PROPERTIES OF THE RIBOSOMES OF BACTERIAL MUTANTS

RESISTANT TO THIOSTREPTON

by

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Preface

This dissertation describes studies of the properties of ribosomes from thioestreptone-resistant mutants of *Bacillus megaterium* conducted between October 1976 and May 1979.

Some of the results presented in this dissertation have already been published or accepted for publication:


"Ribosomes in thioestrepton-resistant strains of *Bacillus megaterium* lacking a single 50S subunit protein"
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(A preliminary account of this work has also appeared: Hoppe-Seyler's Z. Physiol. Chem. (1979) 360, 382)

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"On the biological role of ribosomal protein BM-L11 of *B. megaterium*, homologous with *E.coli* ribosomal protein L11"
J. Mol. Biol. (in press)

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"Binding of thioestrepton to a complex of 23S rRNA with ribosomal protein L11"
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Finally, I am immeasurably grateful to Vanessa Webb for typing this dissertation and for more support and patience than I deserve.

As a member of a research group certain aspects of the work described below have been performed in conjunction with others. I am also particularly grateful to Dr. Georg Stöffler and co-workers in Berlin for providing the results of immunological analyses. Nevertheless, this dissertation records my own unaided work except where specifically stated otherwise and is not the same as one which has been submitted at any other university.

M. J. R. Stark

20th September 1979
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Abbreviations have generally been defined where they first appear in the text. The following abbreviations have been used:-

\[ A_x \] : absorbance at a wavelength of \( x \) nm

AMP, ATP : adenosine 5'-monophosphate, adenosine 5'-triphosphate

butyl-PBD : 2-(4'-tert-butylphenyl)-5-(4''-tert-biphenyl)-1,3,4-oxadiazole

cpm : counts per minute

DMSO : dimethylsulphoxide

DNA : deoxyribonucleic acid

DNase : deoxyribonuclease

DTT : dithiothreitol

EDTA : diaminooethanol tetra-acetic acid

GDP, GTP : guanosine 5'-diphosphate, guanosine 5'-triphosphate

ppGpp, pppGpp : guanosine 5'-diphosphate, 3'-diphosphate; guanosine 5'-triphosphate, 3'-diphosphate

GMPPNP : \( \beta, \gamma \)-imido guanosine 5'-triphosphate

HEPES : N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

MBA : N, N'-methylenebisacrylamide

\( M_r \) : molecular weight (daltons)

PEG : polyethylene glycol

PEI : polyethylenimine

PEP : phosphoenol pyruvate

\( P_i \) : inorganic phosphate

poly U : polyuridylic acid

RNA : ribonucleic acid

rRNA, tRNA, mRNA : ribosomal, transfer and messenger RNA
RNase : ribonuclease
rpm : revolutions per minute
S : Svedberg unit (10^{-13} seconds)
SDS : sodium dodecyl sulphate
TCA : trichloroacetic acid
Tris : tris (hydroxymethyl) aminomethane

Purine and pyrimidine bases have been specified by their initial letters (Ψ = pseudouridine). Trimucleotide codons have been denoted by the initial letters of the bases, e.g. 'AUG' represents 5'-ApUpG-3'.

Enzyme activities have been given in international units ('units').

Ribosomal proteins are denoted according to the standard nomenclature (see e.g. Wittmann et al., 1971) where prefix 'L' denotes a protein from the large ribosomal subunit and prefix 'S' a protein from the small subunit.
This dissertation describes the properties of ribosomes from bacterial mutants resistant to thiostrepton, a potent inhibitor of prokaryotic protein synthesis (Weisblum & Demohn, 1970a). The scope of this work required an appreciation of current knowledge concerning bacterial ribosome structure and function in relation to the molecular events of protein biosynthesis. These fields encompass an immense volume of research and will therefore only be outlined in this introduction, although particular emphasis will be placed on those aspects of direct importance to the present study.

1.1 Ribosomes and Protein Synthesis: A Historical Perspective

The first evidence for the involvement of ribonucleic acid (RNA*) in protein synthesis came from the observations of Brachet (1941) and Caspersson (1941) that tissues actively producing protein were particularly rich in RNA. Much cellular RNA was found to occur as ribonucleoprotein particles in the microsomes (Claude, 1938, 1941) and the proposal that microsomes play a crucial role in protein synthesis (Jeener & Brachet, 1942) was subsequently confirmed (Hultin, 1950; Borsook et al., 1950). However, it was not until later (Palade, 1955) that such ribonucleoprotein particles were shown to represent structures existing as such in cells and the term 'ribosome' (Roberts, 1958) was coined. When analysed by ultracentrifugation (Petermann & Hamilton, 1957), ribosomes from higher organisms were shown to sediment at 80S (where S is the Svedberg unit; $10^{-13}$s) although at low magnesium ion concentrations they dissociated into 40S and 60S components (Chao, 1957).

* see p. viii for a list of commonly-used abbreviations.
These ribosomes were shown to be the site of protein synthesis both in a cell-free system (Zamecnik & Keller, 1954) and in vivo (Littlefield et al., 1955). Only about 1% of the amino acids in ribosomal protein were found to be turning over rapidly, suggesting a steady state in which the rates of formation and release of polypeptide chains were equal (Littlefield et al., 1955).

Although the above studies related to eukaryotic ribosomes, similar ribonucleoprotein particles had also been noted in bacteria (Luria et al., 1943). In early analyses they appeared much smaller than their eukaryotic counterparts (Schachman et al., 1952) but they were shown to sediment as 70S particles (each resulting from the association of a 30S and a 50S component) when the stabilising effect of magnesium ions was discovered (Tissières & Watson, 1958). Ribosomes were shown to be the site of protein synthesis in bacteria by the pulse-chase experiments of McQuillen et al., (1959). From this time onwards much research focused on the simpler bacterial systems and in particular the widely-studied organism Escherichia coli.

Ribosomes were originally thought to be like small plant viruses and were shown to contain RNA molecules of discrete sizes (Kurland, 1960). However, analysis of ribosomal proteins from E.coli indicated a more complex situation (Waller & Harris, 1961), with different proteins in the 30S and 50S ribosomal subunits (Waller, 1964). Subsequent research has continued to reveal the complexity of ribosome structure.

Early studies of the mechanism of protein synthesis showed that amino acids are 'activated' by ATP in a reaction catalysed by enzymes specific for each (Hoagland, 1955), prior to polymerisation. A requirement for GTP in protein synthesis was also found (Keller & Zamecnik, 1956). The elucidation of the structure of DNA (Watson & Crick, 1953) had demonstrated how genetic information could be copied into 'template' polymucleotide chains, prior to translation into the amino acid sequences.
of proteins, but the basis for converting the 'genetic code' of four nucleotides into proteins composed of twenty amino acids was unknown.

Crick (1957) proposed linkage of amino acids to 'adapter' oligonucleotides (which could base-pair with the template) as the means by which the amino acids would be ordered prior to polymerisation. This prediction was fulfilled by the discovery (Hoagland et al., 1958) of a new class of RNA molecules (soluble or 'transfer' RNA). Each transfer RNA (tRNA) molecule was shown to be specific for a given amino acid, to which it became covalently linked by the activating enzyme (Berg & Ofengand, 1958). Such tRNA molecules were shown to bind specifically to ribosomes (Cannon et al., 1963).

Throughout this work it had been assumed that the RNA packaged in the ribosomes (ribosomal or rRNA) was the template containing the genetic information specifying the amino acid sequences of proteins. However, around the time it was shown that the genetic code was 'read' as nucleotide triplets (Crick et al., 1961) a third class of RNA molecules was discovered. The existence of unstable template or 'messenger' RNA (mRNA) molecules (postulated by Jacob & Monod, 1961) was demonstrated by studies of both bacteriophage-infected (Brenner et al., 1961) and uninfected cells (Gros et al., 1961), while even the synthetic polyribonucleotide polyuridylic acid was shown to function as mRNA in vitro (Nirenberg & Matthaei, 1961). With this discovery it soon became possible to summarise the process by which proteins are elaborated (Watson, 1964).

1.2 The Molecular Mechanisms of Bacterial Protein Synthesis

Although much research has been devoted to the subject of bacterial protein synthesis, the overall view of the process has remained unchanged in its essentials for many years. An outline of the molecular mechanisms involved will now be given. While protein synthesis in higher organisms
is in many ways similar to that occurring in bacteria, it differs from the latter in a number of respects (notably in the control of peptide chain initiation) and will not be considered here.

Much of the present knowledge concerning bacterial protein synthesis derives from studies of *E. coli*, although the mechanisms involved are considered likely to have been highly conserved throughout bacteria.

(a) **Activation of amino acids**

Proteins are linear polymers containing some or all of the twenty naturally-occurring amino acids linked together by peptide bonds. As discussed above, tRNA is the adapter molecule whereby mRNA directs the order of the amino acids during the synthesis of a protein. The covalent linkage of each amino acid to a tRNA molecule at the expense of ATP provides the energy subsequently used to drive peptide bond formation and is catalysed by the activating enzyme (or aminoacyl-tRNA synthetase). The reaction, resulting in esterification of the amino acid onto the 2'- or 3'-hydroxyl group of the 3'-terminal ribose of the tRNA chain (Hecht et al., 1959), is generally considered to proceed via a two-step mechanism:-

(1)* \( \text{AA}^i + \text{ATP} + \text{E}^i \rightarrow \text{E}^i + \text{AMP} \)

(2) \( \text{E}^i + \text{tRNA}^i \rightarrow \text{AA}^i\text{-tRNA} + \text{E}^i + \text{AMP} \)

* AA = amino acid or aminoacyl; E = activating enzyme; AA-AMP = aminoacyl adenylate; superscript i denotes a particular amino acid; \(---\) denotes non-covalent interaction.
Due to the degeneracy of the genetic code, in many cases there is more than one 'isoaccepting' species of tRNA for a given amino acid, although there are in total fewer than sixty-one tRNA species since several can recognise more than one codon. There is a single activating enzyme for each amino acid, capable of aminoacylating all of the isoacceptor tRNA species that can recognise codons specifying its cognate amino acid (Loftfield, 1971).

Although tRNA molecules show similar 'cloverleaf' secondary structures and possess certain invariant features (see Ofengand, 1977), the activating enzymes show a high specificity for both the cognate amino acid and the isoacceptor tRNAs. Such specificity is clearly essential since the activated amino acid is subsequently recognised only by the tRNA to which it is attached (Chapeville, 1962).

(b) Protein chain initiation

The specialised reactions of protein chain initiation, catalysed by three 'initiation factors' (Revel et al., 1968), are crucial to protein synthesis since the mRNA must be translated from the precise start of each cistron. Translation is in the 5'→3' direction starting from the methionine codon AUG (sometimes GUG). There are two species of tRNA\textsuperscript{MET} in bacteria (tRNA\textsubscript{F}\textsuperscript{MET} and tRNA\textsubscript{M}\textsuperscript{MET}). Met-tRNA\textsubscript{F}\textsuperscript{MET*} is specifically formylated and fmet-tRNA* is used exclusively during the initiation process. Thus all bacterial proteins have an N-terminal formylmethionine residue, although this is always subsequently cleaved and the methionine residue often removed (Livingston & Leder, 1969).

Dissociation of the ribosomal subunits is an obligatory requirement

* met-tRNA = methionyl-tRNA; fmet-tRNA = formylmethionyl-tRNA\textsubscript{F}\textsuperscript{MET}
The probable sequence of events occurring during polypeptide chain initiation

A description of the events represented in the above scheme can be found in the text. The following abbreviations have been used: 30S, 30S ribosomal subunit; 50S, 50S ribosomal subunit; 70S, 70S ribosome; mRNA, messenger RNA; fmet-tRNA, formylmethionyl-tRNA; GTP, guanosine 5'-triphosphate, IF, initiation factor.
for initiation of protein synthesis on natural mRNA and factor IF 3 is thought to act as an anti-association factor (Subramanian & Davis, 1971), probably in conjunction with factor IF 1 (Noll & Noll, 1972). The three initiation factors (IF 1, IF 2, IF 3) bind cooperatively to the 30S ribosomal subunit and an 'active' 30S subunit carrying all three is thought to be a pre-requisite for initiation (see Revel, 1977). The mRNA and fmet-tRNA now bind to the 'active' 30S subunit in an order yet to be determined, although some evidence (Vermeer et al., 1973) suggests that the mRNA binds first. Initiation factor IF 3 is involved in the latter process (see Revel, 1977) and the 30S subunit is guided to the correct region of the mRNA by an 'initiation signal', which distinguishes the initial AUG codon from others in the mRNA. The molecular basis of this signal may be a short region of the mRNA close to the start of the coded amino acid sequence that is complementary to a nucleotide sequence at the 3' end of the 16S ribosomal RNA molecule (Shine & Dalgarno, 1974, 1975; Steitz & Jakes, 1975). The [mRNA•30S subunit•initiation factor] complex then binds a molecule each of GTP and fmet-tRNA with the loss of IF 3. Binding of fmet-tRNA as a ternary complex with IF 2 and GTP (cf. EF Tu•GTP•AA-tRNA complex below) does not apparently occur. The 30S initiation complex now formed combines with a 50S ribosomal subunit to give the '70S initiation complex', a process resulting in the hydrolysis of GTP and loss of the two remaining initiation factors. The next aminoacyl-tRNA may now bind to the ribosome and protein chain elongation can commence. The complete series of events occurring during initiation is shown in Figure 1.1.

(c) Polypeptide chain elongation

The growing polypeptide chain is always linked to a tRNA molecule (Gilbert, 1963) which can be bound to the ribosome in either a puromycin-reactive or puromycin-refractory state (Traut & Monro, 1964). These states
are considered to represent two ribosomal sites termed respectively the 'peptidyl-tRNA site' (P-site) and the 'aminoacyl-tRNA site' (A-site). The elongation process is a cycle of events revolving around these two sites and promoted by two supernatant factors (Allende et al., 1964; Nishizuka & Lipmann, 1966).

