enzyme (Baldwin & Berg, 1966). It would seem that the unfavourable accommodation of the epoxide side chain terminus of pseudomonic acid, which would place the C-13 hydroxyl function in the hydrophobic isoleucine binding cleft, would require stabilisation through interaction of other functionalities in the antibiotic molecule, with the other amino acid residues in the enzyme (Fig. 31).

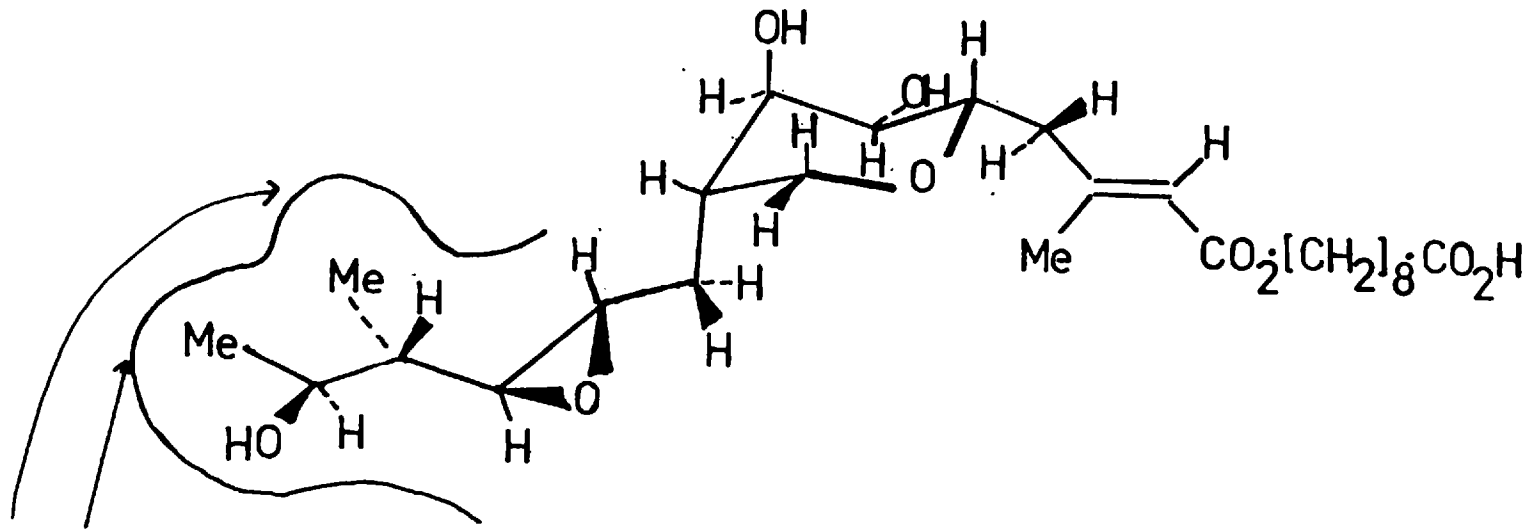
Previous work with isoleucyl-tRNA synthetase from E. coli MRE 600 (Flossdorf et al., 1976b) has shown that the amino group of L-isoleucine is absolutely essential for binding but the enzyme is fairly tolerant against replacement of the carboxyl group by more or less hydrophobic substituents. Pseudomonic acid has no amino group but does have hydrophobic

FIGURE 35. HYDROPHOBIC BINDING DOMAINS ON ISOLEUCYL-tRNA SYNTHETASE.

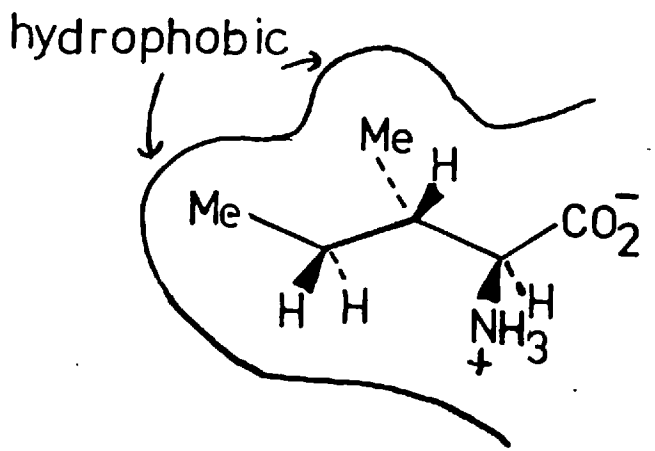
Illustration of hydrophobic binding domains on isoleucyl-tRNA synthetase accommodating a) terminus of epoxide side chain of pseudomonic acid and b) sliphatic side chain of L-isoleucine.

