THE ROLE OF ELONGATION FACTOR - Tu IN BACTERIAL PROTEIN SYNTHESIS

by

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DECLARATION

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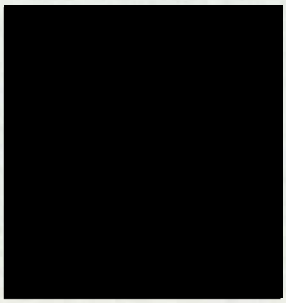


ABSTRACT

1. Elongation factor - Tu (EF-Tu) occurs in large quantities in bacterial cells: as much as 10% in wild-type E.coli, reducing to 4% in a slow-growing mutant strain, E.coli D2216, with a kirromycin-resistant EF-Tu. Isoelectric focussing suggests that of the two E.coli genes for EF-Tu, only one may be functioning in E.coli D2216. Evidence is presented which supports a membrane-association for some of the EF-Tu in the cell.

2. EF-Tu aids the tRNA-mediated translation of mRNA into proteins, through formation of the ternary complex EF-Tu.GTP.aminoacyl-tRNA, which binds to the ribosome.mRNA complex. There, after codon-anticodon interaction, GTP is hydrolyzed and EF-Tu.GDP leaves the ribosome to be recycled. The antibiotic kirromycin binds to EF-Tu in a 1:1 ratio inducing a similar GTPase activity in the elongation factor, which can be further stimulated by ribosomes and/or aminoacyl-tRNA.

3. In the absence of ribosomes and aminoacyl-tRNA, and above 400mM, monovalent cations stimulate the EF-Tu.kirromycin GTPase activity in the order $Li^+ > Na^+ > K^+ > NH_4^+ > Cs^+ > Me_4N^+$, concomitantly changing the K_m for GTP in this reaction. Below 400mM $[M^+]$ ribosomes and aminoacyl-tRNA stimulate this GTPase activity with a monovalent cation specificity: $NH_4^+ > K^+ > Li^+ = Cs^+ > Na^+ > Me_4N^+$, without influencing the K_m for GTP. Ribosomes can completely replace the absolute requirement for monovalent cations in stimulating the EF-Tu.kirromycin GTPase activity. In the physiological EF-Tu GTPase, small monovalent cations (Na^+ and Li^+) also stimulate the GTP hydrolysis at 30mM $[M^+]$, and concomitantly change the K_m for the reaction.

4. Free divalent cations are not absolutely required for EF-Tu. kirromycin GTPase activity, and at 200mM $[M^+]$ only stimulate in the additional presence of ribosomes. In the physiological EF-Tu GTPase increasing divalent cation concentration removes the requirement for mRNA of this reaction. For the kirromycin-induced EF-Tu GTPase activity there is a marked interdependence between pH and monovalent cation concentration in the absence of free Mg^{2+}, supporting the location of a highly charged anionic region close to the catalytic centre for GTP hydrolysis on EF-Tu.

5. EF-Tu interacts with the ribosome largely via the 50S subunit. Although proteins L7/L12 are involved in this interaction, they are dispensible for the expression of the EF-Tu.kirromycin GTPase in the presence of 30S subunits and aminoacyl-tRNA, together with a large number of other ribosomal proteins. These results suggest a complex interaction between EF-Tu and the ribosome, which is supported by other experiments using the antibiotic thiostrepton.

6. The results are discussed in relation to the physiological role of EF-Tu and it is considered that the elongation factor is critically involved in the recognition mechanism whereby miscoding is avoided.

LIST OF ABBREVIATIONS USED IN THE TEXT

1 A_{260} unit	is that quantity of a substance which in a volume of 1 ml and with a path length of 1 cm gives an absorbance at 260nm of 1.0
DMSO	dimethylsulfoxide
DOC	sodium deoxycholate
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis(2-amino-ethylether)N,N'-tetraacetic acid
EF-T	elongation factor - T (= EF-Tu.EF-Ts)
EF-Tu	elongation factor - Tu
EF-Ts	elongation factor - Ts
EF-G	elongation factor - G
IF-1	initiation factor - 1
IF-2	initiation factor - 2
IF-3	initiation factor - 3
K	1000 daltons (molecular weight)
K'	apparent equilibrium constant
K_m	Michaelis constant
M^+	monovalent cations
M^{2+}	divalent cations
Me_4N^+	tetramethylammonium ion
PMSF	phenylmethylsulfonylfluoride
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	non-specific transfer-RNA
$tRNA^{Phe}$	tRNA specifically accepting phenylalanine
$Phe\text{-}tRNA^{Phe}$	$tRNA^{Phe}$ charged with phenylalanine
$tRNA_f^{met}$	tRNA specifically accepting formylmethionine
$fmet\text{-}tRNA_f^{met}$	$tRNA_f^{met}$ charged with formylmethionine - the initiator tRNA
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS - polyacrylamide gel electrophoresis
S30	30000g supernatant
S100	100000g supernatant
A-site	represents the location of the aminoacyl-tRNA on the ribosome after correct codon-anticodon binding and before translocation. The aminoacyl-tRNA in the A-site does not react with puromycin.
P-site	represents the location on the ribosome of the peptidyl-tRNA after translocation and still with codon-anticodon binding. The peptidyl-tRNA in the P-site does react with puromycin.
R-site	precedes and possibly overlaps the A-site, and is the location of the aminoacyl-tRNA during codon-anticodon recognition. Its existence is still in some doubt.

CHAPTER 1. INTRODUCTION.

1.1 General Introduction.

Gene survival and reproduction are now realized to be the axial concept about which all living systems revolve, and upon which Darwinian selection ultimately acts. Though differing in complexity the way genes are expressed is essentially similar in all living systems. Genetic information in the form of DNA gains phenotypic expression via a complex sequence of enzymic reactions. The first step, transcription, consists of the accurate transliteration by RNA polymerase of DNA into messenger RNA (mRNA) molecules, containing nucleotide sequences corresponding to the final protein product, as well as regulatory sequences which are not translated (Fig.1). The subsequent step, translation of the mRNA, is effected by the mediation of highly specific tRNA molecules. These recognize nucleotide triplets in the mRNA, bind to them, and since each tRNA species is specific both for a particular nucleotide triplet (codon) and for a corresponding amino acid, allows the formation of polypeptide chains whose amino acid sequence accurately reflects the coded nucleotide sequence of the original gene. For this translation of mRNA by tRNA to take place in an ordered, efficient and regulated way, large nucleoprotein complexes, the ribosomes, and a variety of other proteins are necessary.

In bacteria, the translation of mRNA can be conveniently divided into three phases: initiation, elongation and termination (reviewed in 1,2,3), all of which require the participation of the ribosomes. Bacterial ribosomes sediment at 70S (total MW 2.7×10^6) and are composed of two subunits sedimenting at 50S (MW 1.8×10^6) and 30S (MW 0.9×10^6). Both subunits comprise structural RNA and a constant

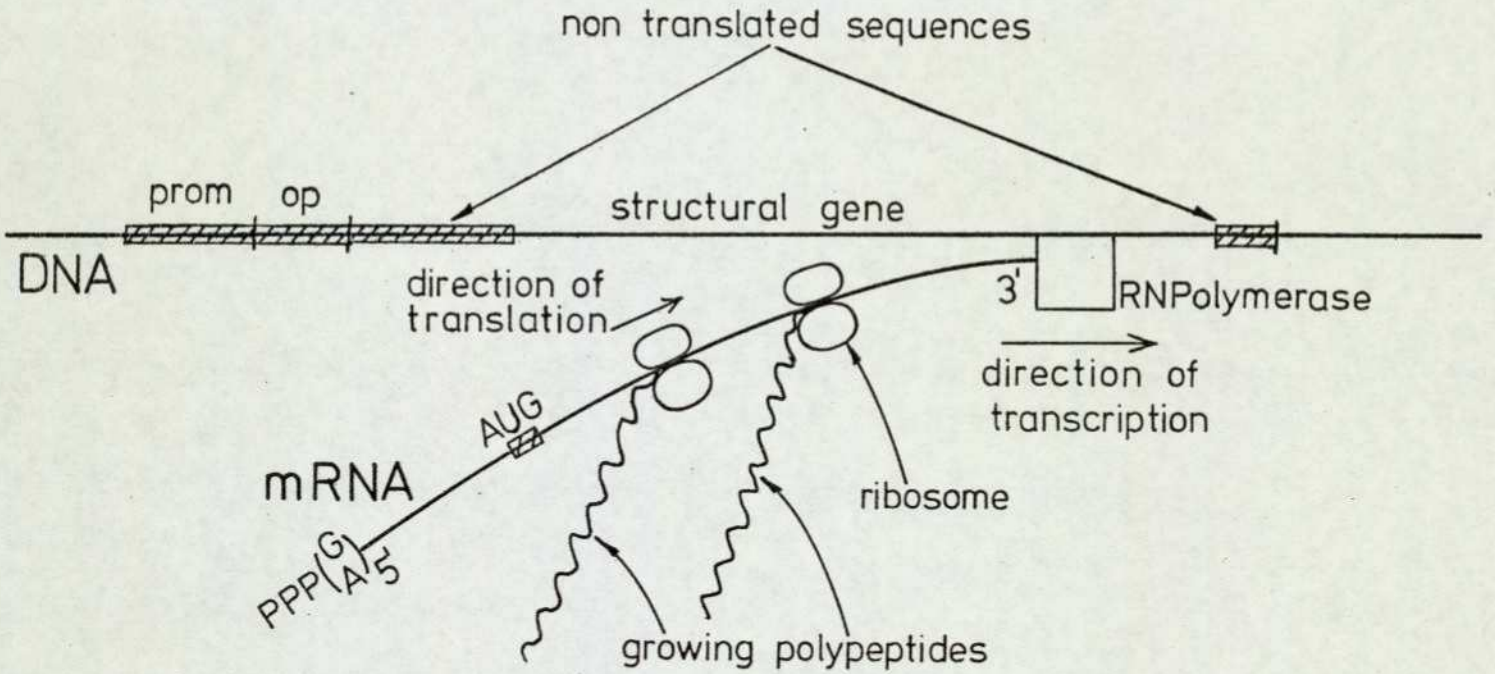
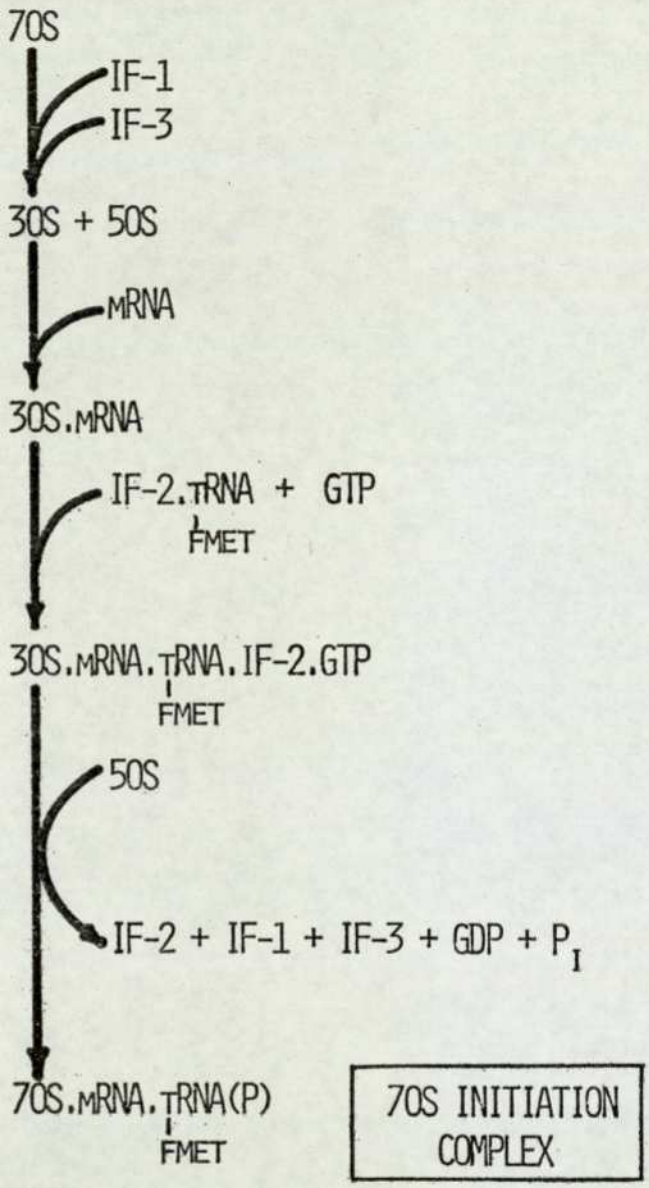


FIGURE 1 DIAGRAM TO ILLUSTRATE THE MECHANISM OF GENE EXPRESSION

(prom = promoter region; op = operator region; other terms explained in the text; based on the model of the lac operon)



INITIATION

FIGURE 2 INITIATION IN PROCARYOTES

(after Van der Hofstad (16))

number of proteins. Two extraribosomal proteins, the initiation factors IF-1 and IF-3, encourage the dissociation of the ribosome into its component subunits. The 30S subunit then binds one molecule of mRNA, close to the 5' terminus, possibly via interaction of a specific sequence of nucleotides (..GUCCA..) on the 16S ribosomal RNA of the small subunit with the nucleotides ..CAGGU.. on the mRNA (4), though other binding mechanisms are also likely. A third initiation factor, IF-2, forms a binary complex with formylmethionyl-tRNA$_f^{met}$ (fmet-tRNA$_f^{met}$) (5,6), which then binds to the 30S.mRNA complex, the anticodon of the tRNA specifically binding to the sequence AUG, the so-called initiator codon, of the mRNA, close to the 5' terminus. The 50S ribosomal subunit then joins the 30S, and, accompanying the hydrolysis of a molecule of GTP, proteins IF-1, IF-2 and IF-3 leave the ribosome, with fmet-tRNA$_f^{met}$ in the so-called ribosomal P-site (Fig.2).

The elongation cycle (Fig.3) repeats for every amino acid added to the growing polypeptide chain. The 70S initiation complex, just described, contains fmet-tRNA$_f^{met}$, or later polypeptidyl-tRNA, in the so-called ribosomal P-site. Aminoacylated tRNA (aa-tRNA) is then introduced to the ribosome as a ternary complex with elongation factor - Tu (EF-Tu) (MW 45000) and GTP, the anticodon of the appropriate aa-tRNA complementing the nucleotide triplet next to the initiator triplet or preceding codon, following the principles of the genetic code. After thus correct encoding of the aa-tRNA in the so-called ribosomal A-site, EF-Tu leaves the ribosome, accompanied by the hydrolysis of a further molecule of GTP. A peptide bond is then formed between the peptidyl chain and the newly imported amino acid, both of which now lie adjacent to one another in the peptidyl transferase centre located on the 50S subunit (7); the now longer peptidyl chain remains

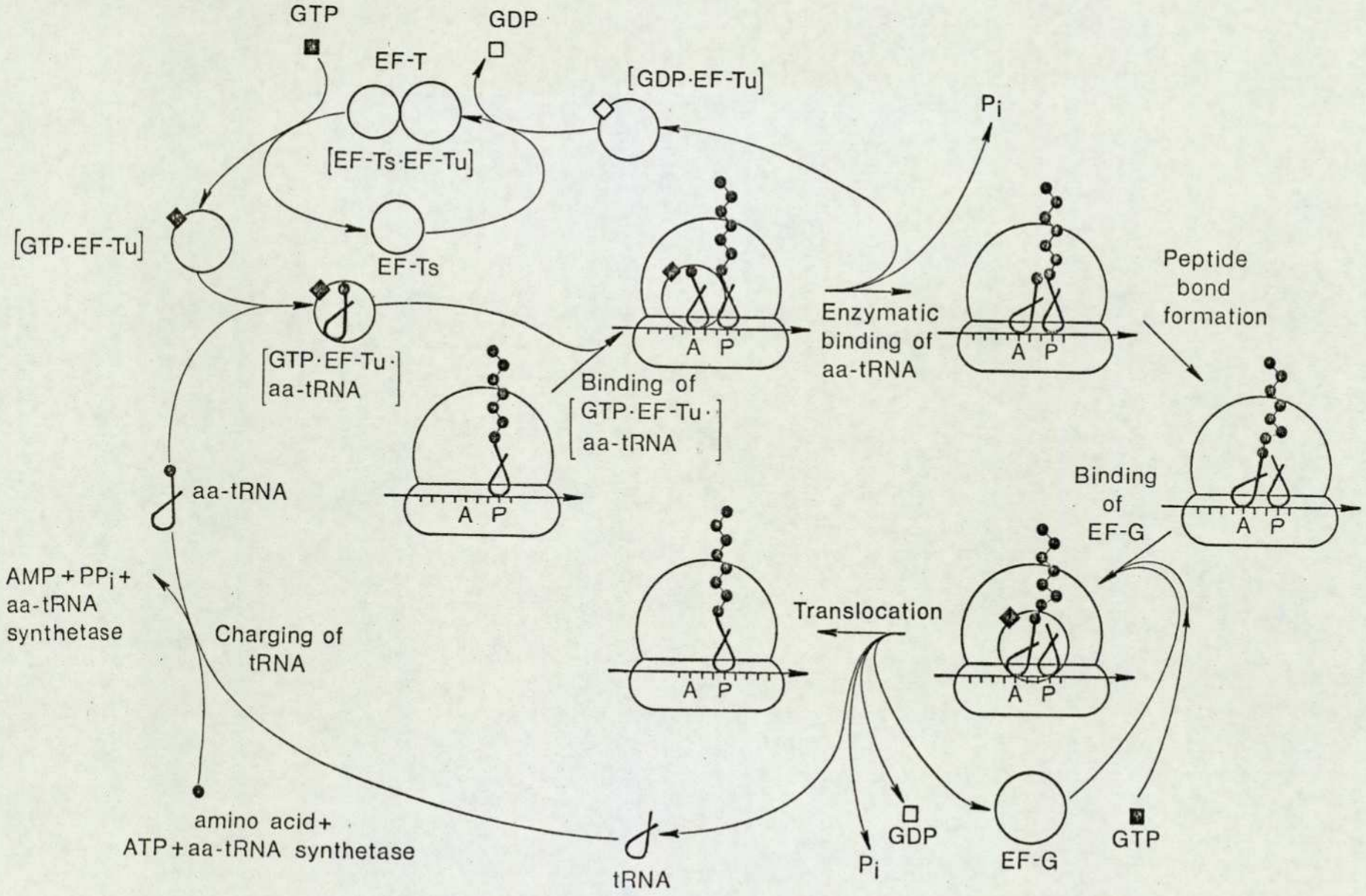


FIGURE 3 THE PROCARYOTIC ELONGATION CYCLE

(for explanation, see text; the arrow attached to the mRNA indicates the direction of movement of the messenger in respect of the ribosome)

attached to the tRNA in the A-site. A second elongation factor, EF-G (MW 84000), as the binary complex with GTP, then interacts with the ribosome; GTP is hydrolyzed to GDP, and the mRNA and the peptidyl-tRNA encoded in the A-site are translocated to the P-site of the ribosome, ousting the now uncharged tRNA. EF-G, GDP and this tRNA simultaneously leave the ribosome, which is now ready to repeat a further cycle of elongation (reviewed in 2,8).

The details of termination are less well known. On encountering the so-called "nonsense" mRNA codons (UAA, UAG or UGA) in the ribosomal A-site, for which there are normally no corresponding tRNA species, certain protein release factors succeed in disengaging the newly formed protein, tRNA, mRNA and ribosomes (3).

In vivo estimates of protein synthetic rates suggest that about 1000 amino acids are incorporated per ribosome per minute (9). In vitro translation of natural messenger RNA is estimated at 200-300 amino acids per ribosome per minute (10,11), though for the standard artificial translation system, poly(U)-directed polyphenylalanine synthesis, amino acid incorporation at the present time varies between 1 and 5 amino acids per ribosome per minute (12,13,personal data). The latter system, however, lacks a proper secondary conformation of the mRNA (14,15,16), and has no specific system for initiation, which may thus be a limiting step (12, see section 2.2.2), and conditions may yet be found which will give higher rates of synthesis. These systems all contain an excess of elongation factors; however, it has been shown (17) that elongation can occur in the poly(U)-directed system in the absence of elongation factors, and hence in the absence of GTP hydrolysis, though the rate of such elongation is at least 100 times slower than the factor-dependent poly(U)-directed polyphenylalanine synthesis (17).

There is sufficient energy contained in the amino acid - tRNA bond, which is formed via hydrolysis of ATP to AMP, to drive peptide bond formation and translocation of the mRNA, in the absence of GTP hydrolysis. The large difference in the velocity of the two systems suggests that GTP hydrolysis may serve to accelerate the elongation cycle, to increase the "power of the molecular machine" (17).

1.2 Molecular and biological properties of elongation factor - Tu.

Elongation factor - Tu was first isolated and crystallized as a binary complex with elongation factor - Ts, known as the transfer factor, EF-T (18,19). The current nomenclature derives from the observation that when the component proteins were separated, EF-Tu was found to be unstable to heating at 60°C, unlike EF-Ts which was relatively more stable. EF-Tu from E.coli has been shown to have a molecular weight of between 42000 and 48000 (20,21,22,23); here a molecular weight of 45000 is assumed. EF-Ts is somewhat smaller (MW 30000 approx.). The amino acid composition of EF-Tu has been determined (20,23), and indicates 3 cysteine residues, and of aromatic amino acids, 2-3 tryptophane residues, 8-10 tyrosine residues and 14-15 phenylalanine residues per molecule. To date, 50% of the amino acid primary sequence, the C-terminal end, has been published (24) and this has been used to indicate some homology with elongation factor - G (25). The publication of the complete sequence, obtained both directly and from DNA sequencing of the genes, is expected shortly.

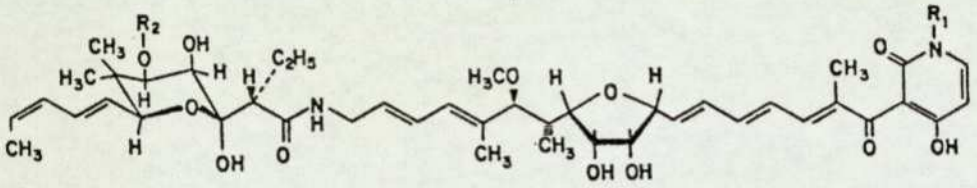
Because EF-Tu.GDP can be readily crystallized to form orthorhombic, bipyramidal, hexagonal or monoclinic crystals, it has also become an object for X-ray crystallographic studies. The first of these (26), of low resolution (6Å), used a trypsin-digested EF-Tu

molecule; consisting of a 39K fragment and a smaller 6K fragment, which would appear to remain in situ. Subsequently, a high resolution model (2.6Å) has appeared of EF-Tu.GDP (27), though the crystals are reported to contain EF-Tu fractured in a comparable way to the trypsin digests. Both models indicate a slightly elongated molecule, approximately 75Å x 50Å x 45Å, made up of three domains, one domain, the "head", separated from the rest of the molecule by a narrower "waist" region. The model of the Danish group (27) is able also to indicate the position of the GDP substrate, which lies close to the outer surface of the "head" domain.

Elongation factor - Tu, free in solution, has a much higher affinity for GDP than for GTP (K'_{GDP} = 1 x 10^{-9}M, K'_{GTP} = 5 x 10^{-7}M at 0°C) (28). On leaving the ribosome after each cycle of elongation, EF-Tu.GDP must interact with another protein elongation factor, EF-Ts. The latter appears to "open" the binding site for GDP (28,29), increasing both its rates of association and dissociation, raising the probability of access to GTP. Aminoacyl-tRNA only binds to EF-Tu.GTP, and in so doing lowers the equilibrium constant for GTP to 10^{-9}M, equivalent to that for GDP, thus locking the GTP into the nucleotide binding site on formation of the ternary complex EF-Tu.GTP.aa-tRNA (28). This complex then binds to the ribosome (enzymatic binding), both EF-Tu and aa-tRNA being involved in the interaction; if there is no proper codon-anticodon interaction, that is, if the tRNA does not complement the mRNA triplet in the A-site, then this quaternary complex formation is fully reversible. If, however, there is appropriate codon-anticodon binding, GTP is hydrolyzed. The affinity of aa-tRNA for EF-Tu, now as EF-Tu.GDP, is thereby drastically reduced, and EF-Tu.GDP leaves the ribosome. Thus a prime function of GTP hydrolysis is to make irreversible the elongation cycle, consequent

only on proper codon-anticodon interaction. Furthermore, EF-Tu.GTP will only bind to aminoacylated tRNA, and does not react with uncharged tRNA, evidently recognizing a particular conformation of the -CCA terminus of tRNA induced by amino acid binding (30). Thus it appears that EF-Tu - dependent binding of aminoacyl-tRNA and the accompanying GTP hydrolysis are playing a very important role in translation by increasing the velocity and efficiency of elongation.

In 1974 the antibiotic kirromycin (MW 794; Fig.4) was shown by the group of Parmeggiani to bind specifically to EF-Tu (31) and to inhibit protein synthesis by causing the elongation factor to be blocked on the ribosome, inhibiting peptide bond formation. In the presence of kirromycin, the affinities of aa-tRNA for EF-Tu and EF-Tu.GDP are raised (28), thus preventing EF-Tu.GDP from leaving the ribosome. mRNA.aa-tRNA complex, as would normally ensue from GTP hydrolysis. It was additionally shown to induce turnover GTPase activity in isolated EF-Tu (31,32,33). These results strongly suggested that the catalytic site for EF-Tu - dependent GTP hydrolysis was on the factor and not on the ribosome. Kirromycin has been shown to compete only with EF-Ts for binding to EF-Tu (32), suggesting an overlap in their binding sites. By contrast, no competition could be demonstrated between the antibiotic and either guanine nucleotides, ribosomes or aa-tRNA. Subsequent work has shown that the antibiotic functions like EF-Ts in "opening" the nucleotide binding site of EF-Tu (28,29), specifically increasing the rates of association and dissociation of GDP, but also that like aa-tRNA it encourages the formation of EF-Tu.GTP, thus establishing the basis for a turnover GTPase activity (28)(Fig.5). Such turnover GTPase activity can otherwise be demonstrated uncoupled from protein synthesis only in the presence of EF-Tu, EF-Ts, mRNA, aa-tRNA and ribosomes (34). In the



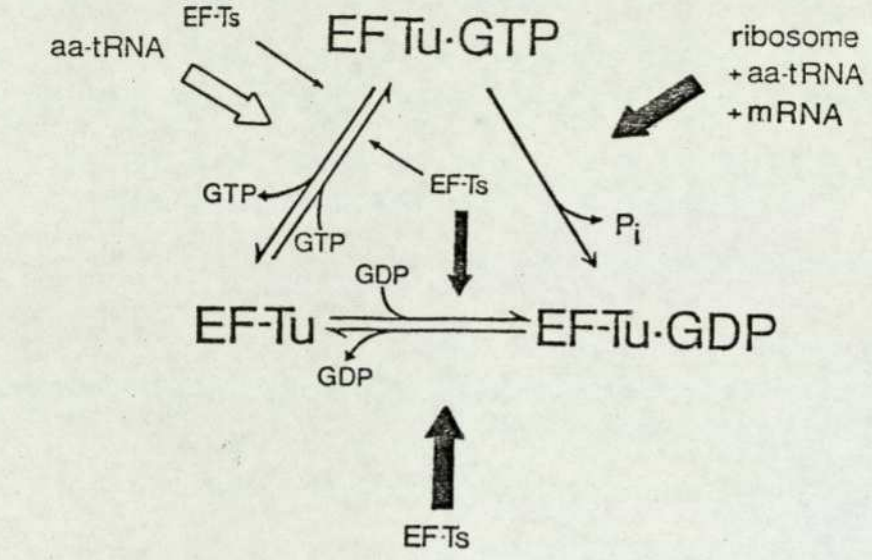
Kirromycin : R_1 = -H ; R_2 = -H ($C_{43}H_{60}O_{12}N_2$; MW 797)

Goldinomycin : R_1 = $-CH_3$; R_2 = -H ($C_{44}H_{62}O_{12}N_2$; MW 811)

Efrotomycin : R_1 = $-CH_3$; R_2 = the Disaccharide $C_{15}H_{27}O_8$ ($C_{59}H_{89}O_{20}N_2$; MW 1146)

FIGURE 4 THE KIRROMYCIN FAMILY OF ANTIBIOTICS

(Kirromycin is also known as mocimycin; goldinomycin as X-5108 or Aurodox; after Vazquez,D (215))



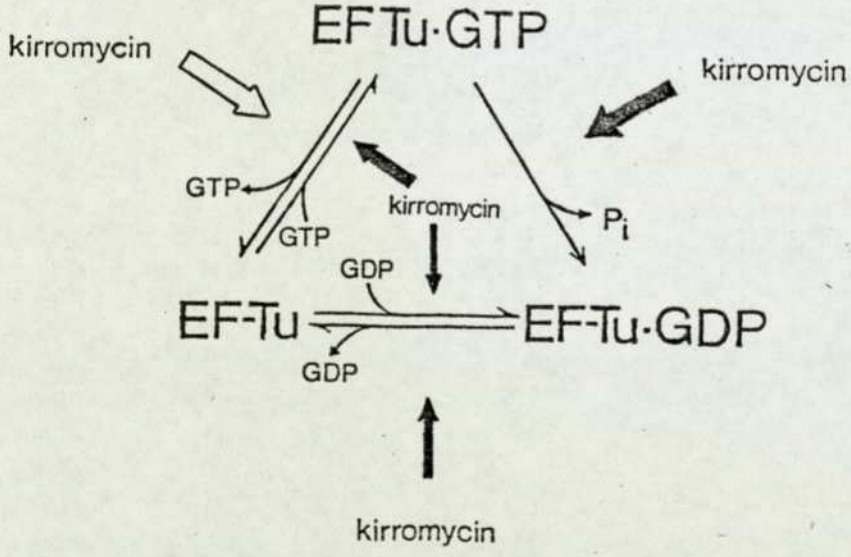


FIGURE 5 SCHEME TO COMPARE THE INFLUENCE OF PHYSIOLOGICAL EFFECTORS AND KIRROMYCIN ON THE EF-Tu: GUANINE NUCLEOTIDE INTERACTIONS.

(dark arrows: stimulation, open arrows: inhibition; the size of the arrows gives a measure of the effect; after Fasano et al. (28))

kirromycin-induced EF-Tu GTPase, no macromolecular effectors additional to EF-Tu are required, though aa-tRNA and/or ribosomes stimulate the activity (28). Further addition of mRNA to the ribosome / EF-Tu / aa-tRNA / kirromycin reaction is inhibitory after one round of GTP hydrolysis since the mRNA - aa-tRNA interaction prevents the ternary complex leaving the ribosome, which in this system would normally appear to accompany the turnover activity (32).

The findings that EF-Tu can be induced by kirromycin to show GTPase activity separately from the protein synthesis system, and that this activity can be modified by interaction of the factor with the other components of the translation system, provide an ideal opportunity to investigate in considerable detail the allosteric regulation of this GTPase activity, and hence also the regulatory role of EF-Tu in the protein synthesis system as a whole.

Monovalent cations are absolutely required for the expression of the EF-Tu.kirromycin GTPase activity; addition of ribosomes, however, eliminates this requirement. Starting from this finding, this GTPase assay system has been used to study the involvement of cations in the hydrolytic activity of EF-Tu and the influence upon this of interaction with the other macromolecular components. These results are then compared with the physiological EF-Tu GTPase system in the absence of the antibiotic. In the presence of kirromycin, small monovalent cations are directly involved in the site of catalysis via a highly charged anionic region. This region can be modulated by a divalent cation, usually Mg^{2+}. The conformation of the factor induced by monovalent cations can also be induced by the physiological effectors, ribosomes and aa-tRNA. Different cations, particularly Mg^{2+} and NH_4^+ or K^+, are involved in the interaction between the elongation factor and the

physiological effectors. The finding that these effectors can be functionally replaced by small cations implies that the effect of the ribosomes and aminoacyl-tRNA is mediated via specific local charges at the sites of interaction.

The ribosomes is an important allosteric effector of EF-Tu. In order to study this EF-Tu - ribosome interaction in greater depth, the ability of ribosomes to stimulate the kirromycin-induced EF-Tu GTPase was used as an assay system. Ribosomes consist of two subunits, 50S and 30S. The 50S subunit is a complex of two RNA molecules, sedimenting at 23S and 5S, together with 33 proteins (L1 - L33). With the exception of proteins L7 and L12, which are identical except that L12 possesses an acetylated NH_2- terminus, and of which altogether there are 4 copies, all the proteins exist in single copies, one each per ribosome. The 30S subunit has a single molecule of RNA, sedimenting at 16S, and 21 proteins (S1 - S21), again one copy of each protein per subunit. By careful treatment with CsCl it is possible to prepare ribosomal subunits lacking several ribosomal proteins (35,36). Using this technique, it was possible to estimate the importance of a number of ribosomal proteins in the EF-Tu - ribosome interaction. As an alternative approach, a specific site on the ribosome, in the neighbourhood of protein L11, may be blocked by the antibiotic thiostrepton (37). Using a variety of conditions known to alter the EF-Tu - ribosome interaction, the influence of thiostrepton was tested on the ability of ribosomes to stimulate EF-Tu GTPase activity, and from these results the relationship between EF-Tu and the thiostrepton binding site could be determined. Both approaches have shown that the binding of EF-Tu to the ribosome is complex, involving a number of different ribosomal proteins, and that this interaction is modified by the presence of aminoacyl-tRNA and by certain cations.

The importance of EF-Tu in the bacterial cell is emphasized by the very large quantities in which it is present: as much as 10% of the total soluble protein (38, section 2.3.2), making it certainly one of the most common proteins in wild type E.coli strains. This means that in vivo there are approximately 10 molecules of EF-Tu for every ribosome. By contrast, the other elongation factors, EF-Ts and EF-G, occur at approximately 1:1 ratios with ribosomes (34). One explanation for this large quantity of EF-Tu suggests that all tRNA present in the cell should be in the form of ternary complex, EF-Tu.GTP.aa-tRNA, to maximize the efficiency of translation (38). Recent reports, however, are suggesting that EF-Tu may have other roles in the bacterial cell. It may have structural properties, possibly analogous to actin or tubulin (39,40), since under certain conditions EF-Tu has been shown to form filaments at concentrations below those reported in vivo. EF-Tu has also been shown to comprise one of the four subunits of the enzyme Qβ replicase (41); EF-Ts and ribosomal protein S1 are two of the remaining subunits. Here it appears to serve a function quite dissimilar from its usual role in protein synthesis, since EF-Tu and EF-Ts can be covalently bound together and yet remain fully functional in this enzyme (42). EF-Tu has been found associated with the bacterial membrane (43,44, section 2.3.4), and has been implicated in the regulation of ribosomal RNA synthesis (45). To account for the large quantity of EF-Tu, it has been shown that, unlike the other elongation factors, EF-Tu demonstrates the very rare property for bacteria of being coded on the Escherichia coli genome by two genes (46), at 72 min (tufA) and 88 min (tufB). In wild-type strains under optimal growth conditions these are expressed in the ratio of 3:1 respectively (47). The gene tufB appears to be regulated by its own promoter (48) even though it occurs in a cistronic region with several

functionally related ribosomal proteins (e.g. L7/L12, L10 and L11). To date no notable differences have been found to distinguish the two gene products, though the DNA sequences currently being determined in various laboratories will clarify this.

Thanks to the action of kirromycin, it has been possible to raise mutant strains of E.coli (49,50) or Bacillus subtilis (51) with altered EF-Tu, resistant to the antibiotic. Because non-resistant EF-Tu is blocked by kirromycin on the ribosomes, and in vivo EF-Tu is in large excess over ribosomes, kirromycin-sensitivity must be a dominant trait (49); thus for kirromycin-resistance to be expressed, either both tuf genes must be mutated, or one gene or its product is inactivated. The presence of a single normal tuf gene product would clearly block the ribosomes, making the cells non-viable. This has been confirmed in a series of tufA mutants produced by the group of Bosch, where it appears that the tufB gene product is inactive (50,52). Bidimensional electrophoresis / isoelectric focussing of lysates of one of these mutants indicate two discrete spots corresponding to EF-Tu, presumably one of which is inactive (52).

As part of a study to investigate the functions and regulation of EF-Tu in vivo, a series of quantitative estimates were made to determine the total quantity of EF-Tu present in various strains including an EF-Tu mutant, E.coli D2216, and a recombinant E.coli strain in which at least 6 copies of tufB were present (53), and its intracellular distribution. Results indicate that the total content of EF-Tu in the cell is regulated not as a function of the number of gene copies, but of growth rate, implying end-product feedback control probably at the transcriptional level. Both pure and crude extracts of EF-Tu from mutant strain D2216 were analysed by isoelectric focussing

and shown to be inseparable from wild-type EF-Tu, with identical isoelectric points, contrasting therefore with another EF-Tu mutant strain HAK 88 (54), in which two EF-Tu species could be separated by isoelectric focussing, as well as with the mutant of Bosch (52). Work is currently in progress to characterize, from an enzymological point of view, the pure mutant EF-Tu from D2216, and to use it as a tool to learn more about the regulating mechanisms of this aspect of the elongation cycle.

1.3 Aims of the investigation.

1. To determine the mechanism of action of the catalytic centre for GTP hydrolysis on EF-Tu, and its allosteric control.
2. To investigate the role of monovalent and divalent cations in the EF-Tu GTPase activity.
3. To study in depth the mechanism of action of the antibiotic kirromycin, and to relate its function to physiological events.
4. To investigate the interaction between EF-Tu and the ribosome and the role of the EF-Tu GTPase activity in the elongation cycle.
5. To characterize from a quantitative and qualitative point of view the EF-Tu from the mutant E.coli strain D2216, and to compare this with wild-type strains.

CHAPTER 2: QUANTITATIVE AND QUALITATIVE ASPECTS OF ELONGATION FACTOR - Tu IN *ESCHERICHIA COLI* STRAINS.

2.1 Introduction.

Elongation factor - Tu is a multifunctional protein, whose principal role appears to be to aid the efficient tRNA-mediated translation of mRNA into polypeptide chains. Of all the proteins involved in gene expression systems, EF-Tu is the only one so far known in *E.coli* to be coded for by two apparently similar genes, located in quite different transcription groups (46). In *Bacillus subtilis* there would appear to be only a single gene coding for EF-Tu (51). As part of a study on *in vivo* aspects of EF-Tu expression, estimates were made of the total quantity of the elongation factor, its distribution and functional character in several *E.coli* strains, including an EF-Tu mutant, resistant to the antibiotic kirromycin (D2216)(49), which for reasons of dominance is believed to have only one functional *tuf* gene, and a recombinant strain, containing between 6 and 8 cloned *tufB* genes (53,55).

2.2 Materials & Methods.

Except where otherwise stated all materials used were of analytical reagent grade.

2.2.1 Nucleotides.

ATP, GTP and GDP were obtained from Boehringer (Mannheim) as the Lithium salts. These were converted to K^+ salts by passing over an AG-50 (Bio-Rad) ion-exchange column (36), previously equilibrated with

KCl. ^{3}H.GDP was from Amersham, U.K., and its purity was periodically checked by thin layer chromatography on POLYGRAM CEL 300 (PEI UV_{254}) plates, developed at room temperature with a buffer of 0.75M KH_2PO_4, pH 3.5 .

2.2.2 Poly(uridylic acid).

Commercial grade poly(uridylic acid) (poly(U)) from Sigma Biochemicals was run on a column of Sephadex G-200 (60cm x 1cm), previously washed with 0.1M NaCl and equilibrated against the elution buffer, at room temperature, eluting with a solution of 10mM Tris.HCl pH 7.8 in 1% sodium dodecyl sulphate (SDS) (56). Fractions were collected as indicated in Figure 6. The poly(U) was concentrated by precipitation with two volumes of absolute ethanol at -15°C for 1 hr. The precipitate was centrifuged at 10000 rpm for 15 min, washed once in ethanol, lyophilized and redissolved in an appropriate quantity of distilled water. Such poly(U) was stable for at least several months, stored frozen at -20°C. The first fractions of the peak contained so-called "long chain" poly(U), which has been shown to contain between 150 and 200 nucleotides per chain, as judged by optical measurements of polysome formation (57). Using the fractions thus obtained in poly(U)-directed polyphenylalanine synthesis showed that the longer fractions could improve rates of polypeptide synthesis by up to 4-fold, by comparison with the commercial grade poly(U) (Fig.6). These results strongly suggest that termination or initiation, and not elongation, are the limiting steps in this artificial protein synthesis system.

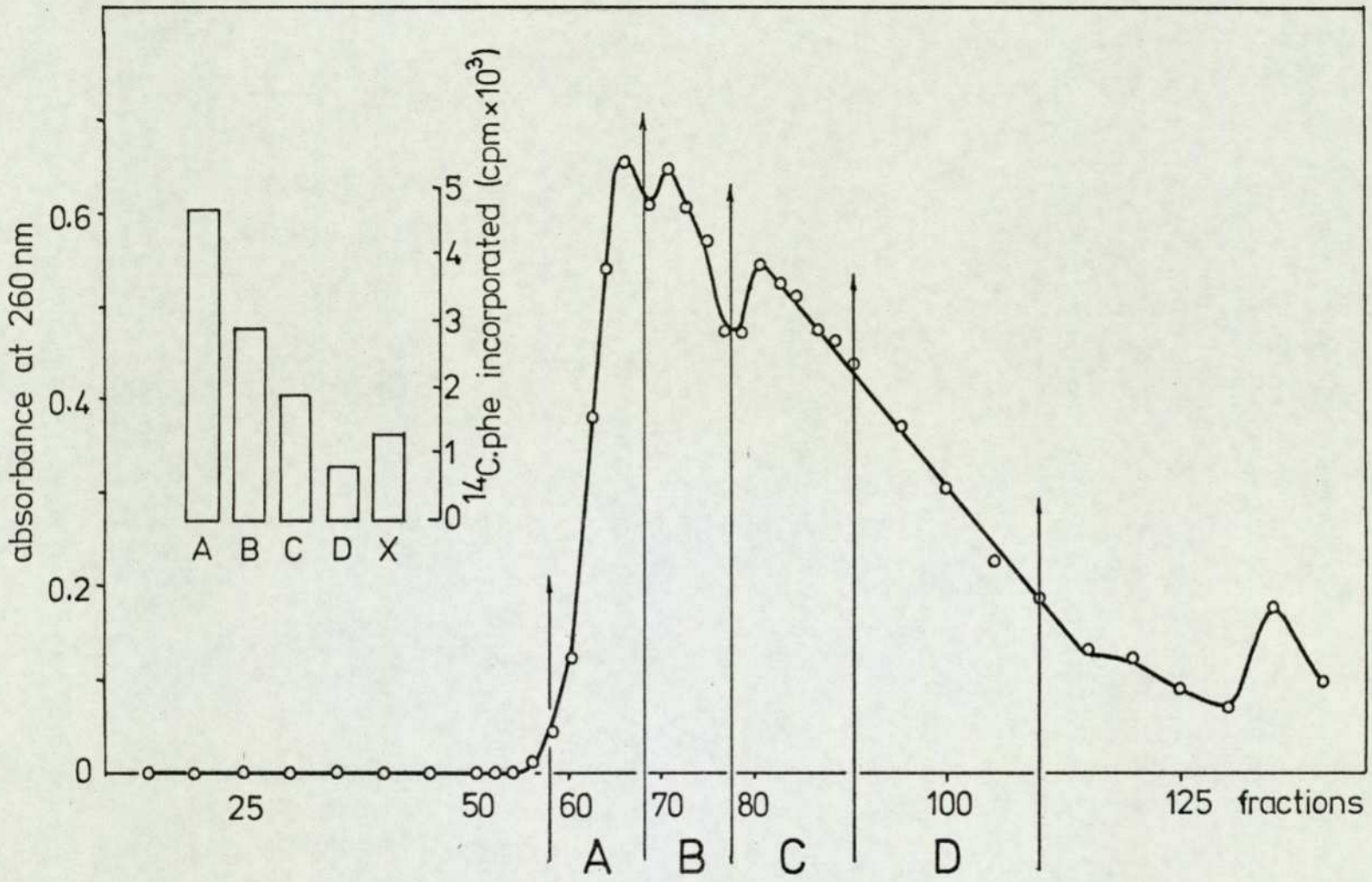


FIGURE 6 PREPARATION OF "LONG CHAIN" POLY(U).
Elution profile of a Sephadex G-200 column, as described in the text. Inset: peptidization activity of the fractions A,B,C and D from the elution profile; X is commercial poly(U) used as a control. Assay conditions as in section 2.2.7, except that specific activity of phenylalanine was 200cpm/pmol, incubation time was 15min. and the reaction mix contained 20pmol EF-Tu and 20pmol ribosomes in a total of 75µl.

2.2.3 Elongation factor - Tu

Electrophoretically homogeneous EF-Tu for analytical purposes was isolated from E.coli strains $BT2^r$, A19 or MRE600, using modifications of described procedures (32,58). A 100000g supernatant was prepared from approximately 300g of cell paste, disrupted by grinding with alumina in a Retsch mill (Retsch, West Germany) in buffer A (150mM KCl, 10mM $MgCl_2$, 20mM Tris.HCl pH 7.8, 7mM mercaptoethanol and 25 mg/l phenylmethylsulfonylfluoride (PMSF)). This was concentrated by precipitation with 70% saturated $(NH_4)_2SO_4$ and the pellet obtained was redissolved in, and dialysed against buffer A plus 10μM GDP, and chromatographed on a DEAE Sephadex A-50 column (5cm x 60cm) eluting with 2 x 2l of a KCl gradient of 150 to 350mM in buffer A plus 10μM GDP. Fractions of about 10ml were collected and assayed for EF-Tu activity by the ability to bind ^{3}H.GDP (see section 2.2.6), for elongation factor - G (EF-G) by GTPase activity in the presence of ribosomes (59) and for EF-Ts by its ability to stimulate EF-Tu.GDP + ^{3}H.GDP exchange at 0°C (28) (Fig.7).

The fractions containing EF-Tu free from EF-Ts were gathered and concentrated using Aquacide II (Calbiochem), and the enzyme solution brought to 40% $(NH_4)_2SO_4$ saturation. The precipitate was extracted twice with 5ml of 35% saturated $(NH_4)_2SO_4$ in 25mM Tris.HCl pH 7.8, 5mM $MgCl_2$, 1mM dithiothreitol, 0.05mM GDP (buffer B), and both supernatants discarded. Extraction of the residual precipitate was repeated twice with 3ml of 25% saturated $(NH_4)_2SO_4$ in the same buffer. Saturated $(NH_4)_2SO_4$ solution was then very slowly added to the combined extracts, raising the percentage $(NH_4)_2SO_4$ saturation to 35% over several days. Crystallization of EF-Tu.GDP started at about 29% $(NH_4)_2SO_4$ saturation. Crystals were harvested and the extraction and crystallization procedure

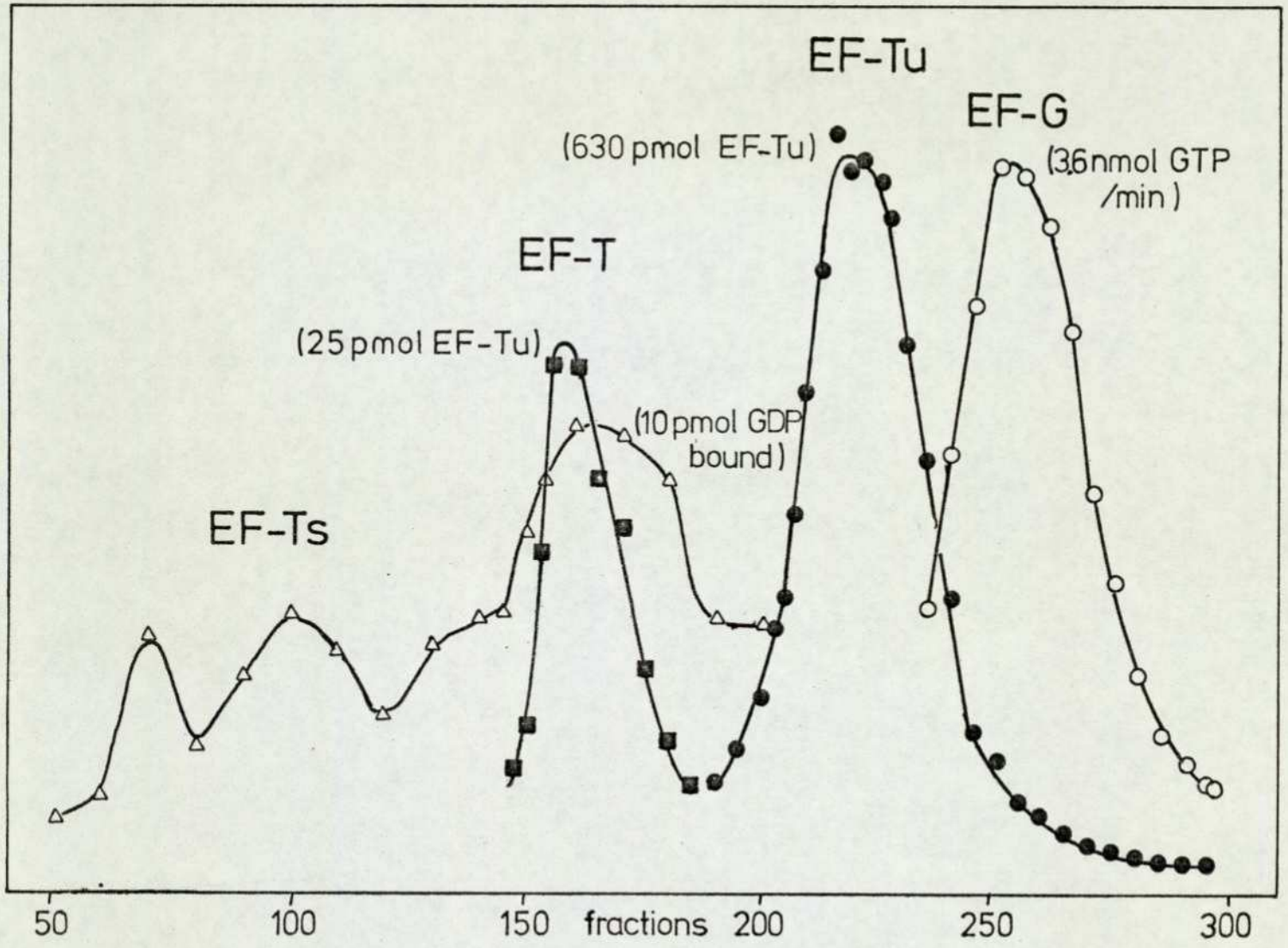


FIGURE 7 PURIFICATION OF EF-Tu.
Elution profile of preparative DEAE Sephadex A-50 column. (Peak assay activities are indicated in brackets. EF-Ts (Δ) was assayed in the presence of 10pmol EF-Tu; EF-Tu (●,■) was assayed as in section 2.2.6; EF-G (o) was measured as GTPase activity in the presence of 50pmol of ribosomes. All data refer to 10μl of original fractions; for further details, see text).

repeated a second time. The crystals were stored in 35% saturated $(NH_4)_2SO_4$ in buffer B at 4°C, and remained stable for at least several months, in that time showing no reduction in biological activity.