(i) **Binding of aminoacyl-tRNA:**

The first step of the 'elongation cycle' is promoted by elongation factor T, which contains two activities (EF Tu and EF Ts). At this point the nascent polypeptide is attached to a tRNA bound at the ribosomal P-site and the codon specifying the next amino acid is available at the A-site. Each aminoacyl-tRNA binds to the ribosome from a ternary complex of the form \([\text{AA-tRNA}\cdot\text{EF Tu}\cdot\text{GTP}]\). Any such complex can bind reversibly to the vacant A-site of the ribosome, but only one containing a cognate tRNA is selected. Although the codon directs selection of the correct tRNA by base-pairing with the anticodon carried by a cognate tRNA, the interaction between a codon and a cognate tRNA is weak. Thus codon-anticodon recognition must specifically initiate a chain of further events before stable binding of the correct aminoacyl-tRNA can occur. It has been envisaged that interaction of the codon with a cognate (but not a non-cognate) tRNA might result in a conformational change exposing binding sites common to all tRNA molecules, which are then responsible for stable interaction of the tRNA with the ribosome (Kurland, 1977a). Stable binding of a cognate aminoacyl-tRNA at the ribosomal A-site may involve interaction of the invariant 'T-Ψ-C-G' region of the tRNA with a complementary region of the 5S rRNA (Erdmann et al., 1973).

* See footnote on p. 4
Tight binding of a cognate aminoacyl-tRNA from the [AA-tRNA\cdot EF Tu\cdot GTP] complex results in the hydrolysis of GTP and the release of [EF Tu\cdot GDP] from the ribosome. EF Tu binds GDP tightly but only interacts with aminoacyl-tRNA when complexed with GTP. Exchange of GDP bound by EF Tu with GTP is catalysed by EF Ts, which therefore serves to recycle EF Tu:

\[
\begin{align*}
&\text{EF Ts} \\
&\text{[EF Tu\cdot GTP]} \xrightarrow{\text{GTP}} [\text{EF Tu\cdot EF Ts}] \xrightarrow{\text{GDP}} [\text{EF Tu\cdot GDP]} \\
&\text{EF Ts}
\end{align*}
\]

(ii) Peptide bond formation:

When the incoming aminoacyl-tRNA is correctly bound at the ribosomal A-site, peptide bond formation occurs by transfer of the nascent peptide (or, initially, formylmethionine) to the \(\alpha\)-amino group of the aminoacyl-tRNA. This process requires no supernatant factors or external energy source (Maden et al., 1968) although a soluble protein (termed EF P) has been reported to stimulate the event \textit{in vitro} (Glick & Ganoza, 1975). Peptide bond formation is associated with a region of the 50S ribosomal subunit known as the peptidyl transferase and results in the presence of deacylated tRNA in the P-site and an elongated peptidyl-tRNA at the A-site.

(iii) Translocation:

To complete the 'elongation cycle' the deacylated tRNA must leave the ribosome, the peptidyl-tRNA must be 'translocated' into the now vacant P-site and the mRNA must move relative to the ribosome to position the next codon at the A-site. These events are tightly coupled and neither the mechanisms nor the correct sequence are known. All three processes require the elongation factor EF G and are associated with EF G-dependent GTP hydrolysis:
The next aminoacyl-tRNA may now bind since the ribosome has returned to the state described in section (i).

(d) Termination

The end of a coded amino acid sequence is signalled by one of the three 'nonsense' codons (UAA, UAG and UGA). These are recognised not by tRNA but by supernatant 'release' factors, which show the following specificities (Scolnick et al., 1968):

RF₁: UAA, UAG
RF₂: UAA, UGA

A third factor (RF₃) appears to stimulate the activity of the other two but is not absolutely required (Milman et al., 1969).

The termination event itself requires the peptidyl transferase centre of the ribosome (Caskey et al., 1971). The mechanism appears to involve modification by the release factors of the specificity of the peptidyl transferase such that the nascent peptide is hydrolysed. The requirements for dissociation of the resulting [tRNA•ribosome•mRNA] complex are not known. However, a 'ribosome-releasing' factor has been isolated that promotes the release of mRNA from the ribosome during termination in a release factor-dependent manner (Ogawa & Kaji, 1975).

1.3 The Ultrastructure of the E.coli Ribosome

The complexity of bacterial ribosomes is hardly surprising in view of the multiplicity of events occurring during protein synthesis.
The proteins of the *Escherichia coli* ribosome were first given a unifying nomenclature on the basis of their mobilities upon two-dimensional polyacrylamide gel electrophoresis (Wittmann et al., 1971). Twenty-one such proteins were identified from the small subunit while the large subunit yielded thirty-four. However, more recent studies have shown that the true total is probably only fifty-two. Thus proteins L7 and L12 are indistinguishable except for acetylation of the N-terminal serine residue in the former (Terhorst et al., 1973) while proteins L26 and S20 are immunologically identical (Stöffler, 1974). In addition, 'protein L8' is a stable complex containing proteins L7/L12 and L10 (Pettersson et al., 1976). All the proteins are present as one copy per ribosome with the exception of L7/L12, of which there are four copies (Hardy, 1975; Subramanian 1975). None of the 52 proteins share common antigenic determinants (Stöffler & Wittmann, 1971a, b; Stöffler, 1974). The primary sequences of at least 35 proteins are known, confirming that there are no extensive sequence homologies between the proteins. That some of the proteins exhibit tertiary structure has been demonstrated (Morrison et al., 1977a, b; Newberry et al., 1977) and stable protein-protein complexes have been isolated (Dijk et al., 1977; Pettersson & Liljas, 1979).

Immunological comparison of *Escherichia coli* ribosomal proteins with those from other species of *Enterobacteriaceae* (Geisser et al., 1973a) and members of the unrelated *Bacillaceae* (Geisser et al., 1973b) have revealed in both cases considerable similarity, indicating that ribosomal proteins have been highly conserved during evolution.

Each *Escherichia coli* ribosome also contains three RNA molecules, two (23S and 5S) in the large subunit and one (16S) in the small subunit (Kurland, 1960; Rosset & Monier, 1963). The sequence of the 5S RNA was determined first (Brownlee et al., 1968) but the complete sequences of both 16S (Brosius et al., 1978) and 23S RNA (H.Noller, reported at the Steenbock Symposium,
1979) have now been determined by the sequencing of rRNA genes.

Although rRNA was first thought to play only a structural role, this is probably not the case. In addition to the possible roles of the 5S and 16S RNA discussed above (section 1.2), the 16S RNA has been implicated in the interaction of tRNA with the small ribosomal subunit (Noller & Chaires, 1972). It has also been shown that fmet-tRNA binds strongly to the 23S RNA by means of a 17-base complementary sequence (Dahlberg et al., 1978), while complementary sequences in the 16S and 23S RNA molecules may be important in ribosomal subunit association (Herr & Noller, 1979; Herr et al., 1979).

Both the 23S RNA (Sloof et al., 1978) and the 16S RNA (Garrett et al., 1977) exhibit tertiary structure and can specifically bind many ribosomal proteins (Littlechild et al., 1977; Marquardt et al., 1979). The 5S RNA also binds proteins specifically (Gray et al., 1973).

The total reconstitution in vitro of both the 30S (Traub & Nomura, 1968) and the 50S ribosomal subunit (Nierhaus & Dohme, 1974) has revealed that the individual components contain sufficient information for self-assembly of the ribosome. Studies of assembly in vitro such as those of Mizushima & Nomura (1970) first emphasised the cooperative interactions of the ribosomal components in the production of ribosomal structure, a cooperativity reflected by studies of ribosomal function (see below).

The shape of the ribosome was first studied by X-ray scattering (Van Holde & Hill, 1974) but recently electron microscopy has proved more revealing. Resulting from such studies three-dimensional models have been proposed for the ribosomal subunits of E. coli (Tischendorf et al., 1975; Lake, 1976; Boublík et al., 1977). However, the superficial similarity of the models is outweighed by marked differences in structural detail. In the version proposed by Lake both subunits are considerably more asymmetric than in the model of Stöffler and co-workers (Tischendorf et al., 1975),
while Boublik and co-workers have depicted a somewhat intermediate situation.

The ultrastructure of the E. coli ribosome has been studied by a number of other techniques. Use of bifunctional cross-linking reagents has revealed protein neighbours in both the small and large subunits (see Kenny & Traut, 1979; Stöffler & Wittmann, 1977, for a summary of data). In the case of the small subunit, many of these results agree with studies of singlet energy transfer between pairs of fluorescently-labelled proteins (Huang et al., 1975), while use of a neutron-scattering technique (Langer et al., 1978) has enabled the three-dimensional positions of a number of 30S subunit proteins to be determined.

Several regions of both 16S (Noller, 1974) and 23S RNA (Herr & Noller, 1978) are exposed at the ribosome surface, confirming earlier results (Miall & Walker, 1967; Panijel & Delaunay, 1967). In addition, use of antisera raised against purified ribosomal proteins has shown that every protein has some exposed antigenic determinants (Stöffler et al., 1973; Morrison et al., 1977c). This latter observation, coupled with the irregularity of both ribosomal subunits when viewed under the electron microscope has enabled the protein topography of the subunit surfaces to be 'mapped'. Thus treatment of subunits with a monospecific antiserum against a single protein results in the production of subunit dimers (linked by the bivalent IgG molecules), revealing the location of antigenic determinants of the protein when subsequently examined by electron microscopy. The studies of Stöffler and co-workers are the most complete to date (see Stöffler & Wittmann, 1977 for a review) embracing both ribosomal subunits, whereas Lake and colleagues have concentrated particularly upon the small subunit (Lake & Kahan, 1975). Such 'immuno electron microscopy' studies have shown that a number of proteins have more than one antigenic determinant exposed, suggesting in several cases somewhat elongated shapes. Immuno electron
microscopy has also revealed that proteins shown to be neighbours by the results of other topographical studies often have antibody sites in close proximity (Stöffler & Wittmann, 1977).

1.4 Investigation of Ribosomal Functions

Many approaches have been used to correlate particular ribosomal functions with individual components of the ribosome. Early attempts to characterise functional impairment caused by omission of single proteins during total reconstitution of E. coli 30S ribosomal subunits (Nomura et al., 1969) failed to be of more than limited use due to the highly cooperative nature of the ribosome. Thus no single ribosomal component has ever been exclusively implicated in any ribosomal activity. The functional sites of the ribosome are more likely to be 'domains' perhaps containing several components (both proteins and rRNA) and not simply individual proteins (Kurland, 1977b). This idea is supported by results presented in this dissertation concerning the functional role of a single ribosomal protein (Chapter 9).

The partial reconstitution technique has proved more useful in implicating ribosomal proteins in particular functions. Thus a few proteins can be removed from the ribosome (eg. by use of high salt concentrations) and added back individually (or in combinations) to the protein-deficient 'core-particles' thereby created, in an attempt to restore particular functions to the latter.

Other more direct methods of study have also been developed. Affinity labelling of ribosomal components using analogues of tRNA, mRNA and other compounds which show specific interactions with ribosomes has been particularly useful (see Pellegrini & Cantor, 1977; Cooperman, 1978 for reviews). Elongation and initiation factors can be cross-linked to ribosomal components (eg. Bollen et al., 1975) and monospecific antibodies
against individual proteins have been used to titrate away particular ribosomal activities (Lelong et al., 1974; Highland et al., 1974). Combination of chemical modification and the total reconstitution technique has also been used with success (Thomas et al., 1975; Dohme & Fahnestock, 1979). Data obtained by these different methods are often in good agreement. Thus the acidic *E. coli* ribosomal protein L7/L12 has been implicated in the interaction of *E. coli* ribosomes with EF Tu and EF G from reconstitution (review: Müller, 1974), antibody (Highland et al., 1973, 1974) and cross-linking studies (Acharya et al., 1973; San José, et al., 1976), while various studies have implicated protein L16 in the peptidyltransferase centre (see section 9.1).

An alternative approach to study of ribosome function has involved the isolation of mutants containing lesions in ribosomal components. Such strains have been obtained most readily by selection using some of the many antibiotic inhibitors of protein synthesis. Where the mode of action of an inhibitor is known, identification of an altered ribosomal component in a drug-resistant mutant provides good evidence for its involvement in the functions inhibited by the drug. Such an approach avoids any theoretical criticisms of the above *in vitro* techniques since it reflects an *in vivo* adaptation, while also complementing *in vitro* study of the ribosomal binding sites of such inhibitors.

One of the antibiotics first studied in this way was streptomycin. In mutant strains of *E. coli* resistance to streptomycin (Ozaki et al., 1969) or dependence on the antibiotic (Birge & Kurland, 1969) was shown to result from alteration in ribosomal protein S12. Thus in both cases it was demonstrated that the streptomycin phenotype of 30S ribosomal subunits reconstituted from mutant and wild-type components was dependent on the origin of protein S12 alone. Streptomycin dependence can be suppressed by further mutation in either protein S4 or S5 (Hasenbank et al., 1973) and
in vitro studies have implicated proteins S3 and S5 in the binding of streptomycin to ribosomes (Schreiner & Nierhaus, 1973). However, streptomycin exerts a multiplicity of effects on various ribosomal functions (Davis et al., 1974) and so particular ribosomal functions cannot readily be associated with the above group of proteins. Since some of these proteins have been shown by other means to be part of the mRNA binding site (see Stößler & Wittmann, 1977) it is perhaps not surprising that streptomycin can affect several aspects of protein synthesis.

Spectinomycin resistance has also been associated with alterations in protein S5 (Bollen et al., 1969). However, different ribosomal proteins have been implicated in resistance phenomena observed using other antibiotics. Thus erythromycin resistance may result from mutation in protein L4 (Otaka et al., 1970; Wittmann et al., 1973) and has been shown to co-transduce with an altered form of protein L22 (Wittmann et al., 1973), although two other proteins (L15 and L16) appear to be important for the binding of the drug to ribosomes (Teraoka & Nierhaus, 1978). Other studies have implicated an altered form of protein L6 in resistance to gentamycin (Kühberger et al., 1979). In an extreme case a streptomycin-dependent strain altered in protein S8 can revert to independence by mutation in a large number of ribosomal proteins from either subunit (Dabbs & Wittmann, 1976). The ability to obtain mutants in every ribosomal protein by this and other means (Dabbs, 1978; Isono et al., 1978) should greatly aid both in vitro and in vivo studies of ribosomal function.