FIGURE 35

a)


OH
H
H
OH
H
H
H
H
O
H
H
H
H
H
Me
H
Me
H
HO
H
O
H
H
Me
CO2[CH2]8CO2H

b)


hydrophobic
Me
H
Me
CO2−
H
H
NH3+
H

components adjacent to the proposed isoleucine carbon skeleton. Flossdorf et al. (1976b) proposed that the carboxyl group specific attachment point is at least in part hydrophobic in nature. Since it appears to be widely accepted that aminoacyl adenylates are true intermediates of amino acid activation, this anhydride has to be protected against the nucleophilic attack of water, which is achieved most simply by a hydrophobic carboxyl binding site. The only alternative is that the intermediate is buried into the interior of the protein and consequently then is also shielded from the nucleophilic attack of the tRNA to be charged. As previously mentioned, pseudomonic acid also has a hydroxyl group in place of hydrogen (at C-13) (Fig. 31) in the proposed area of similarity with L-isoleucine. This again would conflict with the idea of the fully hydrophobic nature of the active site, as defined by Flossdorf et al. (1976b). It has been proposed (Holler et al., 1973) that the active site for isoleucine involved an ion pair that stabilised the binding of the amino and carboxyl groups. It was suggested that this ion pair opened on isoleucine binding. This was proposed to be the initiation of the conformation change which was observed to follow the attachment of L-isoleucine to the enzyme (Yarus & Berg, 1969; Holler et al., 1971; Holler & Calvin, 1972). The ion pair is also opened when ATP alone binds to its specific site. Once the open conformation has been stabilised in this way, some analogues of isoleucine were shown to bind more effectively to IRS (Holler et al., 1973). However, ATP did not increase the ratio of binding of pseudomonic acid to IRS, nor did it appear to affect its stability when subjected to electrophoresis. This suggested that if pseudomonic acid binds at the

isoleucine active site of IRS then it must also open the ion pair gate. This does not appear very likely, since methyl pseudomonate, which does not have a charged group, has a comparable inhibitory effect against IRS to the free acid (i.e. 64% versus 80% inhibition of aminoacylation of $tRNA^{Ile}$ at 100nM inhibitor concentration).

Previous work has shown that the antibiotic was relatively non-toxic to mammals (LD_{50} for rats = 5,000mg/kg, Sutherland et al., 1976). The effect of pseudomonic acid on mammalian IRS was therefore examined to establish whether the lack of toxicity in mammals was due to a pseudomonic acid resistant IRS. A crude preparation of IRS was made from rat liver and the steady state kinetics of yeast $tRNA^{Ile}$ aminoacylation was studied. Pseudomonic acid inhibition of the mammalian IRS was again found to be competitive with respect to isoleucine. The K_m, determined to be 5.4×10^{-6}M (pH 7.4; 37°C, with respect to isoleucine), was very similar to that of the IRS from E. coli B. The K_i (20×10^{-6}M) for the rat liver enzyme however, was considerably higher (8,000x) than that for the bacterial enzyme. As controls, the aminoacylation of three other yeast tRNAs were studied. Interestingly, the phenylalanyl-tRNA synthetase from this crude preparation of synthetases was also slightly inhibited by pseudomonic acid, as was also found with the bacterial enzyme. The antibiotic was without effect on the aminoacylation of yeast $tRNA^{Val}$ and $tRNA^{Leu}$. Thus, it appears that the lack of toxicity towards mammals is at least due in part to the increased resistance shown by the mammalian IRS. The extent to which reduced cellular penetration of the antibiotic contributes to lack of toxicity in mammals is not known.