The remaining fractions of the DEAE Sephadex A-50 eluate were concentrated against Aquacide II, and stored in 50% glycerol at -20°C for later purification.

2.2.4 Ribosomes.

Ribosomes were obtained from the 30000g supernatant of alumina extracts of E.coli strains $BT2^r$, A19 or MRE600. The 30000g supernatant was centrifuged at 100000g for 5 hr to give a 100000g supernatant and a ribosome-rich pellet. This pellet was redissolved in buffer C (10mM $MgCl_2$, 60mM NH_4Cl, 20mM Tris.HCl pH 7.8) and centrifuged in a Spinco 60Ti rotor at 55000 rpm for 6 hr through a 12ml sucrose cushion (18%) (36), after twice washing in a buffer containing 0.5M NH_4Cl, 10mM $MgCl_2$, 20mM Tris.HCl pH 7.8 (Buffer D). Ribosomes were stored at -20°C in buffer C containing also 50% glycerol. Such NH_4Cl-washed ribosomes had high activities in poly(U)-directed polyphenylalanine synthesis, very low elongation factor - independent GTPase activity and comprised at least 80% "tight couples" (60) as assayed by analytical sucrose density gradient centrifugation at 5mM $MgCl_2$ (see also Fig.32). The concentration of the ribosomes in solution was calculated from their absorbance at 260nm (1 A_{260} unit = 25pmol 70S ribosomes).

Purified EF-G, EF-Ts and phenylalanyl-tRNA synthetase were generous gifts from [redacted].

2.2.5 Cell preparation.

All measurements of the quantity and distribution of elongation factor - Tu were made using freshly grown cultures of E.coli. The strains used were A19, MRE600, D2216, an EF-Tu mutant (49), D22, the immediate parental strain of D2216, and a recombinant strain of C600 containing cloned multiple copies of tufB genes, the vector pSF2124 having been used to convey the clone AB80 (53). This recombinant was a generous gift of [redacted].

Cultures were grown in tryptone/NaCl media (13g tryptone (Oxoid), 7g NaCl per litre of deionized water) at 37°C from stock innoculla stored in 50% glycerol at -20°C. Cell growth was monitored by measuring the absorbance at 420nm after 10:1 dilution of a small aliquot in 0.9% NaCl. Except where indicated all cells were harvested in mid-log phase by pouring onto crushed frozen buffer E (3mM $MgCl_2$, 10mM Tris.HCl pH 7.8, 60mM NH_4Cl) at -75°C (500g frozen buffer per litre of culture), and centrifuged in a Beckman JA10 rotor at 9000 rpm for 1hr at 2°C. The pellets were gathered and washed once in the same buffer. The yield was noted. Fresh cells were then resuspended (10ml buffer per g of cells), using a Potter-Elvehjem homogenizer, in either a high salt buffer F (750mM NH_4Cl, 3mM $MgCl_2$, 50mM Tris.HCl pH 7.5, 25 mg/l PMSF, 0.5mM dithiothreitol, 10μM GDP) or a low salt buffer G (as buffer F but 750mM NH_4Cl is replaced by 150mM KCl). This suspension was then sonicated for 3 x 30 seconds (MSE Ultrasonic Disintegrator, 150 watts), with at least 1 min intervals in ice between sonication bouts. Alternative methods of cell rupture had been tested, including reduced times of sonication and the use of osmotic shock treatment prior to, or instead of sonication. The procedure for osmotic shock was modified after standard methods (61,62). 2g of freshly harvested and

washed cells were gently suspended in 25ml of buffer H (33mM Tris.HCl pH 7.8) at 25°C for 10 min. Then, 25ml of buffer I (33mM Tris.HCl pH 7.8, 0.1mM EDTA, 60% sucrose (Schwartz, ribonuclease-free)) were added and the suspension left for a further 30 min at 25°C with constant shaking. After centrifugation at 10000 rpm for 30 min, the supernatant was discarded and the cells resuspended in 50ml of buffer J (0.5mM $MgCl_2$) at 0°C, and shaken for 10 min. at 0°C. However, 3 x 30 seconds sonication alone was found to yield maximum total protein and maximum EF-Tu yields. This cell lysate was then centrifuged at 30000g for 4 hr at 2°C to produce a 30000g supernatant (S30) and pellet. Where high salt buffer F was used the S30 was subsequently dialysed overnight against the low salt buffer G. The pellet was resuspended using the Potter-Elvehjem homogenizer in low salt buffer G plus 0.1% sodium deoxycholate (DOC) and recentrifuged at 30000g for 4 hr at 2°C to give the pellet wash (PW). Where indicated the S30 supernatant was recentrifuged at 100000g for 5 hr at 2°C to yield an S100 supernatant.

2.2.6 EF-Tu.GDP - ^{3}H.GDP exchange assay.

Because EF-Tu has a very high affinity for GDP ($K_d' = 5.5 \times 10^{-9}$M at 30°C (28)), and no other bacterial enzymes are considered to bind GDP to a significant extent under the chosen conditions (44), the EF-Tu.GDP - ^{3}H.GDP exchange assay of Miller & Weissbach (63) was used to estimate the quantity of EF-Tu present in an extract, collecting the EF-Tu.^{3}H.GDP on nitrocellulose filters (Sartorius SM 11306). Enzyme preparations were diluted in the low salt buffer G; 10µl of these dilutions were then incubated with 40µl of an incubation mix containing 27pmol ^{3}H.GDP (specific activity 6500 cpm/pmol), 522pmol GDP, 0.9µg EF-Ts, 7mM $MgCl_2$, 50mM Tris.HCl pH 7.8, 60mM NH_4Cl, for 5 min at 30°C. At the

end of this time 40µl aliquots were pipetted onto nitrocellulose membrane filters, previously soaked for at least 30 min in buffer K (7mM $MgCl_2$, 50mM Tris.HCl pH 7.8, 60mM NH_4Cl), and washed twice with 3ml of the same cold (0°C) buffer. After drying, the radioactivity on the filters was counted in toluene containing 0.5% diphenyloxazol, using an Intertechnique SL4000 liquid scintillation counter. Several different dilutions were made for each extract, with duplicate points for each enzyme concentration. Total EF-Tu content was then determined from the slope of the resultant straight-line plot of protein content versus ^{3}H.GDP bound. The assay system was calibrated using electrophoretically pure crystalline EF-Tu (see section 2.2.3), diluted to various concentrations in low salt buffer G.

2.2.7 Peptidization assay for EF-Tu.

Poly(U)-directed polyphenylalanine synthesis was carried out in conditions limited only by the absence of EF-Tu. In an incubation mix of 75µl were 7mM $MgCl_2$, 35mM KCl, 80mM Tris.HCl pH 7.8, 2.7mM phosphoenolpyruvate, 1µg pyruvate kinase, 20mM mercaptoethanol, 17nmol GTP, 33nmol ATP, 1.7µg "long chain" poly(U) (see section 2.2.2), 140pmol ^{14}C.phenylalanine (Amersham; specific activity ca. 525 cpm/pmol), 1.24nmol phenylalanine, 0.4µg phenylalanyl-tRNA synthetase, 100µg tRNA (Schwartz), 30pmol EF-Ts, 25pmol EF-G and 50pmol ribosomes. After incubation for 15 min at 30°C, the polyphenylalanine produced was precipitated onto paper filters (Schleicher & Schüll) in cold 10% trichloracetic acid, which were then washed successively in hot (100°C) 5% trichloracetic acid, cold 5% trichloracetic acid, 1:1 ethanol/diethyl ether and ether, and then dried. Radioactivity on the filters was counted in 5ml toluene plus 0.5% diphenyloxazol. This assay system

gave a sigmoid plot when calibrated using pure crystalline EF-Tu in the range 0-20pmol EF-Tu.

2.2.8 DEAE Sephadex A-50 chromatography of the 30000g supernatants.

As a control to check the identity of the GDP-binding enzyme, assumed to be EF-Tu, small columns (0.8cm x 20cm) of DEAE Sephadex A-50 were prepared and aliquots of the 30000g supernatants were run, eluting with 2 x 80ml of a KCl gradient of 150-500mM in a buffer system also containing 3mM $MgCl_2$, 50mM Tris.HCl pH 7.5, 25 mg/l PMSF, 0.5mM dithiothreitol and 10μM GDP (buffer L), collecting 1ml fractions. The eluate was assayed both by EF-Tu.GDP - ^{3}H.GDP exchange and by the peptidization assay described above.

2.2.9 Protein concentration.

Total protein concentration was determined by the standard procedure of Lowry et al. (64), using bovine serum albumin (Sigma Biochemicals) as calibration standard.

2.2.10 Intracellular distribution of EF-Tu.

To give a qualitative indication of the intracellular distribution of EF-Tu in the bacterial cell, 1.5ml aliquots of cell lysate from E.coli MRE600, prepared as above (section 2.2.5), using various sonication times, with or without osmotic shock treatment, and containing the equivalent of 60mg fresh weight of cells, were layered onto discontinuous sucrose gradients, containing in buffer L (150mM KCl) 2 x 4ml of 2-28% sucrose overlaying a 3ml cushion of 56% sucrose (65). Membrane fragments accumulate in the heavy sucrose cushion. The sucrose density gradients were centrifuged in a Beckman SW-40 rotor at 27000 rpm for 75 min and at 2°C (65). Fractions were

collected using an ISCO gradient fractionator and Gilson Microcol TDC 80 collector, and assayed for EF-Tu by the EF-Tu.GDP - ^{3}H.GDP exchange assay.

2.2.11 Isoelectric focussing of lysates and pure enzymes.

Isoelectric focussing of cell lysates and pure enzymes was carried out as described by O'Farrell (66). Freshly harvested and washed cells were resuspended in sonication buffer L (150mM KCl) and sonicated for 3 x 20 seconds. DNase and RNase I were then added each to 50 µg/ml and allowed to stand for 5 min at 0°C. Solid urea was then added to bring the final concentration to 9M urea. At room temperature this lysate was then mixed with an equal volume of lysis buffer M (9.5M urea, 2% w/v NP-40 (Shell Chemicals), 1.6% Ampholines (pH range 5-7), 0.4% Ampholines (pH range 3.5-10) (Ampholines supplied by LKB) and 5% mercaptoethanol). Between 5 and 25µl of sample prepared in this way were layered onto first dimension gels made exactly as described by O'Farrell (66). Crystalline EF-Tu in 35% saturated $(NH_4)_2SO_4$ buffer (see section 2.2.3) was diluted in lysis buffer M and dialysed for 6 hr against a buffer containing 9.5M urea, 2% NP-40 and 5% mercaptoethanol (buffer N) to reduce the salt content of the sample which, in controls not shown, markedly influenced the apparent isoelectric point of the final bands. The first dimension gels were run as described (66) for 14 hr at 400 volts followed by 3 hr at 500 volts. Where necessary, these first dimension gels were then stained in 0.1% Coomassie Blue in 50% trichloracetic acid for 15 min and then destained in successive changes of 15% acetic acid / 10% ethanol. For two-dimensional gels, the method of O'Farrell (66) was followed using running gels of 7.5% acrylamide / 0.1% SDS (47). These gels were developed at 20mA constant current for

5-6 hr, stained for 3 hr in 0.125% Coomassie Blue / 50% ethanol / 5% acetic acid, and destained in 15% acetic acid / 10% ethanol. Stained first dimensions were scanned using an ISCO gel scanner fitted with 620nm filters. The pH gradient in the first dimension gels was estimated by cutting a gel, run without sample, immediately after electrofocussing, into 5mm lengths. These gel pieces were placed individually in 0.5ml of quartz distilled water in Parafilm-sealed tubes and shaken overnight. The following morning the pH of the aqueous extract was measured using a standard pH meter (Radiometer, Copenhagen). The resulting pH profile corresponded precisely with the one published by O'Farrell (66) for the same gel/ampholyte system. It should be noted that these pH values, measured in H_2O, are approximately 0.2-0.3 pH units lower than those measured in 9.5M urea (66), the concentration in the gels during isoelectric focussing.

2.3 Results.

2.3.1 Growth rates of various E.coli strains.

Table 1 indicates the growth rates of the strains studied, as doubling times in tryptone / NaCl media, during logarithmic growth, based on measurements of absorbance at 420nm.

The wild-type strains all have a rapid growth, doubling approximately every 35 min. The parental strain D22 is considerably slower, as expected for a strain chosen for the permeability of its membrane to the antibiotic kirromycin (49). The kirromycin-resistant mutant D2216 is slower still; this may reflect not only the known mutation of the elongation factor, but also other mutations incurred by the treatment with the mutagen nitrosoguanidine (49). The

recombinant strain C600pSF2124AB80 showed an intermediate growth rate.

TABLE I GROWTH RATES OF VARIOUS E.COLI STRAINS.

strain	doubling time (hr: mean ± s.d.)
wild-type MRE600, $BT2^r$, A19	0.63 ± 0.08
parent D22	1.33 ± 0.15
mutant D2216	2.84 ± 0.29
C600pSF2124AB80	1.65

2.3.2 Quantities of EF-Tu in various strains assayed by EF-Tu.GDP - ^{3}H.GDP exchange.

In the presence of EF-Ts complete equilibrium is attained in the EF-Tu.GDP - ^{3}H.GDP exchange reaction after only 30 seconds under the chosen conditions (Fig.9). Assays were thus always carried out for 5 min at 30°C in the presence of EF-Ts, though at this temperature the latter is not absolutely necessary (28). Although EF-Tu binds GDP stoichiometrically in a ratio of 1:1, the assays were also externally calibrated against pure crystalline EF-Tu, to cross-check the correction necessary due to radiolysis of the ^{3}H.GDP (Fig.8). Variation in the y-axis is largely due to errors inherent in the protein determination by the Lowry procedure, or in the pipetting of small volumes of the crystal suspension of EF-Tu.

The total quantities of EF-Tu in the various strains were estimated for the combined 30000g supernatant (S30) and pellet wash (PW).

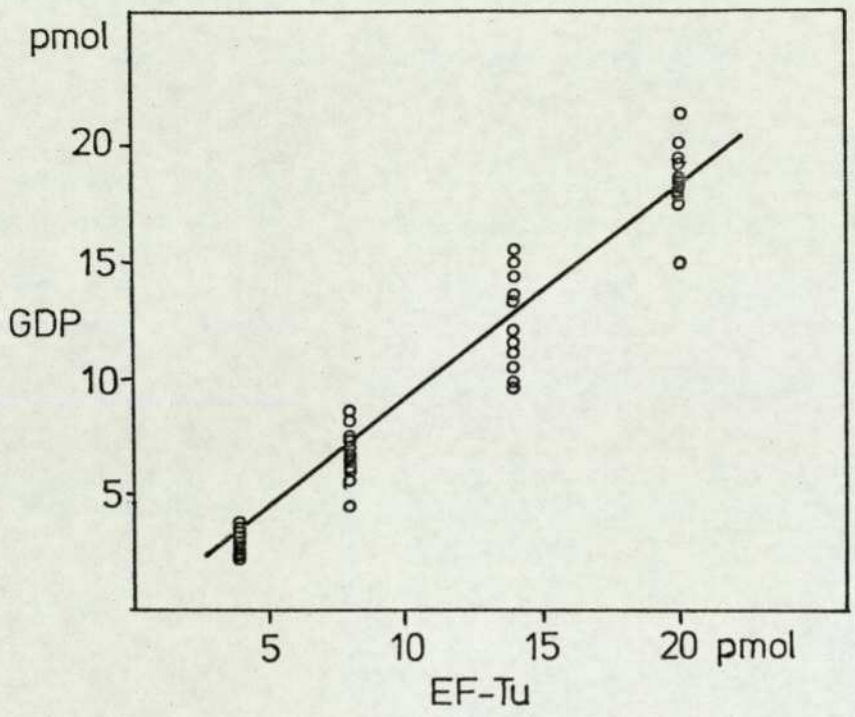


FIGURE 8 CALIBRATION GRAPH FOR THE EF-Tu.GDP - ^{3}H.GDP EXCHANGE ASSAY (see section 2.2.6)

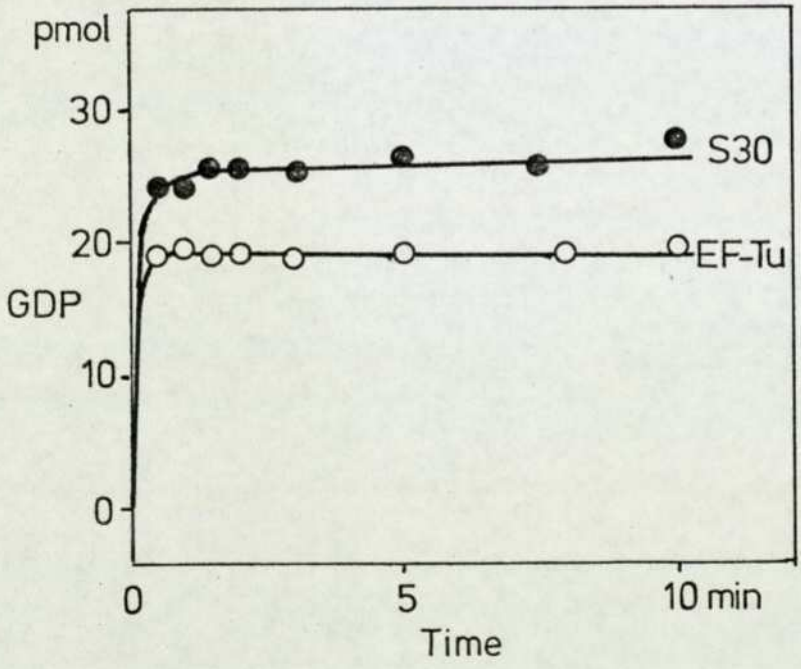


FIGURE 9 KINETIC OF THE EF-Tu.GDP - ^{3}H.GDP EXCHANGE ASSAY AT 30°C

Either 20pmol pure crystalline EF-Tu (o) or 10µl of a wild-type 30000g supernatant (•) were used.

TABLE II QUANTITIES OF EF-Tu AND TOTAL PROTEIN IN VARIOUS STRAINS

	D2216	D22	MRE600 $BT2^r$, A19	C600 pSF2124AB80
total Lowry protein (mg) in S30 + PW per g fresh wt of cells	64.6±13.1	79.3±23.8	80.8±24.0	86.0
nmol GDP bound per mg Lowry protein in the S30 + PW	1.04±0.40	1.66±0.26	2.24±0.19	1.06
total nmol EF-Tu present in S30 + PW per g fresh wt of cells	67	132	181	91
percentage of total protein as EF-Tu in the S30 + PW	4.3%	6.9%	9.3%	4.4%

N.B. This table includes two data obtained for stationary phase cells since these did not differ significantly from values for logarithmically growing cells.

In controls not shown, the presence of small quantities of sodium deoxycholate in the pellet wash did not affect the assay system. The results are tabulated in Table II. Wild-type strains contain approximately 9% of their total soluble protein as EF-Tu, whereas the mutant strain D2216 contains only 4.3%.

2.3.3 DEAE Sephadex A-50 chromatography of the 30000g supernatants.

To confirm the identity of the ^{3}H.GDP-binding protein with EF-Tu, columns of DEAE Sephadex A-50 were run eluting with a gradient buffer of 150-500mM KCl, in the presence of 10μM GDP. In these conditions all EF-T (EF-Tu.EF-Ts) should be separated into EF-Tu.GDP

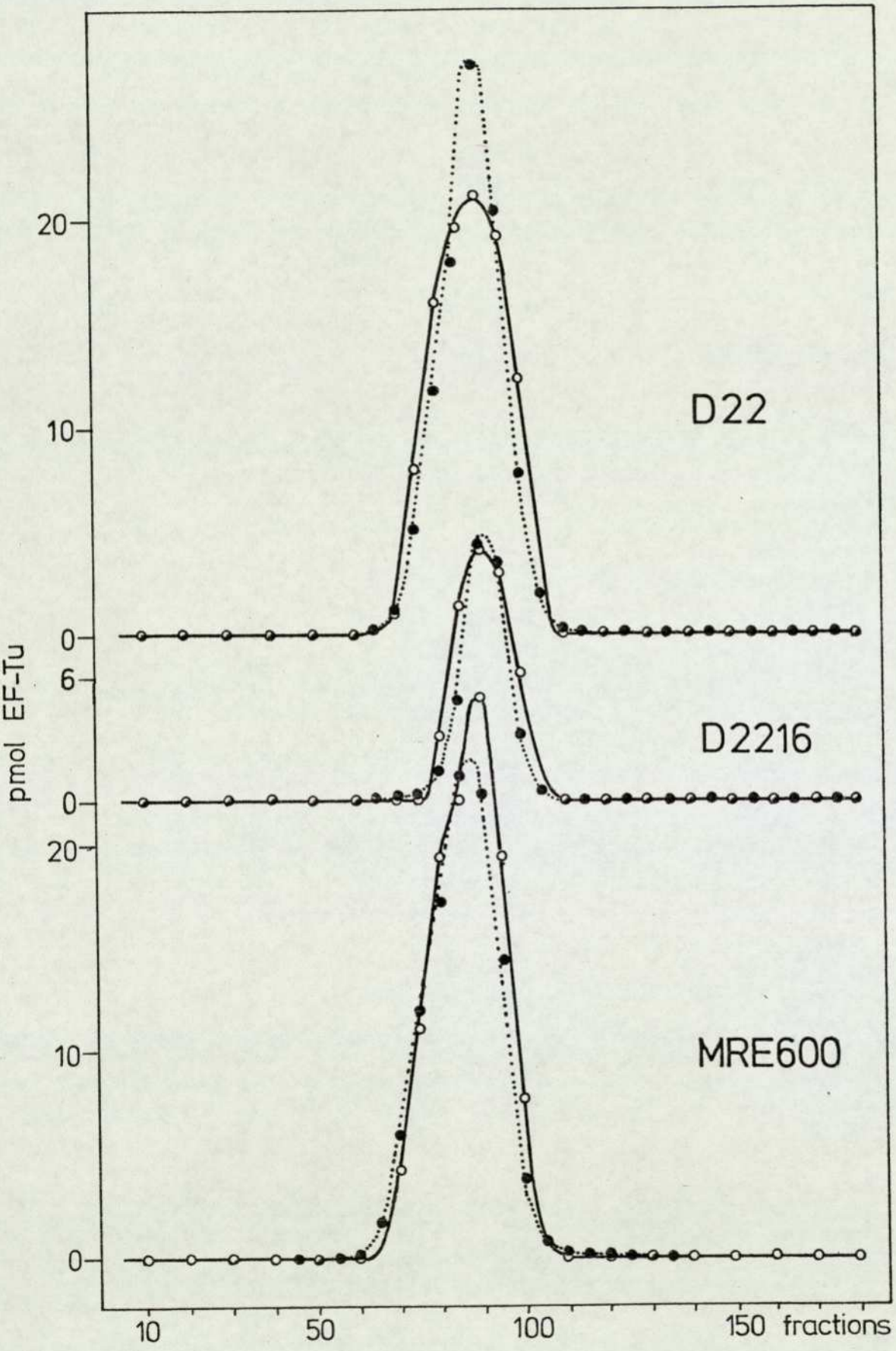


FIGURE 10 EF-Tu ACTIVITY IN THE ELUTION PROFILES OF SMALL DEAE SEPHADEX A-50 COLUMNS, FOR 30000g SUPERNATANTS OF E.coli STRAINS D22, D2216 AND MRE600.
(o) assayed by EF-Tu.GDP - ^{3}H.GDP exchange; (●) assayed by EF-Tu - limited poly(U)-directed polyphenylalanine synthesis.

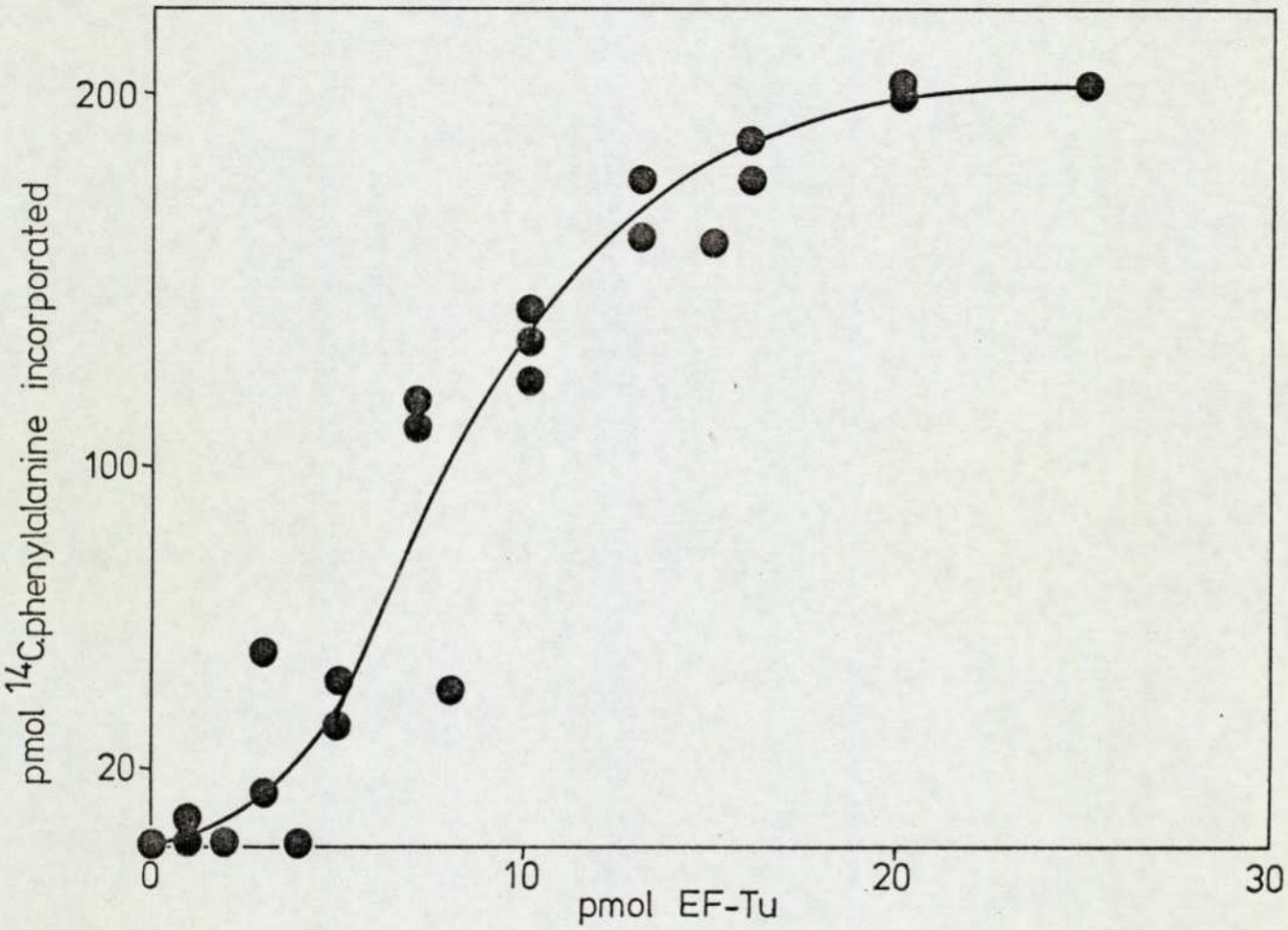


FIGURE 11 CALIBRATION CURVE FOR THE EF-Tu - LIMITED POLY(U) - DIRECTED POLYPHENYLALANINE SYNTHESIS ASSAY SYSTEM (see section 2.2.7)

and EF-Ts (28). All the GDP-binding material emerged as a single peak at about 200mM KCl, in all three strains tested (Fig.10). This peak was also assayed quantitatively by a poly(U)-directed polyphenylalanine synthesis system in which EF-Tu alone was limiting (section 2.2.7). This system was previously calibrated using pure crystalline EF-Tu to give a sigmoid calibration curve (Fig.11). This figure includes data from several independent assays. A comparison of the quantities assayed by this system with those estimated by the EF-Tu.GDP - ^{3}H.GDP exchange assay system indicates that the GDP-binding enzyme was chromatographically homogeneous and identical to EF-Tu, within the limits of experimental error imposed by the assay systems, and therefore supports the use of the GDP-binding assay on crude as well as purified extracts to give reliable estimates of total EF-Tu content.

2.3.4 Intracellular distribution of EF-Tu.

Fig.12 illustrates the distribution of GDP-binding enzyme, that is, EF-Tu, within variously treated MRE600 lysates. The lowermost curve (open circles) is a control indicating the activity of unlysed cells, many of which will have pelleted beyond the sucrose cushion. Since the vertical scale is logarithmic, the figure illustrates that unlysed cells account for at the very most 10% of the GDP-binding activity associated with the cushion material, which must thus comprise EF-Tu bound to membrane, or similar large fragments, as has been elsewhere suggested (43,44). Osmotic shock alone gives relatively low yields, and appears to have little influence when used together with sonication either on the total yield of EF-Tu or in its distribution. Unfortunately, since no estimate could be made of the number of cells

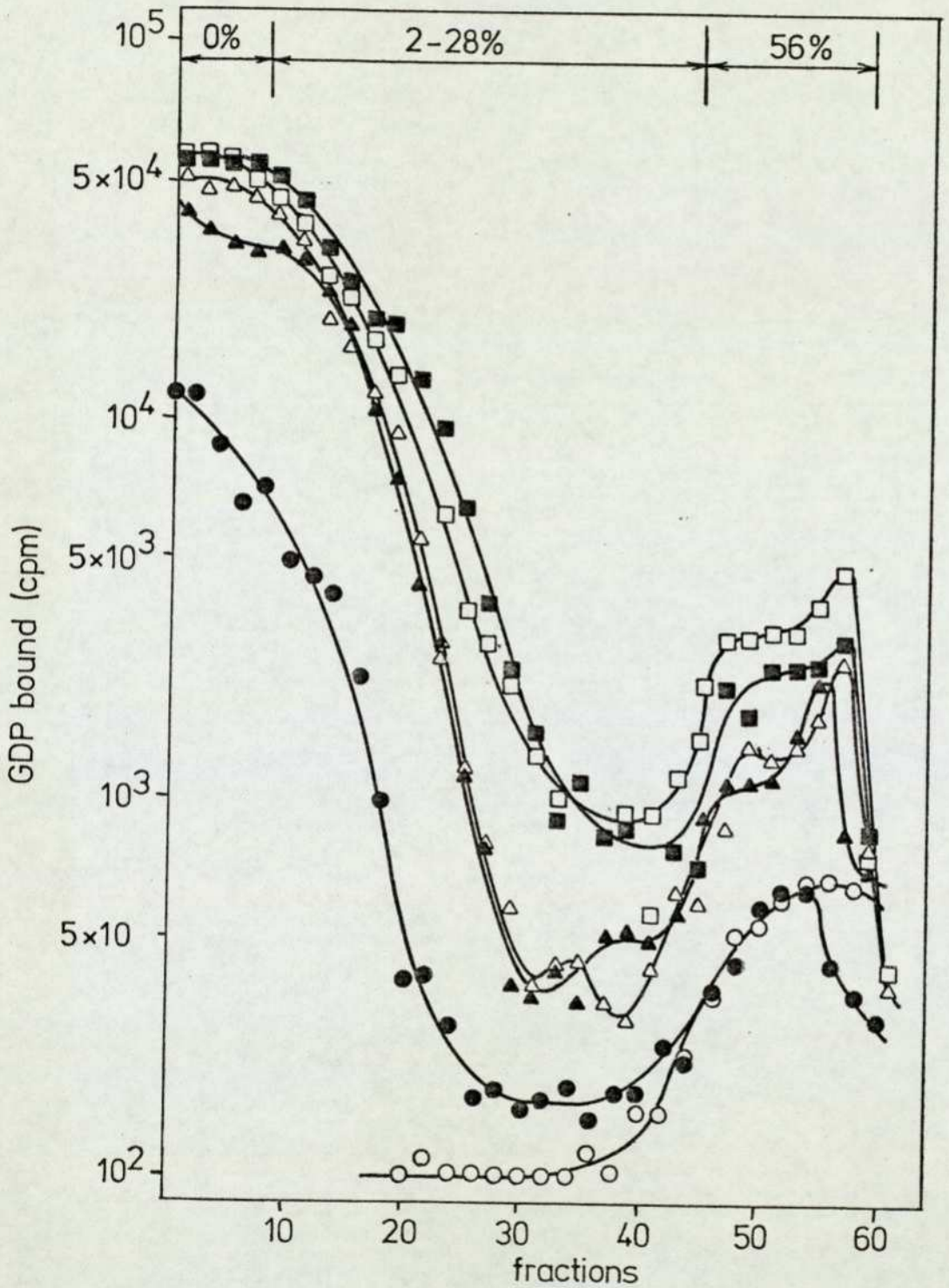


FIGURE 12 EF-Tu ACTIVITY IN SUCROSE DENSITY GRADIENTS OF VARIOUSLY TREATED E.coli MRE600 LYSATES.

(o) not lysed; (●) osmotic shock only; (Δ,▲) 2x10 seconds sonication; (□,■) 3x30 seconds sonication, with (▲,■) or without (Δ,□) prior osmotic shock. Assayed as in section 2.2.6.

remaining intact after lysis, nor how much large material passed through the 56% cushion, these results must be considered only as qualitative evidence for a membrane-associated role for EF-Tu.

Support for these results is provided in Table III, where it can be seen that, independently of the strain, the pellet wash contains more than a quarter of the total GDP-binding protein, and together with the 30000g supernatant accounts for nearly 90% of the cells' total EF-Tu content.

TABLE III PROPORTIONS OF EF-Tu PRESENT IN VARIOUS FRACTIONS OF CELL PREPARATIONS.

Lysate	100%
S30	59.4 ± 4.6 %
PW	29.2 ± 7.5 %
S100	49.5 ± 1.7 %

N.B. Little between-strain differences were found, so data from all strains tested have been pooled.

2.3.5 Isoelectric focussing of cell lysates and pure EF-Tu.

Two-dimensional electrophoresis / isoelectric focussing, made according to the method of O'Farrell (66) (Fig.13), clearly shows only a single spot corresponding to EF-Tu, both in wild-type (MRE600) and the kirromycin-resistant strains. Addition of pure crystalline, wild-type EF-Tu to the lysate samples (Fig.13C) shows that this mutant spot is isoelectrically and electrophoretically indistinguishable from wild-type EF-Tu. This is even more clearly shown in the first dimension

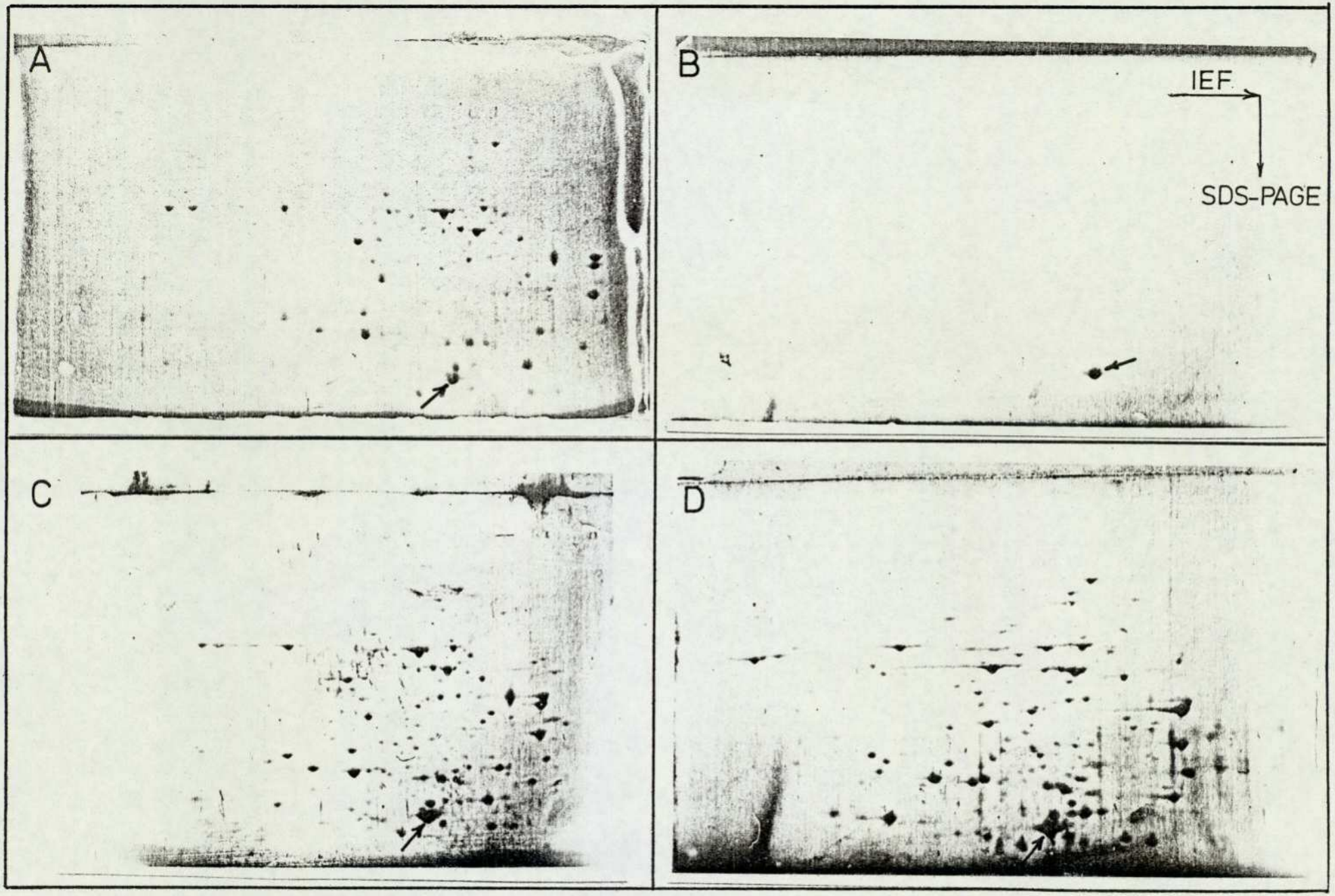


FIGURE 13 BIDIMENSIONAL ELECTROPHORESIS OF CELL LYSATES OR PURE EF-Tu.
Horizontal dimension by isoelectric focussing; vertical dimension by SDS-PAGE. A. D2216 lysate; B. pure crystalline wild-type EF-Tu; C. D2216 lysate + pure crystalline wild-type EF-Tu; D. MRE600 lysate.(arrow = EF-Tu)

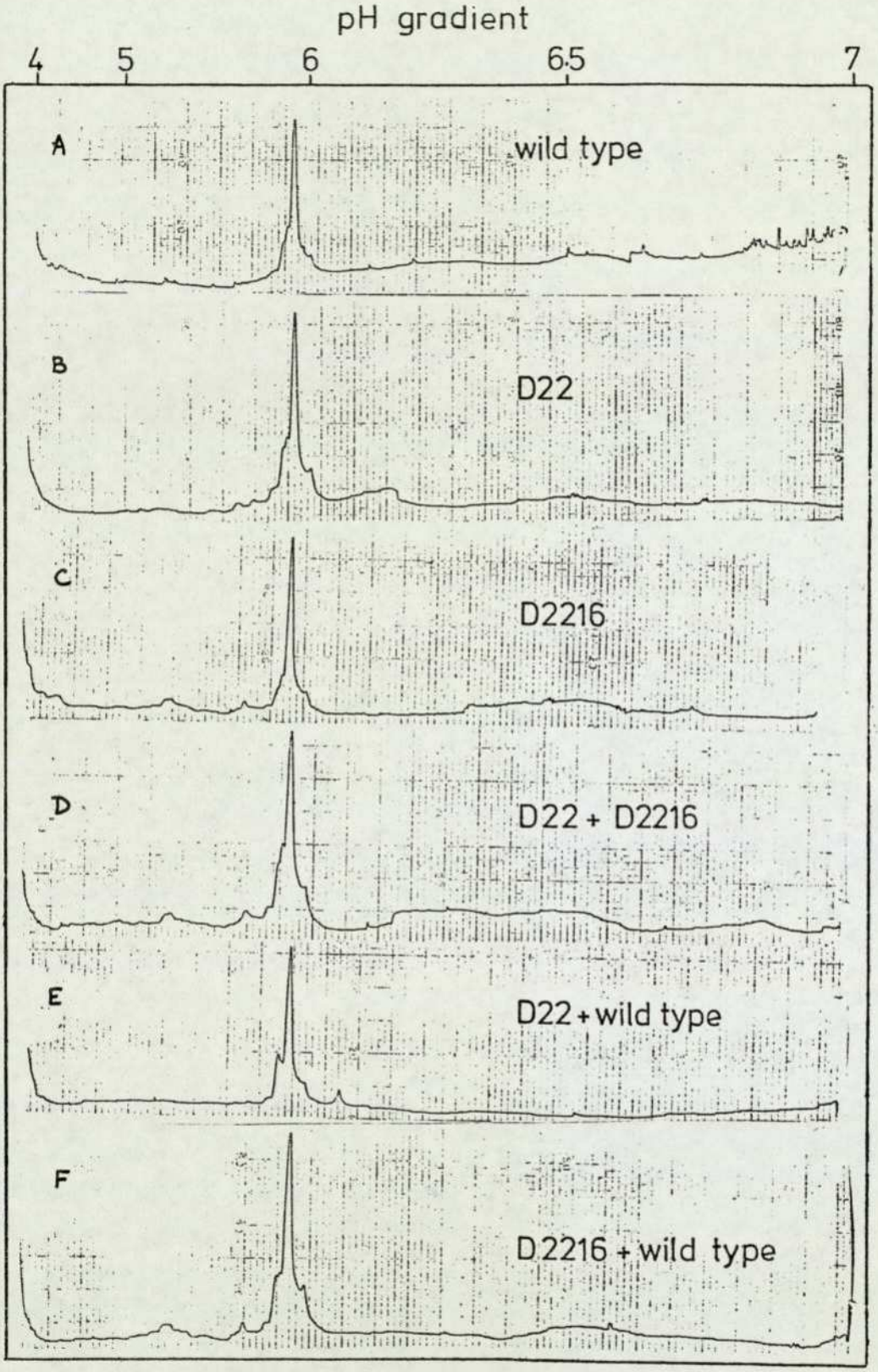


FIGURE 14 SCANNING PATTERNS OF FIRST DIMENSION ISOELECTRIC FOCUSSING GELS OF PURE CRYSTALLINE EF-Tu PREPARED FROM VARIOUS STRAINS AS INDICATED. A-C single samples; D-F mixed samples.

isoelectric focussing patterns of the pure crystalline EF-Tu from the various strains (Fig.14). Each EF-Tu species has only a single band and the mixed sample gels show that the mutant EF-Tu (D2216) comigrates identically with wild-type EF-Tu.

2.4 Discussion.

Quantitative determinations of enzymes are ultimately limited by the efficiency of the techniques used for their extraction. This is especially the case where intracellular distribution is also of importance. In this study, the use of lysozyme or EDTA was prohibited since both can artificially modify the enzyme distribution between the cytoplasm and cell membrane (67,68). Explosive decompression using a high pressure nitrogen bomb (69) was tried but yielded at most 20% of the quantity of EF-Tu provided by alternative methods (data not shown). French pressure cells could give high yields, but are prone to heating and oxidation effects (68). As shown here, osmotic shock alone gave only low total yields of EF-Tu, and had only slight influence in combination with sonication. Maximum total yields were obtained by simple sonication for between 60 and 90 seconds, periodically returning the sample tube to ice to maintain as low a temperature of the lysate as possible. Unfortunately, sonication is known to remove membrane-bound enzymes (68) and this was also the case for EF-Tu: the greatest proportion of EF-Tu demonstrably associated with the membrane was found after only 20 seconds total sonication, and thereafter, although the total yield increased, the absolute quantity in the particulate fraction markedly decreased (data not shown). Thus, although the total EF-Tu estimate is reliable, the data on intracellular distribution can be only considered qualitative, considerably underestimating the

real proportion of the enzyme bound to membrane fragments.

These qualitative results, however, strongly support a membrane-associated role for EF-Tu, as has been already suggested (43, 44), though in the study by Jacobson et al. (43) the controls indicating the quantity of unlysed cells are not given. EF-Tu is not synthesized by ribosomes associated with the membrane (65) and is not among the identified periplasmic proteins (62,68). Further research is clearly necessary to determine whether EF-Tu is functioning structurally as has been recently proposed (39) or in some other capacity.

The isoelectric focussing study of the mutant strain D2216 indicates that the kirromycin-resistant EF-Tu present in this strain is indistinguishable from wild-type EF-Tu and, within the limits of the analytical procedure used, homogeneous. In other mutant EF-Tu strains, such as HAK 88 (54), or the kirromycin-resistant B.subtilis (51), the mutant EF-Tu exhibits an altered net charge, manifest as a shift in the isoelectric focussing band by comparison with wild-type, parental EF-Tu. The group of Bosch (52) has also apparently found two isoelectrically distinct EF-Tu species in a kirromycin-resistant mutant of E.coli. Together with the dominance phenomenon which militates that either both tuf genes must be kirromycin-resistant, or one is resistant and the other inactive, these results point strongly to the conclusion that in strain D2216 only one tuf gene is expressed. A double mutation giving both gene products a net charge identical to one another and to parental EF-Tu seems unlikely, though cannot at this stage be excluded. Genetic studies to clarify this point are in progress in several laboratories.

Making this assumption that in D2216 only one tuf gene is expressed, one can compare the effect of the apparent number of active tuf genes present and the total quantity of EF-Tu in the cell.