Antibiotic resistance is not exclusively confined to alterations in ribosomal proteins. Thus erythromycin resistance can also result from specific methylation of 23S rRNA (Lai & Weisblum, 1971) while kasugamycin resistance may be caused by failure to methylate two adjacent adenine residues close to the 3' end of the 16S rRNA (Helser et al., 1971). The latter result supports the idea that this region of the 16S RNA may be
The structure of thiostrepton, determined by X-ray crystallography (Anderson et al., 1970).
important during polypeptide chain initiation (see section 1.2), since it has been shown that kasugamycin is a specific inhibitor of the initiation process (Okuyama et al., 1971). In addition, it was found recently that viomycin-resistant strains of Mycobacterium smegmatis contain alterations in either the 16S or the 23S rRNA (Yamada et al., 1978).

Methylation of ribosomal RNA is also responsible for the resistance of Streptomyces azureus, the organism that produces thiostrepton, to its antibiotic product (Cundliffe, 1978). Extracts of S. azureus contain an enzyme that methylates the ribose moiety of a specific adenine residue in the 23S RNA, thereby abolishing the ability of the ribosomes to bind thiostrepton (Cundliffe & Thompson, 1979).

The present study is concerned with spontaneously-arising ribosomal resistance to thiostrepton. In contrast to streptomycin and some of the other inhibitors discussed above, the mode of action of thiostrepton is much better understood.

1.5 Thiostrepton and Ribosomes

Thiostrepton is a partly-cyclic polypeptide antibiotic (Figure 1.2). It is a potent inhibitor of protein synthesis and binds tightly to the E.coli 50S ribosomal subunit (Weisblum & Demohn, 1970a) at a single binding site (Sopori & Lengyel, 1972; Highland et al., 1975a). However, most Gram-negative bacteria (including E.coli) are refractory to the drug in vivo since it is excluded by their cell surface.

Initial studies in vitro on the mode of action of thiostrepton showed that it inhibited the EF G-dependent ribosomal GTPase (Pestka, 1970) by preventing the binding of EF G and GTP to the E.coli 50S ribosomal subunit (Bodley et al., 1970a, Weisblum & Demohn, 1970b). In addition, when the antibiotic fusidic acid was used to stabilise the binding of EF G to ribosomes in the presence of GTP, such ribosomes were
protected from inactivation by thiostrepton (Highland et al., 1971).

Since EF G normally functions during the translocation reaction of protein synthesis (see section 1.2) it was concluded from these observations that thiostrepton acts by inhibiting this step of polypeptide chain elongation. However, other in vitro studies revealed that thiostrepton could also inhibit the binding of the [AA-tRNA-EF Tu·GTP]* complex to ribosomes (Modolell et al., 1971).

When the effect of thiostrepton on protein synthesis was examined in vivo (Cundliffe, 1971) and in vitro using a complete system (Cannon & Burns, 1971) peptidyl-tRNA bound to the ribosomes was 'locked' in the post-translocational (i.e. puromycin-reactive) state. It was therefore evident that translocation could proceed and that thiostrepton was inhibiting the binding of aminoacyl-tRNA to the ribosomes. Thus it was proposed (Cundliffe, 1971) that in a complete system thiostrepton only has access to its binding site when the ribosomal A-site is empty (i.e following translocation), whereas in assays of individual functions using salt-washed ribosomes it may bind unhindered.

These observations were explained in terms of a single mode of action of thiostrepton by proposal of the 'single ribosomal GTPase' hypothesis (Cundliffe, 1971; Modolell et al., 1971), which suggested that elongation factors EF Tu and EF G bind alternately to a single ribosomal site during the elongation of a polypeptide. Thus thiostrepton can inhibit the function of either factor when assayed individually. This model was strengthened by the finding that ribosomes cannot interact with EF Tu and EF G simultaneously (Richman & Bodley, 1972; Cabrera et al., 1972; Miller, 1972; Richter, 1972) and that ribosomal protein L7/L12 is required

* see footnote on p.4
for the interaction of both factors with the ribosome (see above).

More recently it has been reported that thiostrepton inhibits ribosomal functions occurring during both polypeptide chain initiation and termination. In the former case conclusions as to the mode of action of the drug have been equivocal. Thus thiostrepton may prevent recycling of IF 2 (Mazumder, 1973; Lockwood et al., 1974a; Sarkar et al., 1974), for which the hydrolysis of GTP is normally required (see section 1.2). However, inhibition of IF 2-dependent GTP hydrolysis by thiostrepton may be indirect and due to prevention of the association of the two ribosomal subunits during the initiation process (Naaktgeboren et al., 1976a). The effect of thiostrepton on the 'termination' event is brought about by prevention of the binding of the release factors to the ribosome (Brot et al., 1974), a process thought to occur in the ribosomal A-site. Both IF 2-dependent GTP hydrolysis (Lockwood et al., 1974b) and the binding of release factors to the ribosome (Brot et al., 1974) require the presence of protein L7/L12.

Thus thiostrepton has been shown to inhibit the interaction of initiation, elongation and release factors with a particular region of the 50S ribosomal subunit. With the exception of release factor binding, the other processes inhibited by thiostrepton are normally coupled to the hydrolysis of GTP.

Although thiostrepton inhibits functions of the *E. coli* ribosome dependent on the presence of protein L7/L12, it can bind perfectly well to ribosomal core-particles lacking the protein (Sopori & Lengyel, 1972; Ballesta & Vazquez, 1972a). However, Highland et al., (1975b) have shown that protein L11 is required for the binding of thioestrepton to ribosomes. Thus although 'core-particles' prepared from *E. coli* 50S ribosomal subunits by treatment with 4M-LiCl did not bind \[^{35}\text{S}]\text{thioestrepton, reconstitution with protein L11 specifically restored this ability. Furthermore,}
addition of antibody (IgG) against protein L11 (but not other ribosomal proteins) to the reconstituted core-particles prevented them from binding \(^{[35}S\)thiostrepton.

The present dissertation describes the characterisation of ribosomes from several spontaneously-occurring thiostrepton-resistant mutants of *Bacillus megaterium* KM. This organism has previously been used in studies of the mode of action of thiostrepton (Cundliffe, 1971) and was chosen since wild-type *E. coli* is insensitive to thiostrepton *in vivo*.

The interaction of thiostrepton with ribosomes from *B. megaterium* wild-type and two thiostrepton-resistant mutant strains has already been investigated (Dixon, 1976). During the present study, knowledge of the nature of ribosomes from these and two other independently-selected thiostrepton-resistant mutants has been considerably extended. Identification of a protein required for high-affinity interaction of thiostrepton with *B. megaterium* ribosomes and of the ribosomal component affected in the mutant strains has been achieved by immunological comparison with the ribosomal proteins of *E. coli*. Study of the minimum requirements for the creation of a high-affinity ribosomal binding site for thiostrepton has also been made. The complete characterisation of ribosomes from one of the mutant strains has enabled the examination *in vitro* of the functional role of the altered ribosomal protein.

The only other work existent similar to that undertaken during this study concerns certain thiostrepton-resistant mutants of *Bacillus subtilis* (Harford & Sueoka, 1970; Goldthwaite & Smith, 1972). When the present study commenced, it had been demonstrated that ribosomes isolated from these mutants do not bind \(^{[35}S\)thiostrepton (Vince et al., 1976) and had been shown that the 50S ribosomal subunit was the site of the mutational alteration (Pestka et al., 1976). Some of the observations made more
recently using these strains are similar to results presented in this dissertation and will accordingly be discussed below.
CHAPTER 2: MICROBIOLOGICAL METHODS

This Chapter describes the growth and maintenance of the bacterial strains employed and the selection of mutant strains resistant to thio streptom.

2.1 Bacterial Strains

The bacterial strains used during this study are listed, together with their sources, in Table 2.1.

2.2 Materials and Media

Casamino acids, malt extract, peptone, tryptone and yeast extract were purchased from Difco Laboratories, Detroit, and agar was obtained from Davis Gelatine (N.Z.) Ltd.

The following media were used for the growth of bacterial strains:

(a) Solid media

(i) Nutrient agar (Difco).

(ii) Minimal agar contained 1.5% (w/v) agar in M9 medium (see below) supplemented with 0.2% (w/v) glucose.

(iii) DYM agar contained (per litre) 4g dextrose, 4g yeast extract, 10g malt extract and 20g agar, adjusted to pH 7.3 using KOH.

(b) Liquid media

(i) ML medium contained (per litre) 10g NaCl, 5g yeast extract, 10g tryptone.

(ii) Nutrient Broth No. 2 (Oxoid Ltd., U.K.)

(iii) M9 medium contained (per litre) 7g $K_2HPO_4$, 3g $KH_2PO_4$, 1g $(NH_4)_2SO_4$, 0.5g sodium citrate, 0.1g $MgSO_4 \cdot 7H_2O$. 
### Table 2.1

**Bacterial strains and their origins**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>MRE 600 (NTCC* 8164)</td>
<td>Microbiological Research Establishment, Porton, U.K.</td>
</tr>
<tr>
<td></td>
<td>prm-1 (K12 1031 relA</td>
<td>Dr. C. Colson, Université de Louvain</td>
</tr>
<tr>
<td></td>
<td>met ara(A)(^{-}) HfrC</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>KM (NCIB* 9521)</td>
<td>Sub-department of Chemical Microbiology, Cambridge</td>
</tr>
<tr>
<td><em>Streptomyces azureus</em></td>
<td>ATCC* 14,921</td>
<td>Dr. E. Meyers, Squibb Institute for Medical Research, Princeton, N.J.</td>
</tr>
</tbody>
</table>

* NCTC : National Collection of Type Cultures (London)

NCIB : National Collection of Industrial Bacteria (Aberdeen)

ATCC : American Type Culture Collection (Rockville, Maryland)
(iv) Low phosphate medium (LPM) contained (per litre) 2g glucose, 2g casamino acids, 0.5g sodium citrate, 0.54mg FeCl$_2$, 6.055g Tris-base, 0.0272g KH$_2$PO$_4$, 0.15g KCl and 0.1g MgSO$_4$·7H$_2$O, adjusted to pH 7.4 using HCl (Reference: Hughes & Mellows, 1978).

(v) Fermentor medium (recipe obtained from Dr. E. Meyers, Squibb Institute for Medical Research, Princeton, N.J.). This contained (per litre) 10g peptone, 15g malt extract, 20g glucose, 3g yeast extract and 20g maltose.

All media were prepared using distilled water. LPM and M9 medium (with or without added glucose or casamino acids) were autoclaved for 10 minutes at 10 lbs/in$^2$, as were stock solutions of 50% (w/v) glucose. All other media (including agars) were autoclaved for 20 minutes at 15 lbs/in$^2$ and sterile glucose solution added subsequently when desired. Autoclaved solutions containing agar were allowed to cool to approximately 60°C before addition of any antibiotic solution (considered sterile) and pouring into plastic vented Petri dishes (approximately 25ml per dish). When set, the plates were inverted and dried at 20°C before use.

2.3 Maintenance of Bacterial Strains

Spore suspensions of S.azureus were kept at -20°C, while other strains were stored as lyophils (at 4°C) and in nutrient agar stabs (at 20°C). Material in lyophils or stabs was recovered by resuspension in a small volume of rich medium which was incubated overnight at 37°C and then plated out onto nutrient agar.

Strains of B.megaterium and E.coli were maintained for daily use on nutrient agar plates (grown at 37°C). Strains of E.coli maintained in this way were stored at 4°C and subcultured every 6 months. However,
B. megaterium strains lost viability under such conditions after prolonged storage and were therefore subcultured every 4 weeks and kept at 20°C.

2.4 Growth of Bacterial Strains

Strains of B. megaterium and E. coli were grown at 37°C from single colonies on nutrient agar plates. Growth was followed by measurement of the increase in optical density of cultures at 600nm. Such measurements could be used to estimate cell yield, since for cultures of B. megaterium wild-type 1 A_{600} unit represents approximately 10^8 viable cells (E. Cundliffe, personal communication).

Small quantities of cells or starter cultures for bulk growth (5 to 400ml) were grown in conical flasks which were vigorously aerated by orbital shaking. During experiments *in vivo*, growth was maintained in exponential phase by periodically subcuturing cells into pre-warmed medium. Starter cultures of E. coli strains were grown overnight in rich medium. However, strains of B. megaterium lost viability at high cell densities during stationary phase and so starter cultures of this organism were normally grown more slowly in minimal medium (M9 medium containing 0.2% (w/v) glucose) and used to inoculate larger flasks before growth had ceased.

Bulk growth of strains was performed in rich medium (ML medium plus 0.5% (w/v) glucose or Nutrient Broth No.2) contained in 2l flasks and shaken vigorously at 37°C as described already. The inoculum was typically 3 to 5% by volume. Growth was continued until the A_{600} of the cultures had reached 0.8 (B. megaterium mutant strains) or 1.0 (B. megaterium wild-type or E. coli), corresponding to late logarithmic phase (Figure 2.1). Cultures were checked under the microscope and then the cells harvested by low-speed centrifugation or by use of a Sharples continuous-flow centrifuge operated at 30,000 rpm. A cell-yield of 2.5 to 3.0g wet weight
The growth of *B. megaterium* in rich medium

Wild-type *B. megaterium* and thiostrepton-resistant mutant MJ1 were grown at 37°C in nutrient broth No.2 containing 0.5% (w/v) glucose. Culture samples were periodically removed and their optical density at 600nm determined (see text).

- wild-type (w.t.)
- mutant MJ1
per litre of culture was routinely obtained. Cells were washed in TMAβ buffer (see Chapter 3) and frozen at -20°C if not immediately required.