One final piece of work is worthy of mention in relation to the interaction of pseudomonic acid with IRS. It was of interest to investigate how the producing organism, Pseudomonas fluorescens NCIB 10586, tolerates high levels of the antibiotic. Mellows & Soughton (unpublished work) have shown, using a crude aminoacyl-tRNA synthetasepreparation from this Pseudomonad, that again pseudomonic acid competitively inhibits the aminoacylation of E. coli $tRNA^{Ile}$ with respect to isoleucine. The K_i (14.5×10^{-3}M) (Fig. 37) was 1,600 fold higher than the K_m (9×10^{-6}M; pH 7.4; 37°C) (Fig. 36) and 6×10^6 fold higher than the K_i for the E. coli B IRS. Thus Pseudomonas fluorescens avoids suicide by producing an IRS which is highly resistant to pseudomonic acid. Again, the extent to which lack of penetration of the antibiotic into the cell contributes to the protection mechanism is unknown.

It has therefore been shown that a variation in sensitivity of isoleucyl-tRNA synthetases to pseudomonic acid exists in three synthetases isolated from different sources. This must reflect, to some extent, a divergence in the evolution of the isoleucyl-tRNA synthetases. Unfortunately, no complete amino acid sequence is yet available for any of the isoleucyl-tRNA synthetases. The sequence of the enzyme from E. coli MRE 600 is currently being determined (M.-R. Kula, Universität Regensburg und Gesellschaft für Biotechnologische Forschung mbH, Braunschweig-Stockheim, West Germany), but it will be 2-3 years before the full primary sequence will be available. It will be even longer before the detailed tertiary structure is known, due to the difficulty in obtaining crystals suitable for X-ray crystallographic studies. The original aim of this

FIGURE 36

Competitive inhibition of Pseudomonas fluorescens (NCIB 10586) IRS:aminoacylation kinetics (Lineweaver-Burke plot)