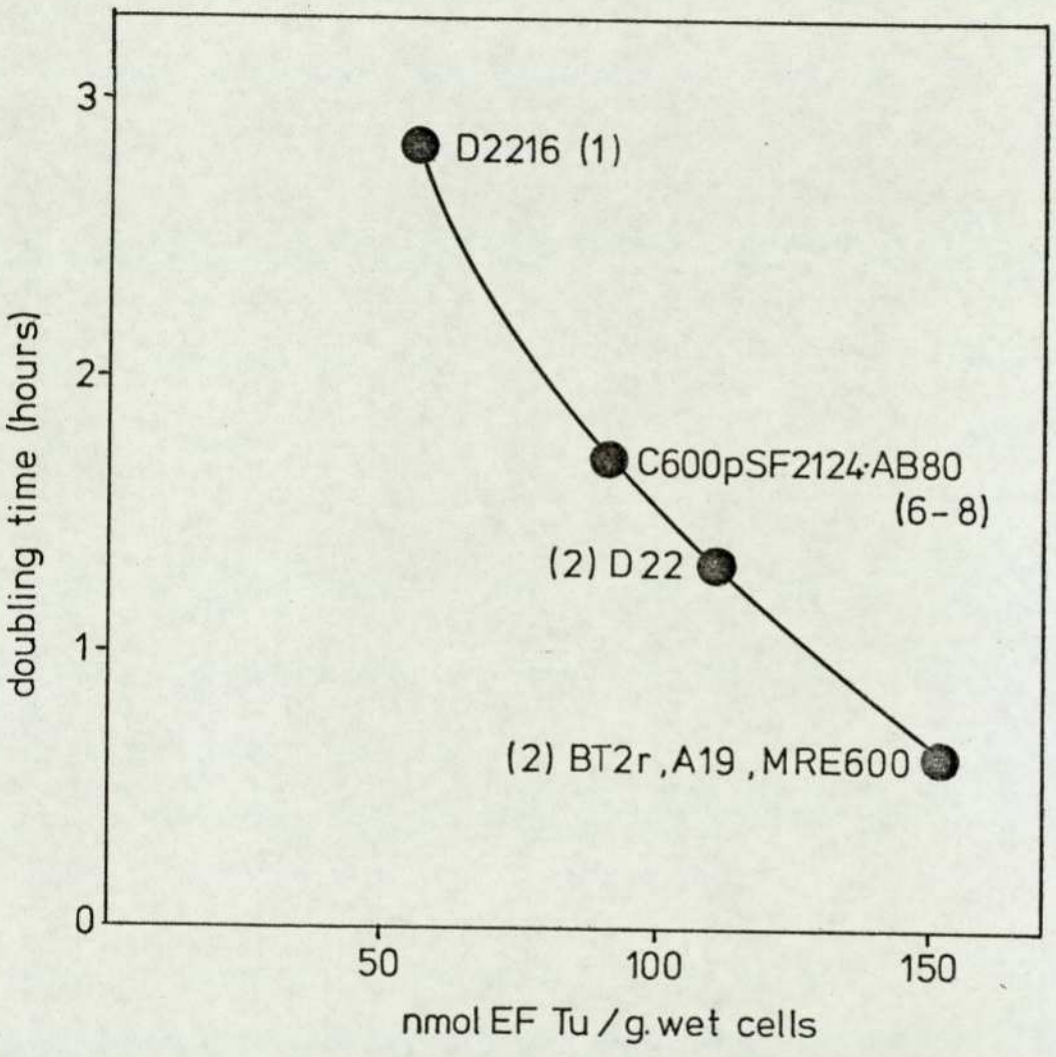


FIGURE 15 RELATIONSHIP BETWEEN GROWTH RATE AND EF-Tu CONTENT FOR VARIOUS *E.coli* STRAINS.

Doubling time is measured in complete tryptone/NaCl media as change in A_{420}. The figures in brackets denote probable number of *tuf* genes present.

Examining the four strains tested, there would appear to be no relationship between the total quantity of EF-Tu and the number of genes actively present; however, there is a good correlation between the quantity of EF-Tu and the growth rate (Fig.15). A similar relationship has been demonstrated within a single strain whose growth rate was varied by changing the composition of the culture medium (70). It seems likely that the quantity of EF-Tu in the cell is determined by an end-product feedback system, possibly at the transcriptional level, since EF-Tu mRNA half-lives are very short (tufA = 3.0 min; tufB = 2.4 min) (71). The gene tufB has now been shown to have its own promoter, independent of the adjacent ribosomal genes (48).

These results have confirmed the very high concentrations of EF-Tu, almost 10% of the total soluble protein, that were first suggested by Furano (38). The question remains, however: why does the bacterial cell invest such a large amount of energy and material to produce such a quantity of a non-exported protein ?

CHAPTER 3. THE IMPORTANCE OF MONOVALENT CATIONS IN THE GTPase ACTIVITY OF ELONGATION FACTOR - Tu.

3.1 Introduction.

During the tRNA-mediated translation of mRNA into amino acid sequences taking place on the ribosome, EF-Tu is responsible for the specific emplacement of an aminoacylated tRNA into the so-called A-site of the ribosome, this being followed by peptide bond formation and translocation of the mRNA. After correct encoding of the aminoacyl-tRNA (aa-tRNA), and prior to peptide bond formation, EF-Tu leaves the ribosome accompanied by the hydrolysis of a molecule of GTP. Earlier studies by the group of Parmeggiani (31,32,33), together with the results presented here, show that the catalytic centre for GTP hydrolysis is located on the factor EF-Tu.

For normal turnover activity, the complex EF-Tu.GDP interacts with a second elongation factor, EF-Ts, which by loosening the tightly bound GDP, encourages the formation of the complex EF-Tu.GTP. This is then stabilized by the binding of a new molecule of aa-tRNA (28).

The antibiotic kirromycin inhibits the elongation of the polypeptide chain through binding specifically to the elongation factor EF-Tu in a stoichiometric ratio of 1:1 (31). As a consequence of antibiotic-induced modifications occurring in the ternary complex, EF-Tu.GTP.aa-tRNA, in the presence of mRNA, EF-Tu is fixed onto the ribosome, effectively preventing peptide bond formation (32). Kirromycin induces a conformation in EF-Tu capable of hydrolyzing GTP in the absence of the normally required physiological effectors, ribosomes and aa-tRNA, though these still stimulate the activity (32,33).

In this chapter the role of monovalent cations in stimulating this EF-Tu GTPase activity is investigated: their importance in the interaction between the factor and ribosomes and aa-tRNA, their involvement in the active site for hydrolysis and the effect of cation species *per se*. Following the stepwise increase in complexity of the system made possible by the use of kirromycin, the role of monovalent cations in the complete EF-Tu - dependent GTPase system in the absence of the antibiotic is then assessed.

Preliminary results are also presented for some parameters of the GTPase activity of EF-Tu purified from the mutant *E.coli* strain D2216. These findings are compared with those relating to wild-type EF-Tu.

3.2 Materials & Methods.

3.2.1 Ribosomes.

Ribosomes washed with 0.5M NH_4Cl were prepared as described (section 2.2.4). Aliquots were then dialysed with several changes against a buffer containing 50% glycerol, 20mM Tris.HCl pH 7.5, 10mM $MgCl_2$ and 60mM of an appropriate monovalent cation, and stored at -25°C. Thus addition of ribosomes did not introduce foreign monovalent cations into assays designed to test the effect of a specific ion, except possibly where very tightly bound. These ribosomes, irrespective of cation, comprised at least 80% "tight couples" as assessed by sucrose density gradient centrifugation in 5mM $MgCl_2$, and retained maximal activity in poly(U)-directed polyphenylalanine synthesis for several weeks, with $[Mg^{2+}]$ optima below 10mM.

To check the effect on the ribosomes of high monovalent

cation concentration, ribosomes were incubated exactly as in normal assays for 10 min at 30°C, in the presence of 2M Li^+, then precipitated with 2 volumes of ethanol. After centrifugation for 20 min at 10000g, the pelleted ribosomes were redissolved in a buffer containing 10mM $MgCl_2$, 20mM Tris.HCl pH 7.8, 60mM NH_4Cl and recentrifuged at 100000g for 4 hr. Aliquots of the final pelleted ribosomes were then analysed by two-dimensional electrophoresis (72) and missing proteins identified.

3.2.2 EF-Tu.

Electrophoretically pure crystalline EF-Tu from E.coli $BT2^r$, prepared as in section 2.2.3, was dialysed with several changes against a buffer containing 50% glycerol, 5mM $MgCl_2$, 25mM Imidazolium acetate pH 7.5, 25 mg/l PMSF and 10µM GDP, and stored at -25°C. In this form the factor was free of monovalent cations and remained stable and active for several months at least. EF-Tu concentration was checked both by the method of Lowry et al. (64) and by measuring maximum binding of EF-Tu.^{3}H.GDP on nitrocellulose filters (see section 2.2.6), which gave similar values.

$EF\text{-}Tu_{D2216}$ was purified from E.coli D2216 to give electrophoretically pure crystals of the protein as described previously (section 2.2.3). Where $EF\text{-}Tu_{D2216}$ was used in assays with wild-type EF-Tu, both elongation factors were freed of monovalent cations and GDP immediately prior to the experiment by chromatography on Sephadex G-25 columns (0.6cm x 8cm) (28). Approximately 400pmol EF-Tu, dissolved in 40µl of a buffer containing 25mM Tris.HCl pH 7.8 and 8mM EDTA were incubated for 15 min at 30°C. This sample was then loaded on a column of Sephadex G-25 as described, and eluted with 25mM Tris.HCl pH 7.8 / 0.2mM EDTA at 4°C. EF-Tu was eluted between 400 and 700µl of

the eluate. This fraction, containing between 0.5 and 1.0 pmol EF-Tu per μl, was used directly in the assays, and the EF-Tu concentration was afterwards determined precisely by the protein assay of Lowry *et al.* (64).

3.2.3 *GTP*.

GTP, obtained as the Li^+ salt (Boehringer, Mannheim) was converted to the K^+ salt by passing over an ion-exchange column as described (section 2.2.1), and was then purified by chromatography on a Whatman DE-52 column, eluting with a 150-300mM linear gradient of triethylammonium bicarbonate (73), precipitated with ethanol and stored in aqueous solution at -25°C. [γ-^{32}P].GTP was prepared by the method of Glynn and Chappell (74) as modified by Sander *et al.*(75). In the presence of GTP, $H_3{}^{32}PO_4$, 3-phosphoglycerate, muscle glyceraldehyde 3-phosphate dehydrogenase and yeast phosphoglycerate kinase, the following reactions occur:

1. 1,3-diphosphoglycerate + glyceraldehyde 3-phosphate dehydrogenase $\rightleftharpoons$ 3-phosphoglyceroyl-enzyme + inorganic phosphate.
2. 3-phosphoglycerate + GTP $\rightleftharpoons$ 1,3-diphosphoglycerate + GDP.

The resulting [γ^{32}P].GTP was then purified as above on a column of DEAE Cellulose (Whatman DE-52). This [γ^{32}P].GTP, which was a generous gift of Dr. A. Parmeggiani, contained less than 1% impurity.

3.2.4 *Kirromycin*.

The antibiotic kirromycin was kindly sent from Tübingen University by [redacted]. Its purity was checked by thin layer chromatography on silica plates (Merck, Kieselgel 60 F_{254}, 0.5mm) eluting with a solution of 50 parts chloroform, 47.5 parts methanol and 1 part concentrated NH_4OH. Pure kirromycin from several sources,

as well as the methylated analogue X-5108 (now also known as Aurodox (76)), is revealed as a UV absorbent spot of RF value 0.7-0.8 . The antibiotic was stored as a stock solution of about 10mM in 100% ethanol at -20°C in the dark, where it was stable for several months. The concentration was determined by UV absorbance at 325nm in a solution of 50% ethanol / 50% 0.1M NaOH (ε_{325} = 28200 M^{-1} cm^{-1}), using a Zeiss DB-4 spectrophotometer.

3.2.5 Phe-$tRNA^{Phe}$.

$tRNA^{Phe}$ was purified from total E.coli tRNA (Schwartz) by chromatography on benzoyl DEAE Cellulose (BD-Cellulose) (77), eluting with a gradient of 0.5 - 0.8M NaCl. The $tRNA^{Phe}$, now 40-60% pure, was then charged with phenylalanine (56). Approximately 100nmol $tRNA^{Phe}$ was incubated for 30 min at 30°C in 5ml of a reaction mixture also containing 200nmol ^{14}C.phenylalanine (Amersham, specific activity 16 cpm/pmol), 11mg impure phenylalanyl-tRNA synthetase (this impure enzyme derives from a DEAE Sephadex A-50 eluate of an E.coli 100000g supernatant, and should also contain a nucleotidyl transferase to repair the -CCA terminus of the $tRNA^{Phe}$), 50mM Tris.HCl pH 7.8 , 20mM KCl, 10mM $MgCl_2$, 1.8µmol CTP, 12µmol ATP and 0.2mM dithiothreitol. After incubation the reaction was stopped by the addition of 0.3ml of 1M potassium acetate pH 4.6, extracted with an equal volume of phenol containing 0.025% quinoline, and after centrifugation the upper aqueous phase was precipitated by the addition of two volumes of ethanol at -25°C for 1 hr. The precipitated RNA was centrifuged, redissolved in 2ml of 50mM potassium acetate pH 4.6 and run on a Sephadex G-25 column to purify the Phe-$tRNA^{Phe}$. The radioactive fractions were gathered and precipitated with two volumes of ethanol

at -25°C for 1 hr. After centrifugation, the pellet was lyophilized, redissolved in minimum H_2O and the specific activity, and hence purity, of the Phe-$tRNA^{Phe}$ determined. 1 A_{260} unit of tRNA was taken to be equivalent to 1600pmol.

3.2.6 Monovalent cations.

Monovalent cations were introduced into the assays as chlorides. LiCl was Suprapur grade (Merck), tetramethylammonium chloride (Me_4NCl) was analytical grade from Fluka A.G., Switzerland. All remaining salts were analytical reagent grade from Merck.

3.2.7 Assay for GTPase activity.

The hydrolysis of [γ^{32}P].GTP was measured as liberation of inorganic phosphate. This was assayed, after addition of 75µl of 1M $HClO_4$ / 1mM KH_2PO_4 to the 75µl of the reaction mixture, by extraction with isopropyl acetate in the presence of sodium molybdate (36). Where cations interfered with this reaction, after arresting the reaction with $HClO_4$ as above, all nucleotides were sequestered by the addition of 0.4ml of 6% activated charcoal in 1M HCl (78), only the ^{32}P liberated by hydrolysis remaining in solution. After centrifugation for 40 min at 5000 rpm, 0.2ml aliquots of the supernatant were added either to 5ml Scintix (Isotec, France) plus 0.3ml H_2O, or to 5ml Aquasol (New England Nuclear), and counted in an Intertechnique SL4000 liquid scintillation counter.

All assays were performed in a buffer containing 50mM Imidazolium acetate pH 7.5, which decreased by less than 0.3 pH units between 0 and 2M M^+. Where ribosomes were included in the assays minimum monovalent cation concentrations achieved were 1mM because

of carry-over with the ribosomes; otherwise the minimum values are less than 0.03mM. In the presence of kirromycin, reaction mixtures were incubated for 10 min at 30°C, during which prior kinetic studies showed the reaction to proceed linearly with time. Incubation in the absence of the antibiotic was for 5 min at 30°C; although this reaction is non-linear, kinetic studies indicated less than 20% departure from linearity up to this time in the chosen assay conditions. All other data concerning reaction mixtures are indicated in the figure legends.

3.2.8 Estimation of K_m.

The K_m (Michaelis constant) for the various assay systems was calculated from Lineweaver-Burk plots, incubating for a time period during which the hydrolysis proceeded linearly in accord with an apparent first order reaction. For the GTPase reaction in the absence of kirromycin, where kinetic studies indicated an apparent second order reaction, initial velocities were estimated from the GTP hydrolysed in the first 30 seconds. All K_m values were calculated at optimum $[Mg^{2+}]$ previously estimated for each set of cation conditions.

3.3 Results.

3.3.1 Effect of monovalent cations on the EF-Tu.kirromycin GTPase.

In this, the simplest system for EF-Tu, giving a turnover GTPase activity in the absence of the physiological effectors, aa-tRNA and ribosomes, monovalent cations are an absolute requirement (Fig.16).

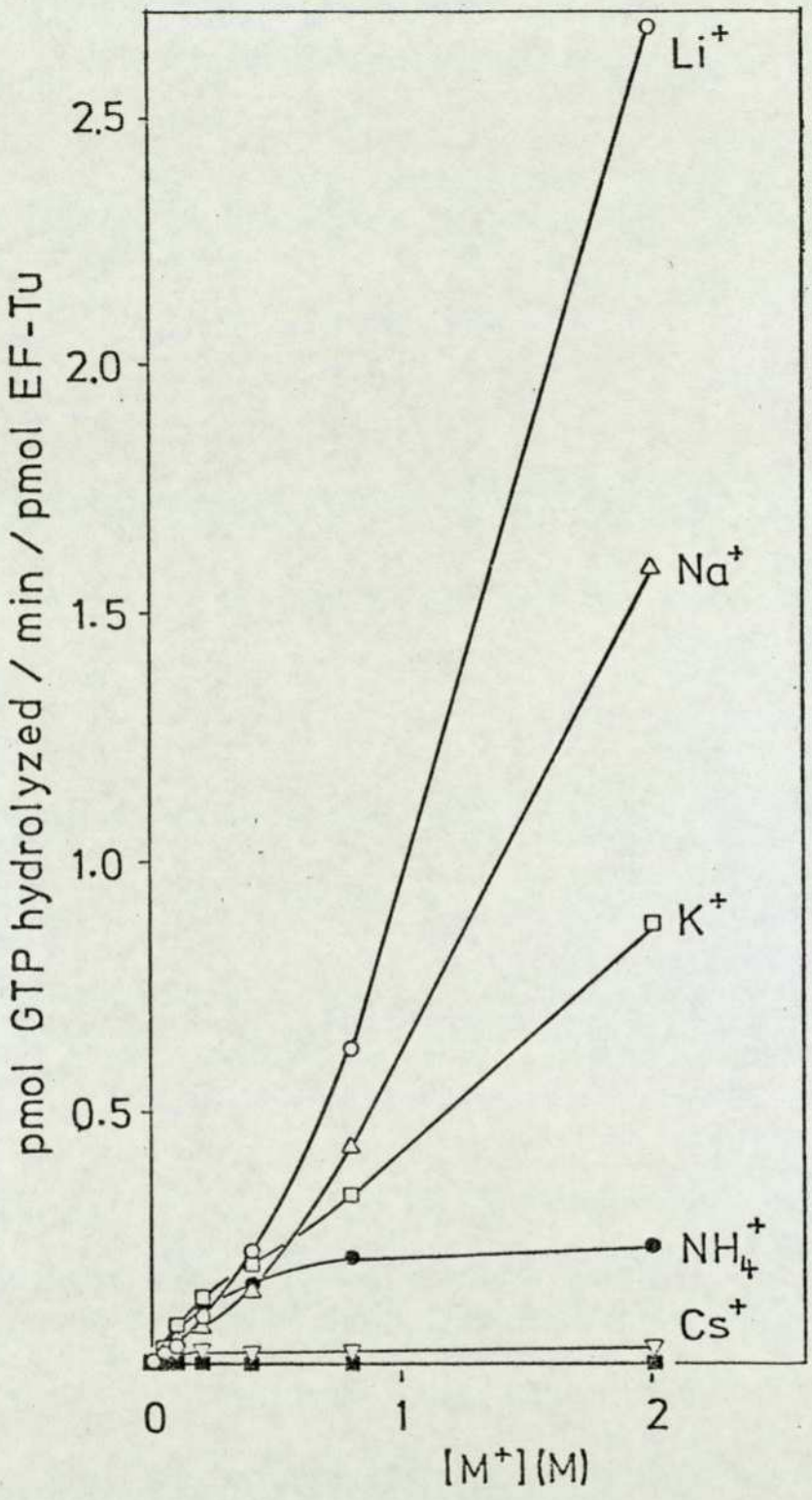


FIGURE 16 EFFECT OF MONOVALENT CATIONS ON THE EF-Tu GTPase IN THE PRESENCE ONLY OF KIRROMYCIN.

Reaction mixtures contained in a volume of 75µl, 10mM $MgCl_2$, 50mM Imidazolium acetate, pH 7.5, 20pmol EF-Tu, 2nmol ^{32}P.GTP (specific activity 100-150 cpm/pmol) and 50µM kirromycin. (o) Li^+, (Δ) Na^+, (□) K^+, (●) NH_4^+, (▽) Cs^+, (■) Me_4N^+.

In general, an increase in monovalent cation concentration brings about a higher GTPase activity. At concentrations higher than 0.4M $[M^+]$, the extent of this stimulation depends upon the cationic species and follows the order $Li^+ > Na^+ > K^+ > NH_4^+ > Cs^+$; Me_4N^+ and $Tris.H^+$ (not shown) do not stimulate, even at 2M concentration. At lower monovalent cation concentrations the differences between the active cations are less pronounced, K^+ and NH_4^+ being, however, the two most efficient followed by Na^+ and Li^+. In the presence of NH_4^+ ion, EF-Tu GTPase activity levels off at between 0.4 and 1M, whereas for Li^+ ion stimulation continues up to a maximum at about 3.5M; at 5M there is almost complete inhibition (not shown). The action of Na^+ and K^+ lies between that of Li^+ and NH_4^+, increasing steadily to near saturation conditions.

These results indicate an effect which is dependent on both decreasing ionic radius and increasing ionic concentration. The former appears to be determinant since the largest cations (e.g. Cs^+, Me_4N^+, $Tris.H^+$) have little or no effect.

3.3.2 Influence of the physiological effectors, ribosomes and aminoacyl-tRNA, on the monovalent cation specificity of the EF-Tu.kirromycin GTPase.

Fig.17 illustrates the effect of interaction between the physiological effectors, aa-tRNA and ribosomes, and specific monovalent cations, on the EF-Tu.kirromycin GTPase. At high monovalent cation concentrations (ca.2M), the physiological effectors stimulate only slightly the activity already generated by the EF-Tu.kirromycin complex alone, the order of activity being inverse to that of ionic radius

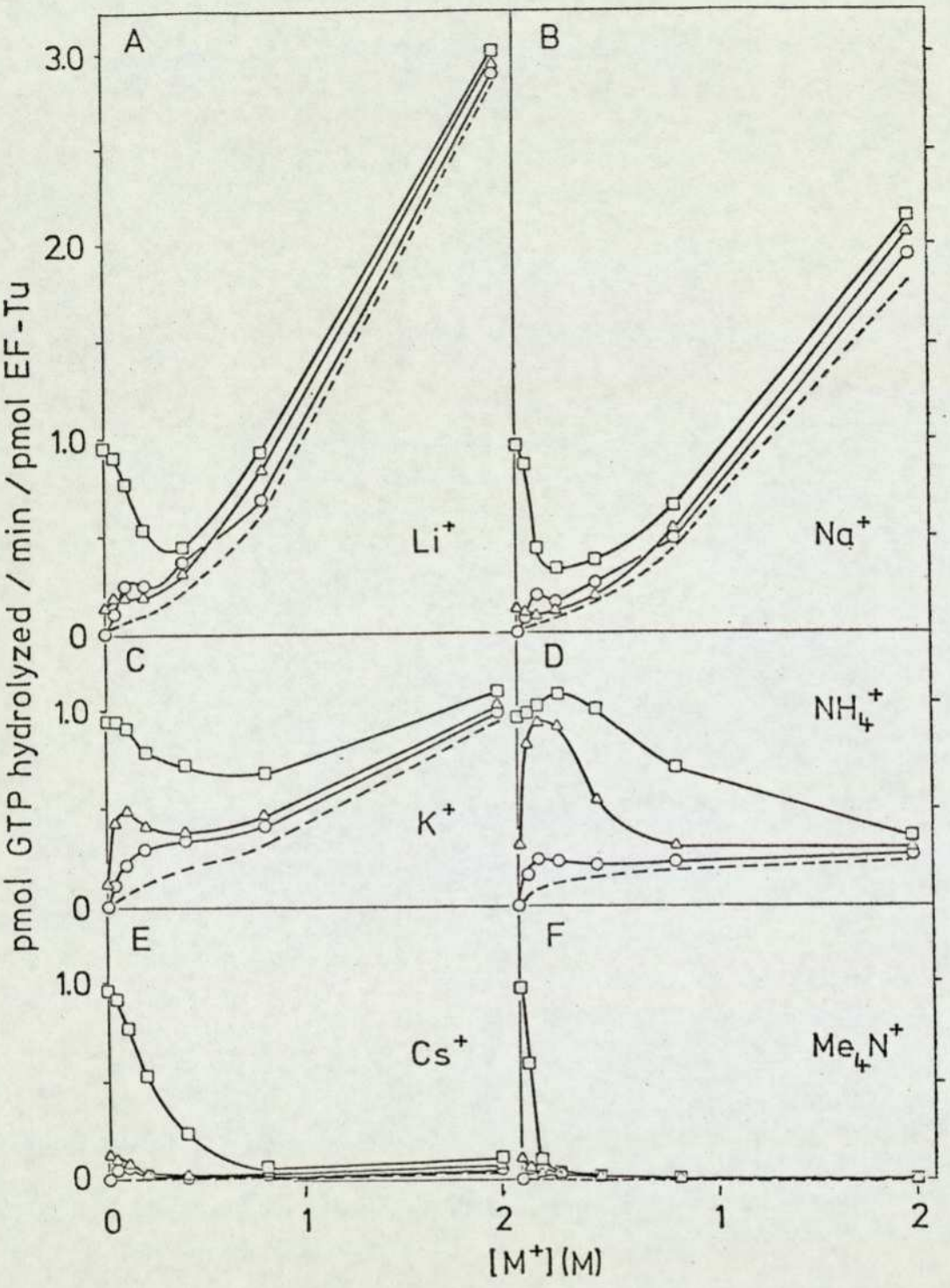


FIGURE 17 EFFECT OF MONOVALENT CATIONS ON THE EF-Tu GTPase IN THE PRESENCE OF KIRROMYCIN AND THE PHYSIOLOGICAL EFFECTORS, RIBOSOMES AND AMINOACYL-tRNA.

A. Li^+, B. Na^+, C. K^+, D. NH_4^+, E. Cs^+, F. Me_4N^+. Reaction mixtures were as in Fig.16 without (dashed line, taken from Fig.16) or with the addition of 100pmol Phe-$tRNA^{Phe}$ (o), 40pmol ribosomes (Δ), or both Phe-$tRNA^{Phe}$ and ribosomes (□).

(viz. $Li^+ > Na^+ > K^+ > NH_4^+ > Cs^+ > Me_4N^+$). At high ionic concentrations, ribosomes are likely to become stripped of some ribosomal proteins. This possibility has been examined for Li^+, the cation of highest charge-to-radius ratio, by two-dimensional electrophoresis (72). At 2M $[Li^+]$ under the incubation conditions used in the assays it was found that proteins S2, S3, S5, S13, S14, S21, L7/L12, L10, L16 and L25 were partially or wholly removed from the ribosomes, though present in solution. The decreasing stimulatory effect of the ribosomes at these high salt concentrations may therefore be in part due to an inactivation of ribosomal function resulting from the loss of component proteins.

Below about 400mM $[M^+]$ both ribosomes and aa-tRNA, individually or together, stimulate the hydrolysis of the GTP in a manner characteristic of the monovalent cation species. For both effectors together, at 200mM $[M^+]$, the order of activity is $NH_4^+ > K^+ > Cs^+ = Li^+ > Na^+ > Me_4N^+$, indicating a specificity for the cations NH_4^+ and K^+, though all of the other cations, except for NH_4^+, show, in the presence of ribosomes and aa-tRNA, higher activity at near zero concentrations than at 200mM. In the presence of ribosomes alone, for Li^+ and K^+ (Fig.17A&C) the optimum monovalent cation concentration (i.e. that concentration inducing the highest GTPase activity) shifts to higher values, suggesting that aa-tRNA is reducing the requirement for these ions of the EF-Tu.kirromycin / ribosome system. In the absence of ribosomes, aa-tRNA stimulates the EF-Tu.kirromycin GTPase activity by a factor of two to three, below 400mM $[M^+]$, though having little effect at higher concentrations. The earlier report (79) that the requirement for NH_4^+ is eliminated by the presence of ribosomes is now confirmed also for the other monovalent cation species. This absolute requirement

is not, however, affected by the presence of aa-tRNA.

3.3.3 Effect of monovalent cations on the K_m of the EF-Tu.kirromycin GTPase in the presence and absence of physiological effectors.

The K_m values for GTP given in Tables IV and V were measured at optimum Mg^{2+} concentrations, which varied between 2 and 5mM at 2M $[M^+]$, and between 5 and 10mM at 200mM and 10mM $[M^+]$. The specific role of divalent cations will be considered in greater detail later (chapter 4).

In the absence of aa-tRNA and ribosomes (Table IV) there is a marked change in K_m between 200mM and 2M for all cations except NH_4^+. At low salt concentrations (200mM M^+), K_m values are all about 0.2μM, with little cation specific variation. However, at 2M concentrations there is a large cation-dependent variation in K_m from 0.2μM for NH_4^+ to about 1.0μM for Li^+. With the exception of NH_4^+, increased cation concentration raises the K_m for GTP, and correspondingly increases the amount of GTP hydrolyzed, the stimulation being greater as the cationic radius becomes smaller.

The presence of the physiological effectors (Table V) has little or no effect on the K_m at 200mM $[M^+]$. Values of K_m are slightly higher at 10mM $[M^+]$ than at 200mM for all cations except NH_4^+, which is also the only cation to stimulate hydrolysis between these concentrations in the presence of aa-tRNA and ribosomes. At concentrations below 1mM M^+, K_m values in the presence of both effectors lie between 0.3 and 0.4μM (not shown), the differences observed probably being due to cations bound to and carried over with the ribosomes. At 2M monovalent cation concentrations, ribosomes and aa-tRNA have little influence on the K_m, reflecting their lack of stimulatory activity at this concentration.

TABLE IV K_m VALUES FOR GTP IN THE EF-Tu.KIRROMYCIN GTPase IN THE ABSENCE OF PHYSIOLOGICAL EFFECTORS.

Cation	Concentration (M^+)		
	10mM	200mM	2M
Li^+	-	0.20 μM	1.06 μM
Na^+	-	0.15	0.78
K^+	-	0.21	0.50
NH_4^+	-	0.20	0.20
Cs^+	-	0.20	0.39
Me_4N^+	-	0.20	-

TABLE V K_m VALUES FOR GTP IN THE EF-Tu.KIRROMYCIN GTPase IN THE PRESENCE OF RIBOSOMES AND AMINOACYL-tRNA.

Cation	Concentration (M^+)		
	10mM	200mM	2M
Li^+	0.23 μM	0.20 μM	1.11 μM
Na^+	0.23	0.16	1.00
K^+	0.40	0.29	0.57
NH_4^+	0.40	0.40	0.26
Cs^+	0.33	0.22	0.40
Me_4N^+	0.23	0.20	-

3.3.4 Influence of monovalent cations on the EF-Tu GTPase in the absence of kirromycin.

GTP is hydrolysed in the presence of EF-Tu, aminoacyl-tRNA and mRNA.ribosome complex. The turnover EF-Tu - dependent activity is greatly stimulated by the presence of elongation factor EF-Ts, which, acting in a similar way to kirromycin (28,29) promotes the exchange of the GDP which is tightly bound to EF-Tu with free GDP, without essentially affecting the equilibrium constant of the complex. However, kirromycin additionally stabilizes the binding of GTP to the factor, mimicking the action of aa-tRNA. In the described assay conditions, the rate of GDP dissociation from the EF-Tu.GDP complex is the limiting factor in the EF-Tu - catalyzed hydrolysis of GTP (28); thus EF-Ts is important in maintaining a turnover GTPase activity which would otherwise be strongly reduced after one round.

To test the effect of ionic concentration on this system, curves of GTPase activity versus increasing $[M^+]$ were constructed (Fig.18). Conditions were chosen to give an EF-Ts : EF-Tu ratio of 4, a value at which EF-Ts was found to be saturating in this system (see later). Except for EF-Tu, all other components were also in excess.

In general, above 30mM $[M^+]$ there is a steady decline in activity with increasing concentration for all cation species, reaching near zero values at 400mM $[M^+]$. In marked contrast to the striking GTPase activity induced in EF-Tu by the antibiotic kirromycin, there is very little activity at higher concentrations. This result probably implies that high ionic concentration, irrespective of the cation species, is strongly inhibitory on this system. For some cations this

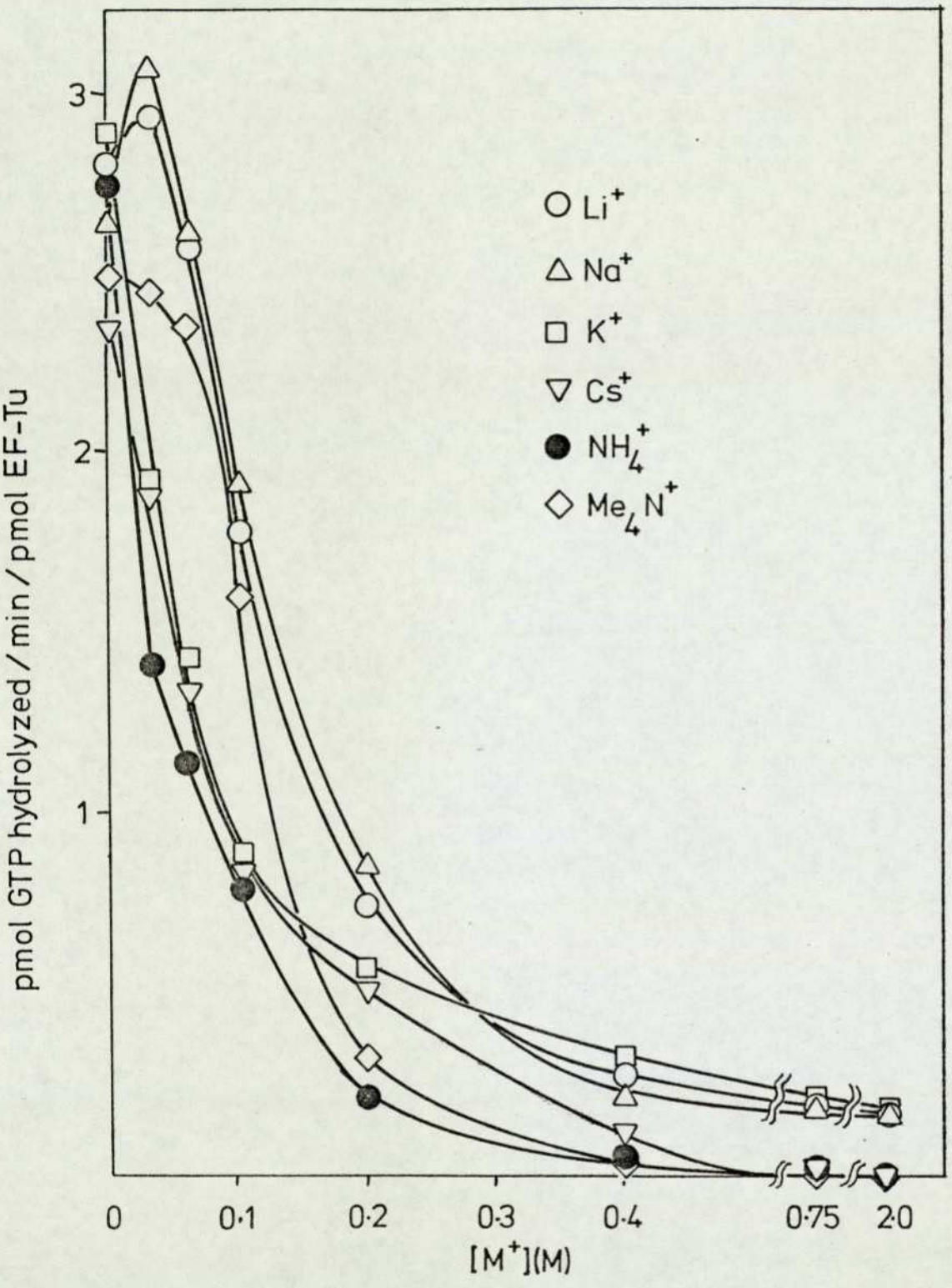


FIGURE 18 EFFECT OF MONOVALENT CATIONS ON THE EF-Tu GTPase INDUCED BY AMINOACYL-tRNA, mRNA, RIBOSOMES AND EF-Ts.

Reaction mixtures contain in a volume of 75µl, 10mM $MgCl_2$, 50mM Imidazolium acetate, pH 7.5, 10pmol EF-Tu, 40pmol EF-Ts, 1 or 2 nmol ^{32}P.GTP (specific activity 100-200 cpm/pmol), 4µg poly(U), 100pmol Phe-tRNA^{Phe} and 20pmol ribosomes. Symbols as indicated in the figure.

inhibition is marginally retarded until slightly higher concentrations.

At concentrations between 0mM and 200mM [M^+] there is a marked cation specificity, with some ions, for example Na^+ and Li^+, stimulating between 0.3 and 30mM. In the case of NH_4^+, and to a lesser extent K^+, there is already from 0.3mM concentration a sharp decline in GTP hydrolysis with increasing cation concentration. At 30mM [M^+], the difference between the cations is greatest with Na^+ and Li^+ stimulating the GTPase activity more than twice as much as the equivalent concentration of NH_4^+. The order of the cations at 30mM, as judged by the physiological EF-Tu GTPase activity they sustain, was $Li^+ = Na^+ > Me_4N^+ > K^+ = Cs^+ > NH_4^+$.

The system at 30mM [M^+] was further explored in experiments carried out to test the influence of monovalent cation species on the stimulatory effect of EF-Ts (Fig.19A). At low ratios of EF-Ts : EF-Tu (e.g. 0.5), there is little difference between the effects of the various cationic species. However, at all higher ratios a cation specificity is evident: Na^+ and Li^+ induce the greatest GTP hydrolysis, rising with increasing [EF-Ts], followed by Me_4N^+, Cs^+ and K^+. The ammonium ion was the only ion tested in whose presence EF-Ts becomes saturating at ratios of EF-Ts : EF-Tu of little more than 1. For Na^+ and Li^+, saturation was eventually reached with about a three-fold excess of EF-Ts over EF-Tu.

In these experiments, there was no uncoupling of the EF-Tu - dependent GTPase from its usual effectors, as occurs in the presence of methanol (80,81) or kirromycin. In experiments not shown, with EF-Ts in a three-fold excess over EF-Tu, it was demonstrated that for all monovalent cations, there was still an absolute requirement for all additional effectors: aminoacyl-tRNA, mRNA and ribosomes.

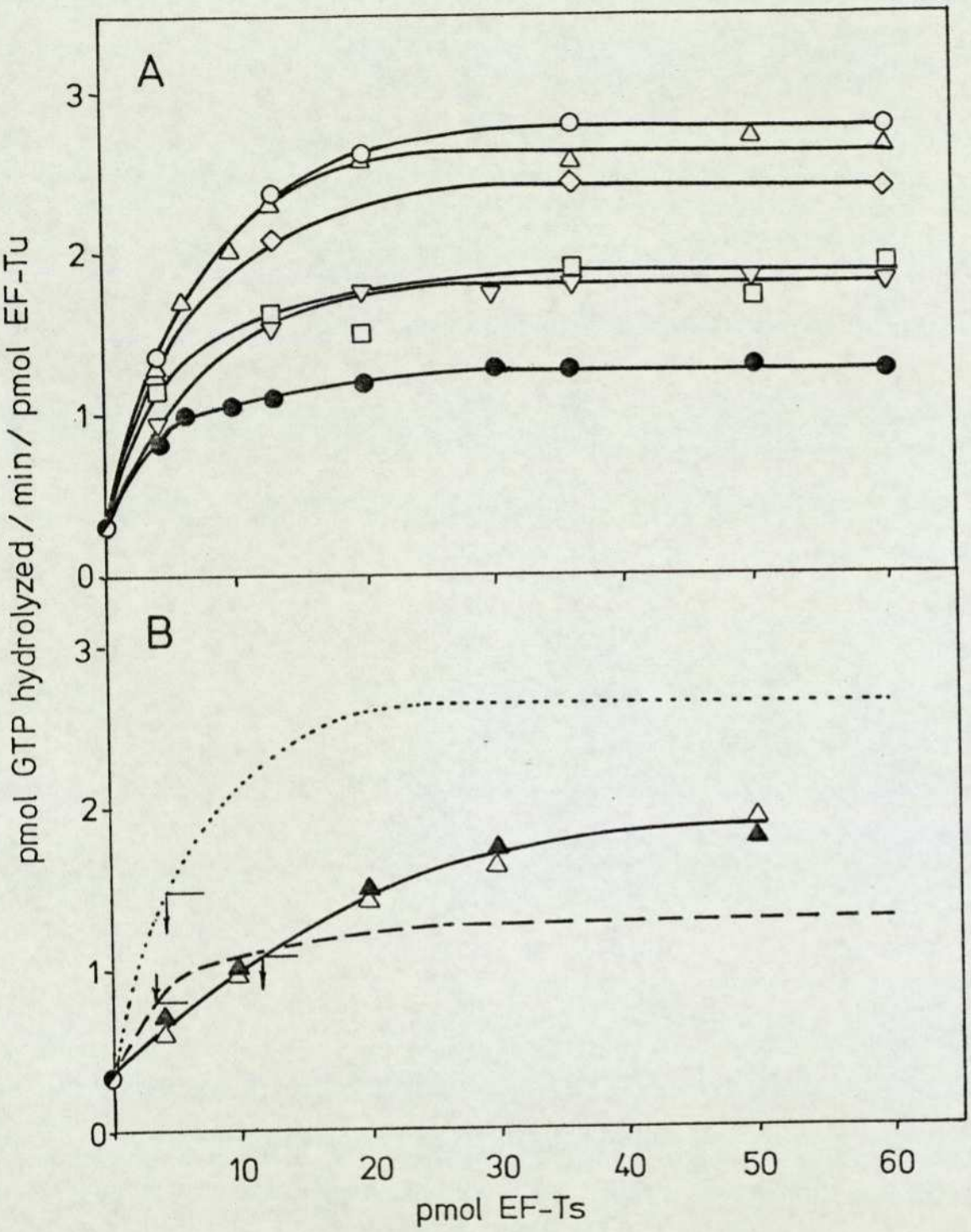


FIGURE 19 INFLUENCE OF MONOVALENT CATIONS ON THE STIMULATION BY EF-Ts OF THE PHYSIOLOGICAL EF-Tu GTPase.

A. Using wild-type EF-Tu. Reaction conditions as in Fig.18. All assays carried out in 30mM monovalent cations. (o) Li^+, (Δ) Na^+, (□) K^+, (●) NH_4^+, (▽) Cs^+, (◇) Me_4N^+.

B. Using mutant EF-Tu_{D2216}. Conditions as above, in the presence of 30mM Na^+ (Δ) or NH_4^+ (▲). Comparable wild-type EF-Tu curves are given by dotted (Na^+) or dashed (NH_4^+) lines. Arrows indicate points of 50% stimulation by EF-Ts.

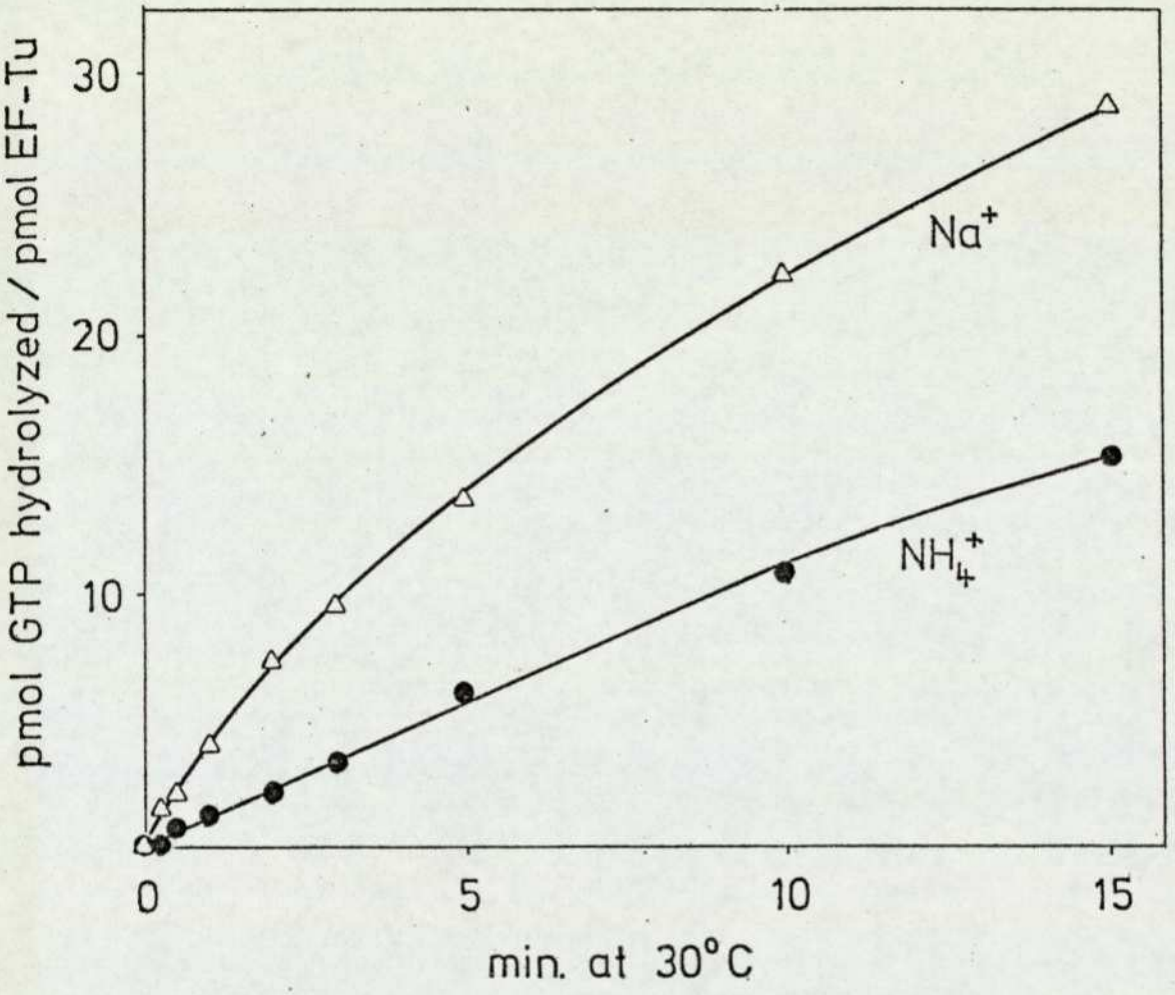


FIGURE 20 KINETIC OF THE EF-Tu GTPase ACTIVITY.

In the presence of 10pmol EF-Tu, 30pmol EF-Ts, 20pmol ribosomes, 50pmol Phe-tRNA^{Phe}, 4µg poly(U) and 25 uM GTP per 75µl reaction mixture, carried out at either 30mM Na^+ (▲) or 30mM NH_4^+ (●). Other conditions as in Fig.18.

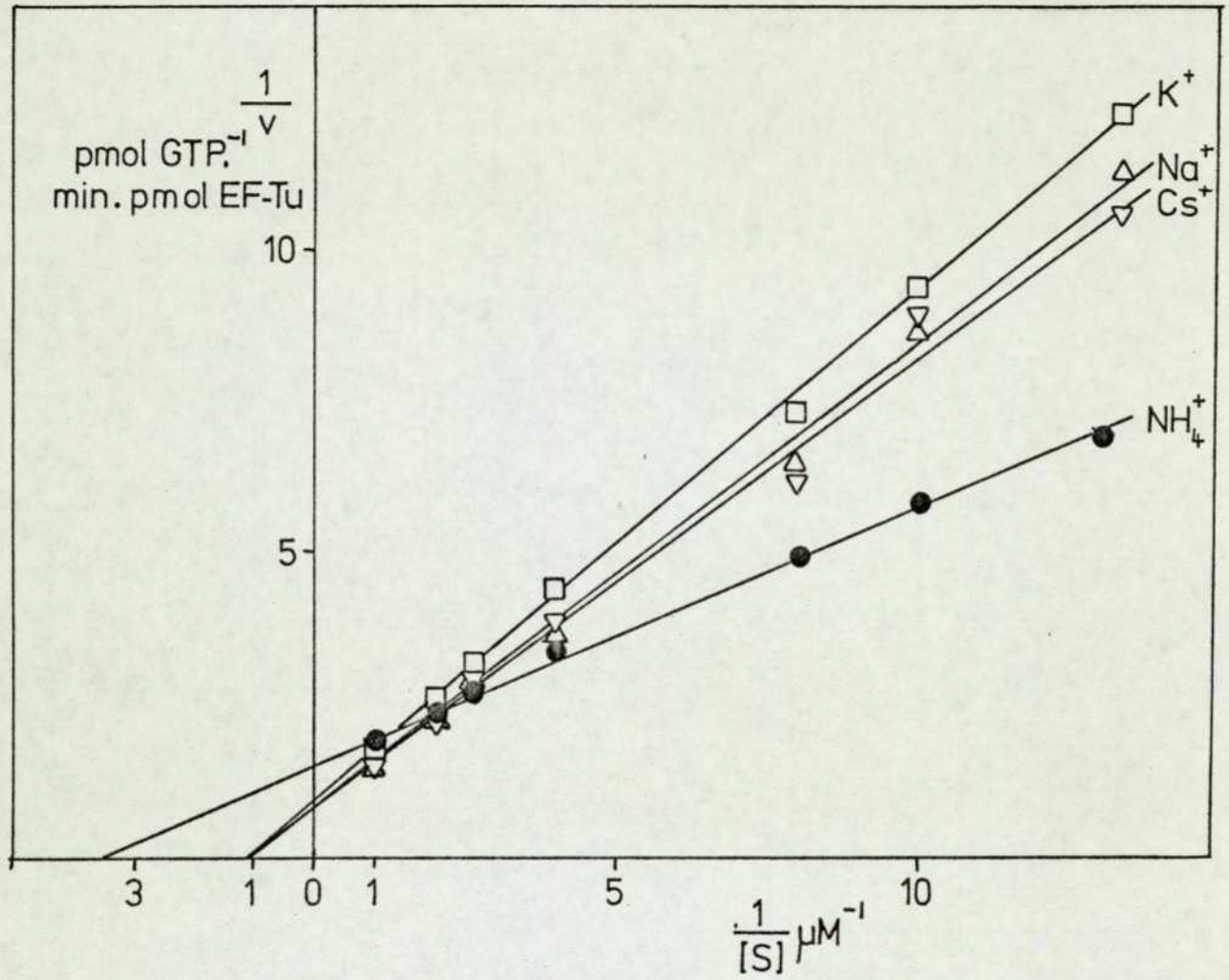


FIGURE 21 LINEWEAVER - BURK PLOTS OF THE EF-Tu GTPase IN THE ABSENCE OF KIRROMYCIN.
Reaction mixtures contained in 75µl 10pmol EF-Tu, 20pmol ribosomes, 50pmol Phe-tRNA^{Phe}, 4µg poly(U) and were carried out in 30mM NH_4^+ (●), Cs^+ (▽), Na^+ (△) or K^+ (□). Other conditions as in Fig.18.