Starter cultures of thioestrepton-resistant *B. megaterium* strains contained thioestrepton at 1.0μg/ml, although bulk growth was necessarily conducted in the absence of the drug. Such cultures were therefore checked after growth in the absence of thioestrepton to ensure that reversion to thioestrepton-sensitivity had not occurred; this was never observed.

Kilogram quantities of bacteria were grown by continuous culture at the MRE, Porton. Wild-type *B. megaterium* KM was grown at 35°C in medium containing 4% (w/v) mannitol, 0.1% (w/v) yeast extract and salts, at a dilution rate of 0.26 h⁻¹. Growth of *E. coli* MRE 600 was conducted similarly but at 37°C with a dilution rate of 0.78 h⁻¹. Cells of both strains were washed in buffer containing 10mM-Tris-Cl (pH 7.4) and 10mM-magnesium acetate, before rapid freezing and storage at -20°C.

The growth of *S. aureus* for the production of [³⁵S]thioestrepton is described in Chapter 6.

2.5 The Selection of Thioestrepton-Resistant Mutants of Bacillus *megaterium* KM

Cells from single colonies of *B. megaterium* KM were grown up overnight at 37°C in M9 medium containing 0.1% (w/v) glucose. Samples (0.2ml) of each culture were plated out onto nutrient agar containing 3μg/ml thioestrepton and incubated at 37°C (48 hours). For *B. megaterium* KM grown on nutrient agar, the minimum growth-inhibitory concentration (MGIC) of thioestrepton is approximately 0.1μg/ml (Dixon, 1976). The selective medium therefore contained approximately 30 times the MGIC of the antibiotic.

Some plates yielded resistant colonies at an estimated
Figure 2.2
Legend to Figure 2.2

Morphology of *B. megaterium* strains

The following strains were grown in nutrient broth No.2 until late logarithmic phase and examined by phase-contrast microscopy:

(a) *B. megaterium* wild-type (x 250)
(b) *B. megaterium* mutant PD1 (x 250)
(c) *B. megaterium* mutant MJ1 (x 250)
(d) *B. megaterium* mutant MJ1 (x 500)

The cells of the mutant strains were found to be long and filamentous, as was first noted in the case of mutant PD1 by Dixon (1976).
frequency of between 1 in $10^9$ and 1 in $10^8$. Such colonies were stored
and maintained as described above but with thioestrepton present at
3μg/ml in nutrient agar on which they were grown.

Mutant strains designated MJ1, MJ3 and MJ5 (the 'MJ' mutants)
obtained by this means from different parental colonies were considered
to have arisen by independent, spontaneous mutational events. Mutant
strains PD1, PD5, PD14 and PD18 (the 'PD' mutants) had been selected by
a similar means using thioestrepton at 10μg/ml (Dixon, 1976). The
preliminary examination of the MJ mutants presented in this section
inevitably followed a course similar to that pursued by Dixon (1976)
using the PD mutants.

The MJ mutant strains were, like the parental strain, Gram-positive
and catalase-positive. However, they showed a long, filamentous morphology
quite unlike the characteristic shape of cells of the wild-type, as has
been noted in the case of the PD mutants (Dixon, 1976). This is
illustrated in Figure 2.2.

The growth-rate of the mutant strains in a variety of liquid media
was always 2 to 3 times lower than that of the wild-type (Table 2.2).
Indeed, their growth-rate was lower still in the presence of thioestrepton,
indicating that the mutant strains were not totally resistant to the drug
but simply tolerant of higher drug concentrations than the wild-type.
This had been noted previously in the case of mutant PD1 (Dixon, 1976).
It was additionally noted that strains MJ1 and PD1 were unable to grow on
minimal agar, although strain MJ1 (but not PD1) could grow in liquid
minimal medium. This difference between the two mutant strains remains
to date the only one detected.

When the differing sensitivity to thioestrepton of the wild-type and
'MJ' mutant strains was examined (Table 2.3), the results suggested that
the mutant strains were up to 100 times more tolerant of thioestrepton.
### Table 2.2

<table>
<thead>
<tr>
<th>medium</th>
<th>doubling time (min)</th>
<th>w.t.</th>
<th>MJ 1</th>
<th>PD 1</th>
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<tbody>
<tr>
<td>ML + 0.5% glucose</td>
<td>30</td>
<td>87</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>M9 + 0.2% glucose</td>
<td>90</td>
<td>170</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>M9 + 0.2% glucose &amp; 0.2% c.am. acids*</td>
<td>47</td>
<td>88</td>
<td>n.d.</td>
<td></td>
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<tr>
<td>LPM</td>
<td>55</td>
<td>95</td>
<td>n.d.</td>
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### Table 2.3

<table>
<thead>
<tr>
<th>µg thiostrepton per disc</th>
<th>zone radius (mm)</th>
<th>w.t.</th>
<th>MJ 1</th>
<th>MJ 3</th>
<th>MJ 5</th>
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<tbody>
<tr>
<td>0.05</td>
<td>-</td>
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<td>0.1</td>
<td>-</td>
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<tr>
<td>0.5</td>
<td>1.0</td>
<td>2.0</td>
<td>6.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0</td>
<td>6.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>6.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>10</td>
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<tr>
<td>50</td>
<td>1.0</td>
<td>1.0</td>
<td>0.5</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>1.5</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>-</td>
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</tbody>
</table>

### Table 2.4

<table>
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<tr>
<th>antibiotic</th>
<th>w.t.</th>
<th>MJ 1</th>
<th>MJ 3</th>
<th>MJ 5</th>
<th>PD 1</th>
<th>PD 5</th>
<th>PD 18</th>
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<tbody>
<tr>
<td>10 µg thiostrepton</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>10 µg thiopeptin</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<td>3 µg micrococcin P</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>10 µg sporangiomycin</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>3 µg siomycin</td>
<td>S</td>
<td>R</td>
<td>R</td>
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<td>R</td>
</tr>
</tbody>
</table>
Legend to Table 2.2
The growth-rates of \textit{B. megaterium} strains in different growth-media

The growth-rates at 37°C of \textit{B. megaterium} wild-type and mutant strains MJ1 and ED1 are expressed as the doubling times (determined from measurements of culture \(A_{600}\)). The composition of the media used can be found in section 2.1.

* C.am. acids: Casamino acids; n.d.: not determined.
Strain ED1 would not grow in M9 medium plus 0.2% (w/v) glucose.

Legend to Table 2.3
Sensitivity to thiostrepton of \textit{B. megaterium} strains in vivo

Samples (0.1ml) from overnight cultures of each organism were mixed with 3.0ml ML medium containing 0.5% (w/v) agar, which had been autoclaved and allowed to cool to 50°C. The mixture was rapidly and evenly poured over the surface of a nutrient agar plate and allowed to set. Paper discs (Whatman AA) containing different quantities of thiostrepton were applied to the surface. The radius of any clear zone of growth inhibition around a disc after 48 hours at 37°C was measured.

Paper discs were impregnated with 20 to 50\(\mu\)l of antibiotic solution and dried at 37°C. Dimethylsulphoxide was used as a solvent for thiostrepton (and most other antibiotics) but control discs revealed no contribution by the solvent to the effects reported.

Legend to Table 2.4
Cross-resistance of thiostrepton-resistant \textit{B. megaterium} strains to other sulphur-containing antibiotics

The experiment was performed as described in the legend to Table 2.3. Sensitivity of \textit{B. megaterium} wild-type (w.t.) or of the thiostrepton-resistant strains to particular antibiotics was scored by the presence of unequivocal clear zones around discs after 48 hours growth at 37°C.

R: resistant; S: sensitive.
Table 2.5

(a) | (b)
---|---
| spiramycin (µg ml⁻¹) | w.t. | MJ1 | erythromycin (µg ml⁻¹) | w.t. | MJ1 |
| 0.5 | ++ | ++ | 0.01 | ++ | ++ |
| 1.0 | ++ | + | 0.05 | ++ | ++ |
| 3.0 | ++ | - | 0.1 | ++ | + |
| 6.0 | ++ | - | 0.5 | + | - |
| 10.0 | + | - | 1.0 | + | - |

Enhanced sensitivity of mutant MJ1 to erythromycin and spiramycin

Samples (0.5ml) from cultures of _B. megaterium_ wild-type (w.t.) or mutant MJ1 were plated out onto nutrient agar containing the indicated concentration of spiramycin III (a) or erythromycin (b). Growth after 48 hours at 37°C was denoted thus:

++ : good;  + : poor;  — : no growth.
Examination of the sensitivity of the mutant strains to other high molecular weight, sulphur-containing antibiotics revealed cross-resistance to siomycin, sporangiomycin, thiopепtin and micrococcin P (Table 2.4) but not to althiomycin (data not shown). These results are therefore in agreement with earlier studies using the PD mutants (Dixon, 1976). This cross-resistance probably indicates the known similarities between the structures of some of these antibiotics (Anderson et al., 1970; Tori et al., 1976), their modes of action (review: Cundliffe, 1979a) and their ribosomal binding sites (Cundliffe & Dixon, 1975). Sensitivity of the mutant strains to althiomycin supports previous results (Fujimoto et al., 1970; Pestka, 1972; Burns & Cundliffe, 1973) showing that this antibiotic is unlike thiostrepton in its mode of action.

The sensitivity of the wild-type and mutant strains to a range of other antibiotics was found, in most cases, to be similar (data not presented). However, closer examination of mutants MJ1 and PD1 revealed an enhanced sensitivity to erythromycin and spiramycin in these strains when compared with the wild-type (Table 2.5; data for mutant PD1 omitted). This effect was first noted by Goldthwaite & Smith (1972) with certain thiostrepton-resistant mutants of B.subtilis. The molecular basis for enhanced sensitivity to these macrolide antibiotics is unknown. However, ribosomes from B.megaterium wild-type and mutant MJ1 were equally sensitive to spiramycin when the drug was used, over a range of concentrations, to inhibit the poly U-dependent synthesis of polyphenylalanine (data not presented). Thus ribosomes from mutant MJ1 apparently do not exhibit enhanced sensitivity to macrolides in vitro.

2.6 Attempts to Select Thiostrepton-Sensitive Revertants from Mutant Strains MJ1 and PD1

The ability to select revertants to antibiotic sensitivity from
antibiotic-resistant mutant strains is useful both in understanding the
nature of the forward mutation and in the characterisation of the lesion.
Thus mutant strains carrying total or partial gene deletions might not be
expected to revert to the wild-type phenotype, whereas nonsense, mis-sense
and frameshift mutations might be expected to revert spontaneously, albeit
with differing frequencies.

Before attempting to isolate thio strepton-sensitive organisms from
cultures of the mutant strains, an additional genetic marker was
introduced. Thus any putative revertants could be distinguished from the
parental wild-type strain and from contaminants. Culture samples of
mutants MJ1 and PD1 were plated out onto nutrient agar containing 50µg/ml
nalidixic acid and a strain of each resistant to the drug was isolated.
These strains (designated MJ1 _nal_ and PD1 _nal_) were maintained on nutrient
agar containing (in addition to 3µg/ml thio strepton) nalidixic acid at
50µg/ml.

The following strategies were used in attempts to isolate thio strepton-
sensitive revertants:-

(a) **Selection using minimal agar**

Since the thio strepton-resistant mutant strains had become unable to
grow on minimal agar it was considered likely that revertant strains would
regain this ability.

Cultures of MJ1 _nal_ and PD1 _nal_ were grown until late logarithmic
phase and then 0.1ml samples (approximately 10^7 cells) were plated out on
minimal agar and incubated at 37°C. Colonies that developed were replica-
plated onto nutrient agar plus or minus thio strepton (at 3µg/ml). No
sensitive colonies were obtained in this way, despite examination of
culture samples totalling several millilitres in both cases.

(b) **Selection by growth-rate**

The mutant strains of _B.megaterium_ grow considerably more slowly...
than the wild-type strain (Table 2.2). Revertant organisms might therefore be expected to out-grow the mutants during continuous culture, given sufficient time.

A chemostat containing 150ml of Nutrient Broth No.2 (supplemented with 0.5% (w/v) glucose) was inoculated with a small culture of either MJ1 nal or FD1 nal and grown at 37°C until late logarithmic phase. The culture was then diluted at a rate of approximately 0.3 h⁻¹ with identical medium, such that cell growth occurred continuously for 5 days. Samples of the culture were then plated out onto minimal agar and incubated at 37°C. Any colonies that developed were examined as above by replica-plating. No revertant colonies were identified from either strain.

(c) Selection using erythromycin

Cultures (5ml) of strains MJ1 nal and FD1 nal were grown at 37°C in Nutrient Broth No.2 until late logarithmic phase. Samples (0.5ml) were plated out onto nutrient agar containing 1.0µg/ml erythromycin and incubated at 37°C. Under these conditions cells of the wild-type but not the mutant strains were able to form colonies (Table 2.5). Thus ability of cells from cultures of the mutants to grow on 1.0µg/ml erythromycin could have indicated reversion to thiostrepton-sensitivity. No such 'erythromycin-resistant' colonies were ever observed from cultures of either mutant strain.

In order to screen more cells of the mutant strains for reversion, a culture of MJ1 nal (250ml) was grown at 37°C in Nutrient Broth No.2. When the A₆₀₀ reached 0.4 the cells were harvested by low-speed centrifugation. The cells (1.0g wet weight) were then re-suspended in Nutrient Broth No.2 (1 l) containing 0.8µg/ml erythromycin, and incubated at 37°C with vigorous shaking. After 24 hours samples (0.1ml) were plated out onto nutrient agar containing 1.0µg/ml erythromycin. No colonies of
B. megaterium were thus isolated.