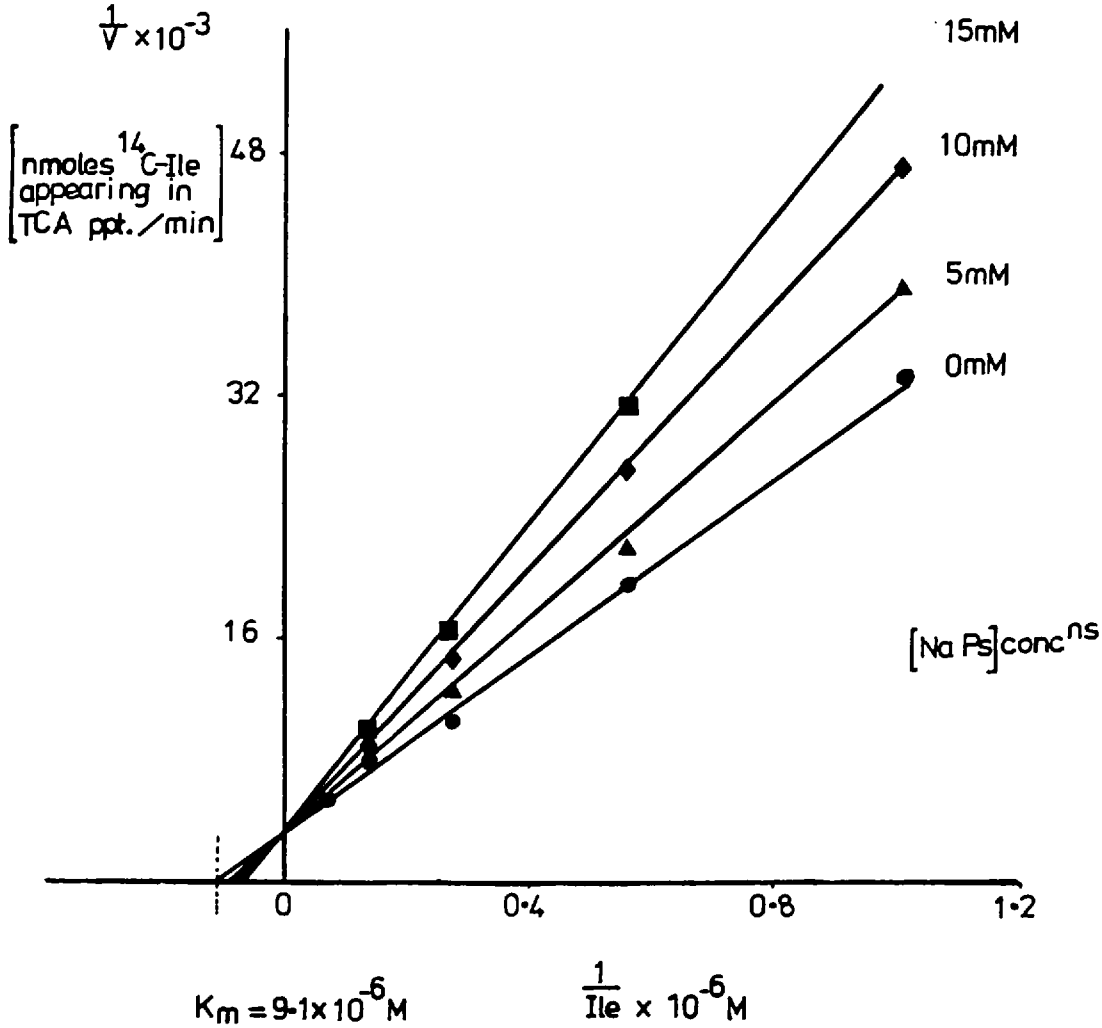
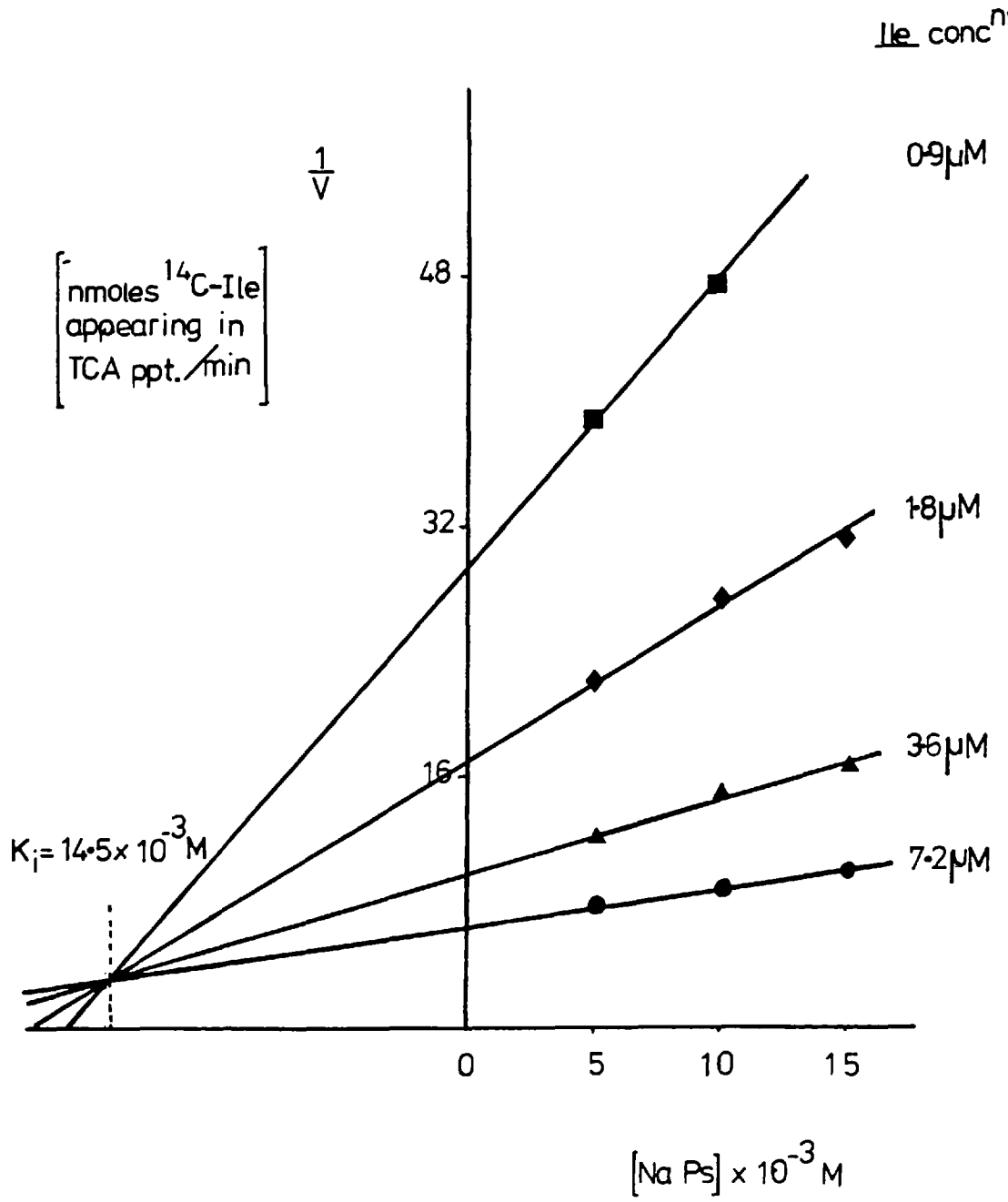