To shed further light on these interesting results, K_m values were determined for all cations at 30mM $[M^+]$ and with an EF-Ts : EF-Tu ratio of 3. Time curves for GTP hydrolysis in the presence of various cations are hyperbolically non-linear even in large excess of GTP (Fig.20), except in the case of ammonium ion, where linearity is maintained for several minutes before becoming eventually asymptotic. Initial velocities were estimated from the GTP hydrolyzed in the first 30 seconds of the reaction. The results were unambiguous (Fig.21); for NH_4^+ the K_m varied between 0.2 and 0.3μM (results from several experiments), a similar value to that calculated at low monovalent cation concentration in the kirromycin-induced system (Tables IV & V). The other cations tested yielded constants of between 0.5 and 1.0μM, similar to the maximum values attained in the kirromycin-induced activity at 2M $[M^+]$. The highest value was consistently recorded for the Na^+ ion, where the K_m was 1.0μM. There appears to be a close correspondence between the K_m values and the estimated GTPase activities, as has also been found with the kirromycin-induced GTPase system.

3.3.5 Characterization of the EF-Tu GTPase activity of $EF\text{-}Tu_{D2216}$.

The $EF\text{-}Tu_{D2216}$ derived from the kirromycin-resistant *E.coli* strain D2216 is able to show GTPase activity when kirromycin is present at a very high concentration (49). In order to check the effect of monovalent cations on this activity, kinetic assays were carried out at chosen monovalent cation concentrations as indicated in Table VI. Although activity was stimulated by kirromycin at 10^{-4}M concentration, maximum GTPase activity was attained only when the kirromycin concentration was raised to 5×10^{-4}M, which is close to the solubility limit of the antibiotic, if the ethanol content of the assay system is

not to exceed 5%.

TABLE VI EF-Tu.KIRROMYCIN GTPase ACTIVITY OF EF-Tu FROM MUTANT AND WILD-TYPE STRAINS.

Strain	[kirromycin]	ionic concentration			
		2M Li^+	2M NH_4^+	200mM Li^+	200mM NH_4^+
D2216	10^{-4}M	0.11	0.06	0.06	0.05
	5×10^{-4}M	0.23	0.07	0.06	0.06
wild-type	10^{-4}M	2.70	0.25	0.15	0.21

N.B. data are given in pmol GTP hydrolyzed/min/pmol EF-Tu.

The results given in Table VI clearly indicate that the monovalent cation effect observed in wild-type EF-Tu is also demonstrable in the mutant EF-Tu from E.coli D2216. At 5×10^{-4}M kirromycin concentration, Li^+ ions at 2M can stimulate the GTPase activity more than three-fold compared with the activity in the presence of an equivalent concentration of NH_4^+ ions.

When tested in the physiological EF-Tu GTPase system in the absence of kirromycin, the mutant $EF\text{-}Tu_{D2216}$ exhibits similar levels of GTPase activity to those observed with wild-type elongation factor. However, there is a significant difference between the two types of EF-Tu where it concerns the influence of monovalent cations on this physiological GTPase activity. In Fig.19B are illustrated the EF-Ts saturation curves for both wild-type and mutant EF-Tu, in

the presence of either Na^+ ion (open triangles, dotted line) or NH_4^+ (filled triangles, dashed line). There appears to be no difference between the effects of the two ions on the mutant EF-Tu_{D2216}, in marked contrast to their effects on wild-type EF-Tu.

The quantity of EF-Ts required to stimulate the EF-Tu GTPase to 50% of the maximum possible (i.e. the activity at saturating quantities of EF-Ts) can be thought of as an index of the affinity of the two elongation factors for one another. This measure is comparable to the Michaelis constant, though conditions for the precise measurement of the latter are not fulfilled in this assay system. For wild-type EF-Tu, both Na^+ and NH_4^+ ions yield similar values of between 4 and 5 pmol EF-Ts. By contrast, the mutant EF-Tu_{D2216} gives a value of approximately 12pmol for both cations. This result probably indicates a changed affinity of the mutant EF-Tu_{D2216} for EF-Ts, consequent on the modifications induced by the mutation in the kirromycin binding site.

Preliminary measurements were also made of the K_m for GTP in both the kirromycin-induced and physiological EF-Tu GTPase systems. In the presence of 200mM NH_4^+ and 5×10^{-4}M kirromycin, and in the absence of ribosomes and aminoacyl-tRNA, mutant EF-Tu_{D2216} yielded a K_m of 0.2μM, a similar figure to that obtained using wild-type EF-Tu. In the physiological EF-Tu GTPase system, in the absence of the antibiotic, and in the presence of either 30mM Na^+, NH_4^+ or K^+, K_m values for GTP were obtained consistently between 0.2 and 0.3μM. Thus, just as in the mutant there are no differences between monovalent cation species in respect to GTPase activity, so also are no differences detectable in the K_m values for GTP, in marked contrast to the wild-type EF-Tu.

3.4 Discussion.

The effect of different cation species on an enzyme reaction sheds light on the involvement, directly or indirectly, of cations in the active site(s) (82). Here the role of monovalent cations in the GTPase associated with EF-Tu has been studied under conditions of progressively increasing complexity, thus shedding light also on the role of cations in the interactions between EF-Tu and its macromolecular effectors.

The EF-Tu GTPase induced by kirromycin shows three different responses to monovalent cations (Fig.22). The first of these is apparent either in the absence of the physiological effectors, aa-tRNA, and ribosomes at all concentrations above 400mM, or, in the presence of these effectors, only at high $[M^+]$ (>1M). Under these conditions activity is proportional inversely to cation radius and directly to cation concentration. Ionic concentration per se has no influence, since Cs^+, Me_4N^+ and $Tris.H^+$ have little or no stimulating effect even at high concentration. The order of activity of the cations, which is the order of increasing hydration energy, suggests that the ions are binding to a site(s) of high anionic field strength (82); and the demonstration that there is a corresponding change in K_m indicates that this site is directly involved with the catalytic centre for GTP hydrolysis. However, the high monovalent cation concentrations necessary to achieve maximum GTPase activities imply that the cation binding site(s) is (are) of relatively low affinity. In the only other enzyme for which a similar cation priority is known, calf brain adenylate deaminase (83), it has been suggested that the cations can substitute for a nucleotide effector (ATP) or for a substrate (AMP) in

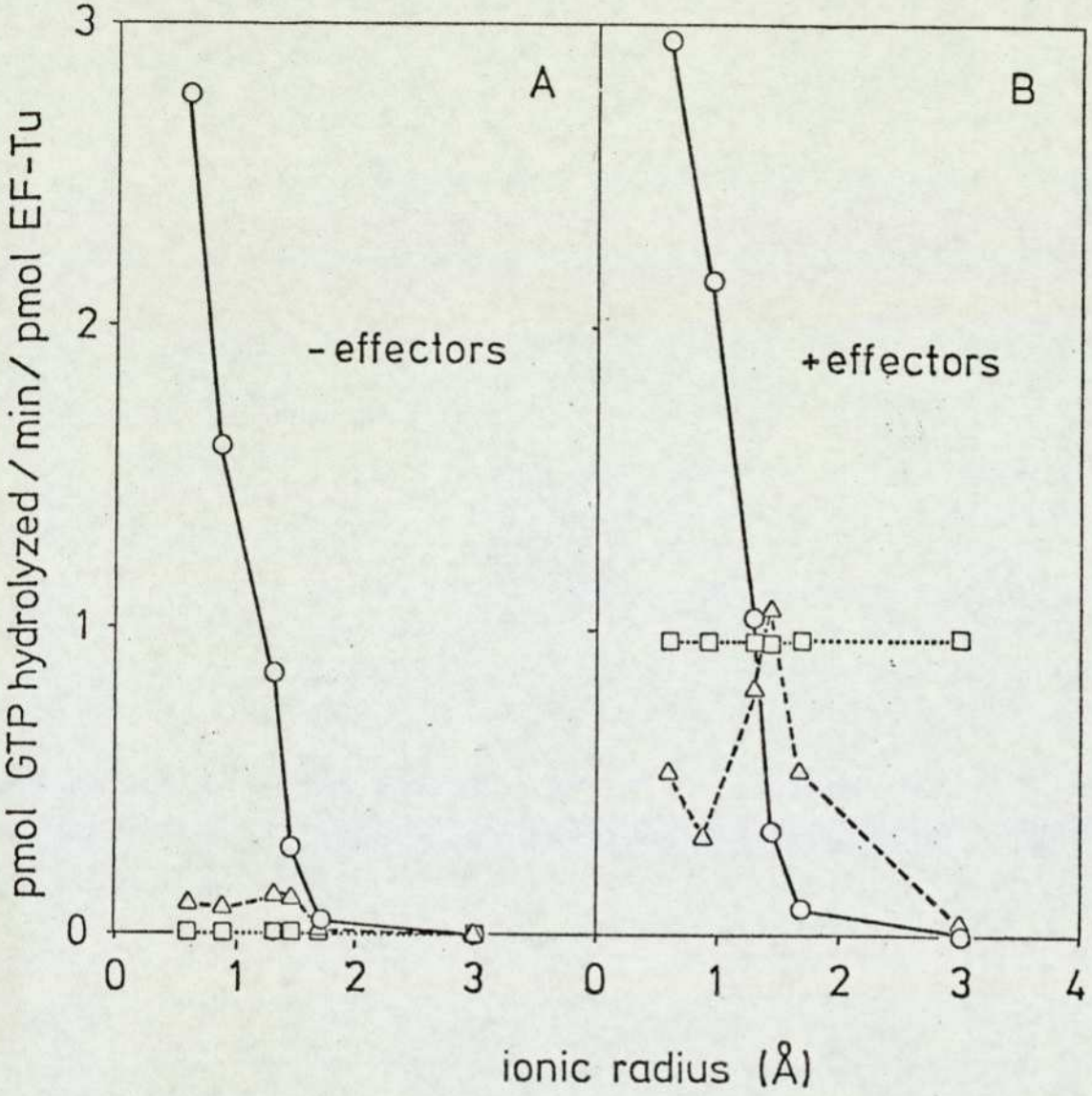


FIGURE 22 MONOVALENT CATION SPECIFICITY IN THE EF-Tu.KIRROMYCIN GTPase IN THE ABSENCE (A) AND PRESENCE (B) OF THE PHYSIOLOGICAL EFFECTORS, RIBOSOMES AND AMINOACYL-tRNA.

GTPase activities extracted from Fig.17 for 2M M^+ (o), 200mM M^+ (Δ) and <1mM M^+ (□).

inducing a conformational change from an inactive to an active enzyme.

The second type of response is most evident at 200mM $[M^+]$. Here NH_4^+ and K^+, and to a lesser degree Li^+ and Cs^+, in the presence of the effectors aa-tRNA and ribosomes (Fig.22B), appear to stabilize selectively the enzyme conformation found at lower monovalent cation concentrations, which is then progressively inhibited at higher concentrations, particularly by Cs^+ and Me_4N^+. A similar, though less pronounced, cation preference is also seen at this salt concentration by EF-Tu and kirromycin alone (Fig.22A). Thus, at 200mM, monovalent cations may be acting directly on the factor and/or via the physiological effectors.

The third response, at very low $[M^+]$ (Fig.22), indicates that the effectors, aa-tRNA and ribosomes, induce identical GTPase activity independently of the cation species. Taken together, these results suggest that, in the presence of kirromycin, the physiological effectors can induce an active conformation in EF-Tu, in the absence of free monovalent cations. This action of the physiological effectors is stabilized by some cations (e.g. NH_4^+ or K^+) but rapidly reduced by others (e.g. Li^+, Na^+, Me_4N^+). A very similar monovalent cation effect has been reported for peptidyl transferase activity of the 50S ribosomal subunit (84), and for non-enzymatic binding of aa-tRNA to the 30S subunit (85), where it was shown that the ribosomes, in the presence of certain cations (Li^+, Na^+) reversibly adopted an inactive conformation. In the case of EF-Tu GTPase activity stimulated by kirromycin and ribosomes, above about 800mM all monovalent cations reduce the activity close to that achieved by the EF-Tu.kirromycin complex alone. At high $[M^+]$ ribosomes and aa-tRNA may be constitutionally

altered due to partial denaturation. That there is little between-cation variation in the K_m below 200mM $[M^+]$ supports this indirect role of the monovalent cations, which thus may be acting largely via the physiological effectors. However, the preference for NH_4^+ and K^+ shown by EF-Tu.kirromycin at 200mM $[M^+]$in the absence of ribosomes and aa-tRNA (Fig.22A) suggests that there may also be a binding site(s) for these ions on the factor, as is found in a variety of other phosphate hydrolases (reviewed in 82). It would be of interest to determine the influence of the different cations on the kinetics of the EF-Tu.GTP and EF-Tu.GDP interactions, namely on their association and dissociation constants in the presence of kirromycin. It is known that the antibiotic "opens" the EF-Tu binding site for GDP, resembling at least qualitatively the action of EF-Ts, and by inhibiting the dissociation of GTP kirromycin raises the affinity of the factor for GTP to a value close to that for GDP, thus locking GTP onto EF-Tu, like aa-tRNA (28,29,86).

In the absence of the antibiotic, turnover GTPase activity requires mRNA bound to the ribosome and is strongly stimulated by EF-Ts. The action of mRNA, however, can be replaced by high $[Mg^{2+}]$ (87, see also section 4.3.7) or by methanol (36,80). Just as in the case of stimulation by ribosomes and aa-tRNA in the kirromycin-induced system, monovalent cations are not necessary for optimal or near-optimal activity, and inhibit the activity at relatively low $[M^+]$ (Fig.18). This strong inhibition occurring at a somewhat lower concentration of monovalent cations than in the presence of kirromycin can be interpreted as a consequence of a more specific series of interactions involving EF-Tu, ribosomes, aa-tRNA and mRNA, necessary to evoke the catalytic conformation of the factor. The increased specificity of

these interactions makes them more vulnerable to the intervention of strongly charged ions which can cause local unfolding in active regions.

At moderate to high ratios of EF-Ts to EF-Tu, as in the kirromycin-induced EF-Tu GTPase system, in the physiological EF-Tu GTPase system cation species of low ionic radius stimulate hydrolysis. In the presence of NH_4^+ ion the K_m for GTP is lower than those for the other cations, similar to the values observed at low $[M^+]$ in the presence of kirromycin, although for none of the cations is there any evidence for uncoupling from physiological effectors, as occurs with the antibiotic. EF-Ts becomes saturating at much lower concentrations in the presence of NH_4^+ than for the other ions, suggesting that NH_4^+ is constraining the EF-Tu in a conformation in which it is not accessible to EF-Ts, the reaction then being limited by other factors. It seems possible, therefore, that monovalent cations are acting, directly or indirectly, on the interaction between EF-Tu and EF-Ts, which encourages the replacement of GDP by GTP in the EF-Tu.guanine nucleotide complex (28,63), possibly via the same cation binding site(s) as on EF-Tu.kirromycin. It is known that NH_4^+ or K^+ are essential to stabilize ribosomal structure and cannot be replaced by Li^+, Na^+ or Cs^+ (88), suggesting that NH_4^+ may also act via a tightening of the ribosome, which in turn imposes a more constrained conformation on EF-Tu.

The results with the mutant $EF\text{-}Tu_{D2216}$ also support the view that the monovalent cations may be acting via the EF-Tu - EF-Ts interaction. EF-Tu from E.coli D2216 has been selected for its resistance to kirromycin. The fact that to inhibit peptidization by the antibiotic to 50% of its original value requires 500 times more kirromycin for the mutant than for the wild-type strain, indicates that the affinity of the EF-Tu

binding site for kirromycin is altered by the mutation. Since kirromycin and EF-Ts are considered to have overlapping binding sites on EF-Tu (32), it is then not suprising to find that the mutation may also have influenced the relationship of EF-Tu with EF-Ts, as is implied by the results presented in Fig.19B. However, it also appears that the same mutation has also influenced the effect of monovalent cations on the physiological EF-Tu GTPase, as measured both by the rate of GTP hydrolysis and by the K_m for GTP in this reaction. This result points to an association between the influence of monovalent cations and the EF-Tu - EF-Ts interaction.

Further research is in progress to clarify this, and to assess the influence of monovalent cations in the partial reactions of EF-Tu.

Comparison of the results for the wild-type EF-Tu GTPase systems in the presence and absence of kirromycin shows that the considerable stimulation by small cations at high $[M^+]$ takes place only in the presence of the antibiotic. Preliminary measurements of kirromycin UV difference spectra suggest that the cations are not acting via a detectable conformational change in the antibiotic. Similar measurements using EF-Tu instead of kirromycin have so far been hampered by the property of EF-Tu to polymerize at relatively low concentration (40). That activity is found at all at such high salt concentrations demonstrates that EF-Tu must be a remarkably stable protein, comparable to enzymes from halophilic bacteria (89).

It is perhaps significant that both K_m and the rate of GTP hydrolysis, in optimal conditions, in the systems with and without kirromycin, tend towards similar values: ca. 1μM and 3pmol GTP hydrolyzed/

min/pmol EF-Tu respectively, implying that the effect induced by several large and complex macromolecules (EF-Ts, ribosomes, aa-tRNA) can be replaced by two types of very small molecule: kirromycin (MW = 794) and a monovalent cation, and by corollary that the physiological effectors function in respect to EF-Tu in a similar way to the cations and kirromycin. Though direct involvement of ribosomal RNA in the EF-Tu GTPase is unlikely (90), cations and polyamines are certainly available, tightly bound to the ribosomes and aa-tRNA (88,91,92).

CHAPTER 4. THE ROLE OF DIVALENT CATIONS IN THE EF-Tu GTPase

4.1 Introduction.

In the presence of the antibiotic kirromycin, elongation factor - Tu hydrolyzes the γ-phosphate from GTP, with the properties of a turnover reaction (31,32), modelling the GTPase activity normally accompanying the enzymatic binding of aminoacyl-tRNA to the ribosome.mRNA complex. Both ribosomes and/or aa-tRNA stimulate the kirromycin-induced activity, though neither are an absolute requirement (31). In the last chapter it was shown that monovalent cations are intimately involved in the catalytic site for this GTP hydrolysis, in a fashion indicative of a highly charged anionic region close to the site of catalysis. Divalent cations are also implicated in a large number of similar hydrolytic reactions (93); in this chapter the role of divalent cations, and in particular of Mg^{2+}, is explored in the EF-Tu GTPase activity, starting with the simplest GTPase system, EF-Tu.kirromycin. Subsequently, the way in which the cation involvement is altered by interaction with ribosomes is looked at, and finally, the role of divalent cations in the complete physiological system, containing EF-Tu, EF-Ts, ribosomes, aa-tRNA and mRNA, is investigated.

4.2 Materials & Methods.

All materials and methods are as previously described (sections 2.2 and 3.2). GTPase activity was assayed by measuring the amount of ^{32}P liberated on hydrolysis of [γ-^{32}P].GTP, after sequestration of all nucleotides by 6% activated charcoal in 1N HCl. All assays

testing the effect of divalent cations also contained 0.5mM EDTA (Prolabo, France), a quantity in excess of the calculated free Mg^{2+} carried over with the biochemical components. Both EDTA and EGTA (Serva, West Germany) were neutralized to pH 7.5 by the addition of 2 moles/mole of Trisma base (Sigma) to avoid the introduction of small cations into the assay systems.

4.3 Results.

4.3.1 Effect of monovalent cations on the Mg^{2+}-requirement of the EF-Tu.kirromycin GTPase in the absence of ribosomes.

At 200mM monovalent cations, in the absence of ribosomes, a condition in which the EF-Tu.kirromycin GTPase shows a preference for K^+ or NH_4^+, closely followed by Li^+ and Na^+, with Cs^+ and Me_4N^+ having little or no effect (section 3.3), Mg^{2+} appears to have only slight influence on the hydrolytic activity. Values in the absence of added Mg^{2+} and in the presence of 0.5mM EDTA are only somewhat lower than those at 10 or 20mM $[Mg^{2+}]$ (Fig.23A). This pattern is parallel for all monovalent cations tested (tetramethylammonium having no stimulatory effect), and suggests that in these conditions free Mg^{2+} can be adequately replaced by a variety of monovalent cations.

At 2M monovalent cations, however (Fig.23C), Mg^{2+} stimulates activity up to 15-fold, though it is important to note that in the absence of Mg^{2+} (0.5mM EDTA) activity is still present, just as at 200mM monovalent cations. With the exception of activity in the presence of Li^+ ion whose $[Mg^{2+}]$ optimum is 5mM, the $[Mg^{2+}]$ optima for the remaining monovalent cations are all less than 2mM. Under these high ionic

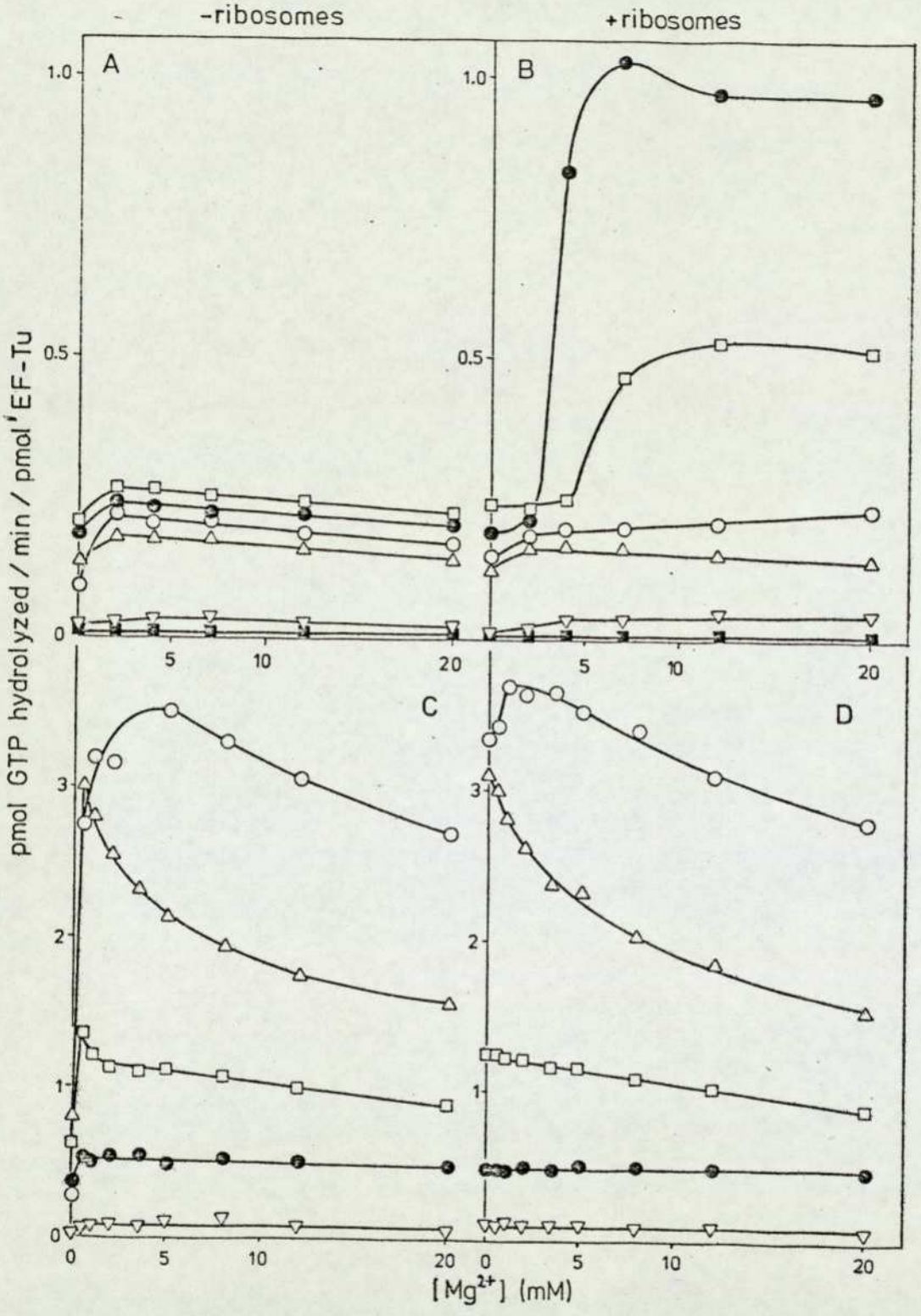


FIGURE 23 EFFECT OF Mg^{2+} ON THE EF-Tu.KIRROMYCIN GTPase IN THE PRESENCE OF VARIOUS MONOVALENT CATIONS. A-B, 200mM M^+, C-D, 2M M^+; A-C, EF-Tu. KIRROMYCIN ONLY, B-D, EF-Tu.KIRROMYCIN + RIBOSOMES.

Reactions were carried out as in Fig.16 in the monovalent cation concentrations indicated. Additionally all reaction mixtures contained 0.5mM EDTA. (o) Li^+, (Δ) Na^+, (□) K^+, (●) NH_4^+, (▽) Cs^+, (■) Me_4N^+.

strength conditions, monovalent cations can evidently not completely replace Mg^{2+} ions, suggesting two discrete roles for monovalent and divalent cations.

4.3.2 Effect of monovalent cations on the Mg^{2+}-requirement of the EF-Tu.kirromycin GTPase in the presence of ribosomes.

Ribosomes bind to EF-Tu at a site (or sites) functionally and structurally separated from the nucleotide binding site, which is also presumably the site of GTP hydrolysis (28,63,94). This interaction with the ribosome takes place principally via the 50S subunit (95, see also chapter 5). At 200mM $[M^+]$, there is a strong monovalent cation specific stimulation (Fig.23B) by ribosomes of the EF-Tu.kirromycin GTPase activity. This stimulation is clearly Mg^{2+}-dependent, being greater for NH_4^+, followed by K^+ and lastly Li^+; Na^+, Cs^+ and Me_4N^+, by comparison with Fig.23A, show scarcely any ribosomal stimulation. Comparing Figs.23A and 23B also shows that this ribosomal stimulation is additive to the Mg^{2+}-independent activity present in the absence of ribosomes, and only occurs at Mg^{2+} concentrations higher than about 5mM.

At 2M monovalent cations, ribosomes have little or no stimulatory effect on the already considerable GTP hydrolysis which takes place in the absence of physiological effectors, and shows a monovalent preference in the order $Li^+ > Na^+ > K^+ > NH_4^+ > Cs^+$. However, ribosomes eliminate entirely the requirement for Mg^{2+} ions to induce maximum activity. The only significant difference between Figs. 23C and 23D is that in the absence of free Mg^{2+} (0.5mM EDTA) ribosomes induce maximum GTPase activity for all cations except Li^+, where the optimum is at 1mM Mg^{2+}. Again, the fact that for all monovalent cations

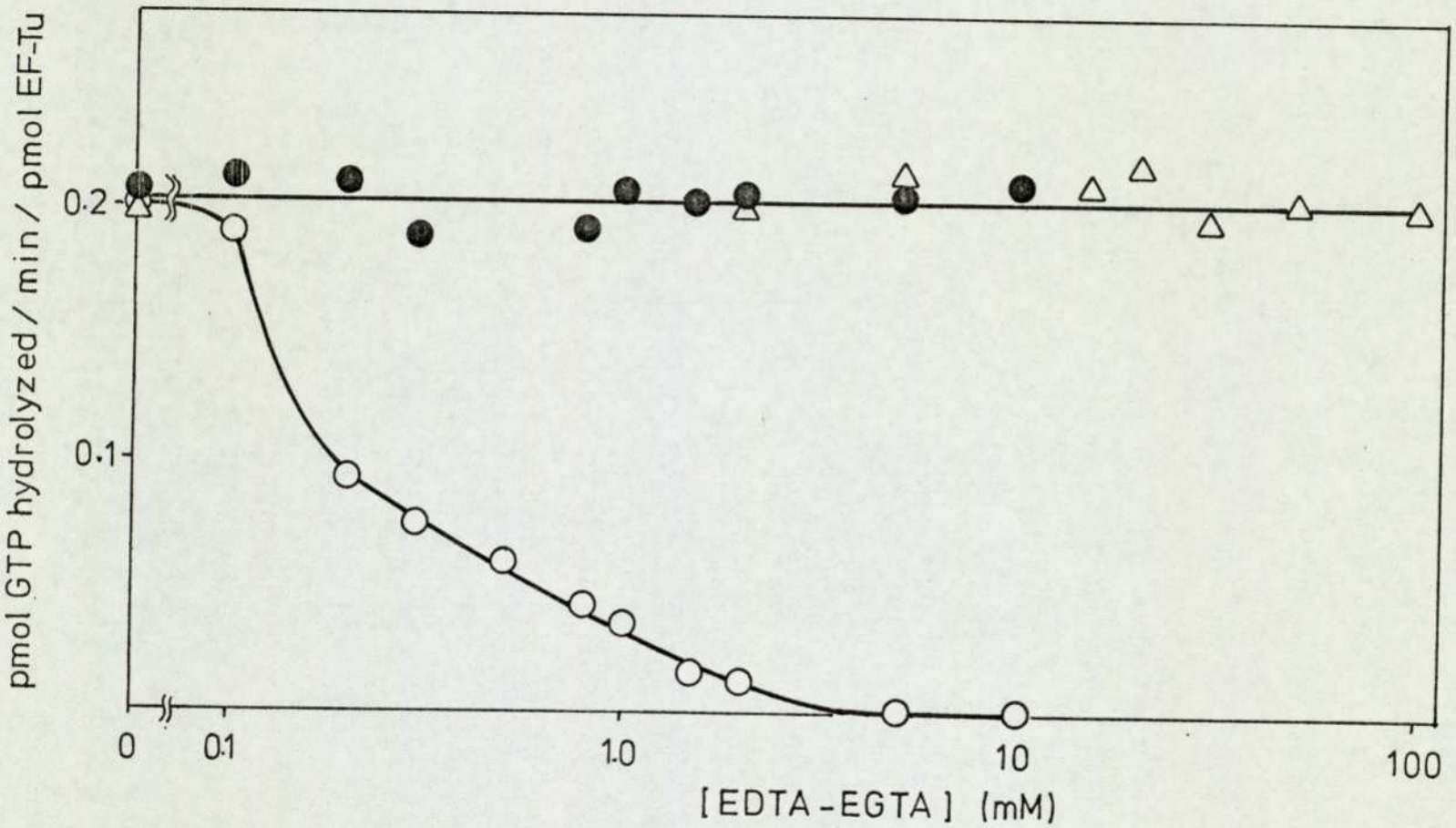


FIGURE 24 EFFECT OF EDTA AND EGTA ON THE EF-Tu.KIRROMYCIN GTPase ACTIVITY IN THE ABSENCE OF FREE Mg^{2+}.

Reaction mixtures contained in 75µl, not more than 0.02mM $MgCl_2$, 50mM Imidazolium acetate pH 7.5, 200mM KCl, 20pmol EF-Tu, 50µM kirromycin and 800pmol GTP (specific activity ca.200 cpm/pmol). (o) EDTA, (Δ) EGTA, (●) EDTA + 10mM $MgCl_2$.

the effect of $[Mg^{2+}]$ (or ribosomes) is similar suggests that monovalent cations cannot replace divalent ones, and that these thus perform mutually exclusive functions.

4.3.3 The EF-Tu.kirromycin GTPase in the absence of free Mg^{2+}.

It is quite unusual for phosphorylase-type enzymes, in which there is P-O cleavage, to show activity in the absence of a divalent metal ion (93). That EF-Tu, shown to have turnover GTPase activity of this type (31), can function in the absence of Mg^{2+} ions (0.5mM EDTA) is thus clearly of interest. This activity was explored by titration against the chelating agents EDTA and EGTA (Fig.24). The results are clear; in the presence of sufficient EDTA all activity is eliminated. That this effect is not due to an inhibition by EDTA itself is shown by the control assay in the presence of excess Mg^{2+}. EGTA has no effect. Since the latter differs functionally from EDTA only in its chelating capacity for Mg^{2+} ions (log K_{EDTA} = 8.9; log K_{EGTA} = 5.4; ref.96), these results show that Mg^{2+} is indeed critically required for EF-Tu GTPase activity. That such large amounts of EDTA are necessary (ca.5mM) for complete inhibition implies, however, that the Mg^{2+} ions must be tightly bound to the EF-Tu.kirromycin complex, and not loosely bound, perhaps via substrate.

4.3.4 The influence of ribosomes on the EF-Tu.kirromycin GTPase in the absence of free Mg^{2+}.

In Fig.23D it was demonstrated that at 2M monovalent cations, ribosomes could completely replace free Mg^{2+}, even in the presence of sufficient EDTA to chelate all free divalent cations. As in the

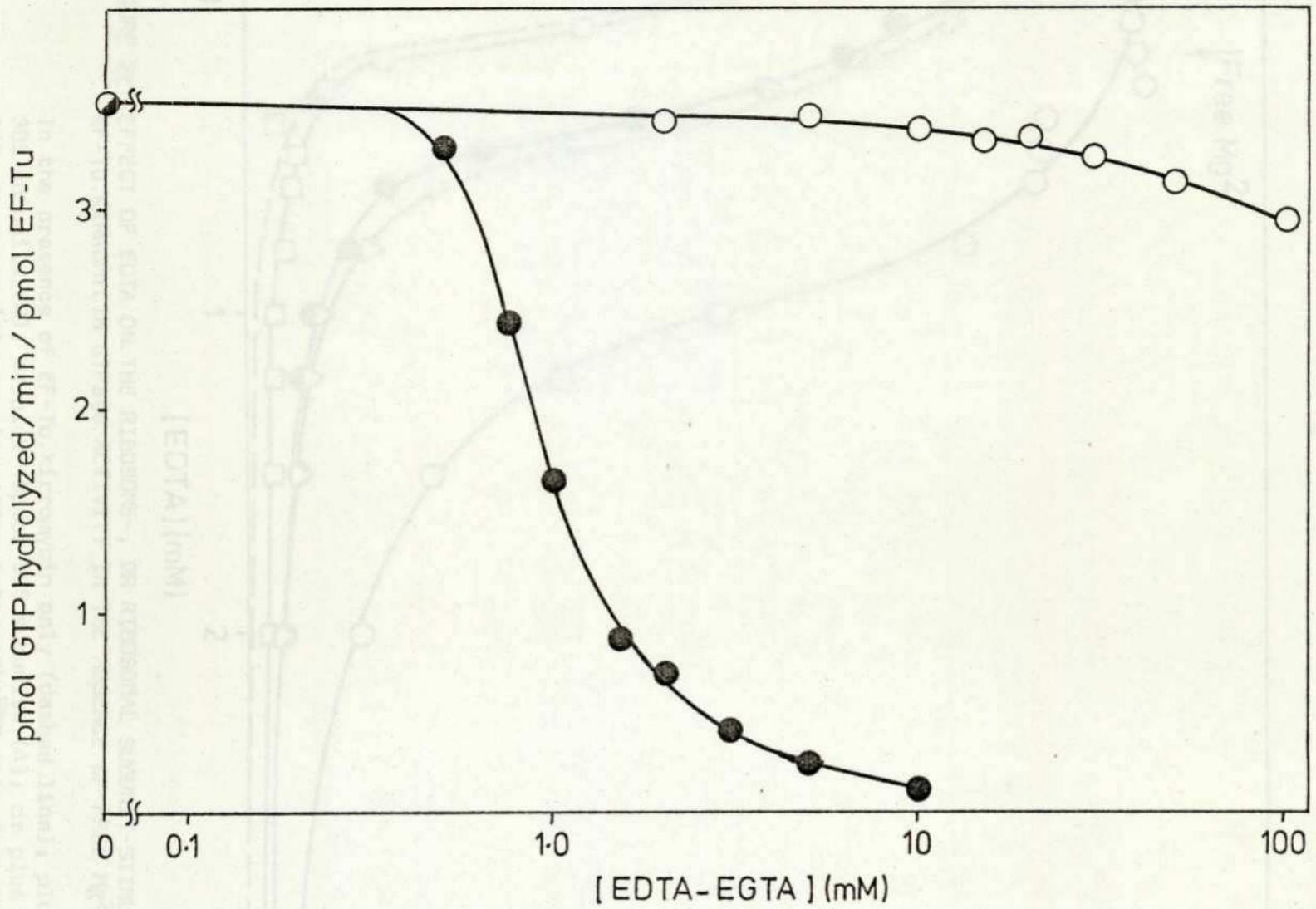


FIGURE 25 EFFECT OF EDTA AND EGTA ON THE EF-Tu.KIRROMYCIN GTPase ACTIVITY AT 2M Li^+ IN THE PRESENCE OF RIBOSOMES AND IN THE ABSENCE OF FREE Mg^{2+}.

Reaction mixtures contained in 75µl, not more than 0.25mM free Mg^{2+}, 2M LiCl, 50mM Imidazolium acetate pH 7.5, 20pmol EF-Tu, 40pmol ribosomes, 50µM kirromycin and 2nmol GTP (specific activity 100-150 cpm/pmol). (•) EDTA, (o) EGTA.

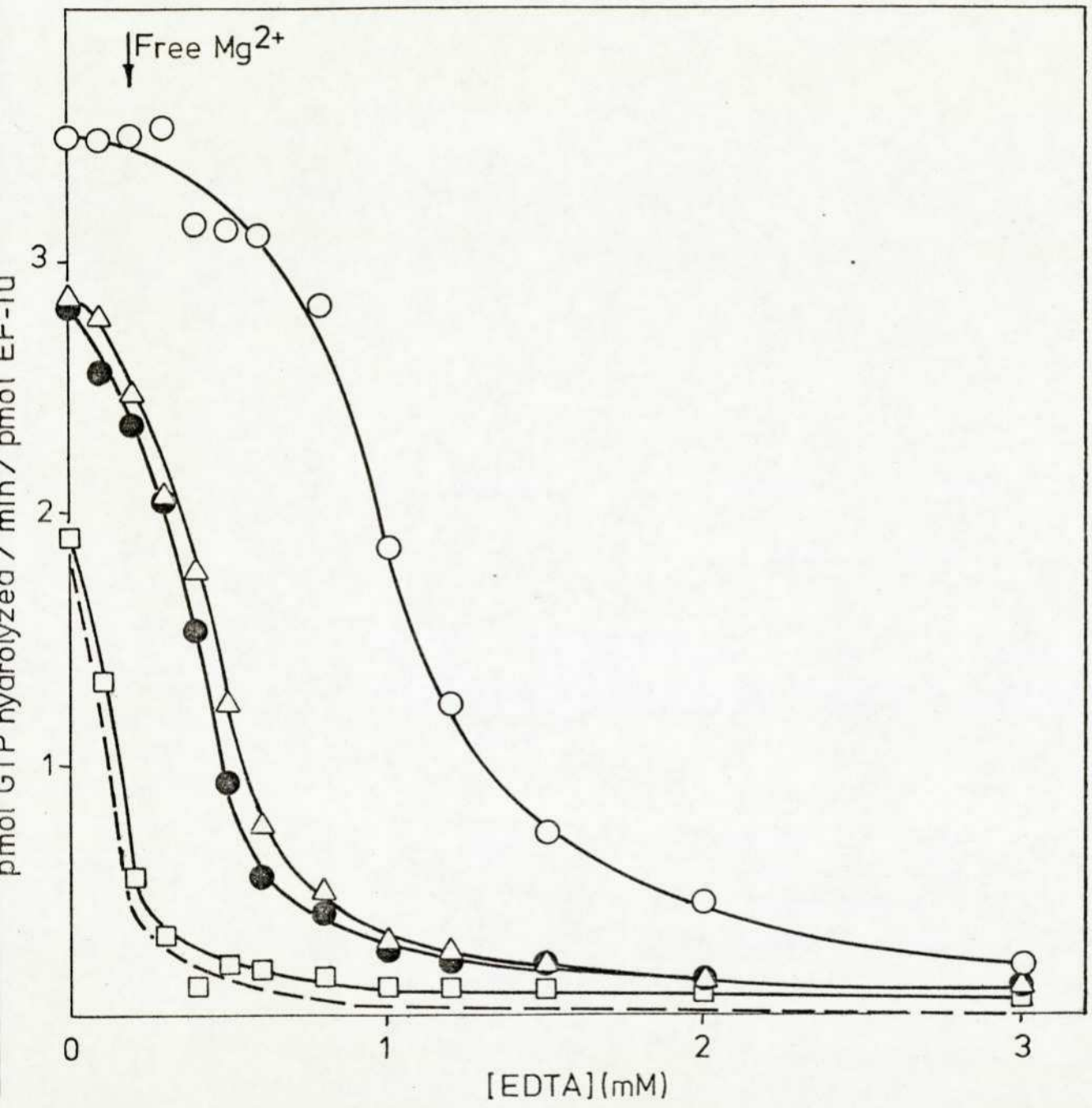


FIGURE 26 EFFECT OF EDTA ON THE RIBOSOME-, OR RIBOSOMAL SUBUNIT-STIMULATED EF-Tu.KIRROMYCIN GTPase ACTIVITY IN THE ABSENCE OF FREE Mg^{2+}.

In the presence of EF-Tu.kirromycin only (dashed line); plus 40pmol 30S subunits (□); or plus 40pmol 50S subunits (Δ); or plus 40pmol 70S ribosomes (○); or plus 100pmol Phe-$tRNA^{Phe}$ (●). Other conditions as in Fig.25 .

preceding section, this situation has been explored by titration with the chelating agents EDTA and EGTA. Fig.25 shows that the primary effect of the ribosomes is via tightly bound Mg^{2+} ions, as in the case of EF-Tu alone. With ribosomes, although only 0.2mM Mg^{2+} is carried over in solution, nearly 10mM EDTA is required for complete inhibition. This effect was further investigated in Fig.26, where ribosomal subunits and aminoacyl-tRNA are also tested for the Mg^{2+} ion-dependence of their stimulatory activity. Neither subunit had the Mg^{2+}-equivalence of the 70S ribosomes, though 50S subunits still substantially retarded the inhibitory effect of EDTA. 30S subunits appeared to have little influence. Aminoacyl-tRNA, known to have at least four tightly bound Mg^{2+} ions per molecule (92), had almost as much effect as the 50S subunits.

It should be remembered that at 2M monovalent cations, the conditions of this assay, ribosomes are probably not in their natural conformation, and in 2M Li^{+} have lost some proteins (see section 3.3.2), though these will be free in solution.

4.3.5 The specificity of divalent cations in the EF-Tu.kirromycin GTPase.

Two assay systems, both in the absence of ribosomes, were used to assess the effect of other divalent cations on the EF-Tu. kirromycin GTPase (FIgs.27A&B). At 200mM $[NH_4^{+}]$, in the absence of divalent cations (0.5mM EDTA) there is already some GTPase activity. At 5mM $[M^{2+}]$, only the Class IIA metals (alkaline earths) (Be^{2+} excepted) and Mn^{2+} stimulate this activity in the order $Mn^{2+} > Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$. All other divalent metal ions tested strongly

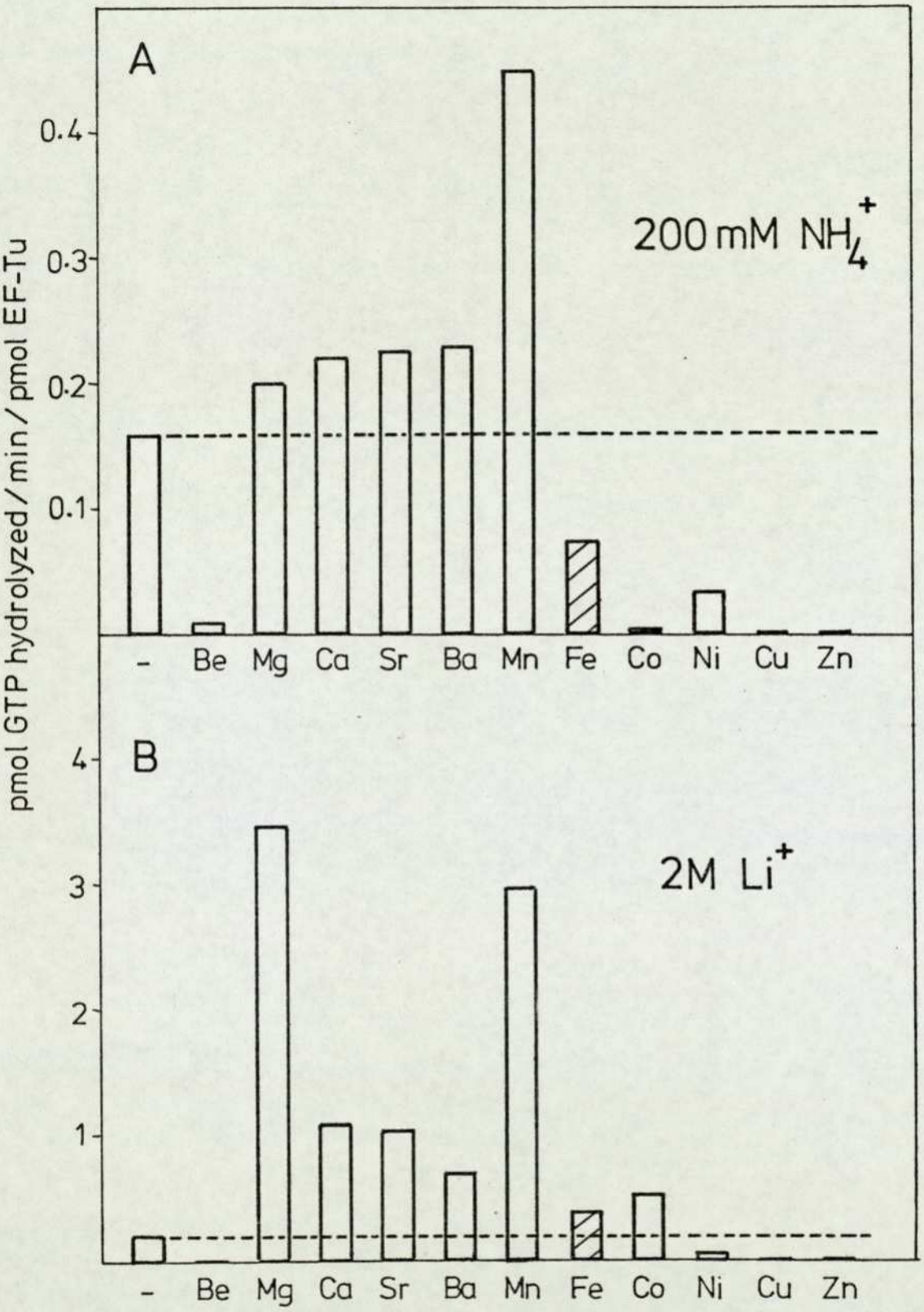


FIGURE 27 EFFECT OF DIVALENT CATIONS ON THE EF-Tu.KIRROMYCIN GTPase.
A. AT 200mM NH_4Cl; B. AT 2M LiCl.

Reaction mixtures contained in 75µl, 5mM $M^{2+}Cl_2$, monovalent cations as indicated, 50mM Imidazolium acetate, pH 7.5, 20pmol EF-Tu, 50µM kirromycin and 1 or 2 pmol GTP (specific activity 100-200 cpm/pmol). The hatched bar for Fe^{2+} indicates that this value did not differ from the blank in the absence of EF-Tu.