Failure to select thiostrepton-sensitive revertants from strains MJ1 _nal_ or PD1 _nal_ could conceivably have resulted from interaction between the nalidixic acid resistance locus and the thiostrepton resistance locus. The procedures described above in sections (a) and (c) were therefore repeated using the parental MJ1 and PD1 strains (i.e. lacking the nalidixic acid marker) but again no revertants were obtained. This does not, of course, indicate that reversion is impossible but it does imply that the frequency of reversion is considerably less than the frequency of mutation to thiostrepton resistance. This is in marked contrast to certain thiostrepton-resistant mutants of _B. subtilis_ which readily yielded revertants using the erythromycin selection procedure (Pestka et al., 1976). Failure to obtain such revertants during the present study renders more likely the possibility that in the mutants of _B. megaterium_ there is a total or partial gene deletion. This would be consistent with the results of experiments yet to be discussed (see Chapters 8 and 10).
CHAPTER 3: GENERAL BIOCHEMICAL METHODS

This chapter describes materials and techniques of general use throughout the study. More specific methods are located with the experiments to which they pertain.

3.1 Materials

(a) Enzymes

The following enzymes were obtained from the Sigma Chemical Co. Ltd., U.K. (Sigma) or Boehringer-Mannheim GmbH, W.Germany (Boehringer):

Glyceraldehyde-3-phosphate dehydrogenase (E.C.1.2.1.12)/3-phosphoglycerate kinase (E.C.2.7.2.3): crystalline suspension (6mg/ml) in 3.2 M-(NH₄)₂SO₄ (Boehringer).

Pyruvate kinase (E.C.2.7.1.40): 10mg/ml in 50% (v/v) glycerol solution (Boehringer).

Deoxyribonuclease I (E.C.3.1.4.5): electrophoretically pure, from bovine pancreas (Sigma).

Ribonuclease A (E.C.3.1.4.22): type XII (Sigma).

Ribonuclease T₁ (E.C.3.1.4.8): grade III, from Aspergillus oryzae (Sigma).

Elongation factor EF Tu was prepared by the method of Miller & Weissbach (1970) and was the generous gift of Dr.E.Cundliffe. It was dissolved in buffer containing 50mM-Tris-HCl(pH 7.6), 10mM-MgCl₂, 1mM-DTT, 50% (v/v) glycerol and stored at -20°C. Elongation factor EF G was the kind gift of Professor James Bodley and was stored at -20°C in 50% (v/v) glycerol solution. It was prepared according to Rohrbach et al. (1974).

(b) Radiochemicals

All radiochemicals were purchased from the Radiochemical Centre,
Amersham, U.K. with the exception of $^{32}P$-adenosine 5'-triphosphate, which was prepared as described below. Their specific radioactivities are given in the legends to Figures and Tables or in the text.

$^{32}P$-adenosine 5'-triphosphate was prepared using an adaptation of the method of Glynn & Chappell (1964). This utilises an enzyme-catalysed exchange reaction between inorganic phosphate and the terminal phosphate group of ATP. To 0.5ml of 200mM-Tris-HCl (pH 8.0) containing 2.4mCi carrier-free $^{32}P$-orthophosphate was added 0.1ml buffer containing 100mM-Tris-HCl (pH 8.0), 4mM-MgCl$_2$, 12mM-DTT, 3mM-3-phosphoglycerate(3-PGA), 1mM-NAD, 10μl 3-PGA kinase/glyceraldehyde-3-phosphate dehydrogenase, followed by 40μl 100mM-ATP. After 20 minutes at 20°C 9 volumes of ice-cold water were added and the reaction mixture loaded onto a DEAE-Sephadex-A25 column (bed volume 1.0ml, equilibrated with water). The column was developed using a zero to 1.0 M gradient of KHCO$_3$ (10ml), collecting 0.5ml fractions. The radioactivity contained in samples of each was determined and those containing the second-eluted peak of radioactivity ([γ-$^{32}P$]ATP) were pooled, diluted to 5.0ml, made 10% (v/v) in triethylamine and lyophilised. After a second lyophilisation from 10% (v/v) triethylamine (5ml), the [γ-$^{32}P$]ATP was dissolved in water and frozen at -70°C. Its purity was determined from chromatography on DEAE-cellulose (see Chapter 10) and by adsorption using activated charcoal. Such analyses showed that 98 to 99% of the radioactivity was present in ATP. The specific radioactivity was determined from measurement of absorbance at 259nm ($\lambda_{\text{max}}$ for ATP), taking $\varepsilon_{\text{max}}$ as 15,400 M$^{-1}$cm$^{-1}$ in the pH range 7 to 11 (Sober, 1968).

(c) Fine chemicals

Adenosine 5'-triphosphate (ATP, disodium salt), cytidine 5'-triphosphate (CTP, trisodium salt), dithiothreitol (DTT) and 3-
phosphoglycerate (3-PGA) were purchased from Boehringer. Uridine was obtained from PL Biochemicals Inc., Milwaukee. L-amino acids, β-mercaptoethanol, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), unfractionated tRNA (from E.coli W, type XXI), polyuridylic acid (MW > 100,000, potassium salt), guanosine 5'-triphosphate (GTP; trilithium salt) and phosphoenol pyruvate (PEP, monopotassium salt) were obtained from Sigma.

(a) Other Chemicals

All chemicals used during the course of this work were of the highest grade available (usually 'AnalaR').

'Atomic absorption' grade lithium chloride (Fisons, U.K.) was used preferentially. When lower grade material was employed, solutions (10M) were treated with activated charcoal and Bentonite (Fisons), filtered and finally passed through a cellulose nitrate disc (Sartorius, 0.45μm pore-size) prior to use.

Polyethylene glycol (PEG) was obtained from Sigma (PEG 20,000) or from Union Carbide (U.K.).

Diaminooethanetetra-acetic acid (EDTA) was stored (at 4°C) as 50mM solutions of the free acid, adjusted to pH 7.6 with KOH. The disodium salt was used only where specified.

Sucrose (Sigma, grade I) was dissolved in stock solutions at 600g/l which were autoclaved for 10 minutes at 10 lbs/in². For some purposes (see text) sucrose solutions were pre-treated with diethyl pyrocarbonate (Sigma) to remove possible ribonuclease activity. Solutions of sucrose (60% (w/v) were made 0.1% (v/v) in diethyl pyrocarbonate and stirred at room temperature for 24 hours. Such solutions were then de-gassed under vacuum and stored at 4°C.

Activated charcoal ('Norit', Sigma) was pre-treated by refluxing with 2M-HCl, then washing with water, ethanolic ammonia solution and finally water, according to Thompson (1960).
(e) Antibiotics

Antibiotics used during this study are listed in Table 3.1, together with their source and the solvent used. Several were of low solubility in water, for example the solubility of thio streptom in water is 88\(\mu\)g/ml (Marsh & Weiss, 1967). In such cases stock solutions were prepared in dimethylsulphoxide (DMSO); for use in biological systems less concentrated solutions were prepared using 25\%(v/v) DMSO (in water).

All the antibiotics used were reported to be of high purity by the suppliers. Of particular relevance to the present study was the purity of the thio streptom used; it was stated to contain 98.5\% thio streptom as determined using a bioassay, but no chemical data was available.

(f) Dialysis tubing

All dialysis tubing was autoclaved in a solution containing 5\%(w/v) NaHCO\(_3\) and 10mM-disodium EDTA, washed exhaustively and stored in water at 4°C. 'Wisking' tubing (Scientific Instrument Centre Ltd., London) was used for most purposes. Spectrapor No.1 tubing (mol.wt. cut-off \(\approx 3,500\)) or No.3 tubing (mol.wt. cut-off \(\approx 6,000\)) was used when handling ribosomal proteins or for dialysis against solutions containing PEG. It was obtained from Raven Scientific Ltd., U.K.

3.2 Buffers

All stock solutions of buffers were adjusted to the appropriate pH at 20°C. A number of standard buffered solutions were used during the present study and are listed below:

TMA\(\beta\) buffer: 10mM-Tris-acetate(pH 7.6), 10mM-magnesium acetate, 50mM-ammonium acetate, 3mM-\(\beta\)-mercaptoethanol.

RS buffer: 10mM-Tris-HCl (pH 7.6), 10mM-MgCl\(_2\), 50mM-NH\(_4\)Cl, 3mM-\(\beta\)-mercaptoethanol.
Table 3.1

Sources of antibiotics

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Solvent</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>water</td>
<td>Sigma Chemical Co.</td>
</tr>
<tr>
<td>Fusidic acid (sodium salt)</td>
<td>water</td>
<td>Leo Pharmaceutical Products, Ballerup, Denmark.</td>
</tr>
<tr>
<td>Pseudomoniac acid (sodium salt)</td>
<td>water</td>
<td>Dr. N. Rodgers, Beechams Pharmaceuticals, Research Division, Betchworth, Surrey.</td>
</tr>
<tr>
<td>Puromycin (hydrochloride)</td>
<td>water</td>
<td>Prof. E. F. Gale, Dept. of Biochemistry, University of Cambridge.</td>
</tr>
<tr>
<td>Siomycin</td>
<td>DMSO</td>
<td>K. Tanaka, Shionogi &amp; Co. Ltd., Osaka.</td>
</tr>
<tr>
<td>Sparsomycin</td>
<td>DMSO</td>
<td>Upjohn, Kalamazoo, Michigan.</td>
</tr>
<tr>
<td>Spiramycin III</td>
<td>water</td>
<td>Rhône-Poulenc, Paris.</td>
</tr>
<tr>
<td>Sporangiomycin</td>
<td>DMSO</td>
<td>Gruppo Lepetit, Milan.</td>
</tr>
<tr>
<td>Thioestrepton</td>
<td>DMSO</td>
<td>Barbara Stearns, Squibb Institute for Medical Research, Princeton, New Jersey.</td>
</tr>
</tbody>
</table>

* DMSO : dimethylsulphoxide
These and all other solutions were prepared using deionised water that was subsequently passed through a Millipore Milli-Q reagent grade water purification apparatus.

3.3 Preparation of Subcellular Fractions from Bacteria

(a) Preparation and fractionation of cell-free extracts

Freshly-harvested cells were resuspended in TMAβ buffer (containing 0.5mM-EDTA) at a concentration of approximately 20mg wet weight per ml buffer and washed at 4°C by centrifugation (8,000 rpm for 10 minutes in the Beckman JA-10 rotor). Alternatively, frozen cell paste was first allowed to thaw in the same buffer before resuspension. The washing procedure was repeated before the cells were resuspended in the same buffer at approximately 500mg (wet weight) per ml and passed twice through a French pressure cell (pre-cooled on ice) at 12,000 lbs/in². After the first passage DNase I was added (5μg/ml final concentration). The cell debris was removed by centrifugation for 40 minutes at 20,000 rpm and 4°C in the Beckman JA-21 rotor (small quantities) or Ti70 rotor (large quantities). The supernatant was decanted and dialysed at 0°C against 3 x 100 volumes of TMAβ buffer.

When desired, the dialysed supernatant ("S-30" fraction) was immediately centrifuged for 5 hours at 50,000 rpm and 2°C in the Beckman Ti70 rotor, yielding a post-ribosomal supernatant ("S-100" fraction) and
a crude ribosomal pellet. The latter was rinsed with TMA\(\beta\) buffer and then resuspended in a small volume of the same.

Crude ribosomes, S-100 and S-30 fractions were stored at \(-70^\circ\text{C}\) in small aliquots, which were frozen rapidly using dry ice/methanol.

(b) Preparation of high-salt-washed ribosomes

'Crude' ribosomes were first obtained from freshly-prepared S-30 fractions, produced as above except that TMA\(\beta\) buffer was replaced by RS buffer throughout. The S-30 fraction was layered over half its volume of RS buffer containing \(40\%\ (w/v)\) sucrose and centrifuged for at least 7 hours at \(50,000\ \text{rpm}\) and \(2^\circ\text{C}\) in the Beckman Ti70 rotor. The resulting pellet ('sucrose-washed' ribosomes) was resuspended in RS buffer and layered over similar buffer containing \(20\%\ (w/v)\) sucrose. Centrifugation for 5 hours at \(50,000\ \text{rpm}\) and \(2^\circ\text{C}\) in the above rotor yielded a pellet of 'high-salt-washed' ribosomes. The high-salt-washing procedure was routinely performed 3 times before the ribosomes were resuspended in and dialysed against RS buffer. Alternatively the final ribosomal pellet was resuspended and diluted at least 200-fold in RS buffer, recovered by centrifugation as above (5 hours) and finally taken up in RS buffer. Ribosomes were stored at \(-70^\circ\text{C}\) in small, rapidly-frozen aliquots.

All ribosomes used during the present work were, unless stated otherwise, prepared in this way (i.e. 3 times 'high-salt-washed').

(c) Preparation of S-100\(\ast\) fraction

Crude ribosome preparations contain a proportion of the soluble protein factors required for protein synthesis; these are removed from the ribosomes by the high-salt-washing procedure just described. For this reason, a supernatant fraction containing the material removed from ribosomes in this way was prepared for complementing high-salt-washed ribosomes when examining protein synthesis \textit{in vitro}.  

Cells were washed (see section (a)) and then broken by grinding with alumina (Staehelin & Maglott, 1971). The cells were mixed with twice their wet weight of levigated alumina (Norton abrasives, U.K.) in a mortar pre-cooled to -20°C. The mixture was ground for approximately 10 minutes until a viscous paste formed. Ice-cold TMAβ buffer (containing 0.5mM-EDTA) was slowly added while grinding continued until approximately 1ml of buffer had been added per g (wet weight) of cells. The mixture was decanted into centrifuge tubes (on ice) and DNase I added to give 5μg/ml final concentration. After 10 minutes the mixture was centrifuged for 60 minutes at 18,000 rpm and 4°C in the Beckman JA-21 rotor. The resulting S-30 fraction was then layered over half its volume of RS buffer containing 20% (w/v) sucrose and centrifuged for at least 7 hours at 50,000 rpm and 2°C in the Beckman Ti70 rotor. The entire supernatant was decanted and dialysed on ice against 2 x 30 volumes of RS buffer, then concentrated 3-fold by dialysis (at 0°C) against RS buffer containing 20% (w/v) PEG 20,000. After a final dialysis against 100 volumes of RS buffer the supernatant (termed S-100* fraction) was rapidly frozen in small aliquots and stored at -70°C.