FIGURE 37

Dixon plot of inhibition of $tRNA^{Ile}$ aminacylation by sodium pseudomonate



project was to gain a detailed understanding at the molecular level on the mode of action of pseudomonic acid. Although it has been proved conclusively that the primary target of action of pseudomonic acid is IRS, the lack of structural information available for the enzyme has precluded further investigations into the molecular detail of the antibiotic-enzyme interaction. A comparison of the amino acid compositions of the enzymes from three strains of E. coli is shown in Table 5. Although the composition of the three enzymes is fairly similar, the enzyme from MRE 600 has only a molecular weight of 102,000, compared with 110,000 for the other two. Durekovic et al., (1973) have also shown that the B and MRE 600 strains have different N-terminal sequences. Variation in the binding sites for the substrates, isoleucine and ATP, would seem unlikely, but possibly the binding site for $tRNA^{Ile}$ may show some variation since different isoacceptor $tRNA^{Ile}$ species exist. It may be that evolutionary changes in the isoleucyl-tRNA synthetases reflect other roles this enzyme plays within the cell. It was speculated earlier that the binding site for pseudomonic acid could be complex. To a certain extent this is substantiated by the fact that all the isoleucyl-tRNA synthetases examined so far are inhibited to some extent by pseudomonic acid. Even the synthetase from the producer organism, upon which there would have been the greatest evolutionary pressure, to evolve a resistant enzyme, still retains some degree of sensitivity.

For future experiments, it has been shown, using a fluorescent reporter group (2-p-toluidinyl naphthalene-6-sulphonate) (Holler, 1971) which binds to IRS in a 1:1 ratio, that a decrease in fluorescence intensity at 443nm is associated

with the binding of isoleucine to the enzyme. This has been interpreted to mean that the conformational change that occurs on isoleucine binding involves at least one tryptophan side chain. It would be very interesting to see whether the same specific decrease in fluorescence was also brought about on pseudomonic acid binding to IRS. If this were shown to occur, then it would provide very good evidence to suggest that pseudomonic acid was binding at the active site. 2-p-Toluidinyl naphthalene-6-sulphonate has also been shown to bind specifically to the active site(s) of E. coli K12 L-phenylalanyl-tRNA synthetase. This contrasts with the binding of this compound to IRS which occurred at a site in close proximity to the isoleucine binding site. Further information on the nature of the molecular interaction of pseudomonic acid with IRS could also be acquired through an antibiotic structure-activity relationship study. Additionally, it may be possible to covalently bind (e.g by photolysis) radiolabelled pseudomonic acid, or a derivative, to the enzyme. After enzymic digestion and peptide purification, the peptide(s) containing the bound antibiotic could be structurally characterised. This would provide information on the amino acid residue(s) at or close to the isoleucine binding site.

One final comment, in conclusion, is that the specificity of pseudomonic acid for isoleucyl-tRNA synthetase could provide the molecular biologists with a useful 'tool' with which to investigate a number of protein synthesis and metabolic phenomena.

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