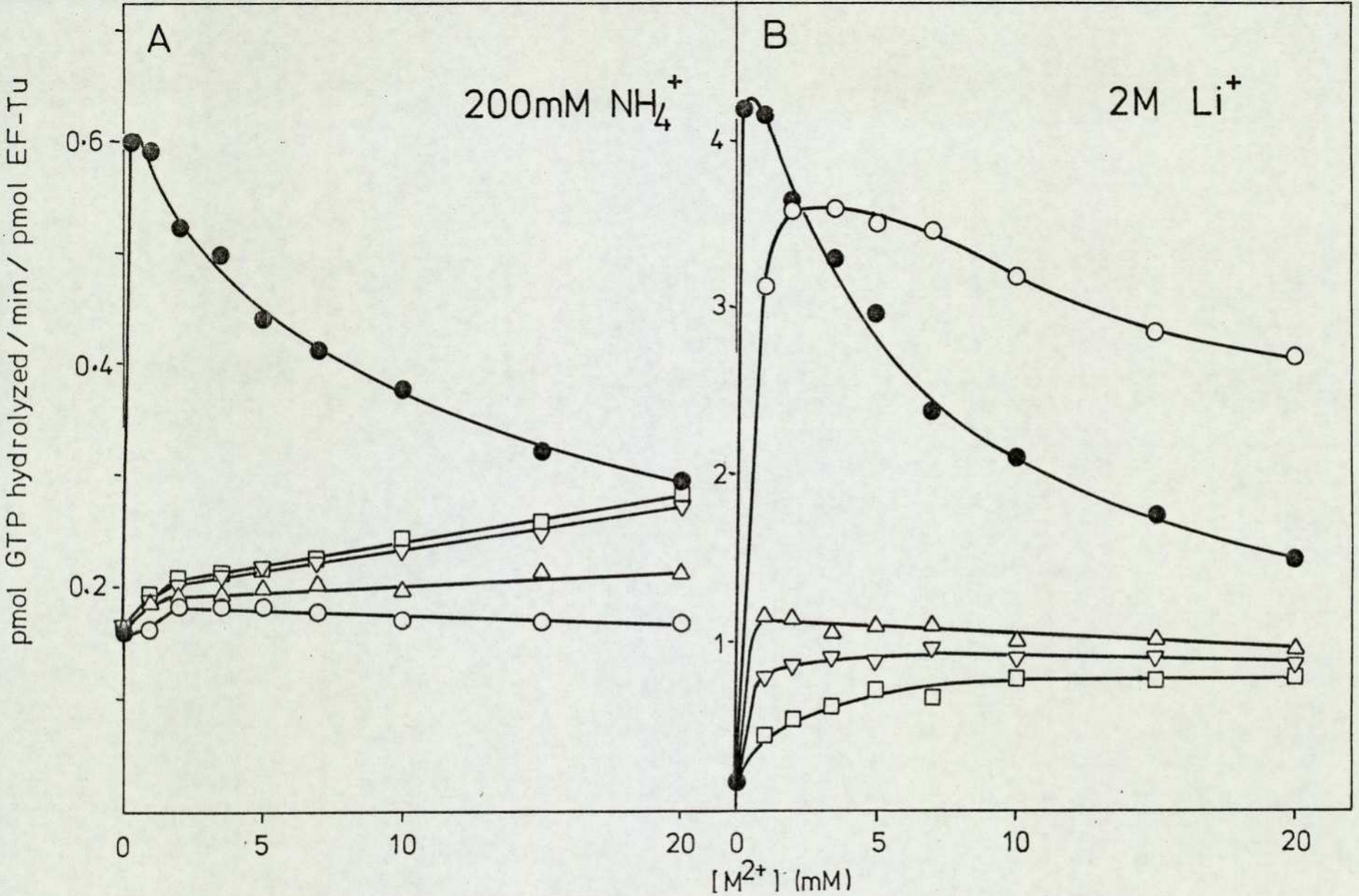


FIGURE 28 CONCENTRATION CURVES FOR THE EFFECT OF DIVALENT CATIONS ON THE EF-Tu.KIRROMYCIN GTPase. A. AT 200mM NH_4Cl; B. AT 2M LiCl.

All reaction mixtures contained 0.5mM EDTA; other conditions as in Fig.27. (o) Mg^{2+}, (Δ) Ca^{2+}, (▽) Sr^{2+}, (□) Ba^{2+}, (●) Mn^{2+}.

inhibited this basal activity (Fig. 27A). The second assay system, at 2M [Li^+], gave similar results, the Class IIA metals (Be^{2+} excepted) and Mn^{2+} stimulating; Co^{2+}, however, which at 200mM [NH_4^+] inhibited, here also shows stimulatory activity. At 2M [Li^+], the order of effectivity of the divalent cations is $Mg^{2+} > Mn^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+} > Co^{2+}$ (Fig 27B).

Control experiments carried out in the absence of EF-Tu showed that with the exception of Fe^{2+}, there was no non-enzymatic hydrolysis of GTP, catalysed directly by the metal ions, as can occur in some model systems (97). It is known, however, that buffers such as Tris.HCl or Imidazolium acetate strongly inhibit such metal-mediated hydrolysis (97).

Concentration curves were made for the Class IIA metal ions (Be^{2+} excepted) and Mn^{2+} at 200mM [NH_4^+] and 2M [Li^+] (Figs.28A&B). In the former condition, the larger the Class IIA ion, the greater the stimulatory activity, and activity optima, with the exception of Mg^{2+} and Mn^{2+}, were greater than 20mM [M^{2+}]. At 2M [Li^+], by contrast, the Class IIA ions had the opposite specificity, Mg^{2+}, the smallest, being the most active, and Ba^{2+} least active; activity optima for these metals were all below 10mM, and corresponded approximately to ion size: Mg^{2+} and Ca^{2+} having the lowest optima, followed by Sr^{2+} and lastly Ba^{2+}. Mn^{2+} behaved in a different way to the Class IIA metals. Firstly, the shape of the Mn^{2+} concentration curve did not follow those of the Class IIA family, in fact, crossing over the Mg^{2+} curve at 2M [Li^+], and appeared to be quite uninfluenced by the monovalent cation condition, with activity optima in both assay systems below 0.2mM.

In experiments not shown, there was no stimulatory effect

at all on the EF-Tu.kirromycin GTPase of the polyamines, putrescine, spermidine and spermine, whether in the presence or absence of additional Mg^{2+} ions.

4.3.6 The effect of pH and Mg^{2+} on the EF-Tu.kirromycin GTPase.

That enzymatic activity could be registered in the apparent absence of free Mg^{2+} allowed the possibility of checking the role of these ions in masking negative charges involved in the catalytic centre, and the extent to which Mg^{2+} could be functionally replaced by a monovalent cation, K^+. K^+ was chosen since it showed least influence on pH when present at high concentration. In Fig.29 are illustrated pH curves at two monovalent cation concentrations, 200mM and 2M K^+, in the presence and absence of free Mg^{2+} ions . In the presence of Mg^{2+} (Fig.29B) curves at high and low $[K^+]$ are similar in form, with optima between pH 7.0 and 8.5. In the absence of free Mg^{2+} (Fig.29A), not only is there a marked shift of pH optima to more acidic values, but there is also a clear influence of monovalent cations, high concentrations of which shift the pH optimum from 6.5 - 7.0 at 200mM $[K^+]$ to 5.8 at 2M $[K^+]$. These observations are completely in accord with the predictions of the polyelectrolyte theory of enzyme regulation (98,99), and imply the existence in the catalytic centre of a strongly charged anionic region, normally masked by Mg^{2+}. In the absence of Mg^{2+}, monovalent cations can modulate the electrostatic potential of this catalytic region.

The converse experiments were also carried out (Fig.30) in which, in the presence and absence of Mg^{2+}, the effect of pH was assessed on the $[K^+]$ optima for the EF-Tu.kirromycin GTPase. In the absence of Mg^{2+} (Fig.30A) the $[K^+]$activity optimum was shifted to

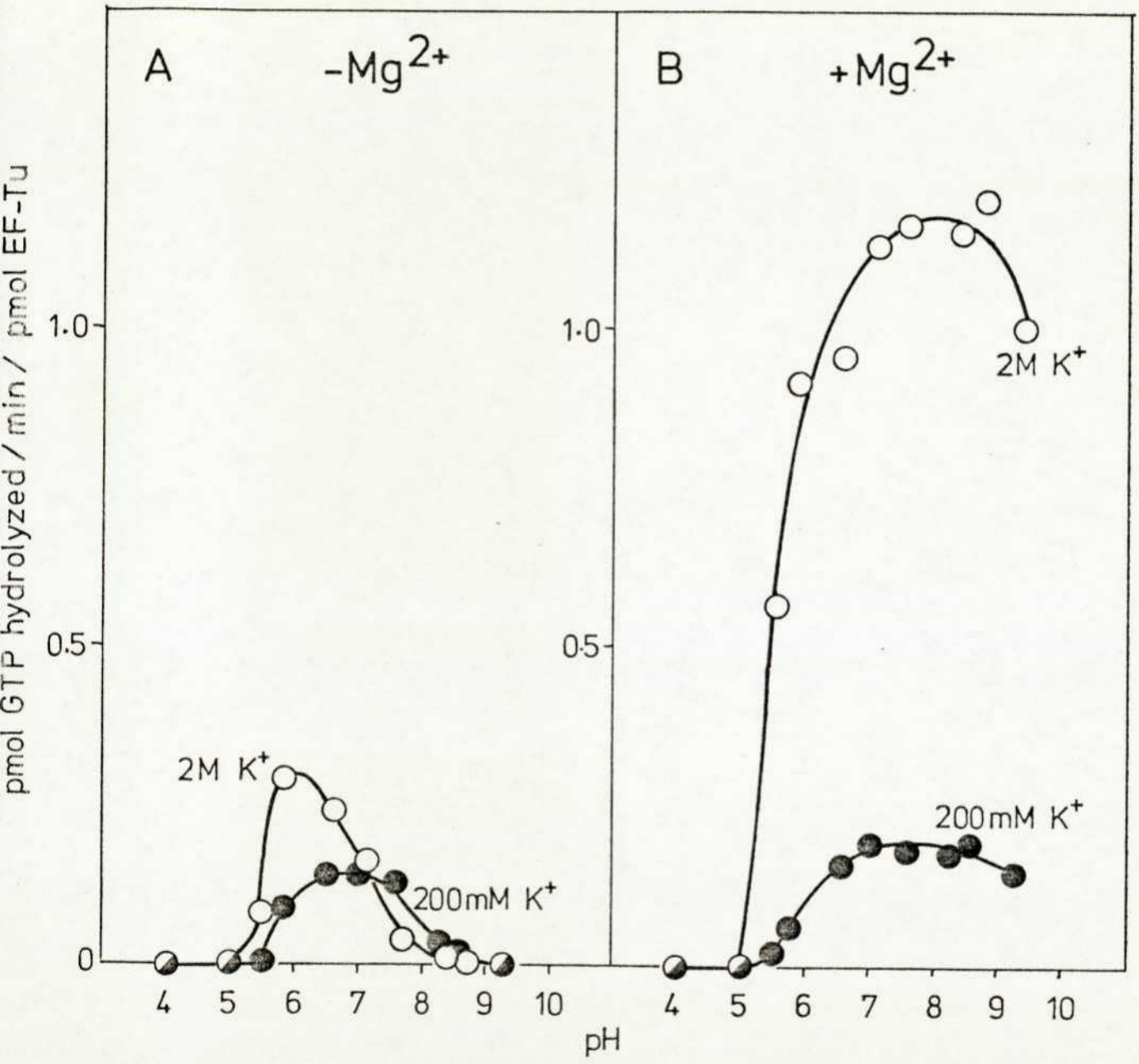


FIGURE 29. EFFECT OF pH ON THE EF-Tu.KIRROMYCIN GTPase AT DIFFERENT MONOVALENT CATION CONCENTRATIONS IN THE ABSENCE (A) OR PRESENCE (B) OF Mg^{2+}.

Reaction mixtures contained in 75µl, 5mM $MgCl_2$ as indicated, either 0.2 or 2M KCl as indicated, 50mM Tris.HCl of appropriate pH when measured under incubation conditions, 0.5mM EDTA, 20pmol EF-Tu, 50µM kirromycin, 1nmol GTP (specific activity 200-250 cpm/pmol). (o) 2M K^+, (•) 200mM K^+.

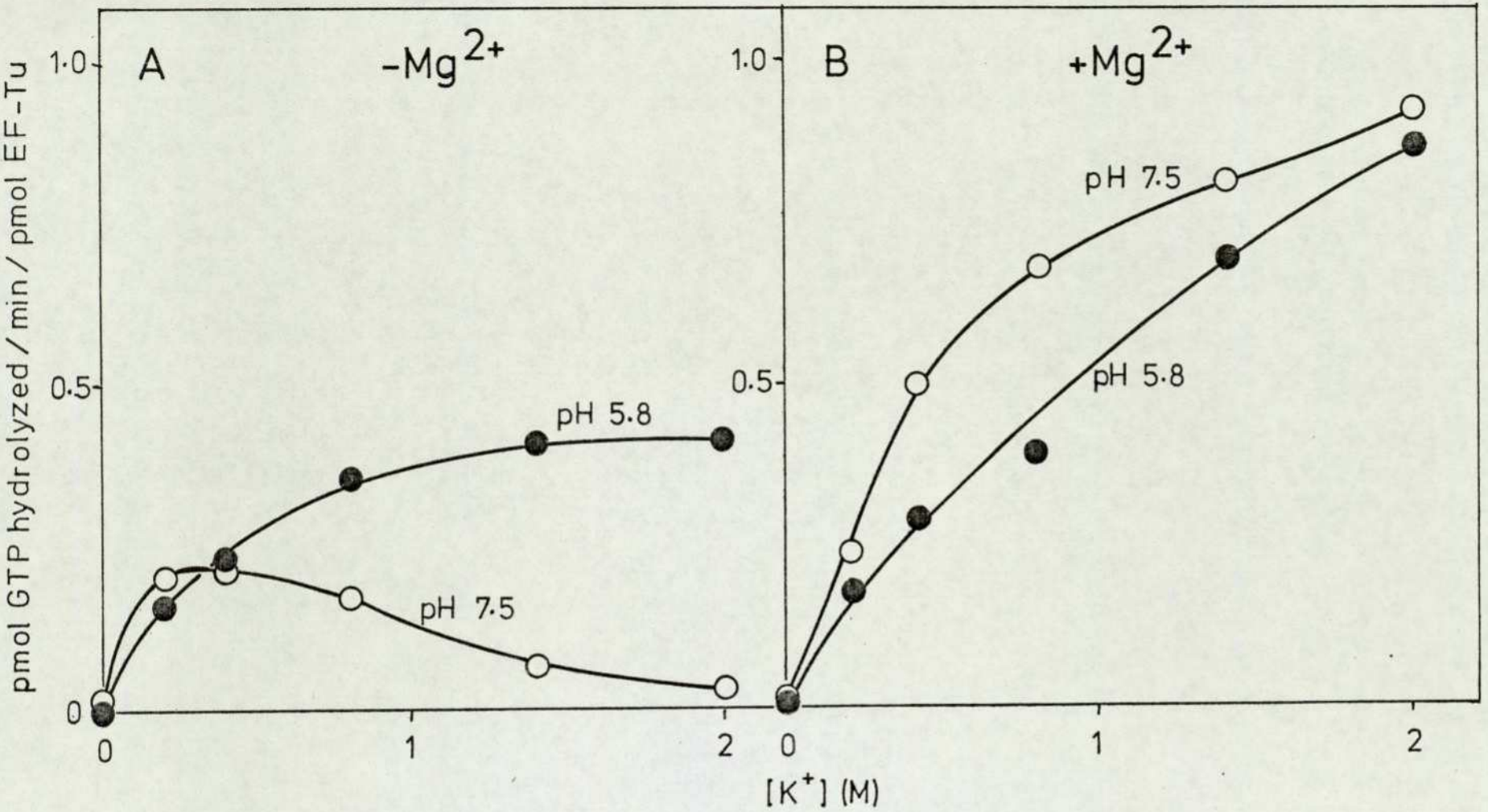


FIGURE 30 EFFECT OF K^+ CONCENTRATION ON THE EF-Tu.KIRROMYCIN GTPase IN DIFFERENT pH CONDITIONS IN THE ABSENCE (A) OR PRESENCE (B) OF Mg^{2+}.

Reaction conditions as in Fig.29. (●) pH 5.8, (○) pH 7.5 .

lower values by raising the pH from 5.8 to 7.5. A similar though far less marked shift is also visible in the presence of Mg^{2+} (Fig.30B).

4.3.7 The influence of divalent cations on the physiological EF-Tu GTPase in the absence of kirromycin.

The presence of Mg^{2+} has been shown to affect the dependence of the EF-Tu GTPase, in the presence also of EF-Ts, ribosomes and aminoacyl-tRNA, on messenger RNA being bound to the ribosomes (87). Therefore in Fig.31 divalent cations have been tested not only for their ability to stimulate the complete physiological system, containing ribosome.mRNA complexes, but also for their influence on the mRNA-dependence of these EF-Tu GTPase activities.

In complete contrast to kirromycin-induced systems, there was no activity registered in the absence of a divalent cation (0.5mM EDTA).

Mg^{2+} (Fig.31A) shows optimal stimulation at about 25mM, though at this concentration there is almost no requirement for mRNA. At 5mM $[Mg^{2+}]$, however, the GTPase activity, albeit lower, is almost entirely dependent on the presence of mRNA. The other divalent cations present a slightly different picture. $[M^{2+}]$ optima in the presence of mRNA are all less than 10mM, Ca^{2+} having the lowest at ca. 5mM, followed by Sr^{2+} (ca. 7mM), Ba^{2+} (ca. 10mM) and Mn^{2+} (ca. 10mM). There is also a large increase in total hydrolytic activity. In the assay system used (10pmol EF-Tu, 30pmol EF-Ts, 20pmol ribosomes, 100pmol Phe-$tRNA^{Phe}$ and, where present, 4µg poly(U) in 50mM Imidazolium acetate pH 7.5 and 30mM NH_4Cl per 75µl of reaction mixture), at optimal M^{2+} concentrations, Ca^{2+}, Sr^{2+} and Ba^{2+} all induce an activity of about 4pmol GTP hydrolyzed/min/pmol EF-Tu; Mn^{2+} hydrolyzed 5.5pmol GTP/min/pmol EF-Tu; Mg^{2+}, in contrast, hydrolyzed only 2pmol GTP/min/pmol EF-Tu.

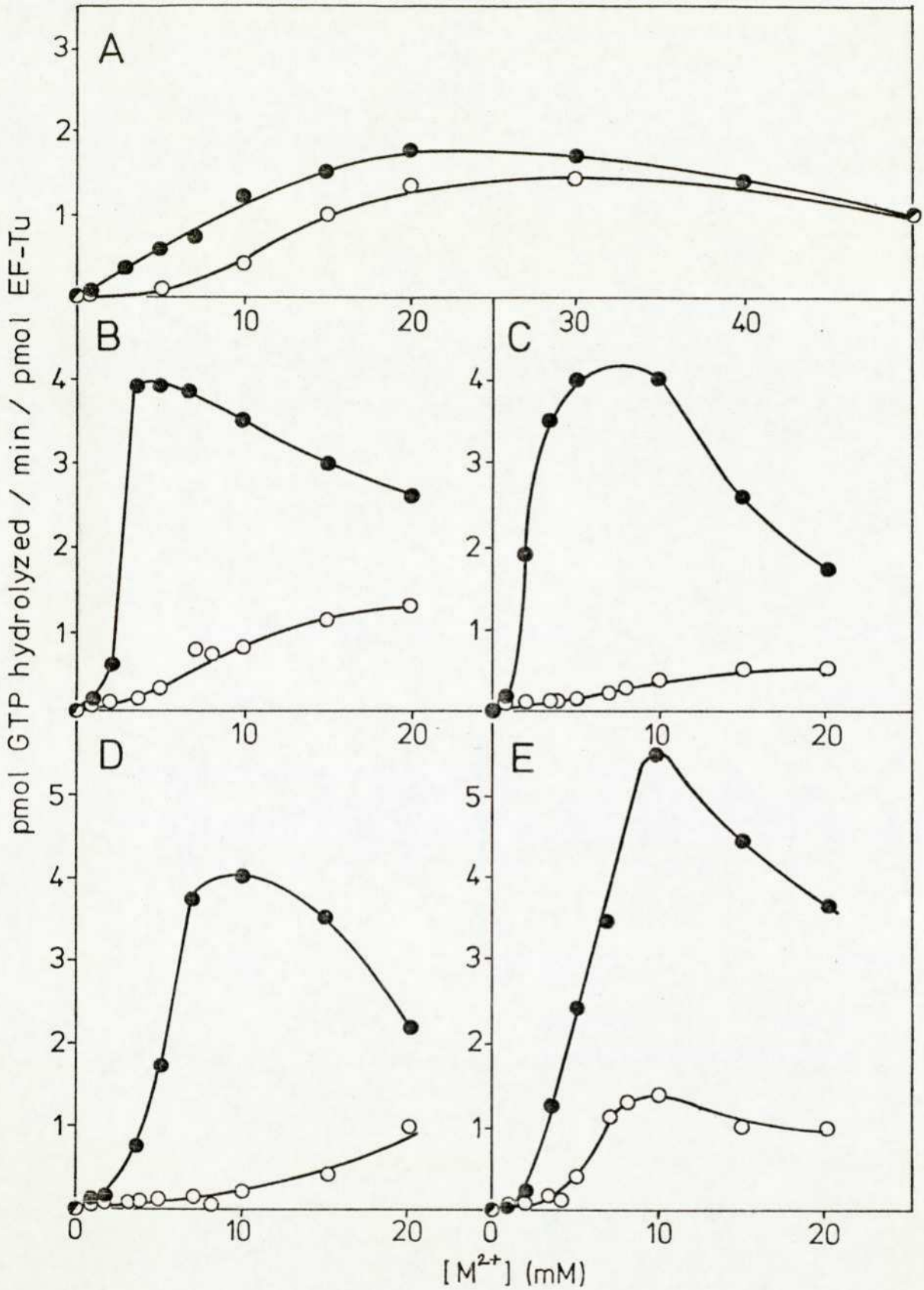


FIGURE 31 EFFECT OF DIVALENT CATIONS ON THE PHYSIOLOGICAL EF-Tu.GTPase, AND ITS REQUIREMENT FOR CODON-ANTICODON INTERACTION.

Reaction mixtures contained in 75µl, 30mM NH_4Cl, 0.5mM EDTA, 50mM Imidazolium acetate, pH 7.5, 2nmol GTP (specific activity 100-150 cpm/pmol), 10pmol EF-Tu, 30pmol EF-Ts, 100pmol Phe-$tRNA^{Phe}$, 20pmol ribosomes, with (●) or without (○) 4µg poly(U).
A. Mg^{2+}, B. Ca^{2+}, C. Sr^{2+}, D. Ba^{2+}, E. Mn^{2+}.

Furthermore, the larger cations are all considerably more dependent on the presence of mRNA, especially at concentrations below 10mM M^{2+}, though, as in the case of Mg^{2+}, increased divalent cation concentration does decrease the absolute requirement for messenger.

4.4 Discussion.

Elongation factor - Tu is a GTPase, catalyzing the hydrolysis of the γ-phosphate of GTP. Normally, a variety of different macromolecular effectors (EF-Ts, ribosomes, mRNA, aa-tRNA) are necessary to put EF-Tu into an active conformation for GTP hydrolysis. Recently, it has been shown that these macromolecules can be functionally replaced by the small antibiotic kirromycin and monovalent cations (79, see section 3.4), thus providing an assay system allowing an assessment of the role of divalent cations firstly in the GTPase, and secondly in the interaction between EF-Tu and ribosomes.

An important finding of this study has been that in moderate ionic conditions (200mM NH_4^+) free Mg^{2+}, or loosely bound Mg^{2+}, are not required for GTPase activity in the presence of kirromycin. In other nucleotide phosphorylase systems, a divalent cation is suggested to coordinate to the substrate via the α- and β-phosphate groups, labilizing the γ-phosphate (93). In several studies on the binding of GDP to EF-Tu it has been shown that such metal-substrate coordination does take place (86,100,101), though the Mg^{2+}, thus bound, can be removed by as little as 0.1mM EDTA in the buffer system (102). In fact, the absence of Mg^{2+} leads to a decreased affinity of the GDP for EF-Tu by 2-3 orders of magnitude (102,103). The ability of Mg^{2+} to encourage binding of the nucleotide can be replaced by Mn^{2+}, Ba^{2+} or Sr^{2+}, but not apparently by either Ca^{2+} or monovalent cations (100).

However, such divalent metal coordination to the substrate is apparently not an absolute prerequisite for EF-Tu GTP hydrolysis and suggests that other mechanisms, in which local charges or monovalent cations are implicated, may be involved, with Mg^{2+} in a modulator role, possibly through masking local anionic charges. Monovalent cations may also be able to perform such a modulatory role. The finding that at high monovalent cation concentrations there is firstly a considerable stimulation by Mg^{2+}, and secondly a greater specificity for this cation, at least in the kirromycin-induced system, among the Class IIA metals, suggests that in these conditions there is a greater stringency in the cation requirement than in moderate ionic conditions, and that Mg^{2+} has here a very specific function. It may well be, that monovalent cations, in inducing a more active enzymatic conformation in the EF-Tu, also impose greater constraints on the active site for catalysis and hence the divalent cation requirement. Mn^{2+} is completely able to replace Mg^{2+} and because of its low optimum evidently has a high affinity binding site, not influenced by monovalent cation concentration. The high affinity of Mn^{2+} for EF-Tu.GDP has already been reported (86, 100) and may reflect a specific function for this metal.

The polyelectrolyte theory of Maurel and Douzou (98,99), in which they suggest that a local polyionic environment, modulated by free ions, may be responsible for the regulation of a variety of enzyme systems, fits the data obtained with the EF-Tu.kirromycin GTPase remarkably well. The marked acidic shift in the absence of Mg^{2+}, which is augmented by high $[M^{+}]$, follows exactly the prediction of this hypothesis based on there being a strong anionic region close to, and intimately involved in, the centre of catalysis. Mg^{2+} ions would then have the role of modulators by variously screening this

highly charged anionic field. These experiments provide independent proof for the conclusions already drawn from the effects of monovalent cations on the EF-Tu.kirromycin GTPase (chapter 3).

Data in the literature (100,102), as well as the effect of 0.5mM EDTA on the pH curves (section 4.3.6) clearly imply that 0.5mM EDTA is more than sufficient to chelate Mg^{2+} or other divalent cations involved in EF-Tu - nucleotide binding; in fact, 0.2mM EDTA is routinely used by some authors to liberate EF-Tu from its bound GDP by passing over a column of Sephadex G-50 or G-25 (100). However, the EDTA/EGTA curves (section 4.3.3) show that there is additionally some strongly bound Mg^{2+} responsible for active function of the EF-Tu.kirromycin GTPase. Wittinghofer & Leberman (100) have shown that both Mg^{2+} and Mg.GDP are effective in inducing an EF-Tu conformation, in the enzyme derived from Bacillus stearothermophilus, which is more resistant to trypsin digestion, and also that Mg^{2+} can influence the modification of -SH groups by **N**-ethyl maleimide. Taken together, these results imply at least two roles for Mg^{2+} ions: a low affinity role involved directly in nucleotide binding and possibly catalysis, and a high affinity role probably concerned in the structural integrity of EF-Tu, much as is the case for $tRNA^{Phe}$ (92).

The role of ribosomes in stimulating the EF-Tu.kirromycin GTPase is especially interesting. At high monovalent cation concentrations, Mg^{2+} has no stimulatory effect in the presence of ribosomes, which in fact eliminate the requirement for Mg^{2+} expressed in their absence. When this phenomenon was studied using EDTA titration (section 4.3.4), the 50S subunit was shown to have the greatest effect implying an interaction between EF-Tu and this subunit. These results may reflect either the direct participation of Mg^{2+} ions, tightly bound to the

ribosomes, or an indirect role whereby a Mg^{2+}-dependent conformation of the ribosomes, by interacting with EF-Tu, protects the latter from the denaturing effects of EDTA. At moderate monovalent cation concentrations (200mM) there is a monovalent cation - dependent interaction between ribosomes and EF-Tu wherein Mg^{2+} has a considerable stimulatory role, implying that in these conditions ribosomes have a more specific effect which cannot be substituted by Mg^{2+} alone. Mg^{2+} is important for ribosomal subunit interaction (104,105), as well as for aminoacyl-tRNA binding (106), probably by modulation of electrostatic forces associated with the phosphate backbone of ribosomal RNA (104). Since there is no suggestion of a direct EF-Tu - rRNA interaction (90), all these results would tend to support the view that in moderate ionic conditions Mg^{2+} may be responsible for an active ribosome conformation which allosterically stimulates the active site for GTP hydrolysis on EF-Tu.

The results from the physiological EF-Tu GTPase in the absence of kirromycin show firstly a complete dependence on divalent cations, unlike the kirromycin-induced system, thus implying that kirromycin alone can, in part, induce a conformation in EF-Tu normally requiring divalent cations. Secondly, divalent cations, particularly Mg^{2+}, can replace the requirement for mRNA if present in high enough concentrations. The consequence of this result is considerable since it shows that EF-Tu - dependent GTP hydrolysis occurs uncoupled from proper codon-anticodon interaction, which at lower M^{2+} levels is considered an absolute prerequisite. If, as is supposed, proper codon-anticodon interaction induces an altered conformation in the aa-tRNA which then influences EF-Tu to hydrolyze GTP, these results imply that divalent cations can achieve the same conformational

changes independently of mRNA. A comparable conclusion has been reached elsewhere, in relation to translocation (107), where the function of EF-G can be inhibited or substituted by choosing appropriate Mg^{2+} concentrations. These results tend to point to the important involvement of divalent cations in the regulation of several elongation processes, and it is reasonable to suppose that macromolecule-induced actions, such as enzymatic binding or translocation, may, in fact, operate by the controlled modulation of local charges by divalent cations or their equivalent.

From the results presented here, Ca^{2+} would appear to have the highest efficiency in the physiological EF-Tu GTPase, since it achieves optimal stimulation at a low concentration, induces a high GTPase activity and at its optimum concentration there is almost complete dependence on mRNA. The high stimulatory activity of Mn^{2+} suggests that the mode of action of this divalent cation may be directly in the catalytic site, since this ion is also one with a very high affinity for EF-Tu.GDP, binding stoichiometrically 1:1 in the GDP binding site on the factor (86). Fortunately, Mn^{2+} lends itself to NMR studies, and it is to be hoped that new data may be forthcoming from this direction.

CHAPTER 5. THE INTERACTION OF ELONGATION FACTOR - Tu WITH THE RIBOSOME.

5.1 Introduction.

In the elongation cycle, the interaction between the ternary complex, EF-Tu.GTP.aminoacyl-tRNA and the ribosome is associated with the hydrolysis of GTP. Even when uncoupled from polypeptide synthesis, this GTPase activity still requires the presence of aminoacyl-tRNA and ribosomes (section 3.3.4). The characterization of the antibiotic kirromycin, which induces GTPase activity in EF-Tu alone, has shown that the elongation factor and not the ribosome bears the catalytic site for GTP hydrolysis. Since, however, this simple EF-Tu.kirromycin GTPase system can be stimulated by both ribosomes and/or aa-tRNA, the antibiotic can be used to probe the involvement of the ribosomal subunits and their components in this important reaction.

The results presented here show the 50S ribosomal subunit to be far more important than the 30S subunit, and that even 50S cores deprived of a large number of ribosomal proteins can stimulate the GTPase activity of EF-Tu; this activity is further augmented by the ribosomal proteins L7/L12 only after reintegration into the 50S ribosomal subunits. The additional presence of aminoacyl-tRNA and of the 30S particles becomes more important as the protein deficiency of the 50S cores increases.

5.2 Materials & Methods.

Electrophoretically pure EF-Tu, 0.5M NH_4Cl-washed ribosomes, Phe-$tRNA^{Phe}$ and kirromycin were as described previously (sections 2.2 and 3.2).

5.2.1 Ribosomal subunits.

50S and 30S ribosomal subunits were prepared by zonal centrifugation in a Beckman Ti15 rotor, using a 5-25% linear sucrose gradient. 600mg of 0.5M NH_4Cl-washed ribosomes were diluted into 120ml of a buffer containing 20mM Tris.HCl pH 7.8, 0.5mM $MgCl_2$ and 60mM NH_4Cl and incubated for 20 min at 30°C. This sample was then layered onto a linear gradient of 5-25% sucrose in the Ti15 rotor spinning at 4000 rpm, and was followed by approximately 100ml of an overlay buffer of the same composition as the sample buffer less the ribosomes. Centrifugation was for 20 hr at 25000 rpm and at 4°C. After deceleration to 4000 rpm the rotor was unloaded using a solution of 35% sucrose, pumped at approximately 30 ml/min. 10ml fractions were collected in an ISCO Golden Retriever collector, monitoring the UV absorbance of the fractions at 260nm with a Gilson Spectrochrom MO spectrophotometer. The discrete subunit fractions were gathered, the Mg^{2+} concentration being adjusted to 10mM, and centrifuged for 6 hr at 2°C in a Beckman 35 rotor at 30000 rpm. The pelleted ribosomal subunits were redissolved in a minimum quantity of a buffer containing 10mM $MgCl_2$, 20mM Tris.HCl pH 7.8 and 60mM NH_4Cl, and dialysed overnight against the same buffer also containing 50% glycerol. Subunits were thus stored at -30°C.

The ribosomal subunits were checked for purity by analytical sucrose density gradient centrifugation (Fig.32), using a gradient of 7-25% sucrose in a buffer containing 10mM Mg^{2+}. Centrifugation was

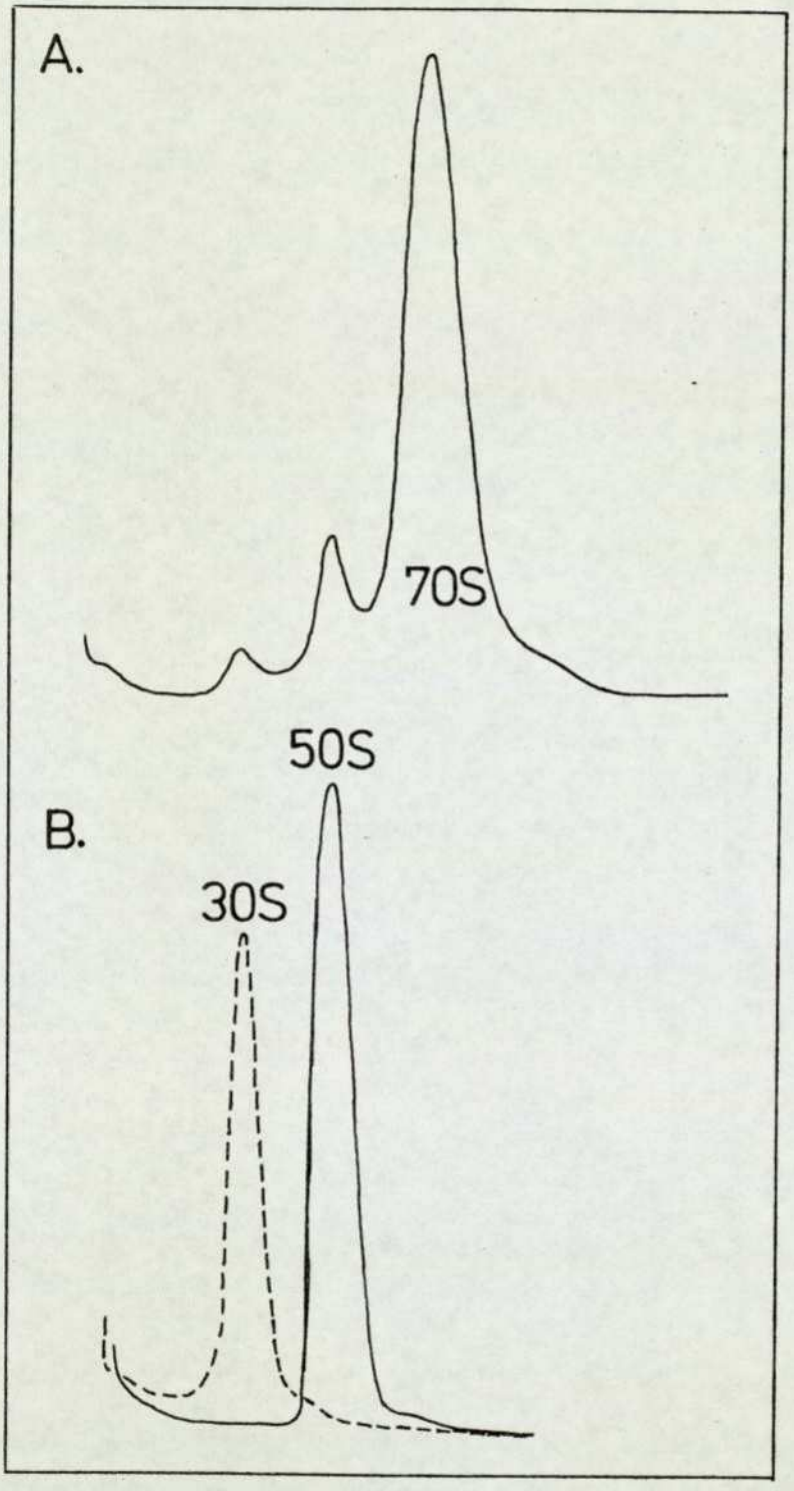


FIGURE 32 ANALYTICAL SUCROSE DENSITY GRADIENTS OF 70S RIBOSOMES (A) AND 50S / 30S RIBOSOMAL SUBUNITS (B).

The 70S ribosomes were centrifuged in 5mM $MgCl_2$, the ribosomal subunits in 10mM $MgCl_2$. The gradient profiles were measured by UV absorbance at 260nm. For other details see text.

for 5 hr at 30000 rpm and at 2°C in a Beckman SW-40 rotor. No contamination was evident in either 50S or 30S subunit preparations. Poly(U)-dependent polyphenylalanine synthesis (see section 2.2.7), using subunits instead of 70S ribosomes, also indicated a complete lack of cross-contamination by the complementary subunits. The concentration of the subunit preparations was calculated after the absorbance at 260nm (50S: 1 A_{260} unit = 39pmol; 30S: 1 A_{260} unit = 67pmol).

5.2.2 Ribosomal proteins L7/L12.

These were extracted from 70S ribosomes, previously tested to contain very little elongation factor - independent endogenous GTPase activity, following the procedure of Hamel et al. (108). 75nmol of 70S ribosomes were diluted in 100ml of a cold (4°C) buffer containing 20mM $MgCl_2$, 20mM Tris.HCl pH 7.8 and 0.5M NH_4Cl. 50ml of cold (4°C) absolute ethanol were then slowly added with constant stirring. After 10 min a further 50ml of cold ethanol were slowly added, precipitating the ribosomes. After centrifugation for 30 min at 10000 rpm in a Beckman JA14 rotor at 2°C, the supernatant was placed to one side and the pellet was redissolved in cold buffer as above and re-extracted. The supernatants from the two extractions were combined and the contained protein allowed to precipitate overnight at -25°C after addition of 2.25 volumes of pure acetone. The obtained proteins L7/L12 were already highly purified, though trace amounts of other ribosomal proteins, particularly L10, were present. These contaminating proteins were removed by DEAE Sephadex A-50 chromatography (78), using an NH_4Cl gradient from 0-500mM in 25mM Tris.HCl pH 7.5 at 4°C. This procedure yields pure L7/L12 as judged by electrophoresis (Fig.33) and isoelectric focussing (Fig.34) with overloaded gels.

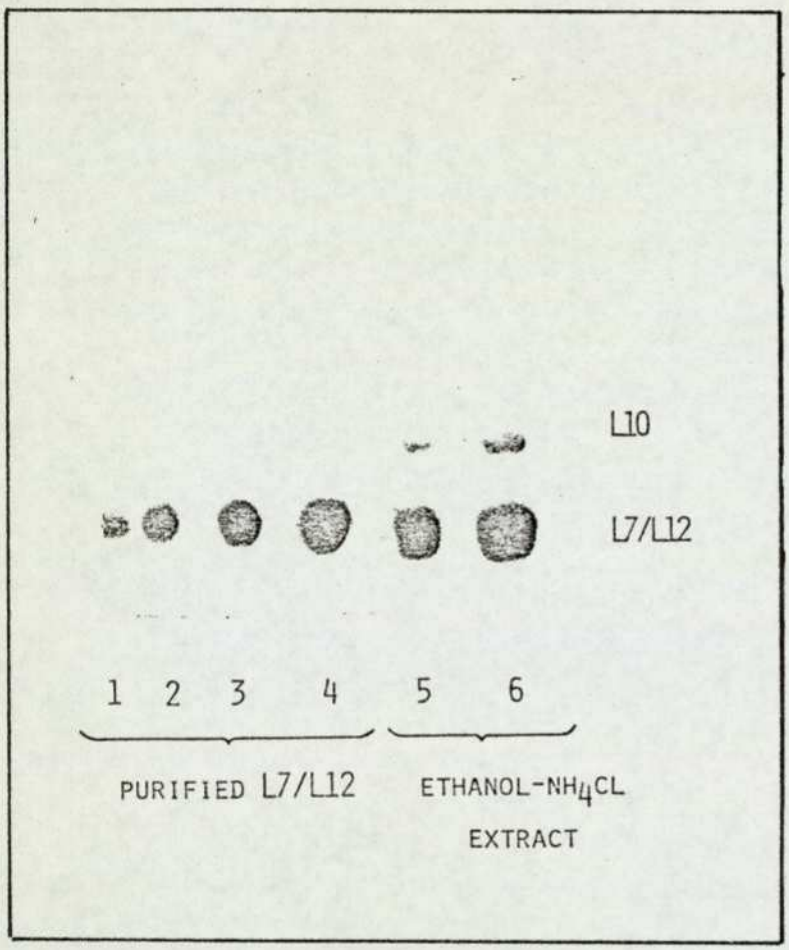


FIGURE 33 SLAB GEL ELECTROPHORESIS IN 18% ACRYLAMIDE AND 0.1% SDS OF PURIFIED PROTEINS L7/L12 (slots 1-4) AND OF RIBOSOMAL NH_4Cl/ ETHANOL EXTRACTS (slots 5-6).

Buffers were essentially those of Davis ; the concentrating gel was 4% acrylamide. Electrophoresis was carried out for 5hr at 75volts in a Savant slab gel apparatus; the gels were stained with 0.1% Coomassie Blue in 50% methanol-7.5% acetic acid and destained by shaking in 5% methanol-10% acetic acid. Slots 1-4: 10, 20, 40 and 80 µg L7/L12 (pure); slots 5-6: 30 and 50 µg respectively of NH_4Cl/ethanol extract (see text). Bidimensional electrophoresis according to Kaltschmidt and Wittmann (72) confirmed the identity of the contaminating spot as protein L10.

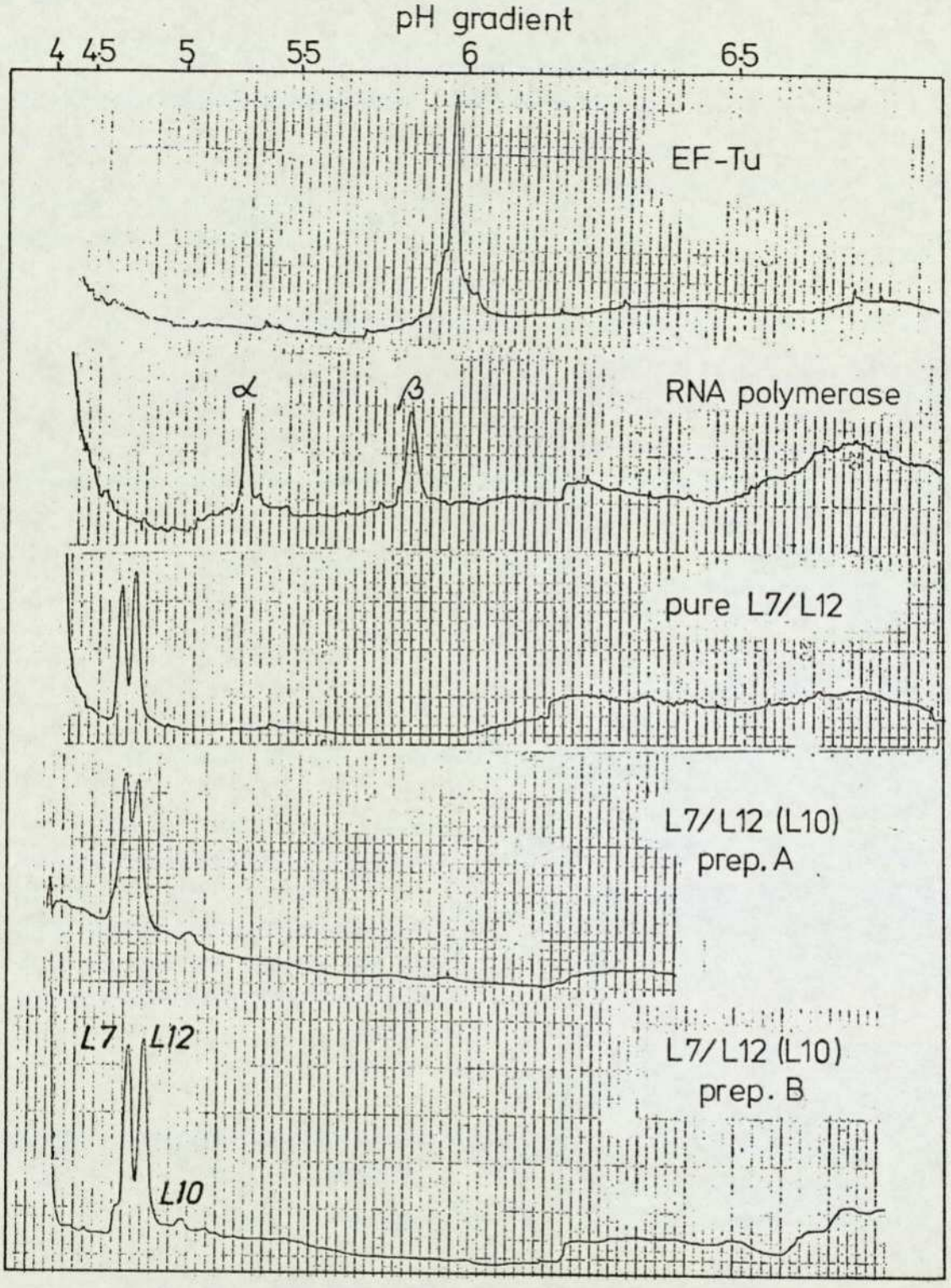


FIGURE 34 SCANNING PATTERNS OF FIRST DIMENSION ISOELECTRIC FOCUSSING GELS OF VARIOUS PREPARATIONS OF PROTEINS L7/L12.

The two upper patterns represent marker proteins, EF-Tu and RNA polymerase, as indicated. The third scan is of purified L7/L12; the remaining two scans of different NH_4Cl/ethanol extracts. The gels were prepared as in section 2.2.11. 25-50 µg of protein were loaded in each gel.

The proteins were stored at -30°C in a buffer containing 10mM $MgCl_2$, 60mM NH_4Cl, 20mM Tris.HCl pH 7.5 and 50% glycerol, and remained functionally stable for at least several months. Amounts of L7/L12 were calculated as the dimeric complex of molecular weight 24400.

5.2.3 Ribosomal cores.

The pelleted L7/L12-deficient 70S ribosomes obtained after the extraction of proteins L7/L12 (see above) were redissolved in 10mM $MgCl_2$, 20mM Tris.HCl pH 7.8 and 60mM NH_4Cl, dialysed overnight against the same buffer containing also 50% glycerol, and stored at -25°C. These NH_4Cl/ethanol cores are equivalent to the P_o cores used by other authors (e.g.109,110).

50S CsCl cores a, b and c were prepared by isopycnic centrifugation in 5M CsCl as described by Sander et al. (36). About 7mg of 50S ribosomal subunits in 0.5ml of 20mM Tris.HCl pH 7.8, 30mM KCl, 30mM NH_4Cl and 20mM $MgCl_2$ were mixed with 12ml of 5.25M CsCl containing 20mM Tris.HCl, 20mM $MgCl_2$, 3.5mM mercaptoethanol and 2mM NaEDTA (final pH 7.4). After centrifugation for 40 hr at 40000 rpm in a Spinco 50Ti rotor at 4°C, 25-30 fractions per tube were collected and the absorption at 260nm measured. There is a single principal band corresponding to core a. The 50S CsCl cores b and c were prepared by a modification of this method in which the 12ml of 5.25M CsCl contained 100mM potassium acetate (pH 6.0), 10mM $MgCl_2$ and 1mM NaEDTA. Two main absorbance peaks are detectable in this variation of the method, the light fraction being core c, the heavier, core b. The 50S CsCl cores were dialysed and stored as for the NH_4Cl/ethanol cores. The CsCl cores used in the following experiments were a kind gift from [redacted] of our laboratory.

5.2.4 Elongation factor - G.

Elongation factor - G (EF-G) was purified to electrophoretic homogeneity from E.coli $BT2^r$ or A19 as described by Parmeggiani (58,111), from whom the preparation used in this chapter was a generous gift.