The different methods of cell breakage employed deserve further explanation. The inconvenience of grinding large quantities of cells with alumina led to use of the French pressure cell when preparing ribosomes. Such ribosomes behaved similarly to those prepared by the alternative method. However, S-100* fractions prepared from cells ground with alumina were routinely of greater activity in the support of protein synthesis in vitro, than were those prepared using the French pressure cell.

(d) Preparation of ribosomal subunits

Ribosomal subunits were prepared from 'sucrose-washed' ribosomes (section (b)). These were resuspended in RS buffer at up to 120mg/ml and
dialysed against 2 x 100 volumes of SUB buffer to effect dissociation into subunits. Two methods of sucrose density-gradient ultracentrifugation were employed for separation of the dissociated ribosomal subunits:—

(i) Small scale: Samples (0.5ml) of dissociated ribosomes in SUB buffer (40 to 50 A_{260} units) were layered onto 16.5ml 5 to 20% (w/v) sucrose density-gradients made up in SUB buffer. After centrifugation for 12 hours at 20,000 rpm and 2°C in the Beckman SW27.1 rotor, the gradients were pumped through an ISCO UA-5 analyser and absorbance at 254nm monitored continuously. Fractions containing 30S and 50S ribosomal subunits were pooled separately, dialysed against RS buffer at 0°C and concentrated by dialysis against similar buffer containing 20% (w/v) PEG 20,000. After further dialysis against RS buffer, subunits were rapidly frozen in small aliquots and stored at -70°C. Subsequent analysis on sucrose density-gradients (see Chapter 9) revealed that all preparations were greater than 95% homogeneous.

(ii) Large scale: Separation was by zonal ultracentrifugation using the MSE B-XIV Ti rotor (volume 649ml). A hyperbolic sucrose density-gradient was designed for this rotor employing the theoretical considerations used by Eikenberry et al. (1970), sucrose density data (Sober, 1968) and published data for the above rotor (Anderson et al., 1967). The density-gradient was generated by an automatic variable gradient-former (MSE, model Z-100) and contained (in SUB buffer) from 7.65% (w/v) sucrose (radius 3.7cm) to 53.5% (w/v) sucrose (radius 6.4cm), the total volume being 425ml. The sample (1000 to 2000 A_{260} units of dissociated ribosomes) occupied 25ml of SUB buffer containing zero to 7.65% (w/v) sucrose (in a zone at radius 3.5 to 3.7cm), the sample itself in an inverse gradient. All solutions used were first cooled to 0°C but centrifugation was performed at 7°C.

The rotor was accelerated to 3,000 rpm and entirely filled with SUB
buffer. The contents of a two-chambered linear gradient-maker were then pumped to the rotor periphery, the output chamber containing the sample (in 12.5ml SUB buffer) and the second chamber SUB buffer containing 7.64% (w/v) sucrose. This was followed by the gradient (425ml) pumped at approximately 3.5ml/min and finally SUB buffer containing 60% (w/v) sucrose. When 530ml of buffer had thus been displaced from the rotor core (indicating correct positioning of the sample zone) the rotor was accelerated to 32,000 rpm. After 12 hours it was decelerated to 3,000 rpm and the gradient displaced from the centre of the rotor by pumping SUB buffer containing 70% (w/v) sucrose to the periphery. The A$_{260}$ of material emerging was monitored continuously using a CE272 spectrophotometer (CECIL Instruments Ltd.) and fractions of 10ml collected. Fractions containing 30S and 50S subunits were pooled separately and material recovered by centrifugation for 12 hours at 50,000 rpm and 2°C in the Beckman Ti70 rotor. Pellets were resuspended in SUB buffer, dialysed against 3 x 100ml RS buffer and stored as described in (i). Analysis of these preparations (as above) revealed no detectable cross-contamination of 30S and 50S ribosomal subunit preparations.

3.4 Preparation of Material from Ribosomes and Ribosomal Subunits of *Bacillus megaterium*

All preparations described in this section employed ribosomal subunits or high-salt-washed ribosomes as the starting material.

(a) Preparation of ribosomal 'core-particles' and 'split-proteins'

Ribosomes (20mg) in RS buffer were mixed with buffer of suitable volume and composition to yield 10ml containing 10mM-Tris-HCl(pH 7.6), 1mM-MgCl$_2$, 1.0M-LiCl, 3mM-β-mercaptoethanol. After 5 hours at 0°C the
mixture was centrifuged for 8 hours at 50,000 rpm and 2°C in the Beckman Ti75 rotor. The supernatant was decanted, dialysed against 100 volumes of RS buffer and then concentrated approximately 35-fold by dialysis against RS buffer containing 20% (w/v) PEG 20,000. After a final dialysis against RS buffer alone (100 volumes) the preparation (termed the '1M-LiCl split-proteins') was frozen at -70°C. For some preparations all the above dialysis steps were conducted in SP buffer (see text). The pellet obtained above was resuspended in and dialysed against RS buffer, before rapid freezing and storage at -70°C. This preparation of protein-deficient ribosomal particles was termed '1M-LiCl core-particles'.

Preparation of 2M-LiCl core-particles was from 1M-LiCl core-particles, by resuspension at 2mg/ml in buffer containing 10mM-Tris-HCl (pH 7.6), 1mM-MgCl₂, 2.0M-LiCl, 3mM-β-mercaptoethanol. After 5 hours at 0°C the 2M-LiCl core-particles thus formed were recovered and treated as described for the 1M-LiCl core-particles.

(b) Complete extraction of protein from ribosomes and ribosomal subunits

The procedure of Fraenkel-Conrat (1957) as modified by Hardy et al., (1969) was used to extract the protein from ribosomal particles. While rRNA cannot be completely de-proteinised in this way, this procedure is known to extract every ribosomal protein from ribosomes (or subunits) of both E.coli and E.stearothermophilus (Wittmann, 1974).

Ribosomes or ribosomal subunits (10mg) in 1.0ml RS buffer containing 0.1M-MgCl₂ were mixed with 2 volumes of cold, glacial acetic acid and kept on ice for 1 hour with frequent agitation. The dense precipitate of ribosomal RNA was removed by centrifugation for 5 minutes at 5,000 rpm and the supernatant decanted. After addition of cold acetone (5 volumes) the proteins were allowed to precipitate for 1 hour at -20°C and were then recovered by centrifugation in a similar manner and dried in vacuo. Storage was at -20°C as a dry precipitate or in a solution containing
8M-urea and 10mM-DTT. Preparations were denoted as follows: TP30, protein extracted from 30S subunits; TP50, protein from 50S subunits; TP70, protein from 70S ribosomes.

(c) Preparation of ribosomal RNA

All solutions for use with ribosomal RNA were autoclaved at 101bs/in$^2$ for 20 minutes, as were all tubes and vessels employed during its preparation. Ribosomal RNA was prepared by phenol extraction of ribosomes, ribosomal subunits or acetic acid-extracted RNA. The latter material was obtained during the preparation of ribosomal proteins (see previous section) and was first washed by resuspension in 66% (v/v) acetic acid followed by centrifugation. The pellet was then washed similarly in water, dissolved in buffer containing 1M-Tris-HCl (pH 7.6) and 50mM-EDTA, then finally diluted 20-fold with water before extraction.

Ribosomes (or ribosomal subunits) in RS buffer (5 to 10mg/ml) or acetic acid-extracted RNA (dissolved as above) were mixed vigorously for 2 minutes with an equal volume of re-distilled phenol (water-saturated). The two phases were clarified by centrifugation at 5,000 rpm for 5 minutes, the upper aqueous phase removed and the lower phase extracted with the same volume of water in a similar manner. The two aqueous phases were pooled, re-extracted by the same procedure with an equal volume of water-saturated phenol and then RNA in the final aqueous phase precipitated by the addition of cold ethanol (3 volumes). After at least 1 hour at -20°C the RNA was recovered by centrifugation at 10,000 rpm for 10 minutes. The pellet was re-dissolved in water, re-precipitated and recovered as above and finally dried in vacuo. It was stored at -70°C as a dry powder or dissolved in buffer containing 10mM-Tris-HCl (pH 7.6), 50mM-NH$_4$Cl.
When samples of rRNA prepared by this means were examined by electrophoresis in gels containing 15% (w/v) acrylamide and 0.1% (w/v) SDS (method of Laemmli, 1970; see below) no protein bands were detectable. Ribosomal RNA thus prepared was denoted as follows: RNA70, RNA prepared from 70S ribosomes; RNA50, RNA prepared from 50S ribosomal subunits; RNA30, RNA prepared from 30S ribosomal subunits.

Fractionated ribosomal RNA from *B. megaterium* (wild-type) and *E. coli* MRE 600 was kindly prepared by Dr. J. Thompson using the method of Harrison et al. (1974) with minor modifications. This involved extraction of ribosomes with phenol and SDS followed by sucrose density-gradient ultracentrifugation.
3.5 Quantitation of Ribosomes and Ribosomal Components

(a) Ribosomes and ribosomal RNA

The concentration of washed ribosomal particles or ribosomal RNA in solution was determined from measurements of optical density at 260nm ($A_{260}$) in quartz cuvettes (1cm path-length) and using a Unicam SP800 spectrophotometer. By considering one $A_{260}$ unit to represent 60μg ribosomal particles or 45.5μg ribosomal RNA and assuming the molecular weights below (see van Holde & Hill, 1974), molar quantities were determined as follows:

<table>
<thead>
<tr>
<th>Material</th>
<th>Molecular weight (daltons x 10^-6)</th>
<th>$A_{260}$ pmol per unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>70S ribosomes</td>
<td>2.70</td>
<td>22.2</td>
</tr>
<tr>
<td>50S ribosomal subunits</td>
<td>1.80</td>
<td>33.3</td>
</tr>
<tr>
<td>30S ribosomal subunits</td>
<td>0.90</td>
<td>66.7</td>
</tr>
<tr>
<td>RNA70 (23S, 16S and 5S RNA)</td>
<td>1.65</td>
<td>27.5</td>
</tr>
<tr>
<td>RNA50 (23S and 5S RNA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23S RNA</td>
<td></td>
<td>41.3</td>
</tr>
<tr>
<td>RNA30 (16S RNA)</td>
<td>0.55</td>
<td>82.6</td>
</tr>
</tbody>
</table>

The concentration of ribosomes in S-30 fractions or crude ribosome preparations cannot be determined due to the presence of other material with absorbance at 260nm. For purposes of comparison the 'nominal' ribosome concentration was determined for such preparations by attributing all the absorbance at 260nm to ribosomes. Subsequent analysis of S-30 fractions using $[^{35}S]$thiostrepton as a probe (Table 6.4) indicated that approximately 90% of their $A_{260}$ was due to ribosomes.
(b) Ribosomal proteins

Quantitation of ribosomal proteins in solution was from measurements (made as above) of optical density at 230nm. It was assumed that one A$_{230}$ unit is equivalent to approximately 200µg of protein. Quantities of 1M-LiCl split-proteins were estimated by assuming negligible loss of material during their preparation. One 'pmol equivalent' was defined as the amount derived from 1 pmol of ribosomes.

More accurate quantitation of protein was according to Nakao et al. (1973) as modified by Cox et al. (1976). This method involved measurement of the quantitative binding of Naphthol Blue Black (Sigma) to samples of the protein, using bovine serum albumen (Sigma) as a standard. The protein-magnesium-dye complex formed during such assays was recovered by filtration through Sartorius cellulose nitrate discs (0.22µm pore-size), re-dissolved and quantitated by its absorbance at 630nm.

3.6 Reconstitution of Ribosomal Particles

Partial reconstitution of 1M- or 2M-LiCl core-particles with ribosomal proteins was according to Highland et al. (1975b). The exact conditions pertaining to particular experiments will be given in the legends to Tables and Figures. Generally core-particles (25 to 50 pmol in RS buffer) were incubated with 50 to 200 pmol (or pmol equivalents) of ribosomal protein(s) for 5 minutes at 0°C followed by 5 minutes at 20°C, prior to assay. This procedure was also adopted for the treatment of ribosomes with ribosomal proteins (see Chapters 6, 9 and 10).

3.7 Analysis of Proteins by One-Dimensional Polyacrylamide Gel

Electrophoresis

Electrophoretic examination of a protein sample in polyacrylamide gels is a useful means of determining its nature and heterogeneity. The
movement of a protein in such gels on application of an electric field is
governed by the ratio of its charge to its mass and thus depends on its
amino acid sequence. However, in gels containing sodium dodecyl sulphate
(SDS) proteins are denatured and bind this detergent quantitatively.
Since SDS is highly anionic the net charge on each protein molecule is
governed primarily by its molecular weight. Such gels can therefore be
used to determine the molecular weights of proteins by comparison of their
electrophoretic mobilities with those of protein standards (Shapiro et al.,
1967).

Two methods of polyacrylamide gel electrophoresis (in one dimension)
have been used during this study to examine ribosomal proteins:—

(a) 0.1% SDS/polyacrylamide gel electrophoresis

Gels containing 13 or 15% (w/v) polyacrylamide and 0.1% (w/v) SDS
were prepared and run according to Laemmli (1970). Protein samples (10 to
40μl) were prepared for electrophoresis by heating for 2 minutes at 90°C
with an equal volume of buffer containing 75mM-Tris-HCl(pH 6.8), 10mM-
DTT, 3% (w/v) SDS, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue.

(b) 8M-urea/polyacrylamide gel electrophoresis

The method used was that of Traub et al. (1971). Separation gels
contained 10% (w/v) acrylamide, 0.15% (w/v) N,N'-methylenebisacrylamide
(MBA), 8M-urea and electrophoresis was conducted at pH 4.5.

Both types of gel were poured as slabs (Reid & Bieleski, 1968) 1.5mm
thick containing separating gels 10cm long. Up to eleven samples could be
run concurrently by this means and higher resolution obtained than with
standard disc-gels. Protein samples were layered under the running buffer
into wells cast into the stacking gels.

Protein bands were visualised according to Reisner et al. (1975) as
modified by Holbrook & Leaver (1976). Gels containing 0.1% (w/v) SDS were, however, first soaked for 1 hour at 37°C in several changes of buffer containing 10% (w/v) TCA and 15% (v/v) methanol to remove much of the SDS. Protein bands were then stained using 0.4% (w/v) Coomassie Brilliant Blue G250 (R.Lamb, London) in 3.5% (w/v) perchloric acid. Finally, gels were destained using several changes of a solution containing 7.5% (v/v) acetic acid and 15% (v/v) methanol.

3.8 Liquid-Scintillation Spectrometry

Radioactivity was estimated by liquid-scintillation spectrometry using a Packard Tri-carb spectrometer and the following scintillation fluorors:

(a) Tol/PBD

This scintillant was used to determine radioactivity present in dry samples (eg. dried filters). It contained 0.4% (w/v) 2-(4'-tert-butyphenyl)-5-(4''-biphenylyl)-1, 3, 4-oxadiazole (butyl-PBD) in toluene.

(b) Tol/PBD/No.1

Aqueous samples (100 to 200μl) were emulsified in 3ml of scintillation fluid prepared by mixing (in the following order) 30g butyl-PBD, 5.0 l toluene, 2.5 l Emulsifier No.1 (Fisons, U.K.) and 0.75 l water.

Counting efficiencies were determined by liquid-scintillation spectrometry of known molar quantities of radiolabelled compounds and are generally expressed at cpm/μmol. The counting efficiency of [35S]thiostrepton was determined as described in Chapter 6. The following efficiencies of liquid-scintillation spectrometry were observed:
<table>
<thead>
<tr>
<th>Radioisotope</th>
<th>Efficiency</th>
<th>Tol/PBD</th>
<th>Tol/PBD/No.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}\text{C}$</td>
<td>~90%</td>
<td></td>
<td>~80%</td>
</tr>
<tr>
<td>$^{35}\text{S}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{3}\text{H}$</td>
<td>25 to 30%</td>
<td></td>
<td>~15%</td>
</tr>
<tr>
<td>$^{32}\text{P}$</td>
<td>not measured</td>
<td></td>
<td>greater than 95%</td>
</tr>
</tbody>
</table>
CHAPTER 4: THE PURIFICATION AND CHARACTERISATION OF A PROTEIN FROM RIBOSOMES OF BACILLUS MEGATERIUM REQUIRED FOR THE BINDING OF THIOSTREPTON

4.1 Introduction

Many methods have been developed in the last fifteen years for the purification of ribosomal proteins from E. coli. Early methods exploited the different electrophoretic mobilities of ribosomal proteins in starch gels (Waller & Harris, 1961) and polyacrylamide gels (Kaltschmidt et al., 1967; Fogel & Sypherd, 1968a) but allowed only a crude separation. Later methods therefore included additional steps (Waller, 1964; Fogel & Sypherd, 1968b) or relied entirely on new strategies. These generally involved, as the first step, reduction of the large number of proteins to be separated by starting with ribosomal subunits (Moore et al., 1968; Mora et al., 1971; Hindennach et al., 1971a, b; Pearson et al., 1972; Zimmermann & Stöffler, 1976), precipitation of the extracted proteins using ammonium sulphate (Mora et al., 1971) or selective removal of proteins from ribosomes using lithium chloride and urea (Traut et al., 1969; Kaltschmidt et al., 1971). Separation of proteins then involved a single step (Otaka et al., 1968; Traut et al., 1969) or multiple steps (Nomura et al., 1969; Kurland et al., 1971; Held et al., 1973; Zimmermann & Stöffler, 1976) of ion-exchange chromatography, or combinations of ion-exchange chromatography and gel-filtration (Moore et al., 1968; Nomura et al., 1969; Traut et al., 1967, 1969; Hardy et al., 1969; Kaltschmidt et al., 1971; Mora et al., 1971; Pearson et al., 1972; Held et al., 1973). Ion-exchange chromatography was generally performed on phosphocellulose (P-cellulose) or carboxymethylcellulose (CM-cellulose) resins using buffers in the pH range 3.0 to 6.5. Since the isoelectric points of ribosomal proteins are generally high (greater than pH 10 in many cases) most are bound under
these conditions. Purification of some 'acidic' ribosomal proteins (which are not bound) requires the use of anion exchanges and will not be considered here (see e.g. Müller et al., 1972). Proteins bound to CM-cellulose or P-cellulose were eluted sequentially using a gradient of concentration of a suitable counterion (e.g. $\text{Na}^+$). Fractions corresponding to 'peaks' of eluted protein were then pooled, concentrated and re-chromatographed as required.

All the methods described so far involve conditions leading to protein denaturation. Thus ribosomal proteins may be extracted using 66% acetic acid, while chromatography is generally performed in buffers containing pyridine-formate (Hindennach et al., 1971a, b) or urea (most other authors) and lyophilisation is used to concentrate protein samples. However, the preparation of ribosomal proteins is not usually an end in itself; such proteins are often required for use in structural and functional studies and harsh preparative techniques may be considered detrimental. Recently gentler procedures have been described for the preparation of ribosomal proteins (Littlechild & Malcolm, 1978; Dijk & Littlechild, 1979). Groups of proteins can be extracted from ribosomes using solutions containing lithium chloride and each fractionated in salt solution in the absence of urea by chromatography on CM-Sephadex G-25 and then Sephadex G-100. Concentration procedures leading to protein denaturation are also avoided. This method has yielded ribosomal proteins that retain more secondary structure than those prepared by earlier methods (Morrison et al., 1977a) and has enabled the identification of additional proteins (including L11) that can bind specifically to ribosomal RNA (Littlechild et al., 1977). Thus some properties of ribosomal proteins may be significantly affected by their mode of preparation (see also Marquardt et al., 1979).

Even though a purified ribosomal protein may appear electrophoretically homogeneous, it can still contain more than one major component. Thus it
may be difficult to establish the purity of such a preparation (see Kurland et al., 1971 and Mora et al., 1971 for discussions). In this study a ribosomal protein purified from *B. megaterium* appeared essentially homogeneous by four independent methods of polyacrylamide gel electrophoresis. Additionally, immunological data attesting to the high purity of the protein will be presented.

### 4.2 A 'Thiostrepton-Binding Protein' from *B. megaterium* Ribosomes

During a previous study (Dixon, 1976) some of the above techniques were used to prepare a protein from ribosomes of *B. megaterium* KM that is responsible for their ability to bind thiostrepton with high affinity. It was found that 1M-LiCl core-particles prepared from such ribosomes had lost this ability, but that reconstitution with the 1M-LiCl split-proteins restored the ability of the core-particles to bind the drug. The same split-protein fraction also restored the binding of $[^{35}S]$thiostrepton to 2M-LiCl core-particles. Similar results were obtained during the present study (Table 4.1). A component of the 1M-LiCl split-proteins responsible for the restoration of thiostrepton-binding to core-particles was purified to electrophoretic homogeneity from approximately 10 other proteins, using a two-step procedure conducted at pH 5.6 in 6M-urea. This comprised ion-exchange chromatography on CM-cellulose (procedure modified from Traut et al., 1969) followed by gel filtration on Sephadex G-75. The protein thus purified from *B. megaterium* 1M-LiCl split-proteins appeared to have similar properties to protein L11 of the *E. coli* ribosome (Highland et al., 1975b), since it also was able to create or complete a binding site for thiostrepton on ribosomal core-particles. It may thus be termed a 'thiostrepton-binding protein'.

In the present study this 'thiostrepton-binding protein' from ribosomes of *B. megaterium* was required in pure form for several purposes. Initially
Table 4.1

<table>
<thead>
<tr>
<th>Ribosomal particles</th>
<th>Additions</th>
<th>pmol TSH* bound per pmol ribosomal particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M-LiCl core-particles</td>
<td>—</td>
<td>0.11</td>
</tr>
<tr>
<td>1M-LiCl core-particles</td>
<td>1M-LiCl split-proteins</td>
<td>1.17</td>
</tr>
<tr>
<td>2M-LiCl core-particles</td>
<td>—</td>
<td>0.02</td>
</tr>
<tr>
<td>2M-LiCl core-particles</td>
<td>1M-LiCl split-proteins</td>
<td>1.19</td>
</tr>
</tbody>
</table>

* TSH : $[^{35}S]$thiostrepton

Ribosomal core-particles (25 pmol in 75μl RS buffer) were incubated with 1M-LiCl split-proteins (100 pmol equivalents in 10μl SP buffer) or SP buffer alone (10μl) for 5 minutes on ice and then 5 minutes at 20°C. $[^{35}S]$thiostrepton (60 pmol in 5μl 50% (v/v) DMSO solution, 370 cpm/pmol) was added and incubation at 20°C continued for 10 minutes. The binding of $[^{35}S]$thiostrepton to the ribosomal particles was then measured using the 'Norit adsorption' technique (see Chapter 6).
the protein was used to investigate its relationship to protein L11 of *E. coli* and also during the characterisation of thiostrepton-resistant strains of *B. megaterium*. Ultimately it was used in studies of its function in the *B. megaterium* ribosome. Since the protein is therefore central to the present study its preparation and characterisation are now described in full. Some of the analytical techniques used (eg. methods for measuring the binding of \[^{35}S\]thiostrepton to ribosomal particles) will not, however, be described until later.

Two methods were employed for the preparation of the *B. megaterium* thiostrepton-binding protein, both yielding material of similar purity. The first was adapted from that of Dixon (1976) and the second was based on the first except that procedures leading to protein denaturation were avoided. Purification under 'nondenaturing' conditions was considered desirable in view of the work discussed above (Littlechild et al., 1977; Morrison et al., 1977) which was published during the present study. However, protein prepared by both methods in fact behaved similarly in most assays (data to be presented).

4.3 Preparation of a Thiostrepton-Binding Protein from *B. megaterium* Ribosomes Under Denaturing Conditions

1M-LiCl split-proteins from *B. megaterium* wild-type were fractionated at ambient temperature (22°C) in CM buffer (6M-urea, 30mM-methylamine, 1mM-DTT, adjusted to pH 5.6 with acetic acid) by chromatography on CM-cellulose followed by gel filtration using Sephadex G-100. Methylamine was present in CM buffer in order to scavenge cyanate ions (urea breakdown products) and thus prevent carbamylation of proteins, while DTT was included to avoid protein oxidation. The thiostrepton-binding protein was located after fractionation by its ability to promote the binding of thiostrepton to core-particles from wild-type *B. megaterium* ribosomes.
The procedure adopted for the purification may be conveniently divided as follows:

(a) **Preparation of CM-cellulose**

Whatman CM-23 cellulose (30g) was resuspended in water (1 l) and allowed to settle for 20 minutes, when the supernatant was discarded. This de-fining procedure was repeated three times and then twice more using 1M-NaOH, before transferring the slurry to a Buchner funnel containing a Whatman No.1 filter paper. The resin was washed with water until the pH of the eluate was less than 8, then resuspended in 1 l of 0.5M-HCl and allowed to settle as before. After two more such cycles through 0.5M-HCl the resin was returned to the Buchner funnel and washed with water until the pH of the eluate was 4.5. The resin was finally stored at 4°C as an aqueous slurry containing 0.06% (v/v) toluene (as an antimicrobial agent).

(b) **Preparation of a CM-cellulose column**

Approximately 50ml of CM-23 slurry was made up to 150ml with CM buffer containing 100mM-sodium acetate (pH 5.6) and allowed to settle. This procedure was repeated twice and then three times more using CM buffer alone. Material thus treated was used to pour a column (14cm x 1cm) of bed volume 11ml. The resin was supported on a glass-fibre disc (Whatman GF/C) and the column poured with the tap open (flow-rate approximately 20ml/hour). When the desired bed volume had been attained the tap was closed and excess slurry removed. The column was then eluted with CM buffer (20ml/hour) until the pH of the eluate was constant at 5.6.

(c) **Preparation of the 1M-LiCl split-proteins**

Ribosomes were prepared from 400g of *B. megaterium* (wild-type) grown by the MRE, Porton (see Chapter 2). The 1M-LiCl split-proteins from 900mg
of these ribosomes were then prepared as described above except that the ribosome concentration was 3.0mg/ml. The split-proteins (300ml) were dialysed against several changes of 2% (v/v) acetic acid at 4°C and then concentrated by dialysis against 2% (v/v) acetic acid containing 20% (w/v) PEG 20,000. The concentrated proteins were finally dialysed exhaustively against CM buffer at 4°C.

(d) Ion-exchange chromatography of 1M-LiCl split-proteins on CM-23 cellulose

The 1M-LiCl split-proteins (in 20ml CM buffer) were allowed to warm up to ambient temperature (22°C) and then loaded on to the CM-23 column at 20ml/hour. The amount of protein loaded (100mg) was estimated as above (section 3.5) from measurement of its optical density at 230nm and was considerably less than the theoretical capacity of the column (700mg protein). The proteins were washed onto the column with 10ml CM buffer and then the column developed (flow rate 20ml/hour) with 100ml CM buffer containing zero to 100mM-sodium acetate as a linear concentration gradient. The conductivity of the column eluate was monitored continuously using a CIM 2f conductivity meter (Radiometer, Copenhagen) and fractions of 1ml collected. Samples of each were analysed by SDS/polyacrylamide gel electrophoresis (section 3.7) and also for their ability to restore to 2M-LiCl core-particles the ability to bind $[^{35}S]$thiostrepton (Figure 4.1). Conductivity measurements were converted into sodium acetate concentrations by reference to standard solutions.