5.2.5 GTPase assays.

GTPase activity was measured as described in section 3.2.7; the composition of the reaction mixtures is given in the figure legends. In the reconstitution assays, ribosomes, core particles and proteins L7/L12 were preincubated for 30 min at 40°C in a buffer containing 30mM Tris.HCl pH 7.8 , 75mM $MgCl_2$ and 300mM NH_4Cl, prior to adding the remaining components of the GTPase assay.

5.3 Results.

5.3.1 Role of the ribosomal subunits in the EF-Tu GTPase activity.

Both ribosomal subunits are needed for the expression of the normal EF-Tu - dependent GTPase activity (36,112). By contrast, each of the two subunits alone can to a certain extent stimulate the kirromycin-induced GTPase activity, particularly at a large ribosome to EF-Tu ratio (33). These phenomena are now studied in more detail, paying particular attention to the effect of the monovalent and divalent cations. Recently, it has been shown that the GTPase activity of the EF-Tu.kirromycin complex strictly depends on monovalent cations, and that this requirement can be overcome by the presence of ribosomes (79, see also chapter 3); thus the Mg^{2+}- dependence of the EF-Tu GTPase activity has been investigated at three concentrations of NH_4^+ (Fig.35). With the simplest assay system, i.e. EF-Tu.kirromycin plus GTP, no

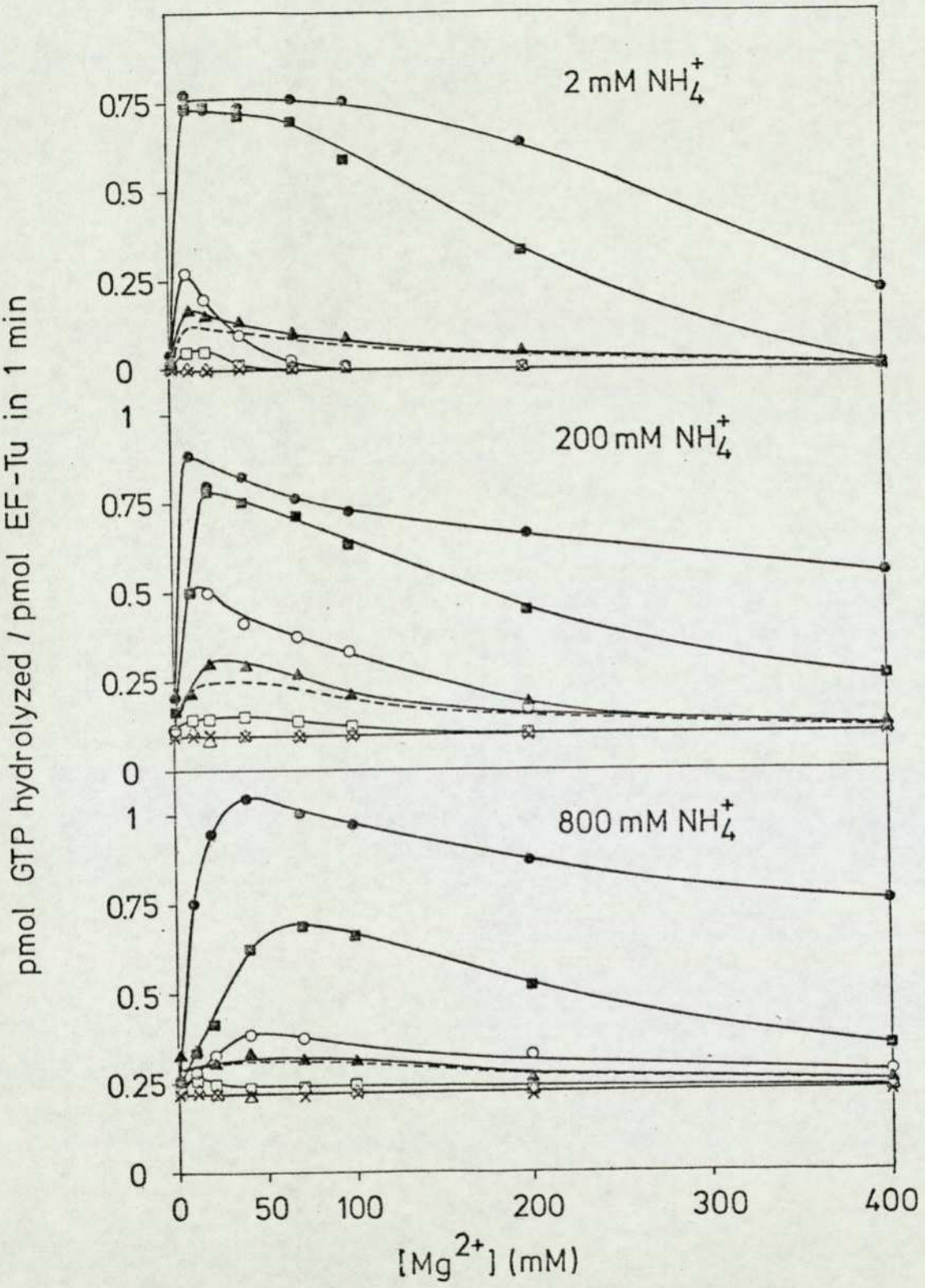


FIGURE 35 DEPENDENCE ON Mg^{2+} OF THE EF-Tu.KIRROMYCIN GTPase AT VARIOUS NH_4^+ CONCENTRATIONS: THE ROLE OF RIBOSOMAL SUBUNITS AND Phe-tRNA^{Phe}.

The 75µl reaction mixtures contained 20pmol EF-Tu, 40pmol 50S and/or 30S subunits and 100pmol Phe-tRNA^{Phe} as indicated, 50µM kirromycin and 1nmol GTP (specific activity 150-200cpm/pmol). (x) EF-Tu; (Δ) EF-Tu plus 30S; (□) EF-Tu plus 50S; (o) EF-Tu plus 50S and 30S. Filled symbols: the same in the presence of Phe-tRNA^{Phe}. Dashed line: activity with EF-Tu plus Phe-tRNA^{Phe} in the absence of ribosomes.

GTPase activity could be detected at 2mM NH_4^+, between 0.1 and 400mM Mg^{2+} (Fig.35A). Thus Mg^{2+} cannot replace NH_4^+ in this reaction. As expected, the GTPase activity with the EF-Tu.kirromycin complex rises progressively with increasing NH_4^+ (compare panels A,B and C, crosses). However, no dependence on Mg^{2+} was observed within the range of $[Mg^{2+}]$ tested.

With both ribosomal subunits present but in the absence of Phe-$tRNA^{Phe}$ (Fig.35, open circles), Mg^{2+} stimulated strongly the GTPase activity, with an optimum near 10mM between 2 and 200mM NH_4^+, and an optimum of 40-50mM at 800mM NH_4^+. Of the ribosomal subunits, only the 50S showed any significant stimulation (open squares).

This stimulation by the 50S subunit of the EF-Tu.kirromycin GTPase reaction increases greatly in the presence of Phe-$tRNA^{Phe}$ (Fig.35, filled squares), in fact, almost reaching the level of the controls (filled circles), in appropriate conditions. By contrast, the 30S subunit only stimulated the reaction when Phe-$tRNA^{Phe}$ was also present, and then only slightly beyond the level reached by Phe-$tRNA^{Phe}$ alone (compare dashed line and filled triangles). This finding was explored using freshly reactivated 30S subunits (84), with increasing subunit concentrations (not shown), but no stimulation of the EF-Tu. kirromycin GTPase could be induced except in the presence of Phe-$tRNA^{Phe}$; and maximum stimulation was attained with an excess of between 1.5- and 2-fold 30S subunits over EF-Tu, with or without the reactivation treatment. This demonstrates that the 50S subunit has a much greater impact on the EF-Tu GTPase reaction than the 30S subunit. As in the absence of Phe-$tRNA^{Phe}$, also in its presence, there is a strong stimulation by Mg^{2+}. Increasing NH_4^+ concentration again shifted the $[Mg^{2+}]$ optimum towards higher values, particularly with the 50S subunit.

Such an interdependence has been shown to exist also for the EF-G - dependent GTPase activity (113) and poly(U)-directed polyphenylalanine synthesis (114).

5.3.2 Components of the 50S subunit implicated in EF-Tu - dependent GTPase activity: I. Action of NH_4Cl/ethanol cores.

The 50S subunits of the NH_4Cl/ethanol cores are deficient in almost all proteins L7/L12, and otherwise lack only a small percentage ($<5\%$) of protein L10 (108). These cores were checked for the presence of L7/L12 using the EF-G GTPase assay system (36, 115), with EF-G limiting (Fig.36). Under these conditions, even a small quantity of L7/L12 should lead to a considerable stimulation of GTPase activity. In the absence of L7/L12 both core a and the NH_4Cl/ethanol core show the same low EF-G - dependent GTPase activity; since CsCl core a completely lacks indigenous L7/L12, Fig.36 must demonstrate also the complete absence of proteins L7/L12 in the NH_4Cl/ethanol cores prepared as described. Of interest is also the observation that purified L7/L12, lacking protein L10, fail to stimulate CsCl cores a, b or c in the conditions used in this assay.

Figure 37 illustrates the effect of the NH_4Cl/ethanol core in the EF-Tu.kirromycin GTPase activity. In the presence both of aa-tRNA and of 30S subunits, the deficiency of L7/L12 is of no evident importance; full activity is reached whether L7/L12 is added or not. In the ionic conditions of this assay (200mM NH_4^+, 50mM Mg^{2+}), the addition of 30S subunits is also superfluous in the presence of aa-tRNA. The picture is different, however, when Phe-$tRNA^{Phe}$ is not present (open diamonds and squares). Then, purified L7/L12 stimulate the NH_4Cl/ethanol core; this effect is particularly noticeable if no extra 30S subunits are added.

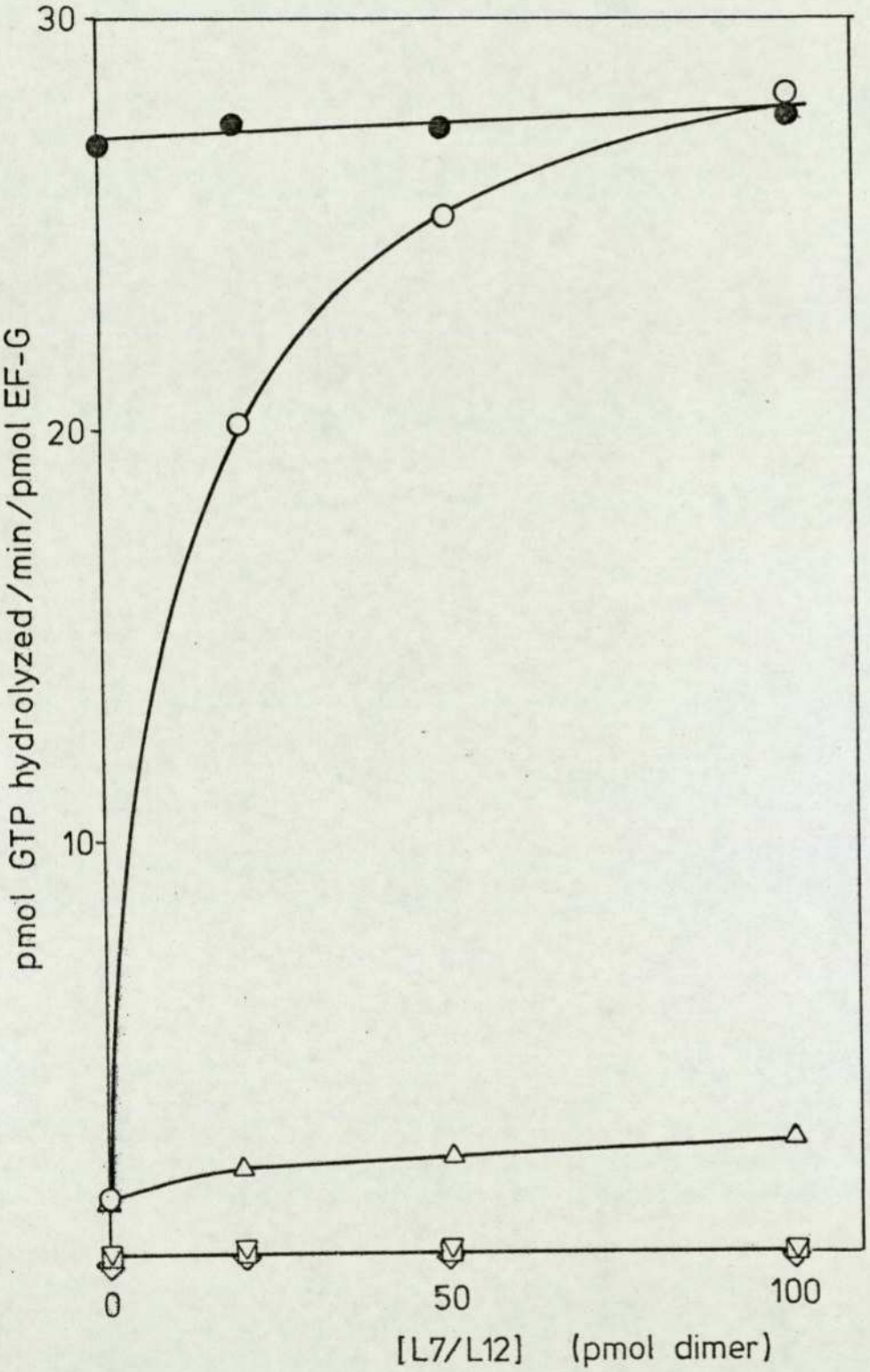


FIGURE 36 EFFECT OF 50S RIBOSOMAL SUBUNITS AND 50S CORES ON THE EF-G GTPase ACTIVITY. INFLUENCE OF PURE L7/L12.

Reaction mixtures contained in 75µl, 80mM NH_4Cl, 15mM $MgCl_2$, 10pmol EF-G, 20pmol 50S subunits or 50S cores as indicated, 30pmol 30S subunits, 10nmol GTP (specific activity ca. 25cpm/pmol). (●) 50S subunits, (o) NH_4Cl/ethanol cores, (Δ) CsCl core a, (▽) CsCl core b, (□) CsCl core c, (◇) 30S subunits alone.

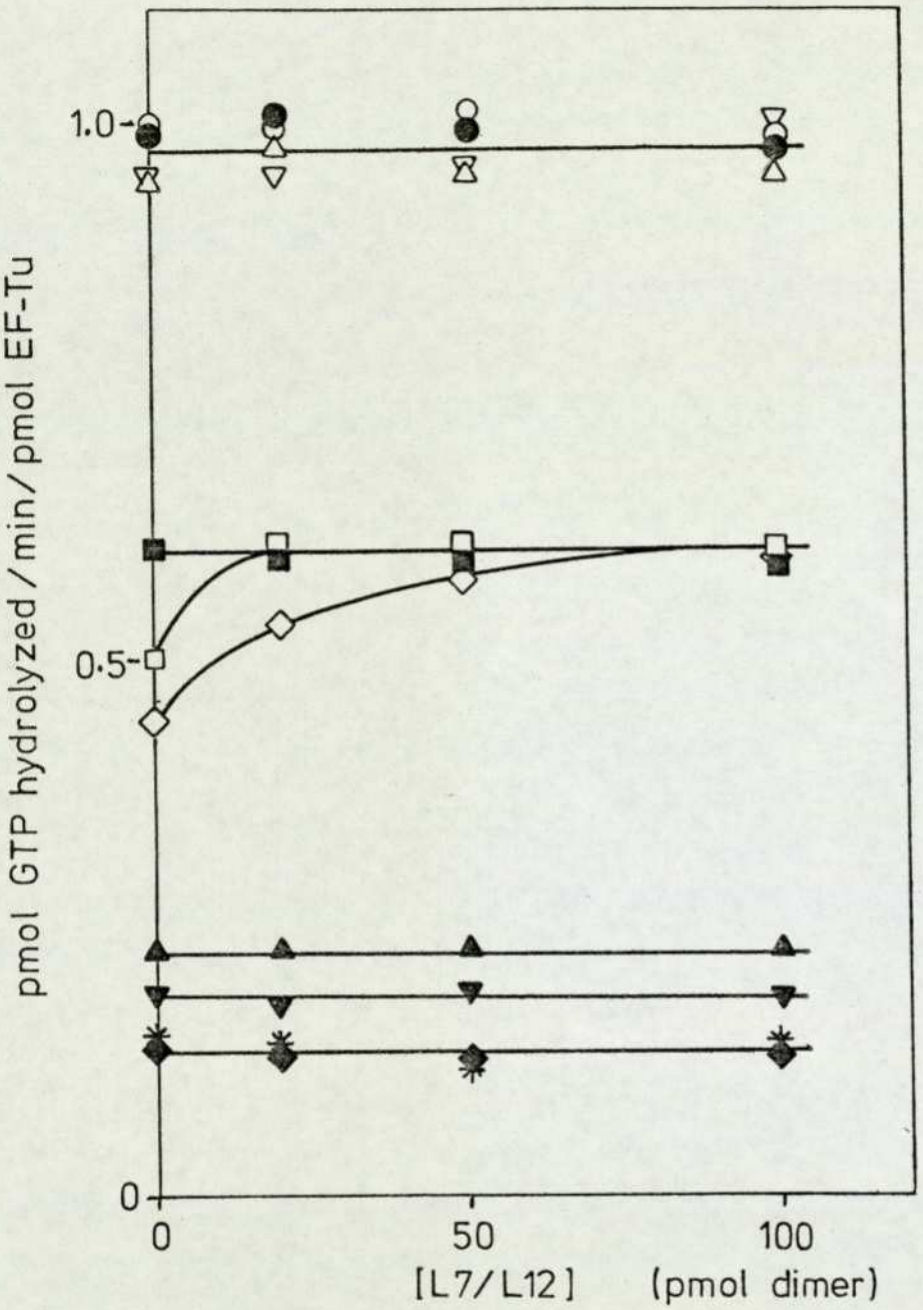


FIGURE 37 EFFECT OF NH_4Cl/ETHANOL CORES ON THE EF-Tu.KIRROMYCIN GTPase. INFLUENCE OF PURE L7/L12.

Where indicated reaction mixtures contained in 75µl, 200mM NH_4Cl, 50mM $MgCl_2$, 20mM Tris.HCl pH 7.8, 0.5mM dithiothreitol, 50µM kirromycin, 10pmol EF-Tu, 20pmol 50S subunits/cores, 30pmol 30S subunits, 50pmol Phe-tRNA^{Phe}, 1nmol GTP (specific activity 150 cpm/pmol). Additions to EF-Tu.kirromycin: (●) 50S + 30S + Phe-tRNA^{Phe}, (Δ) NH_4Cl/ethanol cores + 30S + Phe-tRNA^{Phe}, (▽) NH_4Cl/ethanol cores + 30S + Phe-tRNA^{Phe} + L10, (○) NH_4Cl/ethanol cores + Phe-tRNA^{Phe}, (■) 50S + 30S, (□) NH_4Cl/ethanol cores + 30S, (◇) NH_4Cl/ethanol cores only, (▲) 30S + Phe-tRNA^{Phe}, (▼) Phe-tRNA^{Phe} only, (◆) 30S only, (✱) none.

5.3.3 Components of the 50S subunit implicated in EF-Tu - dependent GTPase activity: II Action of 50S CsCl cores.

The protein-deficient 50S subparticles prepared by isopycnic centrifugation in 5M CsCl, named CsCl cores a, b and c, have been previously described by Sander et al. (36); their protein composition is shown in Table VII. These cores contain no proteins L7/L12 or L10. All of them contain normal amounts of 5S RNA and are able to associate with 30S subunits (36). Their stimulation of the EF-Tu. kirromycin GTPase reaction in the presence of 30S subunits and Phe-tRNA^{Phe} is shown in Fig.38. At sufficiently high [Mg^{2+}], the presence of each of these cores increased the GTPase activity beyond that found with EF-Tu.kirromycin plus only 30S subunits and Phe-tRNA^{Phe}; the core a was most active followed by cores b and c. This behaviour resembles the EF-T GTPase activities previously obtained with the same CsCl cores in the presence of excess L7/L12, also containing a little L10 (36, and see later). It should be noted that proteins L7/L12 were neither added in the experiments of Fig.38, nor present in the cores, as judged by bidimensional electrophoresis with overloaded gels (72).

Thus in the presence of kirromycin, L7/L12 are no longer essential to stimulate GTPase activity in the presence of 30S subunits, 50S CsCl cores and Phe-tRNA^{Phe}.

The kinetics of the EF-Tu GTPase activity stimulated by the 50S CsCl cores and kirromycin at 50mM Mg^{2+} and 200mM NH_4^+ is shown in Fig.39. The reaction was linear up to the 10 min incubation time chosen for all of the other experiments. The figure also illustrates that, in contrast to the 50S subunit, stimulation by the 50S CsCl cores in the presence of Phe-tRNA^{Phe} depends strongly on the 30S subunit (compare panels A and B). Without Phe-tRNA^{Phe} stimulation of the EF-Tu.

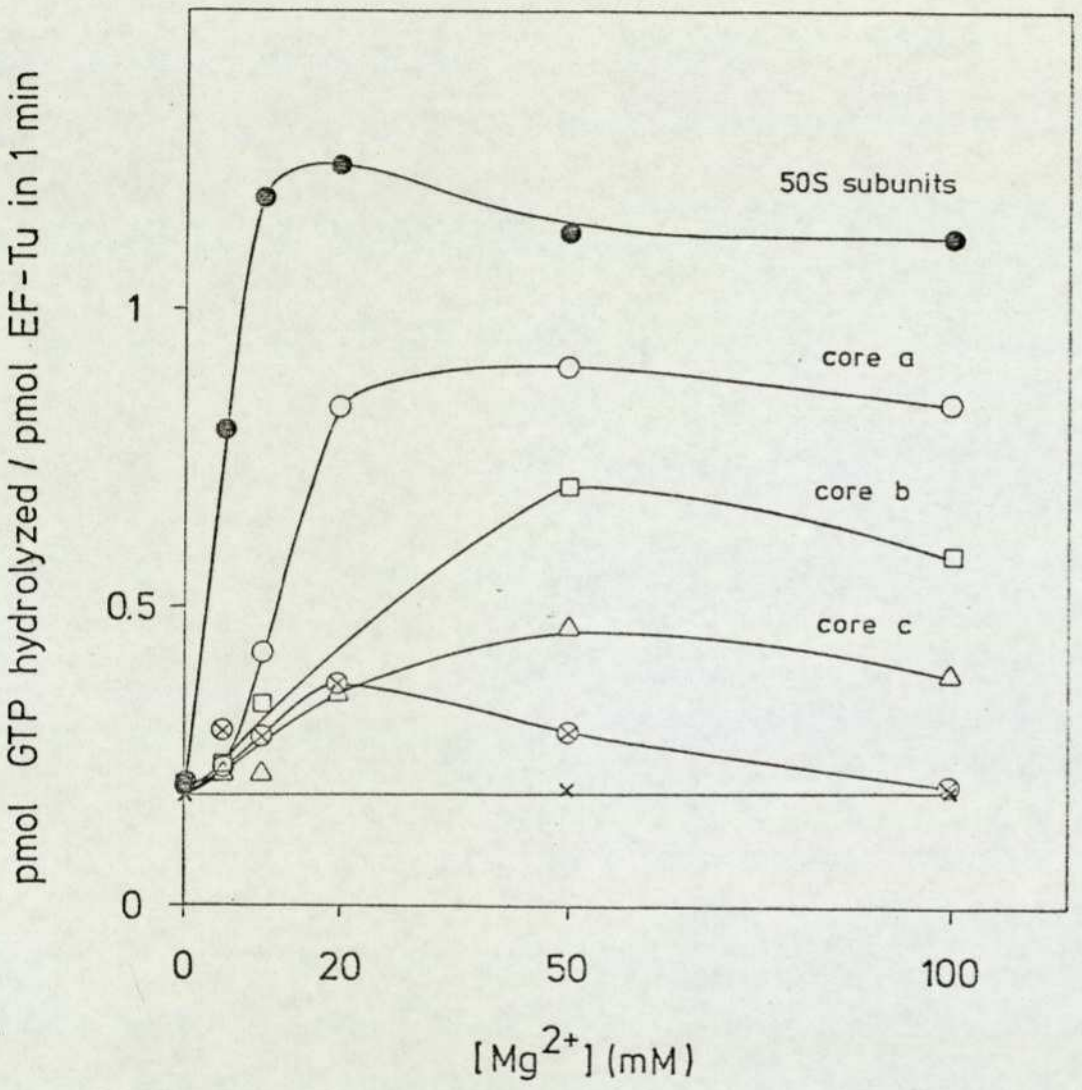


FIGURE 38 STIMULATION BY VARIOUS CsCl CORES OF THE KIRROMYCIN-INDUCED EF-Tu GTPase AS A FUNCTION OF Mg^{2+}.

Reaction mixtures contained in 75µl, 200mM NH_4Cl, 20mM Tris.HCl pH 7.8, 10pmol EF-Tu, 20pmol 50S subunits or CsCl cores, 30pmol 30S subunits and 50pmol Phe-$tRNA^{Phe}$ as indicated, 50µM kirromycin and 1nmol GTP. (x) EF-Tu alone; all other assays with Phe-$tRNA^{Phe}$, plus: (⊗) 30S subunits, (●) 50S subunits, (○) 50S CsCl core a + 30S subunits, (□) 50S CsCl core b + 30S subunits, (△) 50S CsCl core c + 30S subunits.

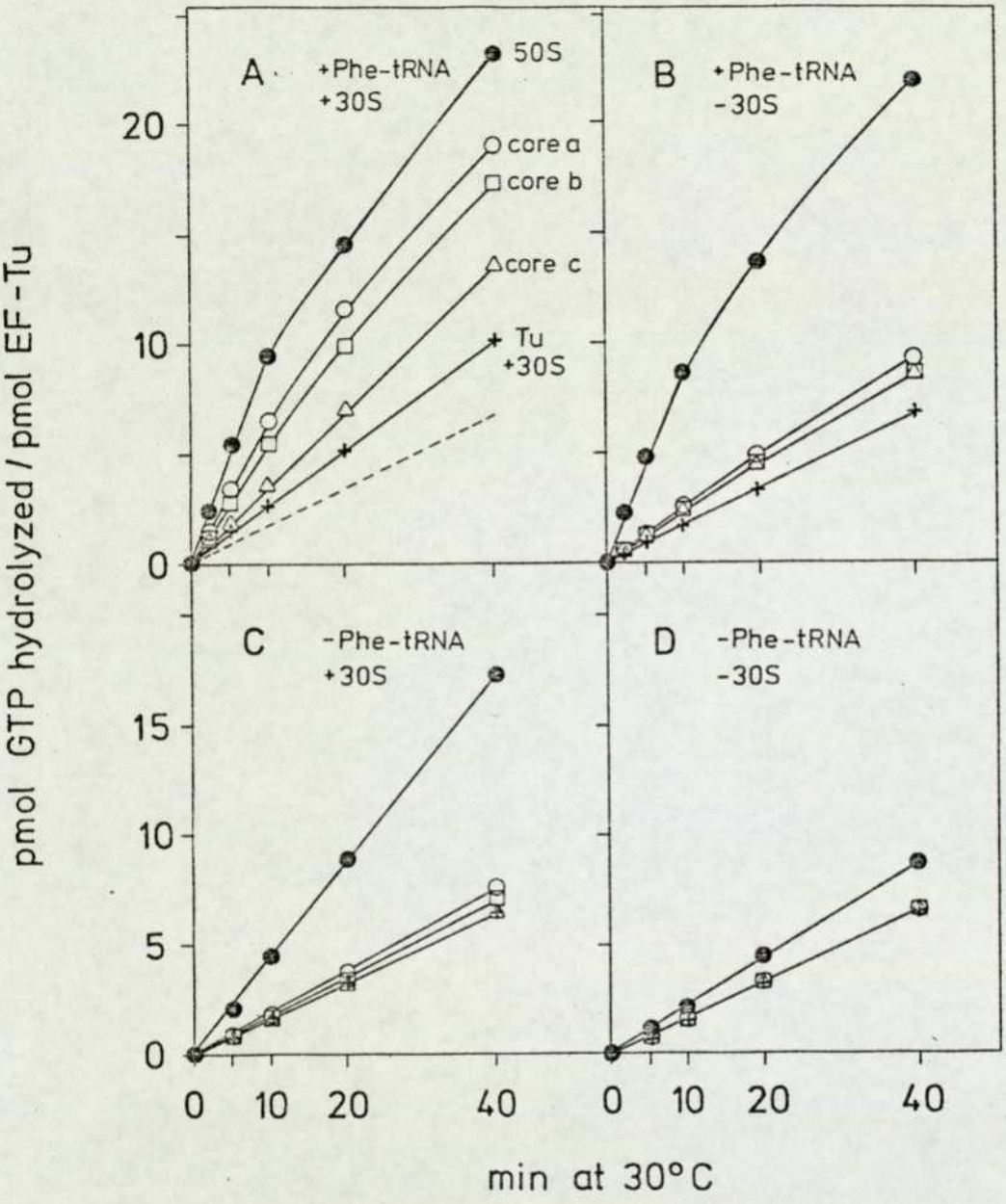


FIGURE 39 KINETICS OF EF-Tu.KIRROMYCIN GTPase ACTIVITY STIMULATED BY 50S CsCl CORES AT 200mM NH_4^+ AND 50mM Mg^{2+}.

Reaction mixtures contained 10pmol EF-Tu, 1.4nmol GTP and 50μM kirromycin, 20pmol 50S particles, 20pmol 30S subunits and 50pmol Phe-$tRNA^{Phe}$ as indicated. All 50S particles were pre-incubated for 30min at 30°C in 20mM Tris.HCl pH 7.8 containing 300mM NH_4Cl and 10mM $MgCl_2$. (+) EF-Tu, (●)plus 50S subunits, (o) plus 50S CsCl core a, (□) plus 50S CsCl core b, (△) plus 50S CsCl core c. Additions: A. 30S subunits and Phe-$tRNA^{Phe}$, B. Phe-$tRNA^{Phe}$, C. 30S subunits, D. none. Dashed line: EF-Tu. kirromycin in the absence of ribosomal particles.

kirromycin GTPase activity by the cores was very low in the presence of 30S subunits and zero in their absence (panels C and D).

TABLE VII. PROTEIN COMPOSITION OF VARIOUS 50S RIBOSOMAL CORES

Protein	NH_4Cl/ethanol core	CsCl core a	CsCl core b	CsCl core c
L1	+	-	-	-
L2	+	+	+	+
L3	+	+	+	+
L4	+	+	+	+
L5	+	+	+	-
L6	+	(+)	-	-
L7	-	-	-	-
L8	+	-	-	-
L9	+	+	+	(+)
L10	+	-	-	-
L11	+	(+)	(+)	(+)
L12	-	-	-	-
L13	+	+	+	+
L14	+	+	+	(+)
L15	+	+	+	-
L16	+	-	-	-
L17	+	+	+	+
L18	+	+	+	-
L19	+	+	+	(+)
L20	+	+	+	+
L21	+	+	+	(+)
L22	+	+	+	+
L23	+	+	+	+
L24	+	+	+	+
L25	+	-	-	-
L27	+	+	+	-
L28	+	+	+	-
L29	+	+	+	+
L30	+	+	+	-
L32	+	+	(+)	+
L33	+	-	(+)	-

N.B. Protein composition was determined using two-dimensional electrophoresis according to Kaltschmidt & Wittmann (72). Symbols: +, present in normal amount; (+), present in reduced amount (L6,L11 and L33: 40-70%; other proteins: 10-30%); -, not present. Neither L26 nor L31 were ever observed. L8 is a complex of L7/L12 and L10 (116).

5.3.4 Reconstitution of GTPase activity to 50S CsCl cores by Proteins L7/L12.

Proteins L7/L12 purified on DEAE Sephadex A-50 contained no protein L10 (Figs.33 and 34; ref.78). This protein has been described as essential for the reintegration of L7/L12 into the 50S particle (115,117,118,119,120), and indeed any effect of L7/L12 prepared by this method on the EF-Tu.kirromycin GTPase stimulated by 50S CsCl cores or subunits (Fig.40) was not visible. By contrast, by using an excess of proteins L7/L12 obtained with the NH_4Cl/ethanol extraction (108), which is known to remove some protein L10 from the ribosome, there was an almost total reconstitution of the EF-Tu.kirromycin GTPase activity with all the 50S CsCl cores (Fig.40B). The previous experiments of Sander et al. (36) were also repeated without kirromycin (Fig.41). Reconstitution of the EF-Tu GTPase activity to the reported levels (core a: 80%, core b: 50%, core c: 20% of the activity with the 50S subunit at 30mM $MgCl_2$) is possible only with the preparation containing contaminating protein L10. Apparently the proteins L7/L12 utilized for the experiments reported by Sander et al. (36) contained some protein L10.

The importance of protein L10 for reintegrating proteins L7/L12 into the ribosome was also evident from the experiments using the NH_4Cl/ethanol cores (section 5.3.2), which contain L10 (108,118, 121): proteins L7/L12, purified as described, stimulated both the EF-Tu and EF-G - dependent GTPase activities (Figs.36,37 & 41).

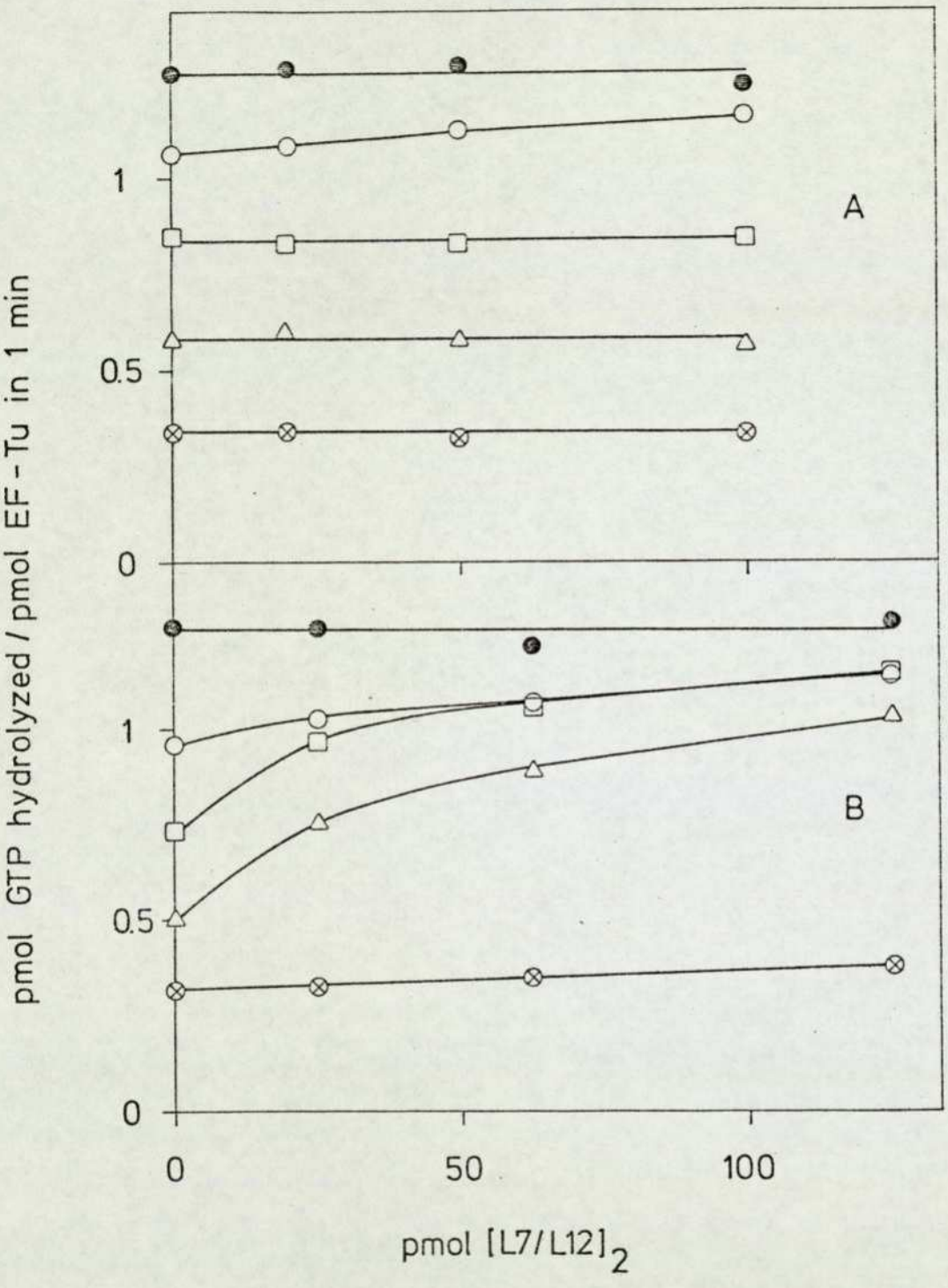


FIGURE 40 COMPLEMENTATION OF 50S CsCl CORES WITH PURE L7/L12 (A) OR L7/L12 WITH L10 (B) AT 200mM NH_4^+ AND 50mM Mg^{2+}.

The reaction mixture contained 10pmol EF-Tu, 30pmol 30S subunits with or without 20pmol 50S subunits or 50S CsCl cores, 50pmol Phe-$tRNA^{Phe}$, 1nmol GTP (specific activity 200 cpm/pmol) and 50 μM kirromycin, plus the indicated amounts of L7/L12. (⊗) 30S subunits; (●) 50S plus 30S subunits; (○) 50S CsCl core a, (□) 50S CsCl core b, (△) 50S CsCl core c, all with 30S subunits.

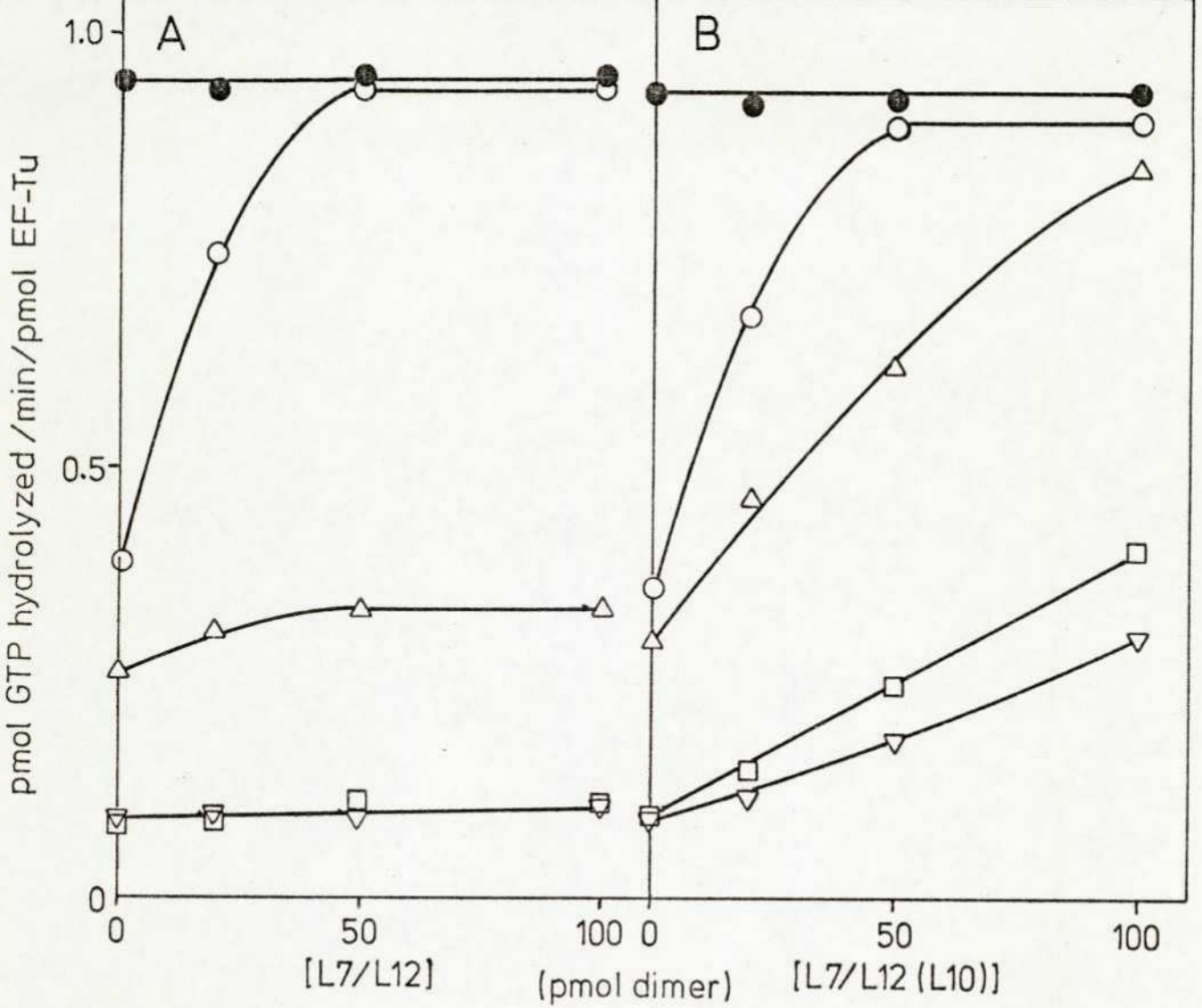


FIGURE 41 EFFECT OF PURE L7/L12 (A) OR L7/L12(L10) (B) ON THE PHYSIOLOGICAL EF-Tu GTPase IN THE PRESENCE OF VARIOUS RIBOSOMAL CORES.

Where indicated, reaction mixtures contained in 75µl, 30mM NH_4Cl, 30mM $MgCl_2$, 20mM Tris.HCl pH 7.5, 10pmol EF-Tu, 30pmol EF-Ts, 20pmol 50S subunits/cores, 40pmol 30S subunits, 4µg poly(U), 100pmol Phe-$tRNA^{Phe}$ and 1nmol GTP (specific activity 200 cpm/pmol). In the presence of (●) 50S subunits, (○) NH_4Cl/ethanol cores, (△) CsCl core a, (□) CsCl core b, (▽) CsCl core c.

5.3.5 Effect of proteins L7/L12 on the EF-Tu GTPase activity in the absence of ribosomal particles.

In a recent publication Kurland and coworkers have reported that purified L7/L12 are able to induce the EF-Tu GTPase reaction in the absence of ribosomes (78). This activity was stimulated up to 10 times by the addition of EF-Ts. These experiments have been carefully repeated (Fig.42A) and no effect could be found by L7/L12 over the whole concentration range examined, whereas these authors have reported values of 0.4pmol GTP hydrolyzed per pmol EF-Tu in 1 min. Nor was any activity found in the additional presence of 30S subunits and/or aa-tRNA. An important difference between the system presented here and that of Kurland and coworkers is their high GTPase activity already obtained with EF-Tu plus EF-Ts in the absence of L7/L12 (0.1pmol GTP hydrolyzed per pmol EF-Tu in 1 min under the same conditions as used in Fig.42A). In the presence of kirromycin (filled symbols, Fig.42), proteins L7/L12, at approximately equimolar concentration to EF-Tu, marginally stimulated the GTPase activity of EF-Tu, whereas they slightly inhibited the reaction when present in a large excess. This is particularly interesting, since the ionic conditions of the experiments described in Fig.42A are the same as those used by Kurland and coworkers.

In a further series of experiments, no GTPase activity could be detected when I) L7/L12 were added in great excess to EF-Tu alone over a large range of monovalent cation concentrations (Fig.42B), II) increasing concentrations of EF-Tu (up to 20 times) were added to 10pmol dimeric L7/L12 (not shown). These experiments are critical for denying the existence of a catalytic site for GTP hydrolysis located

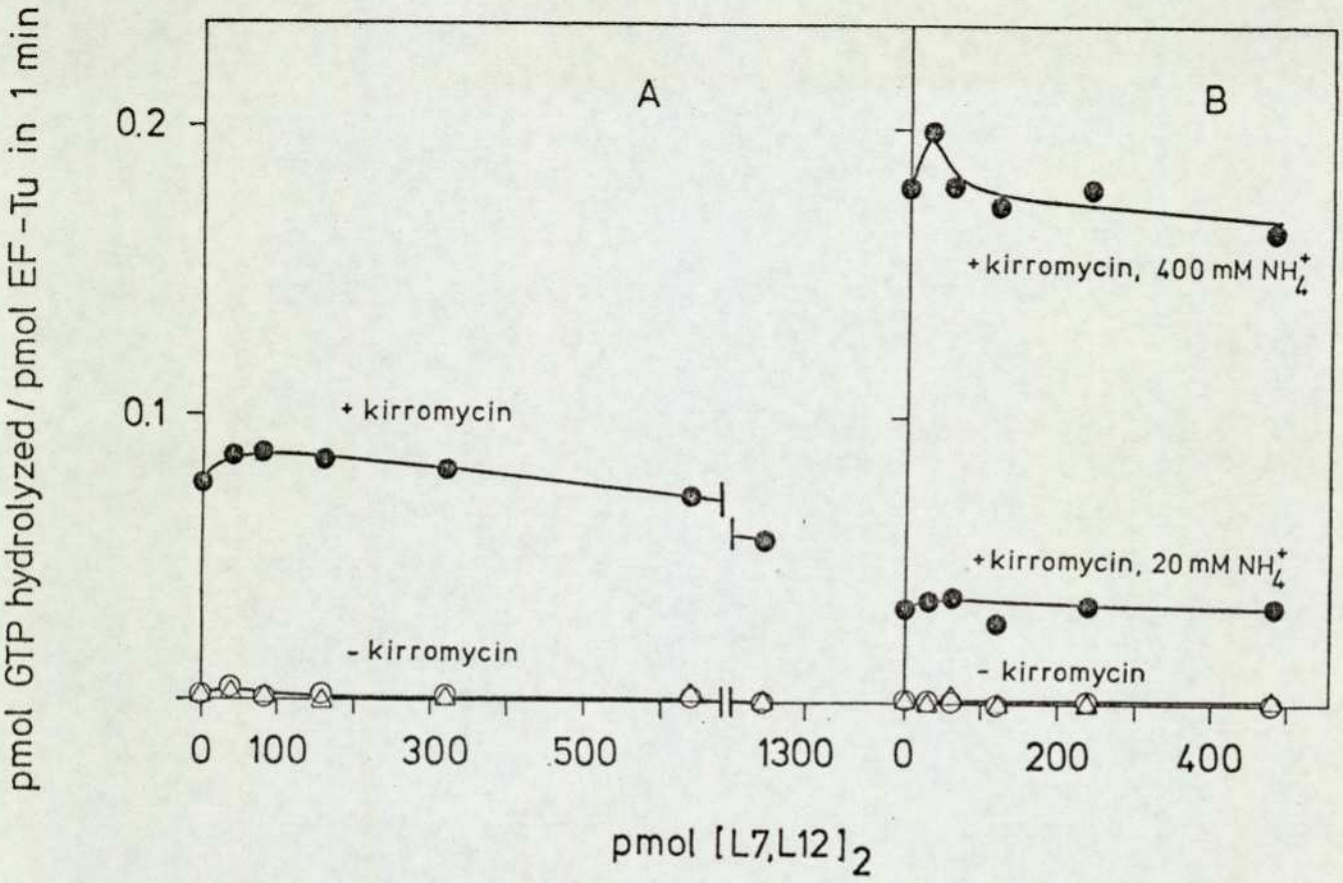


FIGURE 42 PROTEINS L7/L12 DO NOT INDUCE EF-Tu GTPase ACTIVITY.

A. The 75µl reaction mixtures contained, in 20mM Tris.HCl pH 7.5, 30mM KCl, 30mM NH_4Cl, 10mM $MgCl_2$, 1mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride and 150 pmol GTP (specific activity 2500 cpm/pmol). (o) 50pmol EF-Tu; (Δ) 50pmol EF-Tu plus 500pmol EF-Ts; (●) 50pmol EF-Tu plus 50µM kirromycin.

B. The 75µl reaction mixtures contained, in 30mM Tris.HCl pH 7.5, 10mM $MgCl_2$, the indicated NH_4Cl concentration, 1mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride and 150pmol GTP (specific activity 1400 cpm/pmol). (o) 20pmol EF-Tu, (Δ) 20pmol EF-Tu plus 20pmol EF-Ts, (●) 20pmol EF-Tu and 50uM kirromycin. The blanks obtained with proteins L7/L12 alone (maximally 0.01pmol GTP hydrolyzed/min/tube) have been subtracted.

on L7/L12, as suggested by the experiments of Kurland and coworkers (78).

In conclusion, isolated proteins L7/L12 are unable to induce the GTPase activity of EF-Tu in the presence or absence of EF-Ts. The same results were found whether the L7/L12 preparation contained some protein L10 or not (not shown).

5.4 Discussion.

Thanks to the action of kirromycin, which in the absence of mRNA induces a turnover GTPase activity (33, see also chapter 4), it has been possible to define more clearly the interacting regions of the EF-Tu molecule and the ribosome. The results suggest that this region is composed of several sites. There would appear to be at most a weak interaction with the 30S subunit, which is in evidence only when the subunits are in excess over EF-Tu and in the presence of aa-tRNA.

The interaction with the 50S subunit is much stronger and is independent of, although stimulated by, aa-tRNA (see Figs.35 & 39). That the GTPase activity with kirromycin is reduced progressively, but not abolished completely, on passing from 50S subunits, through the NH_4Cl/ethanol core, to CsCl cores a, b and c implies a complex interaction between EF-Tu and the subunit, involving several different proteins. The pivotal role of L6 (the only one lost upon going from core a to core b) is again evident (36,95,115), together with the relative unimportance of proteins L1, L10, L16, L25 and L33, which are missing from CsCl core a, in addition to proteins L7/L12. It should be emphasized that the relative activities of the cores a, b and c, in the absence of proteins L7/L12 but with kirromycin, follow the

same order as those found previously with L7/L12 in the absence of the antibiotic (36; see also Fig.41), and suggest that in the kirromycin-induced system, proteins L7/L12 may only be acting in an auxiliary role. This is especially obvious when the NH_4Cl/ethanol cores are considered; in the presence of aa-tRNA, the absence of L7/L12 causes no detectable reduction in activity. These results confirm that protein L10 is essential for the reintegration of proteins L7/L12 into the 50S particles, and is not directly implicated in the EF-Tu GTPase reaction (see also 115,119,120).

Proteins L7/L12 present curious properties. On the one hand, they are apparently the most important ribosomal proteins implicated in the physiological EF-Tu GTPase reaction (36,108), though this property pertains only when they are an integral part of the 50S ribosomal subunit (Fig.42). In properly chosen conditions, on the other hand, in the absence of these proteins, not only the EF-Tu GTPase reaction functions well, but also polypeptide synthesis in vitro has been reported (122,123). At present there appears no way to explain the considerable discrepancy between the results presented here and those of the group of Kurland, following my failure to observe any effect on EF-Tu GTPase by the proteins L7/L12 separated from the 50S particle. In this regard it should be stressed that these experiments have been repeated a large number of times with different preparations.

The CsCl core c contains only 11 major proteins (L2, L3, L4, L13, L17, L21, L22, L23, L24, L29, L32), the ribosomal RNA thus forming over 80% of the particle. Yet EF-Tu.kirromycin GTPase activity is stimulated considerably by its presence, even in the absence of proteins L7/L12 and L10, raising the possibility of a direct participation by 23S RNA and/or 5S RNA. However, this seems unlikely since in the

intact particles there is no interdependence between the optima for monovalent cations and pH (90), as might be expected in an enzymatic system having RNA in the neighbourhood of the catalytic site (98,99). Moreover, 16S, 23S and 5S RNA, when tested in the EF-Tu.kirromycin system, gave no stimulation of the GTPase activity (124).

In the system containing kirromycin, the other EF-Tu effector, aminoacyl-tRNA, appears to contribute independently towards the conformation of the factor leading to GTP hydrolysis, such that, when acting in concert, ribosomes and aa-tRNA stimulate more than the sum of each acting individually and are always required together for maximum GTPase activity.

Taken together, these results demonstrate the synergistic functioning of a very complex ribosomal system, which is responsible for triggering the GTPase activity of EF-Tu, and deny a simplistic ribosome - EF-Tu interaction, as has been sometimes suggested (78,125, 126).

CHAPTER 6. INFLUENCE OF THIOSTREPTON ON THE EF-Tu - RIBOSOME INTERACTION.

6.1 Introduction.

Thiostrepton is one of a family of antibiotics (also including siomycin and thiopeptin) which bind specifically to the 50S ribosomal subunit in a 1:1 stoichiometric ratio (127), apparently inhibiting EF-G and EF-T - dependent GTPase activities (128,129) and aminoacyl-tRNA binding to the ribosomal A-site (129,130). Additionally, it has been shown to influence initiation (131) and termination (132) steps. The binding site for thiostrepton in the intact ribosome appears to be protein L11 (37,133), though the antibiotic does not bind to this protein when it is separated from the ribosome and free in solution (37). Studies of thiostrepton-resistant mutant bacteria indicate changes in either BS-L11 of Bacillus subtilis or BM-L11 of B.megaterium (equivalent to protein L11 of E.coli)(134,135) or L5 in E.coli (136). Antibody studies show that only anti-L11 blocks ^{35}S.thiostrepton to the ribosome (37). Thus the large amount of data point to ribosomal protein L11 as the target for the antibiotic. This protein binds directly to the 23S ribosomal RNA and aids binding to protein-deficient subunits of L10 and subsequently L7/L12 (137,138), which appear to be close neighbours (139). Together with proteins L16 and L4, L11 has also been implicated in the peptidyl transferase centre (140), though direct involvement of L11 seems to be excluded, since peptidyl transferase activity can also be demonstrated in L11-deficient ribosomal cores (110,121,133). Additionally, photoaffinity labelling has revealed L11 to be close to the site of EF-G interaction with the ribosome (141).

The EF-T - dependent GTPase activity, shown to be completely inhibited by thiostrepton (128,129), can be uncoupled from its macromolecular effectors to varying extents in different systems. In this chapter advantage is taken of the specific action of thiostrepton to investigate the relationship between EF-Tu and the ribosome in several such uncoupled systems. As in the preceding chapter results indicate a complex ribosome - EF-Tu interaction.

6.2 Materials & Methods.

The EF-Tu - dependent GTPase activity was used as the basic assay system, the influence of the thiostrepton binding site being measured as the extent to which GTPase activity could be inhibited by the antibiotic. GTPase activity was assayed as previously described (section 3.2.7). Thiostrepton (Squibb, New Jersey) was stored as a 0.75mM stock solution in 100% dimethylsulfoxide (DMSO) at 4°C, and added to the incubation mixtures to give a final concentration of 20μM (142), which was shown to give maximal inhibition of the physiological EF-Tu - dependent GTPase (data not shown). Reaction tubes lacking the antibiotic contained an appropriate quantity (2.6%) of DMSO. Other details of assays and assay conditions are included in the results sections and figure legends.

6.3 Results.

6.3.1 The methanol-induced EF-Tu GTPase.

In the presence of 20% methanol EF-T shows GTPase activity with ribosomes only (142,143). Since these early reports concerned EF-T preparations, preliminary experiments were carried out using

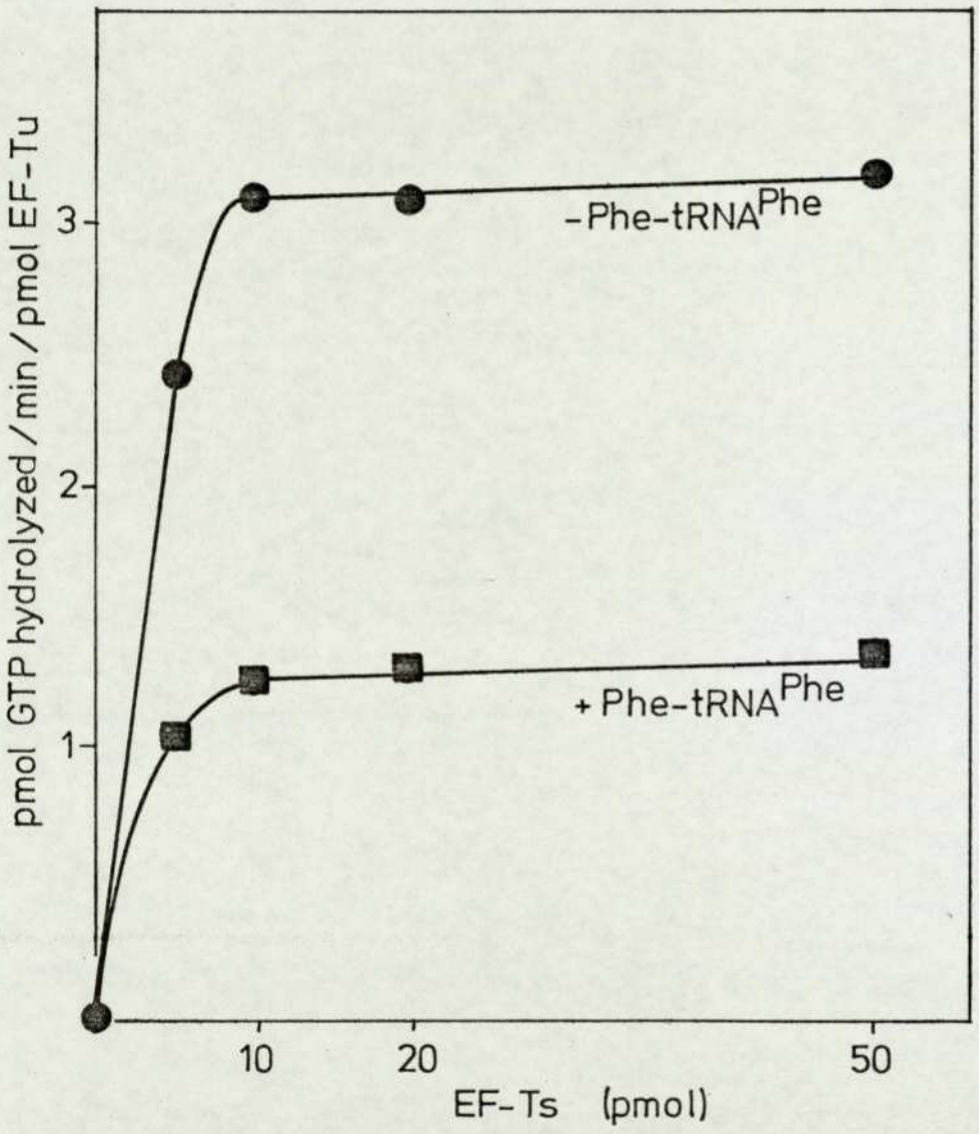


FIGURE 43 EFFECT OF EF-Ts ON THE METHANOL-INDUCED EF-Tu GTPase.

Reaction mixtures contained in 75µl, 20% methanol, 20mM Imidazolium acetate pH 7.5, 5mM $MgCl_2$, 100mM NH_4Cl, 0.5mM dithiothreitol, 10pmol EF-Tu, 20pmol ribosomes, 2nmol GTP (specific activity ca.200 cpm/pmol) with (■) or without (●) 100pmol Phe-tRNA^{Phe}.

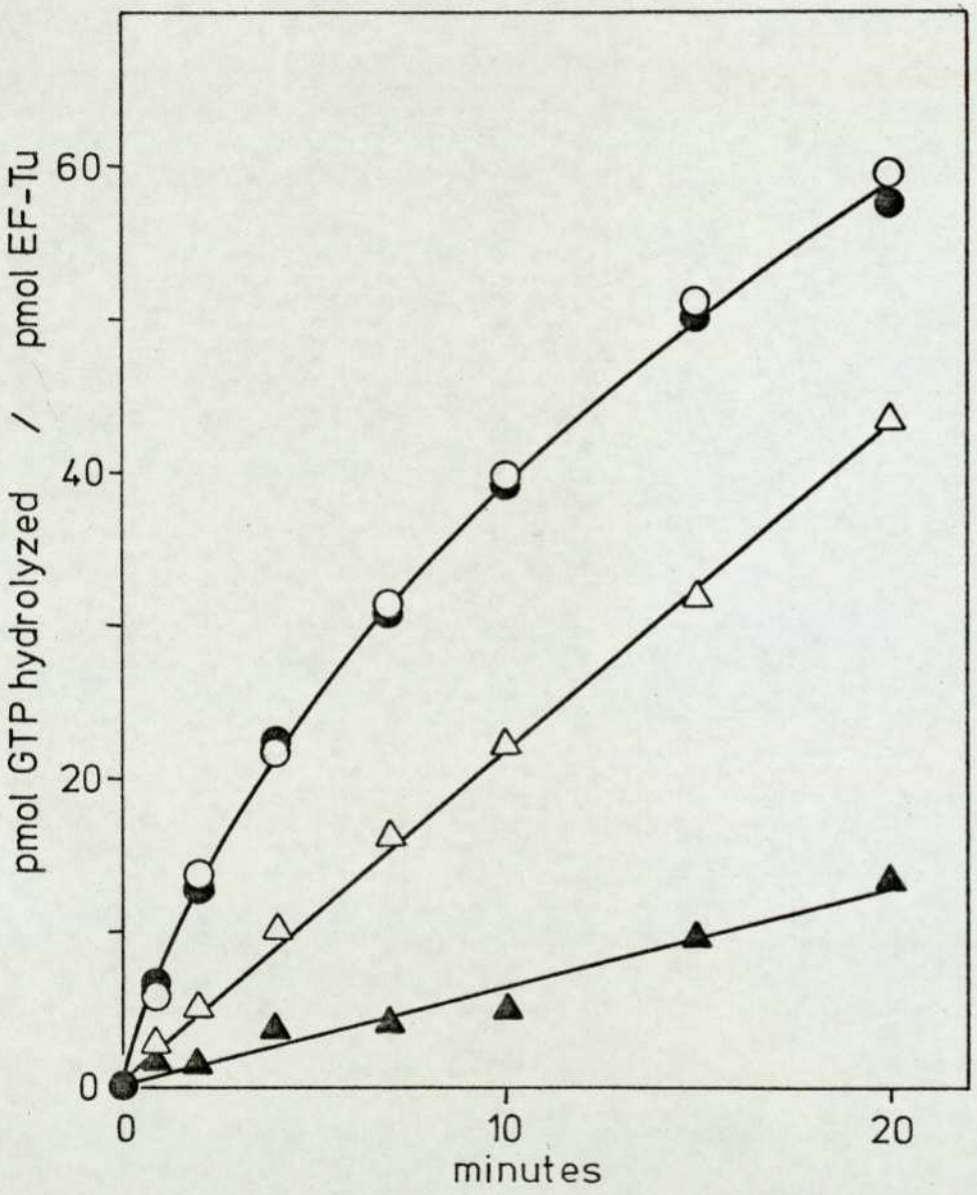


FIGURE 44 KINETIC ASSAY TO SHOW THE EFFECT OF THIOSTREPTON ON THE METHANOL-INDUCED EF-Tu GTPase IN THE PRESENCE AND ABSENCE OF AMINOACYL-tRNA.

Reaction mixtures as in Fig.43 plus 30pmol EF-Ts. (o) ribosomes only, (●)ribosomes + 20uM thiostrepton, (Δ) ribosomes + Phe-tRNA^{Phe}, (▲) ribosomes + Phe-tRNA^{Phe} + 20μM thiostrepton.

purified EF-Tu and EF-Ts to test the requirement for the latter in this system (Fig.43). GTPase activity is absolutely dependent upon EF-Ts, presumably to effect the recycling of EF-Tu.GTP from EF-Tu.GDP. Addition of Phe-tRNA^{Phe} to this system, which also contains 20mM Imidazolium acetate, pH 7.5, 100mM NH_4Cl and 5mM $MgCl_2$ (142), causes a marked inhibition of activity, as has been previously reported in these ionic conditions (143)(Fig.43). Using this system, with a saturating quantity of EF-Ts, the effect of thiostrepton was tested as a function of time (Fig.44). As shown previously for EF-T (142), in the absence of aa-tRNA the antibiotic has no inhibitory effect; but in the presence of aa-tRNA, there is a considerable, though not complete, reduction in activity.

6.3.2 The kirromycin-induced EF-Tu GTPase.

Kirromycin is able to activate the GTPase centre of EF-Tu independently of other normally required effectors (see section 3.3.1). However, ribosomes and/or aa-tRNA can stimulate this hydrolytic activity. Making use of this stimulatory effect, thiostrepton was tested kinetically for its inhibitory capacity in the kirromycin-induced EF-Tu GTPase in the presence of ribosomes with or without added Phe-tRNA^{Phe} (Fig.45). Just as in the preceding section, in the presence of ribosomes only, the EF-Tu GTPase was not at all inhibited by thiostrepton. Addition of Phe-tRNA^{Phe} to the ribosomes in the absence of thiostrepton induced no stimulation of the GTPase, as otherwise might have been expected in the presence of kirromycin (see section 3.3.2; but see below). The reason for this is that these experiments were carried out at 5mM Mg^{2+}, the optimum concentration for the ribosome-only stimulation of the EF-Tu.kirromycin GTPase, and

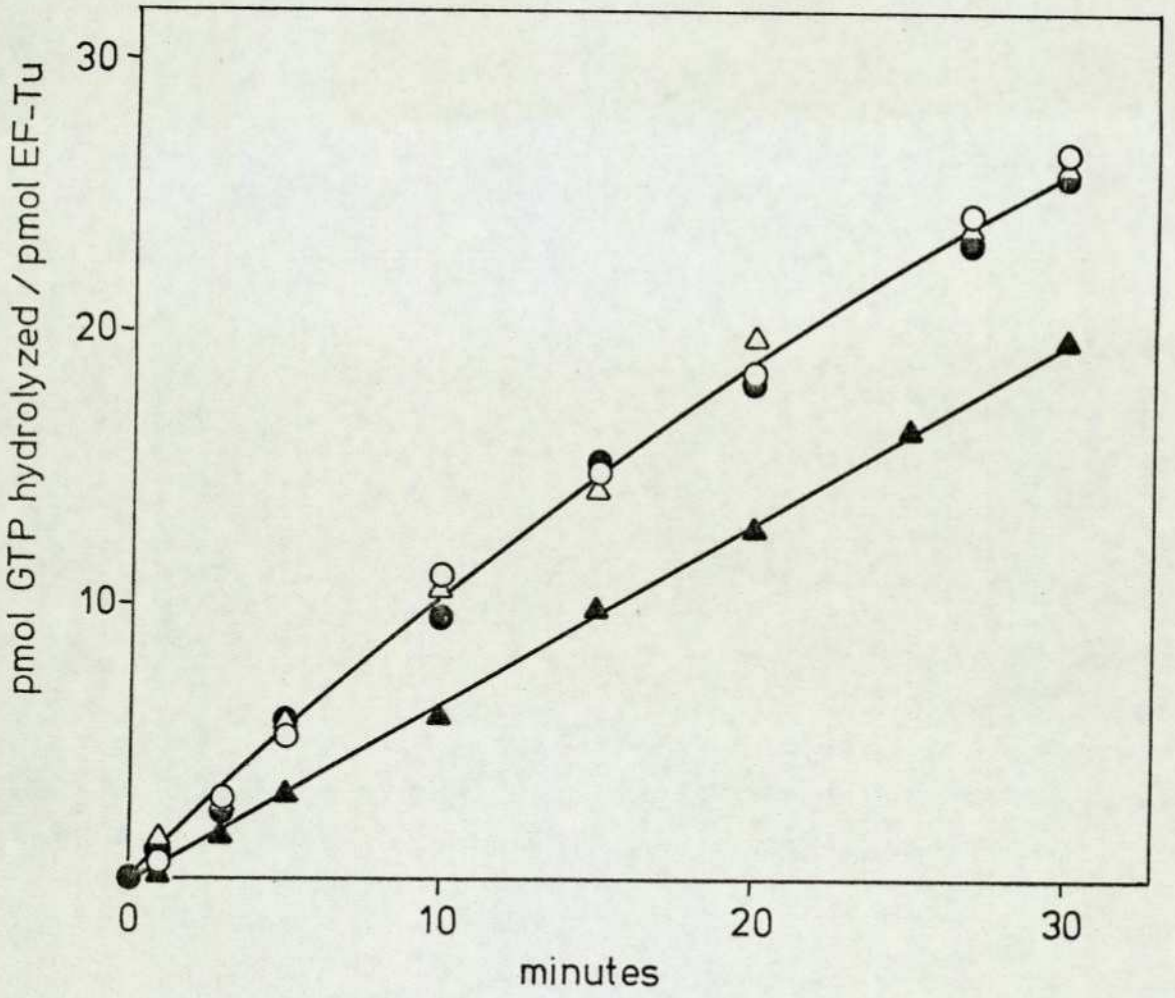


FIGURE 45 KINETIC ASSAY TO SHOW THE EFFECT OF THIOSTREPTON ON THE RIBOSOME-STIMULATED EF-Tu.KIRROMYCIN GTPase: INFLUENCE OF AMINOACYL-tRNA.

Reaction mixtures contained per 75µl, 200mM NH_4Cl, 5mM $MgCl_2$, 0.5mM dithiothreitol, 25mM Imidazolium acetate pH 7.5, 10pmol EF-Tu, 20 pmol ribosomes, 1nmol GTP (specific activity 150 cpm/pmol), with (Δ,▲) or without (○,●) 100pmol Phe-$tRNA^{Phe}$. Open symbols: no thiostrepton; filled symbols: plus 20µM thiostrepton.

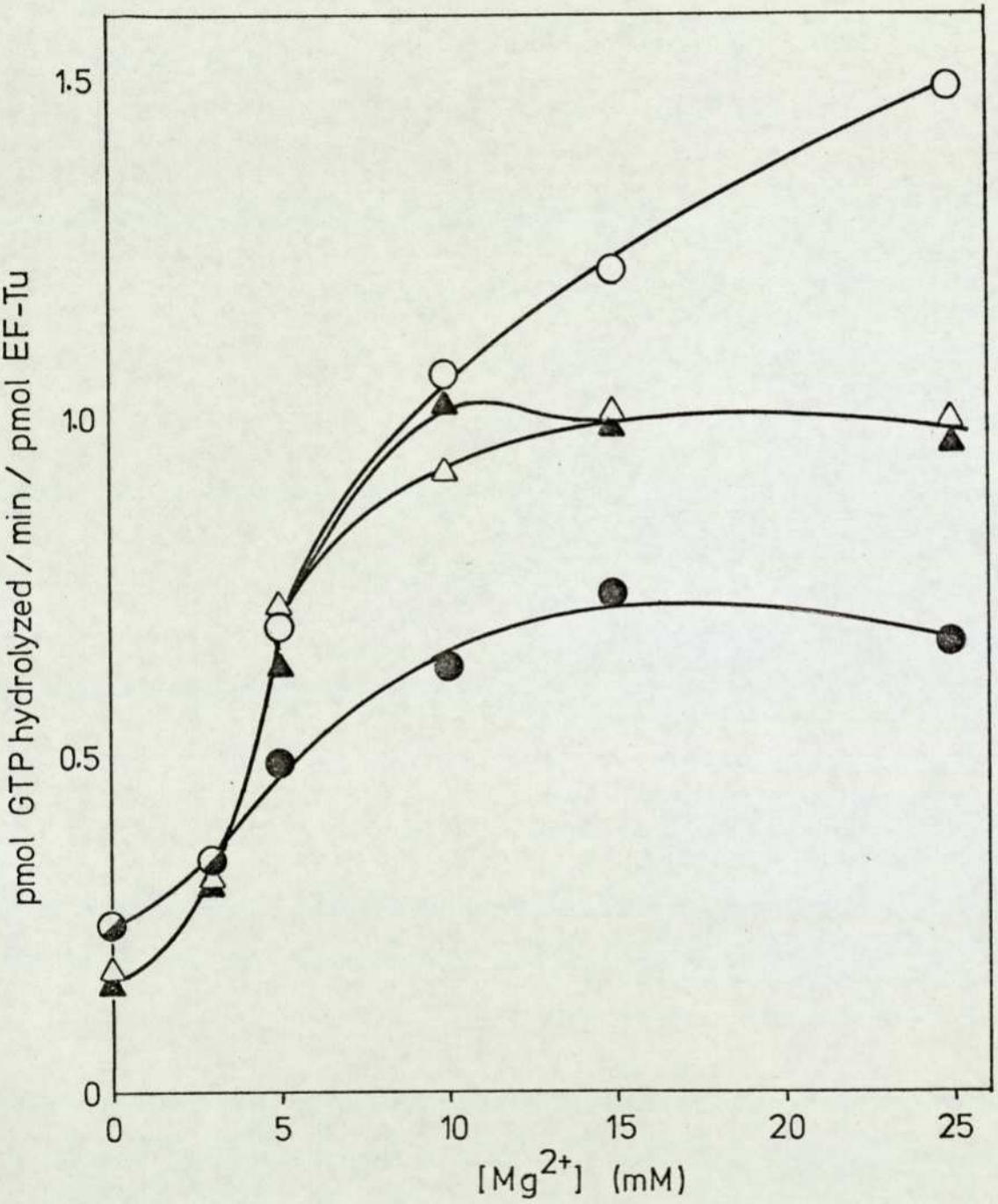


FIGURE 46 Mg^{2+}-DEPENDENCE OF THE RIBOSOME-STIMULATED EF-Tu.KIRROMYCIN GTPase: EFFECT OF AMINOACYL-tRNA AND THIOSTREPTON.

Reaction mixtures as in Fig.45. Additions to EF-Tu.kirromycin: (△) ribosomes only, (▲) ribosomes + 20μM thiostrepton, (○) Phe-$tRNA^{Phe}$ + ribosomes, (●) Phe-$tRNA^{Phe}$ + ribosomes + 20μM thiostrepton.

not at 10mM Mg^{2+} as previously (section 3.3.2). In these conditions, thiostrepton caused only a limited inhibition of activity (ca. 25%), in contrast to the large inhibition incurred on addition of aa-tRNA to the methanol-induced system. It should be noted, though, that the absolute level of hydrolysis attained in the presence of thiostrepton and aa-tRNA is similar in both systems (i.e. approx. 0.6pmol GTP hydrolyzed/min/pmol EF-Tu).

In the absence of thiostrepton, aa-tRNA only stimulates the EF-Tu.kirromycin GTPase in the presence of ribosomes at Mg^{2+} concentrations higher than 5mM (Fig.46), increasing from no stimulation at 5mM to 50% stimulation at 25mM $[Mg^{2+}]$. Thus the same concentration of divalent cations which can relieve aa-tRNA of the necessity for codon-anticodon binding in the physiological EF-Tu GTPase (see section 4.3.7) also induces a greater stimulatory effect in the kirromycin-induced system. Since Mg^{2+} ions can evidently influence the ability of aa-tRNA to stimulate the EF-Tu/ribosome/kirromycin GTPase, the thiostrepton inhibition of this system with and without added aa-tRNA was tested as a function of Mg^{2+} concentration (Fig.46).

As reported above, thiostrepton only exerts an inhibitory effect in the additional presence of aa-tRNA. This inhibition occurs only above 3mM Mg^{2+} and increases to 50% inhibition at 25mM $[Mg^{2+}]$. In the presence of ribosomes and EF-Tu, without aminoacyl-tRNA, thiostrepton not only does not inhibit the kirromycin-induced GTPase at all Mg^{2+} concentrations tested, but even stimulates slightly at 10mM Mg^{2+}, as has been previously remarked (142) for the methanol-induced EF-T GTPase.

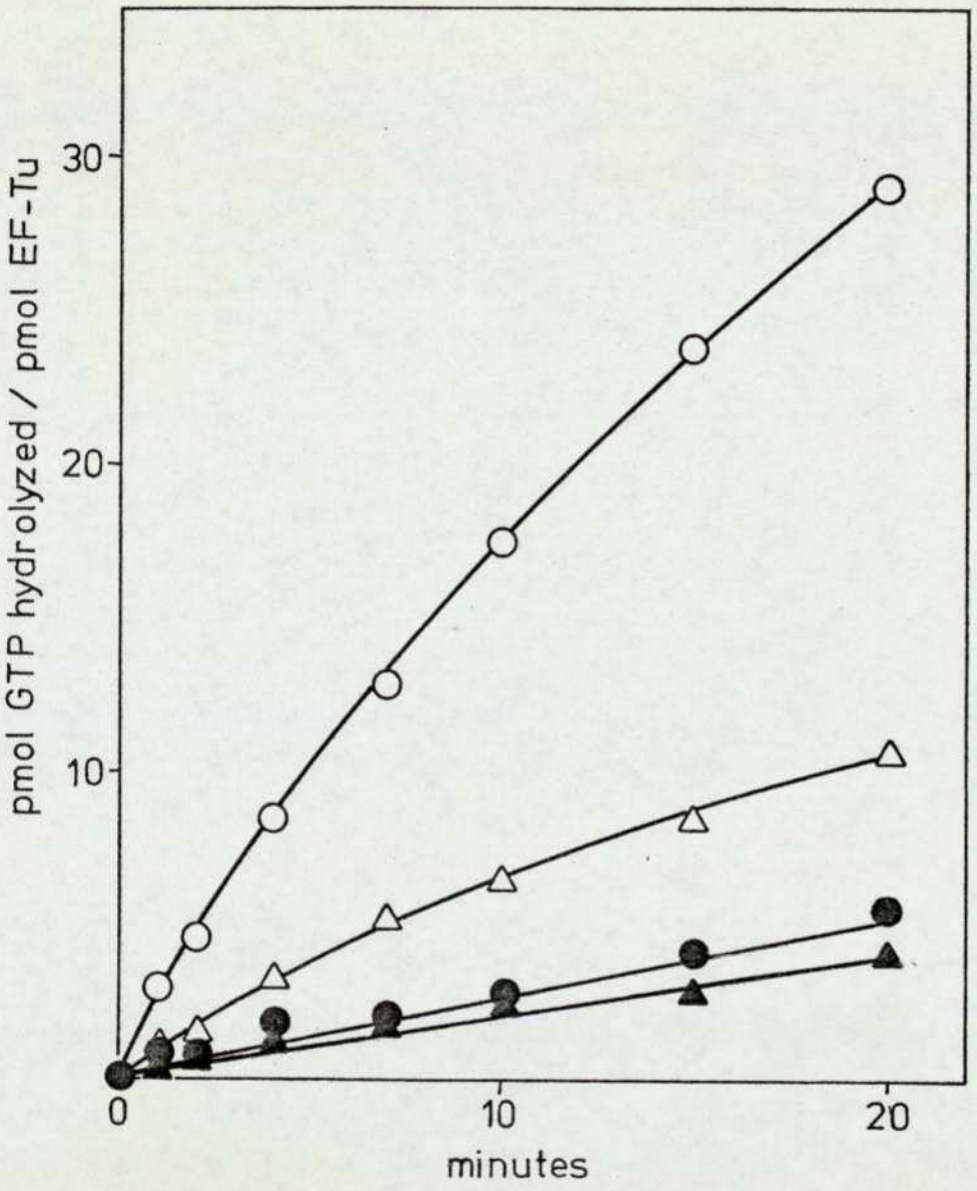


FIGURE 47 KINETIC ASSAY TO SHOW THE EFFECT OF THIOSTREPTON ON THE PHYSIOLOGICAL EF-Tu GTPase: INFLUENCE OF CODON-ANTICODON INTERACTION.

Reaction mixtures contained in 75µl, 30mM NH_4Cl, 25mM Imidazolium acetate pH 7.5, 10pmol EF-Tu, 20pmol ribosomes, 30pmol EF-Ts, 100pmol Phe-$tRNA^{Phe}$, 1nmol GTP (specific activity 150 cpm/pmol) and either 5mM $MgCl_2$ plus 4µg poly(U) (Δ,▲) or 25mM $MgCl_2$ only (○,●). Open symbols: no thiostrepton added; filled symbols: plus 20µM thiostrepton.

6.3.3 The effect of mRNA-dependence on thiostrepton susceptibility of the physiological EF-Tu GTPase.

In the absence of either methanol or kirromycin, for the EF-Tu GTPase to be expressed, ribosomes, aa-tRNA, mRNA and EF-Ts are also necessary. As shown in section 4.3.7, this requirement for mRNA can be waived by increasing the Mg^{2+} level of the incubation, thus freeing the aa-tRNA of codon-anticodon interaction. Using thiostrepton as a probe, the influence of this independence of mRNA was tested on the EF-Tu - ribosome interaction (Fig.47). Assays in the absence of poly(U) were carried out at 25mM Mg^{2+}; in its presence, at 5mM Mg^{2+}, under which conditions activity was completely mRNA-dependent (see section 4.3.7). Irrespective of the experimental conditions chosen, thiostrepton reduced all activity to the same level (approx. 0.2pmol GTP hydrolyzed/min/pmol EF-Tu) which is three times less than the lowest level achieved by thiostrepton in the methanol-induced system or in the kirromycin-induced system. Thus it appears that codon-anticodon binding has no influence on the thiostrepton inhibition of the EF-Tu GTPase.

6.3.4 The effect of monovalent cations on thiostrepton susceptibility of the physiological EF-Tu GTPase.

In this variant of the physiological EF-Tu GTPase, the ability of certain small cations to stimulate the hydrolysis of GTP in this system, as well as raising the K_m for GTP, is exploited. Thiostrepton inhibition was tested in the presence of either 30mM LiCl or 30mM NH_4Cl (Fig.48), with a three-fold excess of EF-Ts, repeating the conditions elaborated in section 3.3.4. In the presence

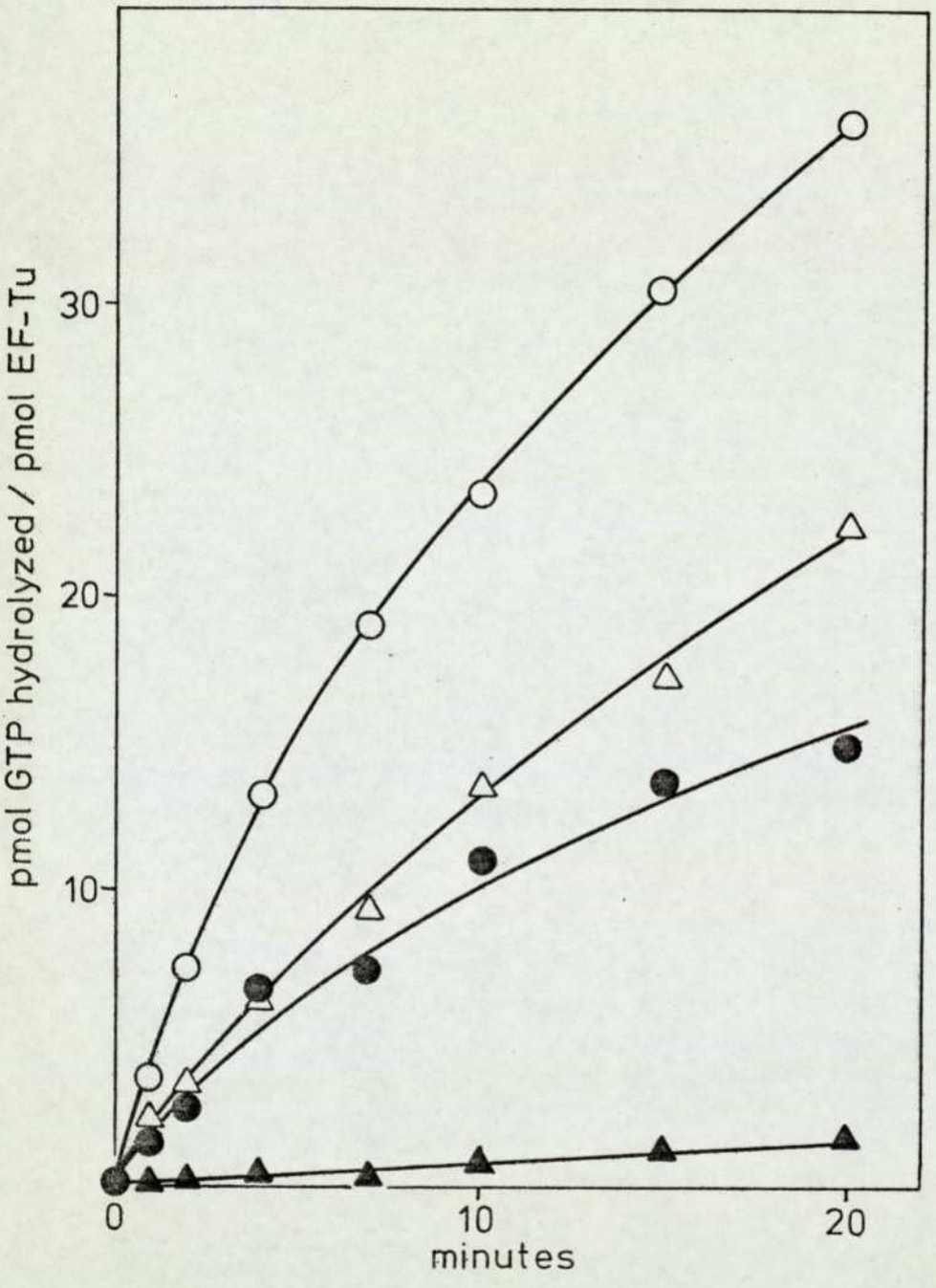


FIGURE 48 KINETIC ASSAY TO SHOW THE EFFECT OF THIOSTREPTON ON THE PHYSIOLOGICAL EF-Tu GTPase: INFLUENCE OF Li^+ AND NH_4^+.

Reaction mixtures contained in 75µl, 30mM NH_4Cl (Δ,▲) or 30mM LiCl (o,●), 5mM $MgCl_2$, 25mM Imidazolium acetate pH 7.5, 10pmol EF-Tu, 20pmol ribosomes, 30pmol EF-Ts, 4µg poly(U), 100pmol Phe-$tRNA^{Phe}$, 1nmol GTP (specific activity 150-200 cpm/pmol). Open symbols: no thiostrepton added; filled symbols: plus 20µM thiostrepton.

of NH_4^+, thiostrepton completely inhibits GTPase activity. By contrast, in the presence of Li^+, activity is reduced by only 50% of the maximum achieved in the absence of the antibiotic. Thus the conformational change in the physiological EF-Tu GTPase system caused by Li^+ is such as also to change the relation of the components to the thiostrepton binding site.

6.4 Discussion.

In the previous chapter, EF-Tu was shown to interact with the ribosome in a complex fashion, GTP being hydrolyzed in the presence of kirromycin, aa-tRNA and ribosomes, with the last reduced by a large number of proteins, though still stimulating activity. The important role of L7/L12 in the presence of L10 was again emphasized. These proteins have also been shown to be critical for the physiological EF-Tu GTPase in the absence of kirromycin (section 5.3.4). Protein L11, the supposed binding site for thiostrepton, appears to be closely connected functionally and physically with proteins L10 and L7/L12 (137,138,139,144,145); the effect of thiostrepton on the EF-Tu GTPase, as presented here, is therefore of considerable interest in indicating the precise relations between the elongation factor and this functionally complex region of the ribosome.

All the experiments show clearly that thiostrepton inhibition occurs only when aa-tRNA is also present, as has been previously suggested for the methanol-induced EF-T system only (142). The extent of this inhibition is variable. In the systems uncoupled from mRNA, induced by methanol or kirromycin, inhibition is not complete, reducing activity to the same value of about 0.6pmol GTP hydrolyzed/min/pmol EF-Tu. Thus, although the antibiotic clearly imposes a constraint on

the aa-tRNA, this is not such as to entirely inhibit the GTPase activity of EF-Tu. By contrast, in the physiological system, in the presence of NH_4^+, thiostrepton inhibition is much more marked reducing GTPase activities to less than 0.3pmol GTP hydrolyzed/min/pmol EF-Tu. Since the existence of codon-anticodon interaction appears to have no influence on this inhibition (Fig.47), this increased effect of thiostrepton appears not to be due to an added constraint on the tRNA in the region of the anticodon loop, imposed by the mRNA.

The effect of Li^+ on the physiological EF-Tu GTPase system is interesting. In the absence of thiostrepton, Li^+ at 30mM can stimulate the EF-Tu GTPase to as much as 3pmol GTP hydrolyzed/min/pmol EF-Tu (see also section 3.3.4). Thiostrepton only inhibits this activity to about 40% of the original: still an appreciable hydrolysis. This effect is clearly comparable to the limited inhibition seen with methanol or kirromycin in the presence of aa-tRNA, though in the case of Li^+, there is no uncoupling from codon-anticodon interaction (see section 3.3.4). This suggests that the cation is inducing a conformation in the components of the system similar to that induced by methanol or by kirromycin, such that the topographic relationship between the tRNA and the thiostrepton binding site is altered. Perhaps also the conformation of the aa-tRNA, in being bound to EF-Tu, is less susceptible to the blockage of L11. In view of the stimulating effect of Li^+ on the EF-Tu.kirromycin GTPase in the absence of ribosomes, it is tempting to support the latter idea.

Taking the physiological EF-Tu GTPase in the presence of NH_4^+ as the control system, one must also explain why both methanol- and kirromycin-induced systems are less susceptible to thiostrepton.

One explanation could be, as above, that the ternary complex, EF-Tu.GTP.aa-tRNA, is more immune to the inhibiting effect of blocking protein L11. Certainly, kirromycin induces a conformation in EF-Tu, which is active in a variety of conditions where otherwise no activity is measurable, conditions such as high ionic strength which are inimical to the majority of biological systems. Methanol may achieve a similar effect, via both hydrophobic and hydrophilic actions, by reducing the degree of freedom of what is otherwise a conformationally "wobbly" system, which normally requires a constellation of cations and specific biological effectors to invoke the enzymatically active conformation in the elongation factor. Such an effect of organic solvents in ribosomal systems has been recently suggested (146).

It should be noted that in the assay conditions used (5-10mM Mg^{2+}) the presence of uncharged tRNA carried over with the aminoacyl-tRNA is of little importance. Firstly, in the presence of mRNA, cognate codon-anticodon interaction of the ternary complex in the A-site of the ribosome has a higher binding affinity than cognate but uncharged $tRNA^{Phe}$ (130,147), the latter, therefore, always being ousted. Secondly, the method used to prepare Phe-$tRNA^{Phe}$ (see section 3.2.5) is such as to acylate virtually all $tRNA^{Phe}$, the remaining uncharged tRNA being of non-cognate species, which in the presence of poly(U) should not bind to the ribosome. In the kirromycin-induced system it has been shown that uncharged tRNA has no effect on the GTPase activity (31) and interacts with the ribosomal P-site only weakly in the absence of mRNA (130).

The fact that such diverse reagents as kirromycin, methanol or Li^{+} can achieve closely comparable results in a highly complex and specific biological system, illustrates without doubt the identity

of the enzymatic activity concerned - the EF-Tu GTPase. These results confirm absolutely the location of the enzymatic centre for GTP hydrolysis on the elongation factor, and demonstrate that this activity can be uncoupled to varying degrees from its normal physiological effectors by a variety of different agents.

Footnote: Thompson, Cundliffe & Stark (148) have recently shown that thiostrepton will bind to protein L11 in the presence only of 23S RNA. This confirms that L11 is the site for thiostrepton binding on the E.coli ribosome.

CHAPTER 7. DISCUSSION: SYNTHESIS AND SPECULATIONS.

Throughout this study close attention has been paid to the ionic environment of the reactions investigated. It is therefore important to be able to relate these results using *in vitro* systems to the environment which pertains within the bacterial cell *in vivo*. In Table VIII are gathered a variety of data, collated from the literature, relating to the constitution of the bacterial cytoplasm. Since most data refer to total quantities as measured by destructive methods or exchange of radioactive isotopes, great care should be taken in transferring such *in vivo* data to the *in vitro* environment. In the case of divalent cations, for example, it is almost certain that all the ions will be chelated to the great variety of macromolecules existing in high concentration within the cell. Nucleotides and nucleic acids, particularly, will chelate Mg^{2+}. Since large proportions of the ions will be in bound form, the chemistry of enzymic reactions will depend not on simple cation concentrations, as measured *in vitro*, but more probably on topology of the ionic environment - can ions bound to one macromolecule (or membrane) have an influence (local or long distance) over another macromolecule ? The effective concentration of any ion will be a resultant of the chelating capacities of all macromolecules for all ions. Thus the data on cation concentration presented in Table VIII should be regarded with a certain scepticism. Despite this, it is probably of significance that optimal concentrations of monovalent and divalent cations *in vitro* ($< 200mM$ $[M^+]$; $4-10mM$ $[M^{2+}]$) do correspond approximately to the order found *in vivo*.

TABLE VIII TOTAL QUANTITIES OF VARIOUS MOLECULES INVOLVED IN PROTEIN SYNTHESIS IN EXPONENTIAL, RAPIDLY GROWING STRAINS OF ESCHERICHIA COLI GROWN IN COMPLETE RICH MEDIA.

	No. of molecules per cell	Approximate molarity	Reference
DNA	4-5	-	149,150
tRNA	190000-300000	0.10-0.20mM	38,150
Ribosomes	32000	0.015-0.020mM	149,150
EF-Tu	300000	0.15-0.20mM	38,53,§
EF-G	40000	0.020mM	53
EF-Ts	30000	0.015mM	53
RNApolymerase α	6500	0.003mM	53
RNApolymerase β	18000	0.009mM	53
ribosomal protein S1	45000	0.023mM	53
stringent factor	150-200	-	151
release factors	600-700	-	151
all nucleotides	3×10^7	15mM	149
ATP	-	1-2mM	152
GTP	-	0.3-1.0mM	152
ppGpp	-	0.02-0.06mM	152
K^+	-	200-250mM	153,154,155, 156,157
Na^+	-	20-80mM	153,154,157
NH_4^+	-	5-10mM	156
Mg^{2+}	-	20-30mM	153,154,156
Ca^{2+}	-	5mM	153,154
putrescine	-	13mM	158
spermidine	-	5mM	158

§ this thesis.
Approximate molarity assumes that the total content measured is in free solution in a volume corresponding to the size of an E.coli cell.

In the translation of mRNA into polypeptide chains, the central role is played by the tRNA adaptor molecule, the anticodon loop of which binds highly specifically to the nucleotide triplets carrying the coded information for single amino acids. Since from a theoretical point of view little more is required of such an adaptor molecule than the anticodon trinucleotide sequence, one is forced to ask why the remaining part of the molecule, the ribosome on which it functions and the EF-Tu.GTP to which it binds as a ternary complex and which is an essential accompaniment of reliable translation, are necessary. Part of the answer lies in the relatively low affinity of the anticodon triplet for the appropriate, or cognate, codon, when free in solution ($K' = 10^{-3}$M at 0°C)(159); this affinity is only about 10 times higher than for the non-cognate codon (159). If translation depended only upon such a discrimination system it has been estimated that over half the codons of a mRNA sequence would be translated with an accuracy of less than 99% (160). Since miscoding *in vivo* is of the order of one nucleotide misread per 10000 (161), the additional molecular machinery must be involved primarily in increasing the reliability of the translation without necessarily impairing its speed.

Since EF-Tu is closely involved with most of the reactions by which tRNA succeeds in encoding amino acids into polypeptide chains, it would be logical to consider the part EF-Tu may play in increasing the speed and fidelity of translation. In the following discussion the functional properties of EF-Tu will be examined with this particular point in mind. In this study effort has been concentrated on the EF-Tu -dependent hydrolysis of GTP, and the role that this GTPase activity may play in translation. The antibiotic kirromycin has been especially

valuable, since it allows a study of the interaction of EF-Tu with various effectors, singly or together, by its remarkable property of inducing the EF-Tu GTPase, whose catalytic centre, as a result of this study has been shown clearly to reside on the elongation factor and not as once thought on the ribosome (162,163). EF-Tu is an interesting protein in that it interacts with a variety of macromolecules, as many as four simultaneously, as well as with both monovalent and divalent cations. All of these influence the conformation of the elongation factor such as to promote or inhibit the hydrolysis of GTP. In the following sections the interactions between EF-Tu and individual effectors are reviewed, so as to analyse individual steps in the mechanism by which EF-Tu may be said to aid translation.

7.1 Interaction of EF-Tu with guanine nucleotides.

EF-Tu is able to form a binary complex with one molecule of either GTP or GDP, as well as with a variety of analogues such as ppGpp, GMPPNP, GMPP(CH_2)P, GMP(S)P, etc., (73,101,102,164) which share the common feature that the GDP core is essentially unaltered. Other purines or pyrimidines have little or no affinity for the elongation factor (32,100). The location of the guanine nucleotide binding site, near to the C-terminus in the primary sequence (27), is proposed to be on the external surface of the denser "head" region of the X-ray model (27), and not as has been suggested buried within the molecule (86). In addition to the high specificity for the purine moiety, there appears also to be a strict requirement in relation to the phosphate chain. From studies using diastereoisomers of GMP(S)P, it has been shown that EF-Tu preferentially binds the A-stereoisomer of the α-phosphate, implying that the β-phosphate is involved in

coordination to EF-Tu (101). Furthermore, the γ-phosphate of the nucleotide appears to lie in an exposed position, since it can be removed by phosphatase action when the GTP is bound to EF-Tu (165), and it has been shown that a variety of sometimes large substitutions may be made in the γ-position without grossly influencing the affinity of the nucleotide for the factor (164). EF-Tu contains three -SH groups, two of which are available for reaction with thiol reagents (63). One of these groups appears to be involved in the binding of guanine nucleotides (100), though more recent information suggests only an indirect role for this -SH group (166). Taken together, all this information points to a highly specific binding site for guanine nucleotides.

In order to understand the significance of the hydrolysis of GTP when bound to EF-Tu, it is necessary to consider the dramatic change in affinity of the elongation factor for the two guanine nucleotides GTP and GDP. Fasano et al. (28) in a detailed study showed that the equilibrium constant for GDP is two to three orders of magnitude lower than for GTP, such that GDP, because of its very slow rate of dissociation, is effectively "locked" into the nucleotide binding site. Not only does this conformational change on hydrolysis of GTP to GDP locally influence the nucleotide binding site, but it has a great influence on the binding of other, macromolecular effectors, particularly aa-tRNA and EF-Ts. Though these effectors will be discussed in more detail in later sections, one allosteric effect of GTP hydrolysis is to reduce dramatically (by 5 orders of magnitude) the affinity of the elongation factor for aa-tRNA, such that when GTP is hydrolysed to GDP in the nucleotide binding site, the liaison between aa-tRNA and EF-Tu is effectively broken. This is of great significance in discussing

the function of the GTPase activity in relation to the translation of mRNA. The binding of a guanine nucleotide also appears to be essential to maintain the stability of the EF-Tu molecule when not associated with other effectors; EF-Tu liberated from GDP consistently and irreversibly loses activity (102,167).

Since the GTPase activity is such a central function of the EF-Tu involvement in the translation process, special attention has been given in this investigation to this enzymatic reaction, particularly concentrating on the way it is modulated either by cations or by other macromolecular effectors. Making use of kirromycin to stimulate the hydrolytic centre of EF-Tu, it was shown in chapter 3 that monovalent cations are implicated in the GTPase activity. In the absence of the other physiological effectors and at moderately high monovalent cation concentrations (>400mM) there is an order of stimulation: $Li^+ > Na^+ > K^+ > NH_4^+ > Cs^+ > Me_4N^+$. Such a series suggests a highly charged anionic region influencing the catalytic centre. The relationship would appear to be direct rather than indirect since under these conditions there is also a concomitant change in the K_m for the GTPase, which is a measure of the affinity for GTP of this reaction system. However, the smaller monovalent cations appear not to influence the binding of GDP to EF-Tu (100). Results with divalent cations (chapter 4), showing a marked cation-dependent pH shift in the kirromycin-induced EF-Tu GTPase, also implicated the involvement in the catalytic centre of a highly charged anionic region, which could be masked by Mg^{2+}. The requirement for divalent cations for stimulating the EF-Tu GTPase activity in the presence only of kirromycin and the elongation factor was most marked at high monovalent cation concentrations. It was also demonstrated, however, that the GTPase centre was able to

function in the absence of free divalent cations, albeit at a low rate of activity. Nevertheless, increasing the concentration of EDTA in the reaction indicated that Mg^{2+} was additionally involved in maintaining an active conformation of the elongation factor. Similar conclusions were reached using GDP-binding studies (100), where it was shown that divalent cations, particularly Mg^{2+} and Mn^{2+}, increased the affinity of the elongation factor for GDP, and that Mg^{2+} helped maintain integrity of the protein structure, increasing resistance to tryptic digestion. Proton relaxation studies (86) also indicated that Mn^{2+} aided GDP binding.

These results show that local charges are clearly involved in the regulation of the GTPase centre. These charges can be influenced by cations or by physiological effectors, particularly ribosomes, which were also shown to be functionally equivalent in some conditions to monovalent or divalent cations.

7.2 Interaction of EF-Tu with EF-Ts.

EF-Tu reacts with EF-Ts to form a stable complex EF-T (EF-Tu.EF-Ts). In cell lysates most EF-Ts is recovered in the form of EF-T, unless an excess of GDP is added to the cell extract. This complex, EF-T, also forms a stable component of Qβ replicase (41), and this suggests that it may have other functions *in vivo* than those concerning polypeptide elongation. On interaction of EF-Ts with EF-Tu, the nucleotide binding site is "opened", both association and dissociation rate constants for GDP and GTP being increased (28). The importance of this effect is that it allows GDP to dissociate from the EF-Tu, to which previously it had been very tightly bound, and thus provides conditions for an exchange of guanine nucleotides. Conversely, an

excess of GDP can cause the dissociation of EF-Tu from EF-Ts. This competition between GDP and EF-Ts has been further demonstrated using a covalently cross-linked EF-Tu.EF-Ts complex in which GDP binding activity was almost completely inhibited (42). However, in the thermophilic bacteria, Thermus thermophilus, the ternary complex EF-Tu.EF-Ts.guanine nucleotide has been demonstrated (168), and it seems probable that guanine nucleotides and EF-Ts compete allosterically, without necessarily occupying overlapping sites in EF-Tu. Further work is necessary to clarify this complex interaction.

In the physiological EF-Tu GTPase, because it encourages the exchange of guanine nucleotides and thus makes possible the binding of aa-tRNA to EF-Tu.GTP, EF-Ts is essential to recycle the EF-Tu.GDP into ternary complex, EF-Tu.GTP.aa-tRNA. In chapter 3 it was shown that the saturation curve for EF-Ts of this GTPase activity could be modified by monovalent cations. And even more interesting, monovalent cations simultaneously altered the K_m for GTP of this reaction. This result implies that monovalent cations can modulate the affinity of EF-Tu for EF-Ts, at least in this system, and likewise influence the catalytic centre, suggesting that there is an allosteric link between the two sites which is possibly regulated by monovalent cations. In an attempt to explore this situation further, similar assays were carried out using EF-Tu purified from the mutant E.coli strain D2216. This $EF\text{-}Tu_{D2216}$ has an altered affinity for kirromycin and because of their overlapping binding sites on the factor possibly also for EF-Ts. Results showed that the monovalent cation effect on the EF-Ts saturation curves disappeared, both Na^+ and NH_4^+ behaving similarly. Likewise, there was no measurable difference in the K_m for GTP, dependent on the cation species, as found with the wild-type.

This strongly suggests that the EF-Tu - EF-Ts interaction is influenced by monovalent cations. Work is currently in progress to clarify these points; there are still many aspects of the role of EF-Ts which remain unclear.

7.3 Interaction of EF-Tu with kirromycin.

The antibiotic kirromycin interacts with EF-Tu at a site which appears to overlap directly the EF-Ts binding site, since there is clear evidence of direct competition between the two effectors (31). Kirromycin has also been shown to be a potent inhibitor of the Qβ replicase enzyme when the latter is dissociated into its component parts, evidently blocking the reformation of the EF-Tu.EF-Ts complex which comprises half the enzyme (42). In the mutant EF-Tu from strain D2216, not only is there a decreased affinity for kirromycin by about 2.5 orders of magnitude (Fig.49), but its relationship to EF-Ts also appears to be altered (section 3.3.5). In wild-type EF-Tu, kirromycin increases both association and dissociation rate constants for GDP, just as in the case of EF-Ts (28). Additionally, however, kirromycin decreases strongly the equilibrium constant for EF-Tu.GTP from approximately 600nM to 1.4nM at 0°C, in this way mimicking the action of aa-tRNA in stabilizing the formation of EF-Tu.GTP complex, and providing the prerequisite conditions for a turnover GTPase activity. Furthermore, the antibiotic activates the catalytic centre of the elongation factor. This GTPase activity, occurring in the presence only of kirromycin, EF-Tu and GTP is completely dependent on monovalent cations. The smaller the monovalent cation and the higher its concentration, the more the stimulatory effect. Stimulation of this type and magnitude only occurs in the presence of the antibiotic,

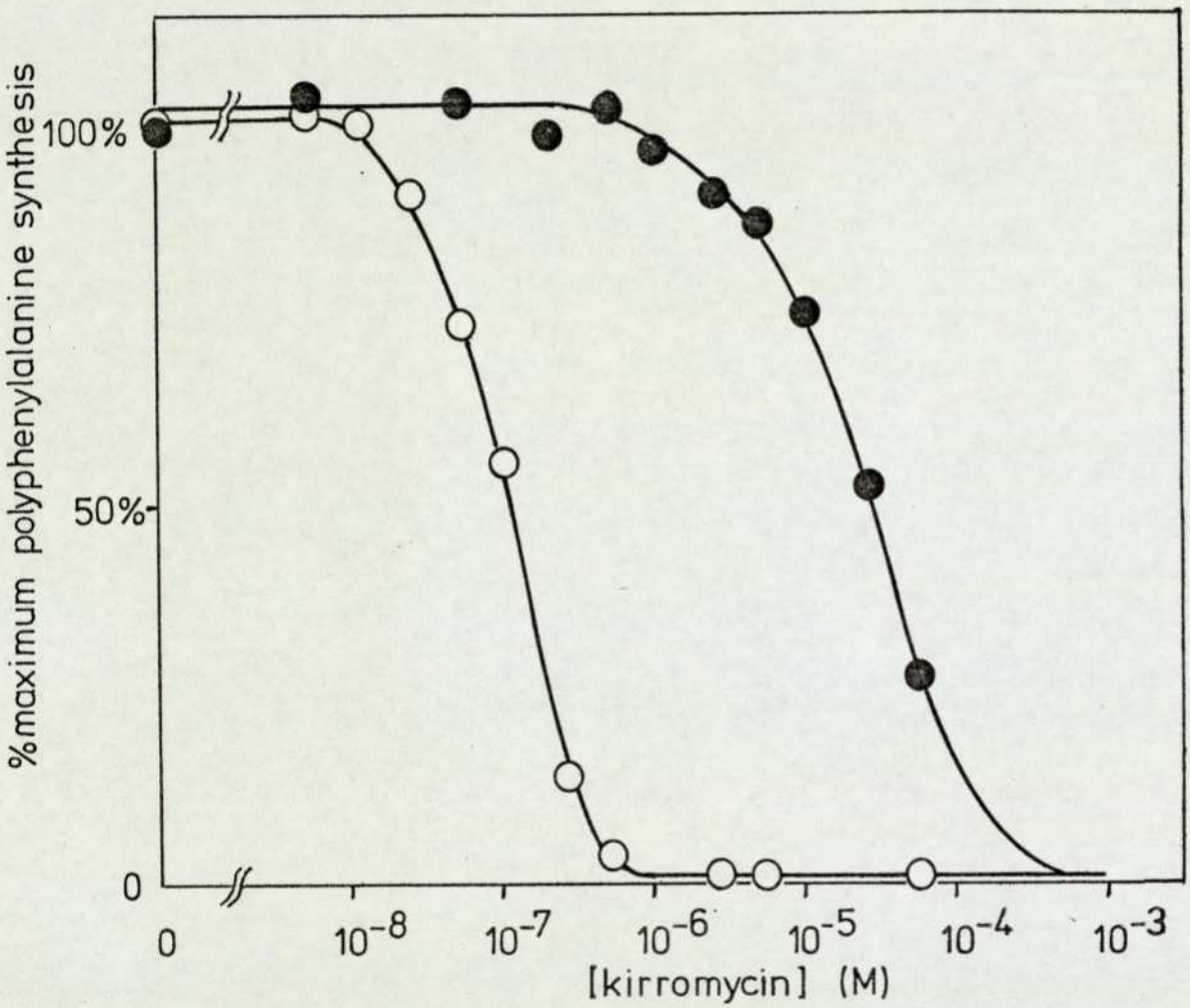


FIGURE 49 INHIBITION BY KIRROMYCIN OF POLYPEPTIDE SYNTHESIS IN E.coli PARENTAL (D22) AND MUTANT (D2216) STRAINS.

Assays were carried out as described in section 2.2.7. EF-Tu was supplied as the 30000g supernatant from strains D22 (o) or D2216 (●).

though a considerably lesser stimulating effect by small monovalent cations is seen at 30mM in the physiological EF-Tu GTPase which includes EF-Ts, as described in the preceding section. Evidently, the active catalytic conformation of EF-Tu which is induced by the kirromycin is much more tolerant of a wide range of cation concentrations. It is tempting to propose that the monovalent cations are acting in similar ways by influencing a common allosteric pathway linking kirromycin (EF-Ts) and the catalytic centre: in both the kirromycin-induced and physiological systems the K_m for GTP appears to be influenced in the same direction and to the same extent by the monovalent cations. Because of the negative effects of small cations on the other components of the physiological EF-Tu GTPase, it is hardly suprising that high concentrations of Li^+ or Na^+ are inhibitory in this system. Kirromycin thus appears functionally to resemble both EF-Ts and aa-tRNA, apparently inducing a conformation in EF-Tu not only similar to that induced by these effectors but one that is additionally much more resistant to the negative effects of high ionic concentrations.

7.4 Interaction of EF-Tu with aminoacyl-tRNA.

The formation of the ternary complex EF-Tu.GTP.aa-tRNA is an essential intermediate step in the EF-Tu - dependent GTPase activity accompanying the encoding of aa-tRNA on the ribosome.mRNA complex (63, 94). Aminoacyl-tRNA binds only to the conformation of EF-Tu induced by interaction of the factor with GTP (in the absence of kirromycin), which via a reciprocal interaction it then locks into the nucleotide binding site, by decreasing the EF-Tu.GTP dissociation rate constant about 130 times (28). Thus, although topologically separate, the binding sites for aa-tRNA and guanine nucleotides are sterically linked. EF-Tu appears

only to recognize part of the -CCA end of the aa-tRNA, and only in the configuration adopted by being aminoacylated via either 2' or 3' ester linkage at the terminal adenosine (169,170); amide linkage of the amino acid is not recognized. However, spin-labelling and other modifications of the nucleotides of the -CCA terminus do appear possible without grossly affecting EF-Tu - aa-tRNA recognition (30). It has been additionally shown that if the tRNA has been misacylated by the tRNA-synthetase, the EF-Tu will fail to discriminate the mistake (171), such that any editing of this error must occur prior to ternary complex formation. This misacylation refers to the substitution of the correct amino acid proper to a tRNA species by one that is inappropriate, and does not include the formylated methionine-$tRNA_f^{met}$, important in initiation, which is not recognized and bound by EF-Tu (172,173). An -SH group on EF-Tu has been implicated in this interaction with aa-tRNA (63,165). The ternary complex thus formed appears to be only moderately stable, but can be isolated by Sephadex G-100 chromatography (174). It has also been demonstrated by protection experiments in which EF-Tu inhibits the hydrolytic deacylation of aa-tRNA at 25°C (175). The two molecules, EF-Tu and aa-tRNA, although of different molecular weight, have similar lengths and yield a complex of closely similar chromatographic characteristics to the component molecules (32). This is an important point to recall when considering the topology of tRNA binding to the ribosome. Fig.50 gives an artist's impression of the relative sizes and shapes of the molecules discussed, based on recent models (26,27, 92,176,177,178).

In the presence of kirromycin, which induces a monovalent cation - dependent GTPase activity in EF-Tu, the addition of aa-tRNA stimulates GTP hydrolysis, thus showing that the conformation of the

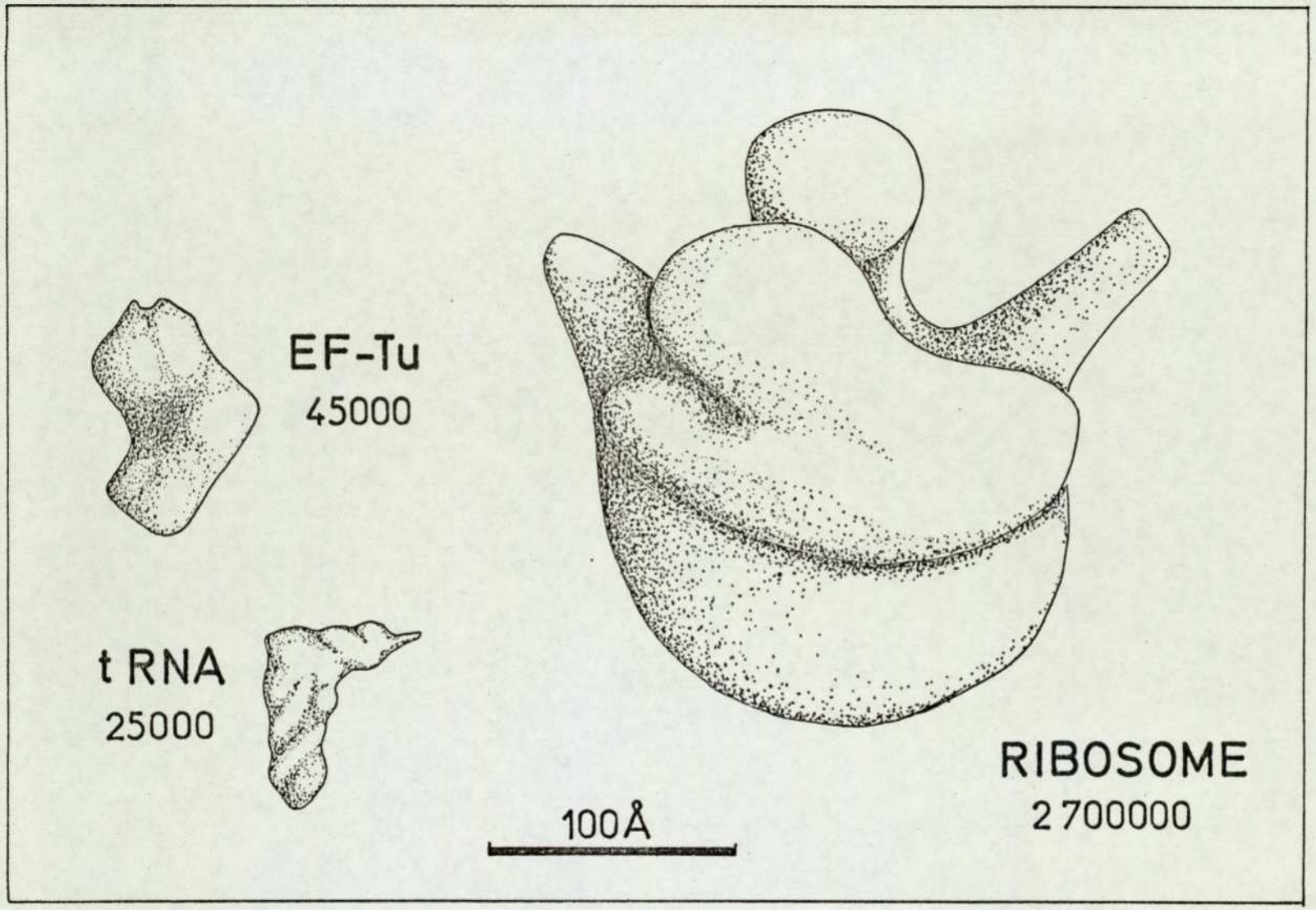


FIGURE 50 ARTIST'S IMPRESSION OF THE RIBOSOME, EF-Tu AND tRNA MOLECULES.

Based on published models (see text)

EF-Tu is changed to a more catalytic one just by the binding of aa-tRNA. Though increasing the rate of GTP hydrolysis, this binding does not affect the K_m for GTP (data not shown), suggesting that the allosteric pathway involved is independent of the one linking the catalytic centre and the kirromycin binding site. Neither does this interaction influence the monovalent cation requirements of the GTPase activity.

Aminoacyl-tRNA binding to EF-Tu also induces a change in the conformation of the tRNA molecule itself. Clark (166) has postulated that there is a loosening of structure in the -TΨCG- loop of the aa-tRNA on ternary complex formation, and at the same time there is an increase in the affinity of the anticodon loop for the complementary, cognate codon, either in the presence or in the absence of 30S ribosomal subunits (179). Thus, the formation of the ternary complex induces reciprocally a conformational change in the two major components involved, and at the same time augments the discriminating capacity of the tRNA-codon interaction.

7.5 Interaction of EF-Tu with the ribosome in the absence of aa-tRNA.

There is not a great deal of information relating to the interaction of EF-Tu with the ribosome in the absence of aminoacyl-tRNA. Cross-linking studies of EF-Tu to ribosomal proteins, made in the presence of aa-tRNA, have implicated a variety of 50S proteins in the EF-Tu neighbourhood (180,181), though the lack of consistency in the results allow few conclusions to be drawn. Published studies relate to the EF-T GTPase activity which can be induced by 20% methanol in the additional presence only of ribosomes (143). Using this system it was shown that high rates of GTPase activity could be induced in

the presence of the 50S subunit only (182). Proteins L7/L12 were essential for full activity; on using 50S ribosomal cores depleted of these proteins GTPase activity was reduced by 65% (182). In an interesting series of experiments Ringer et al. (183) showed that an aminoacyl-dinucleoside analogue of aa-tRNA would bind to the ribosomal A-site and inhibit the normal physiological EF-Tu GTPase activity. They then repeated the experiments in the absence of aa-tRNA, but in the presence of 20% methanol, conditions suitable for the uncoupled EF-T GTPase activity. The aminoacyl-dinucleoside inhibited as much in this assay as previously, and since in the presence of methanol the analogue could not bind to EF-Tu, this demonstrates that the inhibitory effect is acting via the ribosome - EF-Tu interaction.

Using the kirromycin - induced EF-Tu GTPase, it was shown in chapter 5 that the 50S subunit, in the absence of aa-tRNA, alone could stimulate activity. The 30S proved inert. Then, making use of ribosomal cores depleted of various proteins of the large subunit, it was demonstrated that, in the absence of aa-tRNA, relatively large activities were obtained only with the NH_4Cl/ethanol cores, and that this activity could be increased to the same value as intact ribosomes on the reintegration of proteins L7/L12. Thus, as in the physiological EF-Tu GTPase system (36, section 5.3.4), L7/L12 appear to play an important role in the binding of EF-Tu to the ribosome. Other ribosomal components must also be involved since, in the absence of L7/L12, more than 60% of the maximal activity without aa-tRNA can be measured with the NH_4Cl/ethanol cores. However, on passing to CsCl cores a, b and c, this activity is largely lost (Fig.39), implying that the proteins lost in passing from NH_4Cl/ethanol cores to CsCl core a (i.e. L1, L10,

(L11), L16, L25 and L33) are implicated in the EF-Tu-ribosome interaction sufficiently to influence the GTPase activity.

To help clarify this situation, experiments were carried out using the antibiotic thiostrepton, which is considered to bind on protein L11 (37,148). In both methanol- and kirromycin-induced systems thiostrepton had no effect in the absence of aa-tRNA. Thus the immediate neighbourhood of protein L11 is not involved in the binding of EF-Tu to the ribosome. These results lend weight to the similarity between the kirromycin-induced systems for generating EF-Tu GTPase activity, leaving very little doubt as to the identity of the enzymatic activities.

Because ribosomes could stimulate EF-Tu GTPase activity in the presence of kirromycin it was possible to assess the roles of monovalent and divalent cations in this stimulation. Ribosomes were able to replace completely the requirement for monovalent cations otherwise essential for the EF-Tu.kirromycin activity. At moderate monovalent cation concentrations (200mM) this ribosomal stimulation of the EF-Tu.kirromycin GTPase was completely dependent on Mg^{2+}. Activities were highest in the presence of NH_4^+, followed by K^+ and Li^+; Cs^+, Na^+ and Me_4N^+ allowed no ribosomal stimulation at 200mM. This is in marked contrast to the activities at high $[M^+]$, where ribosomes eliminated any requirement of the EF-Tu alone for divalent cations. This ionic substitution effect of the ribosomes at 2M M^+ was shown to be sensitive to high concentrations of EDTA, and thus was due either directly to Mg^{2+} tightly bound to the ribosomes, to a Mg^{2+}-dependent ribosome conformation, or to protection by the ribosomes of the Mg^{2+}-dependent active conformation of EF-Tu. Further research is necessary to clarify this point.

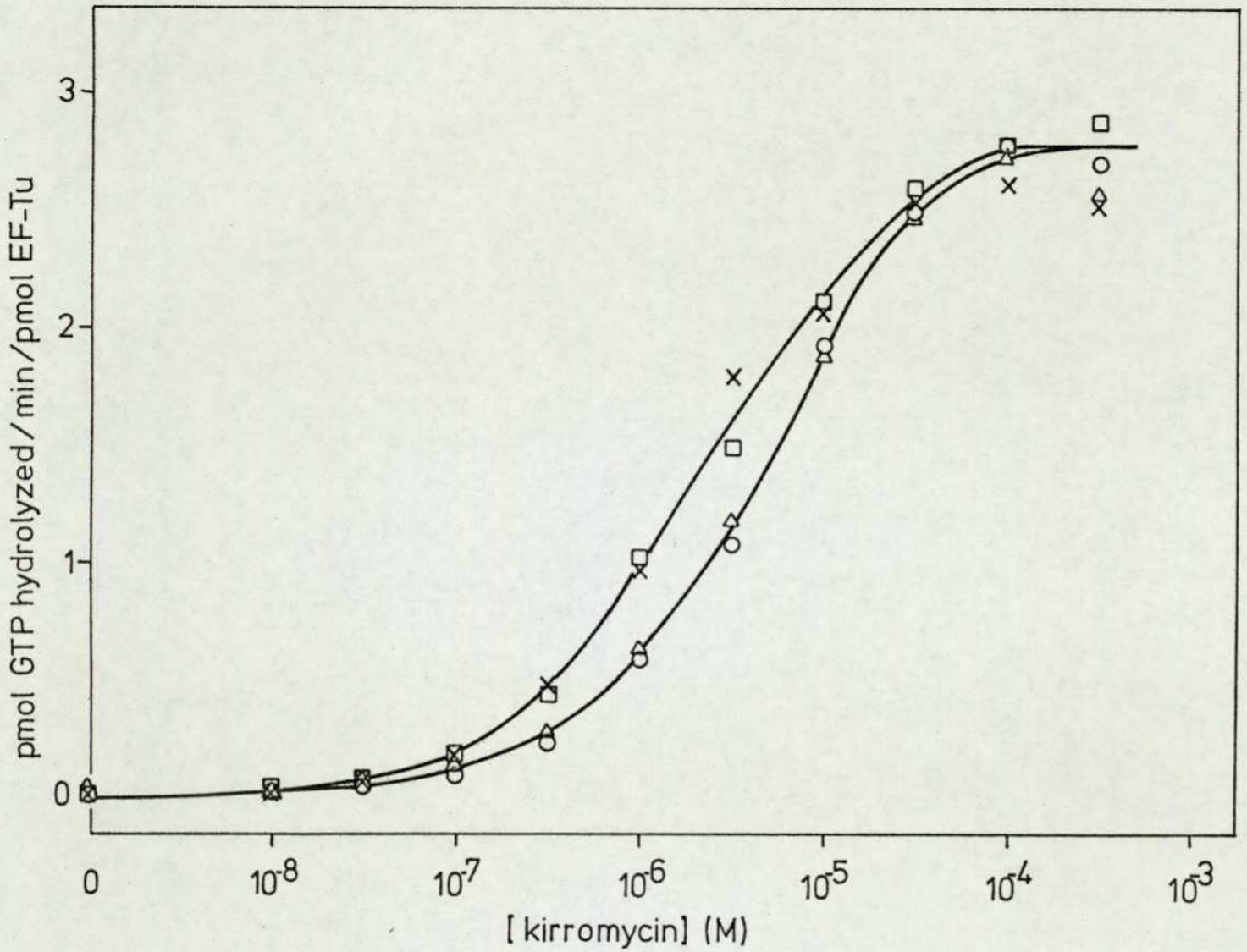


FIGURE 51 STIMULATION BY KIRROMYCIN OF THE EF-Tu GTPase AT 2M Li^+ IN THE PRESENCE AND ABSENCE OF AMINOACYL-tRNA AND/OR RIBOSOMES.

Reaction mixtures contained in 75µl, 2M LiCl, 50mM Imidazolium acetate pH 7.5, 5mM $MgCl_2$, 0.5mM dithiothreitol, 20pmol EF-Tu, kirromycin as indicated, 2.0% ethanol carried over with the kirromycin, 2nmol GTP (specific activity 150 cpm/pmol). Additions: (o) none, (Δ) 100pmol Phe-$tRNA^{Phe}$, (□) 40pmol ribosomes, (x) 40pmol ribosomes plus 100pmol Phe-$tRNA^{Phe}$.

At 2M Li^+, there is little stimulation by ribosomes of the already high EF-Tu.kirromycin GTPase activity. However, it was shown that the presence of ribosomes, irrespective of the additional presence of aa-tRNA, caused a reduction in the amount of kirromycin required to give 50% of the maximum EF-Tu.kirromycin GTPase activity (Fig.51), by about three-fold. Thus ribosomes can influence the EF-Tu - kirromycin binding site, increasing the affinity for the antibiotic. This is an interesting result, not only because of the light it throws on EF-Tu function, but because it offers a part explanation of the anomaly that whereas 10^{-6}M kirromycin is required to induce 50% of the maximum GTPase activity, only $5x10^{-8}$ - 10^{-7}M are required to inhibit protein synthesis by 50% (Fig.49). Since these two activities are functionally related, one would expect the kirromycin titration curves to be mirror-images of one another. The result presented above suggests that the affinity of EF-Tu for kirromycin can be modified by interaction with other effectors.

7.6 Interaction of the ternary complex, EF-Tu.GTP.aa-tRNA, with the ribosome.

"Enzymatic binding" is defined as the union of the ternary complex with the ribosome, dependent on proper codon-anticodon interaction and accompanied by the hydrolysis of one molecule of GTP. The aa-tRNA is then in the pretranslocational position on the peptidyl transferase centre, the so-called A-site. Evidence suggests that aa-tRNA binds to the ribosome in at least three different places. Firstly, there is codon-anticodon interaction, Watson-Crick base pairing occurring between at least two of the three pairs of nucleotides in the anticodon loop of the tRNA and on the mRNA (184). Secondly, there

is probably interaction of the tRNA in the neighbourhood of the 16S RNA of the small subunit (185,186,187). Then, there is some evidence in favour of an interaction between the -TΨCG- loop of the tRNA and the -CGAA- sequence at positions 43-46 on the 5S RNA of the 50S subunit (183,188,189), which has been shown to lie in an exposed position on the surface of the ribosomal subunit (190). Finally, there is interaction of the aminoacyl terminus of the tRNA with the peptidyl transferase centre (191,192). This series of interactions is almost certainly not simultaneous, but sequential, and takes no account of the role additionally played by EF-Tu, which remains bound to aa-tRNA and also interacts with the large ribosomal subunit.

Considering firstly the codon-anticodon interaction, as stated earlier, the affinity of aa-tRNA for free, cognate trinucleotides is relatively low (equilibrium constant, K' = ca. 10^{-3}M). This affinity is already increased by binding in the ternary complex (179); but it is vastly increased on binding to the ribosome (cognate codon-anticodon affinity (measured as affinity constant,K) is reported to be 10^{-11}M (147)). Evidence is accumulating for a conformational change in the anticodon loop of the aa-tRNA, consequent on cognate binding (189,193, 194,195,196), with nucleotide stacking possibly reversing from a 5' to a 3' arrangement (197). A consequence of this change in stacking would be to bring the -TΨCG- loop into juxtaposition with the 5S RNA sequence -CGAA- (188,189). The group of Gassen (179,188) originally supposed that the -TΨCG- loop was also everted from an internal position in the tRNA during this conformational change, but latest information suggests that in the ternary complex, at least, this sequence may be already exposed (166). These arguments, which relate to the conformational change of aa-tRNA upon proper codon-anticodon interaction have given

new support to the notion of a third ribosomal - tRNA binding state, the recognition, or R-site (197), discrete functionally from the A- and P- sites. Strong evidence for such a site was provided as early as 1969 when it was shown that puromycin can still react with peptidyl-tRNA in the P-site, even when aa-tRNA is enzymatically bound in the then so-called A-site, using the non-hydrolyzable GTP analogue $GMPP(CH_2)P$ (198). The hydrolysis of GTP by EF-Tu would appear to accompany the switch of the aa-tRNA from the R-site to the A-site, preparatory to peptide bond formation, and because of the changed affinity of EF-Tu.GDP for aa-tRNA, the former dissociates from the ribosome.

Considerable information on the relationship between aa-tRNA and the 30S subunit has resulted from the many studies of misreading of the genetic code evoked by some aminoglycoside antibiotics, particularly streptomycin (199), and the functionally equivalent ram mutants, involving protein S4. Both of these modifications act in such a way as to antagonize the normal ribosome - aa-tRNA interaction which prevents the miscoding of one of the codon bases. In the usual situation the small ribosomal subunit evidently imposes a constraint on the tRNA such that correct codon-anticodon interaction is enforced. Streptomycin or ram mutation removes this constraint. That this is probably effected not via the anticodon itself but via another part of the tRNA is strongly suggested by UGA suppressor trp-$tRNA^{trp}$ molecules which have a normal trp. anticodon (i.e. not UGA), but are mutated at another site in the tRNA (200,201), and consequently bind specifically to the UGA nonsense codon.

An important facet of many of the studies now being done on the relationship of aa-tRNA to the ribosome is the key role being

played by divalent cations, largely Mg^{2+}, which regulate the non-enzymatic binding of tRNA to the ribosomal A- and P-sites (106,130,202). At low $[Mg^{2+}]$ (<10mM) tRNA binds almost exclusively to the ribosomal P-site; at higher Mg^{2+} concentrations (>10mM) the ribosomal A-site is also occupied. Enzymatic binding (87) and the mRNA - specific physiological EF-Tu GTPase (chapter 4) are optimal at low $[Mg^{2+}]$ (ca. 5mM). Furthermore, it has been shown that the postulated conformational change in the aa-tRNA anticodon loop is also Mg^{2+}-dependent, and that binding of EF-Tu to the aa-tRNA can reduce this requirement for Mg^{2+}, as well as raise the affinity of the anticodon for the codon (189). EF-Tu also reduces the Mg^{2+} requirement for the binding of the oligonucleotide CGAA to $Phe\text{-}tRNA^{Phe}$, an analogous system, it is supposed, to the interaction with the 5S RNA (189). Thus, it appears that there is an intimate steric interaction between EF-Tu and aa-tRNA which influences the requirement of this complex for divalent cations. In an interesting series of experiments using the bacteriocin, colicin E3, which bisects the 16S RNA of the small subunit, but leaves the severed fragments in situ, Sander (87,203) has shown that at 5mM Mg^{2+} EF-Tu GTPase activity is stimulated 5-fold, whereas enzymatic binding is reduced 4-fold. This effect is eliminated by raising the $[Mg^{2+}]$ to 25mM. He suggests that the colicin induces an altered mRNA conformation via its binding to 16S RNA, but that high $[Mg^{2+}]$ stabilizes the consequent "wobble" induced in the codon-anticodon interaction.

In chapter 4, experiments are reported which test the effect of divalent cations on the physiological EF-Tu GTPase, as a function of dependence on mRNA. Increasing the divalent cation concentration removed the requirement for proper codon-anticodon interaction. This effect was greatest for Mg^{2+} at 25mM. At 5mM all divalent cations tested

were completely mRNA-dependent, with Ca^{2+} being the most active and Mg^{2+} least active at this concentration. In the kirromycin-induced activity, which contains no mRNA, in the presence of aa-tRNA and ribosomes, the induced EF-Tu GTPase reaches maximum activity at between 5 and 30mM $[Mg^{2+}]$, depending upon the monovalent cation concentration, and remains active even at relatively very high $[Mg^{2+}]$. These experiments show that codon-anticodon interaction is not an essential prerequisite for EF-Tu GTPase activity, provided sufficient divalent cations, or kirromycin, are present. In the absence of kirromycin, however, at 5mM $[M^{2+}]$, proper codon-anticodon binding is essential. If mRNA is added to the kirromycin system, cognate codon-anticodon binding occurs, but on hydrolysis of GTP, the complex EF-Tu.GDP.aa-tRNA.kirromycin remains bound to the ribosome, and there is no further hydrolysis (32). In the absence of codon-anticodon interaction, the EF-Tu.kirromycin. guanine nucleotide.aa-tRNA complex would appear to recycle in relation to the ribosome between each round of GTP hydrolysis (32).

Using a Mg^{2+} level (5mM) where the physiological EF-Tu GTPase was completely dependent on mRNA, Thompson & Stone (160) showed that if Phe-$tRNA^{Phe}$, the cognate aa-tRNA for poly(U) messenger, was substituted by Leu-$tRNA^{Leu}$ or Ileu-$tRNA^{Ileu}$, wherein only two of the three anticodon nucleotides were complementary to the codon, there was still some GTP hydrolysis, albeit much lower than in the cognate situation, but none of the non-cognate amino acids reached the A-site for reaction with a peptidyl-tRNA in the P-site. This suggests that GTP hydrolysis is playing a role in recognition, and that it occurs between the R-state and the A-state of the aa-tRNA, supporting the view that it accompanies the conformational change in the aa-tRNA which ensues from codon-anticodon recognition.

In both the methanol-induced uncoupled EF-Tu GTPase (143,182), and in the physiological EF-Tu GTPase (36, see section 5.3.4) as well as now with the kirromycin-induced GTPase in the presence of ribosomes only, the central role of the 50S subunit and in particular of proteins L7/L12 has been emphasized. The addition of aa-tRNA to the latter system considerably changes this picture. Firstly, L7/L12 are no longer essential: NH_4Cl/ethanol cores, lacking these proteins are just as active in stimulating the GTPase activity as entire 70S ribosomes. Secondly, CsCl cores a, b and c, lacking a progressively larger number of proteins, though retaining full complements of 5S and 23S RNA, are still active, though with reduced activities. These cores can be complemented by L7/L12 (as long as L10 is present to aid reintegration) to give higher GTPase activities. It is important to note that core c, which in addition to rRNA contains only 12 ribosomal proteins, can still markedly stimulate the EF-Tu.kirromycin GTPase. These activities are drastically reduced in the absence of aa-tRNA; thus it appears likely that it is via this effector that the stimulation of EF-Tu takes place. There may be a connection between this result and that of the group of Ofengand (183) who show that addition of the complementary oligonucleotide TØCG to the physiological EF-Tu GTPase system inhibits the hydrolysis of GTP, presumably via binding to the -CGAA- sequence of 5S RNA and thus preventing proper binding of the aa-tRNA. That this effect is due to steric hindrance of a necessary aa-tRNA conformation was suggested by the parallel experiment in which an aminoacyl-dinucleoside analogue of the -CCA terminus of aa-tRNA was bound to the A-site; the oligonucleotide TØCG failed to induce any EF-Tu GTPase activity in a system lacking aa-tRNA (183). Thus, in spite of tRNA analogues being bound to two of the apparently

important sites of aa-tRNA interaction with the 50S subunit, EF-Tu could not be stimulated to hydrolyze GTP.

A similar conclusion may be reached in relation to the small ribosomal subunit. The 30S particle is only shown to stimulate the EF-Tu.kirromycin GTPase in the additional presence of aa-tRNA. Even in the EDTA titration experiments performed to demonstrate the Mg^{2+}-like effect of ribosomes and their subunits at 2M Li^{+} (chapter 4) in the EF-Tu.kirromycin GTPase, 30S subunits had no effect; the EDTA titration curves in the presence of the subunit and control EF-Tu were little different (Fig.26). Since 30S is known to contain much tightly bound Mg^{2+} (204) this figure also demonstrates that the Mg^{2+}-like effect of ribosomes is not due to a "chasing" of Mg^{2+} from the subunit by Li^{+} ions, but to a direct interaction between the components.

These results underline the importance of aa-tRNA in inducing in EF-Tu a conformation which is susceptible to a productive interaction with the ribosomes or their components.

On the basis of the preceding results, the following scheme can be proposed as a working hypothesis for the steps involved in enzymatic binding of aa-tRNA to the A-site. It is inevitably speculative, since there are still large gaps in our knowledge of the system. It tries to take account of the minimum requirements consistent with the results of this study and other published data, and offers several predictions which could be tested in the near future.

Step 1. EF-Tu.GTP.aa-tRNA approaches the ribosome, making initial contact via codon-anticodon interaction. Possibly, there is already contact of EF-Tu with the ribosome, maybe via proteins L7/L12. This represents the recognition, or R-site.

Step 2. Following proper codon-anticodon binding, there appears to be a conformational change in the anticodon loop of the aa-tRNA, possibly caused by a reversal of the anticodon loop stacking.

Step 3. This conformational change causes a large change in orientation and conformation of the aa-tRNA. The -TΨCG- loop comes into juxtaposition with the 5S RNA of the large subunit. Simultaneously, as a consequence of these steric events, which are conveyed via the aa-tRNA to the EF-Tu, the catalytic centre for GTP hydrolysis on EF-Tu is activated.

Step 4. GTP is hydrolysed to GDP. EF-Tu.GDP, having no affinity for aa-tRNA, and possibly only a tenuous one with the ribosome at this stage, leaves the ribosome.

Step 5. The aa-tRNA is now located in the A-site, the aminoacyl terminus lying in the pretranslocative position of the peptidyl transferase centre possibly via L16, L4 or 23S RNA (205,206, 207). The A- and R-sites may be partially overlapping.

Step 6. Peptide bond formation occurs.

Step 7. EF-G intervenes via proteins L7/L12 (36) and in some way involving hydrolysis of GTP, assists the translocation of peptidyl-tRNA, and the mRNA to which it remains bound (208,209) from the A-site to the P-site, consequently ejecting the deacylated tRNA from the P-site.

Further support for steps 3-5 is given by the work of Lucas-Lenard and Rychlik (198,210) who have confirmed not only that without GTP hydrolysis (i.e. by using non-hydrolyzable GTP analogues) there can be no peptide bond formation, even though the ternary complex is bound to the ribosome via a coded interaction with the mRNA, but also that until EF-Tu leaves the complex, the α-amino group of the aminoacyl-tRNA remains protected by the elongation factor and unavailable for peptide bond formation (210).

In the presence of kirromycin the relationships between the components of the GTPase system are slightly different. For example, in this system codon-anticodon interaction is inimical since it prevents the ternary complex from leaving the ribosome which appears to be an essential prerequisite for a turnover GTPase activity (32).

The questions that are generated by the proposed sequence of events leading to the binding of aa-tRNA in the A-site are many. However, it leads to the central conclusion that in the physiological system the chief effector on the EF-Tu is aa-tRNA, and that it is the varied allosteric influence of this effector that finally triggers the EF-Tu GTPase centre. This underplays the role of the ribosomes which in the absence of aa-tRNA certainly can stimulate the EF-Tu GTPase. Of relevance here is the fact that by themselves ribosomes are inactive; proteins L7/L12, the only proteins for which a direct connection to EF-Tu has been proved when isolated from the ribosomes and other components of the system, are likewise totally without effect. Only in the presence of kirromycin or methanol can EF-Tu GTPase activity be induced by ribosomes. By altering the dissociation constant of EF-Tu for GTP, kirromycin has been shown to function very much as an analogue of aa-tRNA.

Methanol is less simple. It has been shown to accelerate the physiological EF-T and EF-G GTPase activities (36,143). In the methanol-induced EF-Tu GTPase Phe-tRNA^{Phe} actually inhibits at 5mM Mg^{2+} (see chapter 6), suggesting some sort of competition between the two effectors. Methanol, in high concentration (33%), is also essential for the "fragment reaction" whereby CACCA-Leu.Ac in the P-site is transferred to puromycin in the A-site (211), suggesting again that methanol can substitute functionally for the missing parts of the tRNA analogues in permitting peptidyl transfer; for normal peptidyl transfer, tRNA in both A- and P-sites should have proper codon-anticodon interaction with adjacent triplets of the mRNA (208,209). It is possible to conceive, therefore, that in these aa-tRNA - free systems, methanol and kirromycin can, in conjunction with the ribosome, functionally replace aa-tRNA: also in inducing the EF-Tu conformation active in GTP hydrolysis.

7.7 The role of enzymatic binding in translation fidelity.

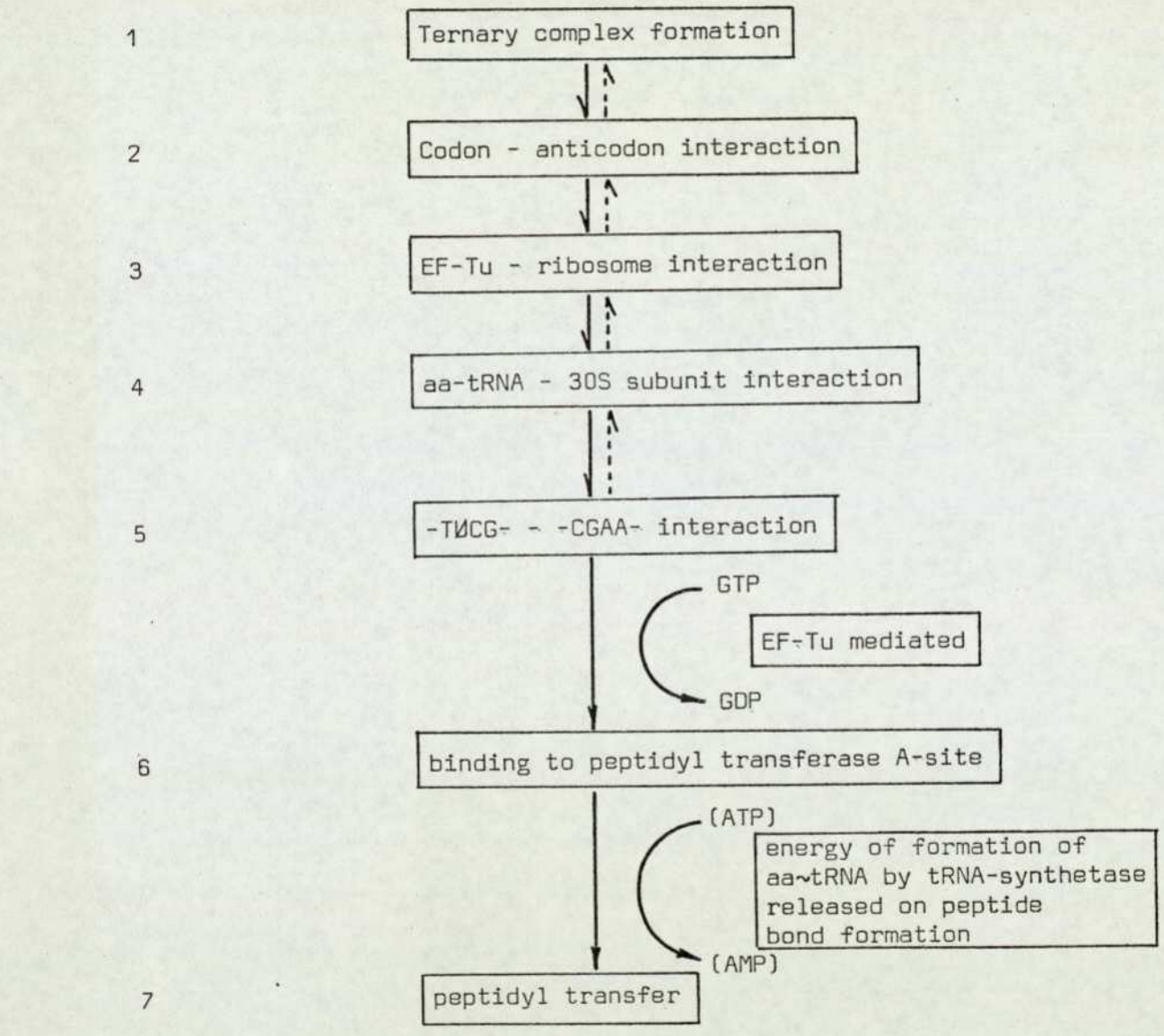
Recently, several theoretical arguments have been proposed supporting a proof-reading step in the tRNA-mediated translation of mRNA (159,212,213). Two essential principles are invoked. The first, is straightforward geometric discrimination, such as occurs in codon-anticodon binding. The second is a kinetic discrimination, whereby for proper recognition the association rate constant (k_{+1}) must be greater than the dissociation rate constant (k_{-1}); but as this is only evident in an equilibrium situation, reaction time plays a critical role and delay steps may have to be introduced into a recognition sequence (213). A third idea has been introduced by Hopfield (212) to overcome the latter problem. He invokes a coupled exothermic reaction, such as a nucleotide triphosphate hydrolysis, which coupled to a

geometric discrimination step forces the reaction in one direction, thus permitting a non-equilibrium discrimination. In Fig.52 a scheme based on known or surmised interactions of the enzymatic binding process is given. It is evident that there are several discriminating steps based on geometric principles, as well as the important GTP hydrolysis step which allows non-equilibrium discrimination to occur at a relatively rapid rate. Clearly, the distinction between geometric and kinetic discrimination is arbitrary, and both can probably be modified by the involvement of cations. In the absence of EF-Tu, recognition would probably have to proceed via equilibrium kinetics only, and although there is very little miscoding in this system (214), velocities are also extremely low. The interactions that occur after codon-anticodon interaction would be disturbed if the tRNA is in any way distorted by false encoding on the mRNA. Thus there are probably at least seven discriminating steps, concomitant with proper codon-anticodon binding, the latter two of which are strongly exothermic. The more the number of discriminating steps, the more effective the system (159), and it is also interesting to note that the exothermic steps come after several other discriminating stages as predicted by Hopfield (212).

EF-Tu influences at least three of these discriminating steps, and certainly permits non-equilibrium discrimination, allowing translation to proceed more rapidly and probably also more accurately than in its absence. EF-Tu can therefore be said to be a key element in the protein synthesis recognition system.

FIGURE 52 HYPOTHETICAL RECOGNITION SEQUENCE IN BACTERIAL TRANSLATION

(the order of the stages is not intended to be a precise temporal sequence of events)



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