Figure 4.1 shows that fractions 32 to 45 (i.e. eluted between 25mM and 38mM-sodium acetate) contained most of the material capable of restoring the binding of $[^{35}S]$thiostrepton to the core-particles. These results are therefore in agreement with those of Dixon (1976) who eluted the thiostrepton-binding protein under similar conditions at between 35mM
Legend to Figure 4.1

The Chromatography of 1M-LiCl Split-Proteins on Carboxymethyl Cellulose

1M-LiCl split-proteins prepared from ribosomes of *B. megaterium* (wild-type) were chromatographed on CM23 cellulose (see text). Fractions (1ml) were collected and analysed as follows:

(a) **Conductivity:** The conductivity of the column eluate was measured continuously and the Na\(^{+}\)-concentration in each fraction estimated by reference to standard solutions of sodium acetate in the buffer used (□).

(b) **Ability to restore binding of thiostrepton to core-particles:** Assay mixtures contained (in 100µl of RS buffer) 45 pmol (●) or 450 pmol (○) 2M-LiCl core-particles (from *B. megaterium* wild-type) and 15µl column fraction. After 5 minutes at 0°C followed by 5 minutes at 20°C, 360 pmol (●) or 1.0 nmol (○) \(^{35}\text{S}\)thiostrepton (in 5µl of 50% (v/v) DMSO solution) was added and incubation continued at 20°C for 10 minutes. The amount of \(^{35}\text{S}\)thiostrepton bound to the core-particles was determined by gel filtration (Chapter 6). The presence of 0.9M-urea (from the column fraction) did not disrupt the assays.

(c) **Protein Content:** Samples (25µl) of fractions were analysed by 0.1% SDS/polyacrylamide gel electrophoresis (see section 3.7). Separation gels contained 13% (w/v) acrylamide, 0.35% (w/v) MBA and 0.1% (w/v)SDS. Creatine kinase (mol. wt. 40,000), chymotrypsin (mol. wt. 24,500) and lysozyme (mol. wt. 14,500) were used as molecular weight markers. A sample of the unfractonated split-proteins (S) was also analysed.

Fractions were pooled prior to the second stage of purification as denoted by the numerals I and II (see text).
and 43 mM-sodium acetate. Dixon found the protein to have a molecular weight of approximately 13,000 as judged by SDS/polyacrylamide gel electrophoresis. Examination of the gel profiles of fractions 32 to 45 (Figure 4.1) showed that 'activity' correlated with the appearance and disappearance of a protein of this size (mol. wt. 14,000 to 16,000).

It was therefore apparent that this protein was the thio strepton-binding protein and it was accordingly purified further from the contaminating proteins present in the same fractions. Fractions 33 to 42 were pooled and then desalted over a column of Sephadex G-25 (25 cm x 1.0 cm). The resin was swollen in water for 3 hours at 90°C prior to pouring the column, which was equilibrated with 0.1% (v/v) acetic acid (100 ml) before use. The pooled fractions were loaded and eluted with 0.1% (v/v) acetic acid at ambient temperature; meanwhile the A230 of the column eluate was monitored continuously using a CE272 spectrophotometer (CECIL Instruments). Eluate containing protein was lyophilised and re-dissolved in CM buffer (1.0 ml) prior to the final purification step.

(e) Gel filtration on Sephadex G-100

Following ion-exchange chromatography and desalting as above, the concentrated proteins were further fractionated over Sephadex G-100. This means of separation was chosen in view of the relatively large difference in molecular weight between the presumed thio strepton-binding protein (14,000 to 16,000) and the other proteins present (approximately 23,000 and 28,000; see Figure 4.1).

Sephadex G-100 (40 to 120 µm bead-size) was swollen in CM buffer for 4 hours at 98°C and poured (when cool) into a column 100 cm x 1.4 cm (bed volume 155 ml) with the tap open. After reaching the desired bed volume, excess slurry was removed and the column washed with 200 ml CM buffer. Fractionation of marker proteins was then performed to determine the
column parameters \((V_o', V_t')\) and to test its resolution (data not shown). The column was finally washed with 100ml CM buffer.

The pooled, desalted and concentrated fractions from the ion-exchange chromatography (in 1.0ml CM buffer) were loaded onto the drained surface of the column and eluted with CM buffer at 12ml/hour. Fractions (1.0ml) were collected and samples analysed by electrophoresis as above (data not given). Fractions containing only material of molecular weight 14,000 to 16,000 were pooled, dialysed exhaustively at 2°C against 0.1% (v/v) acetic acid, lyophilised and re-dissolved in RS buffer (700μl) before storage at -70°C. A yield of 1.75mg of protein was shown using the method of Nakao et al. (1973) as described above (section 3.5). When the ability of the purified protein to restore thioestrepton-binding to core-particles was tested it was found to restore drug-binding quantitatively (Table 4.2, compare lines a and b).

(f) The 'minor binding protein'

When the 1M-LiCl split-proteins were chromatographed on CM-cellulose, material capable of restoring the binding of \([^{35}S]\)thioestrepton to core-particles was mainly recovered in those fractions (denoted by 'I' in Figure 4.1) subjected to further separation as described. However, some such material was present as a small peak (denoted by 'II' in Figure 4.1) which eluted after the bulk of the material. The 'activity' of these fractions also appeared to be associated with the reappearance of material in the 14,000 to 16,000 molecular weight region of the gel. Since in both \(B.\) megaterium (Dixon, 1976) and \(E.\) coli (Highland et al., 1975b) only a single thioestrepton-binding protein was isolated from the ribosomes, it was decided to purify and characterise the active component from these later fractions. Thus fractions 48 to 54 from the ion-exchange chromatography were pooled, desalted and chromatographed over Sephadex G-100 as described above. Fractions were analysed as before and those containing material in
Table 4.2
Stimulation of the binding of $[^{35}S]$thiostrepton by 2M-LiCl core-particles

<table>
<thead>
<tr>
<th>Additions</th>
<th>pmol THS$^*$ bound per pmol ribosomal particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. None</td>
<td>0.08</td>
</tr>
<tr>
<td>b. 120 pmol 'thiostrepton binding protein' (major)</td>
<td>1.08</td>
</tr>
<tr>
<td>c. 120 pmol 'minor binding protein'</td>
<td>0.42</td>
</tr>
</tbody>
</table>

* THS : $[^{35}S]$thiostrepton

Wild-type *B. megaterium* 2M-LiCl core-particles (50 pmol) in 42.5μl RS buffer were mixed with 2.5μl of RS buffer containing 120 pmol 'thiostrepton binding protein', 120 pmol 'minor binding protein'. (in 2.5μl 0.1% (v/v) acetic acid; see text) or with RS buffer alone (2.5μl). After incubation for 5 minutes at 0°C followed by 5 minutes at 20°C, $[^{35}S]$thiostrepton (65 pmol in 5μl 50% (v/v) DMSO solution, 230 cpm/pmol) was added and incubation continued for 10 minutes at 20°C. The amount of $[^{35}S]$thiostrepton bound by the core-particles was determined by gel filtration (see Chapter 6).
the molecular weight range 14,000 to 16,000 were pooled, dialysed against 0.1% (v/v) acetic acid and lyophilised. The lyophilised protein was, however, insoluble in RS buffer and also in buffers used by others for dissolving ribosomal proteins (see eg. Fahnestock et al., 1974; Moore et al., 1975; Cohberg & Nomura, 1976). It was finally dissolved in 0.1% (v/v) acetic acid (700μl) and stored at -70°C. A yield of 400μg was determined as above. The purified protein (henceforth referred to as the 'minor binding protein') was able to restore the binding of [35S]thiostrepton to core-particles, although under the particular assay conditions employed it appeared less active than the 'major' binding protein on a mole for mole basis (Table 4.2).

(g) Analysis of the purified proteins

The purity of the protein preparations was initially examined using two methods of polyacrylamide gel electrophoresis, in the presence of 0.1% (w/v) SDS or 8M-urea. Both methods revealed the major thiostrepton-binding protein and the minor binding protein to be greater than 95% pure, as judged from the relative staining intensity of protein bands on such gels (Figure 4.2). In the case of the major protein, this conclusion was also supported by the results of two-dimensional polyacrylamide gel electrophoresis (Chapter 7). The nature of the traces of contaminating protein in the two preparations is not known. However, a faint band visible in the 'minor binding protein' when analysed using 8M-urea/polyacrylamide gels (Figure 4.2(a) co-migrated with the major thiostrepton-binding protein.

The molecular weights of the two proteins were determined from their migration in 0.1% SDS gels (Figure 4.2(b). Since such determinations from single gels are only accurate to about plus or minus 10% (Traut et al., 1969), several gels were examined in each case. This revealed molecular weights of approximately 15,500 (major thiostrepton-binding protein) and
Electrophoretic analysis of *B. megaterium* 'thiostrepton binding proteins'

The major thiostrepton binding protein (1) and (see text) the 'minor binding protein' (2) were examined by two methods of polyacrylamide gel electrophoresis. Both gels received 5.0μg of each protein.

(a) Analysis by the urea/polyacrylamide method of Traub et al. (1971).

The separation gel contained 10% (w/v) acrylamide, 0.15% (w/v) MBA, 8M-urea. Electrophoresis was at pH 4.5.

(b) Analysis by 0.1% SDS/polyacrylamide gel electrophoresis (Laemmli, 1970). Separation gels contained 15% (w/v) acrylamide, 0.4% (w/v) MBA and 0.1% (w/v) SDS. The following proteins were used as molecular weight markers (10μg each): chymotrypsin (24,500), myoglobin (17,000), lysozyme (14,500), RNase A (13,700).
approximately 14,800 (minor binding protein).

4.4 Preparation of a Thiostrepton-Binding Protein from \textit{B. megaterium} \\
Ribosomes Under Nondenaturing Conditions \\
(a) Preparation of the 1M-LiCl split-proteins \\
Ribosomes were prepared from freshly-grown cells (100g) of \textit{B. megaterium} \\
(wild-type) and 1M-LiCl split-proteins were obtained from 1.0g of the 
ribosomes as already described (section 4.3). The split-proteins were then 
dialysed exhaustively at 2°C against 3 x 15 volumes of 10mM-sodium acetate 
(pH 5.6 with acetic acid). A fine protein precipitate that resulted from 
this treatment was removed by centrifugation in the Beckman Ti70 rotor for 
20 minutes at 10,000 rpm and 2°C. The pH of the split-proteins (5.6) was 
then checked.

(b) Chromatography on CM-cellulose \\
The split-proteins were fractionated at 4°C using CM-cellulose. All 
solutions of sodium acetate were adjusted to pH 5.6 (at 4°C) using acetic 
acid.

CM-cellulose (Whatman CM-23) was pre-cycled as described above 
(section 4.3(a) and then 50ml of slurry were resuspended in 200mM-sodium 
acetate (200ml) and allowed to settle. This procedure was repeated twice 
and finally 3 more times using 10mM-sodium acetate. A column (14cm x 1.0cm) 
was then poured as described above (section 4.3(b) and washed with 200ml 
10mM-sodium acetate.

The split-proteins (in 330ml of 10mM-sodium acetate) were loaded onto 
the column and washed on with 20ml 10mM-sodium acetate. The amount of 
protein loaded (approximately 45mg) was determined as above (section 4.3(c)). 
The column was developed with 200ml of a 10mM to 200mM linear concentration 
gradient of sodium acetate, collecting 2.5ml fractions. Samples of each
Figure 4.3

[Graph showing binding levels across fraction numbers.]
Carboxymethyl cellulose chromatography of 1M-LiCl split-proteins in sodium acetate solution (pH 5.6)

1M-LiCl split-proteins were prepared from ribosomes of *E. coli* (wild-type) and chromatographed on CM23 cellulose as described in the text. Fractions (2.5mL) were collected and analysed as follows:

(a) Activity in restoring the binding of thioestrepton to core-particles:
Assay mixtures containing 40µL column fraction together with 30 pmol of 1M-LiCl core-particles (wild-type) in 45µL RS buffer were incubated for 5 minutes at 0°C followed by 5 minutes at 20°C. After addition of 5µL [³⁵S]thioestrepton (40 pmol, in 50% (v/v) DMSO solution) incubation was continued for 10 minutes at 20°C. The amount of drug bound by the core-particles was then determined using the 'Norit adsorption' technique (see Chapter 6). Samples of particularly active fractions were suitably diluted with 10mM-sodium acetate solution prior to assay.

(b) Protein content: Samples (25µL) of each fraction were analysed by electrophoresis in 0.1% SDS/polyacrylamide gels as described in the legend to Figure 4.1.
were analysed for their ability to restore the binding of thiostrepton to core-particles and also by SDS/polyacrylamide gel electrophoresis (Figure 4.3). Almost all the 'activity' was contained within fractions 38 to 78, which contained only a single protein as judged by the electrophoretic analysis. There was no evidence for the presence of a 'minor binding protein'. Accordingly, these fractions were pooled, dialysed against 3 x 100 volumes of RS buffer (at 0°C) and then concentrated by dialysis against similar buffer containing 20% (w/v) PEG 20,000. When the volume was approximately 4ml, the protein solution was dialysed finally against 200 volumes of RS buffer (at 0°C) and then rapidly frozen before storage at -70°C. The concentration of the protein was measured as before and showed a yield of 6.0mg.

(c) Analysis of the purified protein

The thiostrepton-binding protein purified in this manner was compared with the major protein isolated as described above (section 4.3) by two methods of electrophoresis (Figure 4.4). Both preparations showed the same electrophoretic mobility using either technique, confirming their identity. The protein prepared under non-denaturing conditions was also judged to be at least 95% pure (from the staining intensities of the protein bands) but it was apparently more heterogeneous than that prepared in 6M-urea (Figure 4.4). This is not surprising since the 'non-denatured' protein was not subjected to gel filtration.

The two preparations of the protein were further compared in their ability to restore the binding of [35S]thiostrepton to core-particles. There was very little difference between the two preparations in this respect; either could stimulate the core-particles to bind the drug quantitatively when reconstituted in the presence of 3 to 4 pmol of protein per pmol of core-particles (Figure 4.5).
Legend to Figure 4.4

Electrophoretic analyses of purified *B. megaterium* thio strepton-binding protein

'Thio strepton-binding protein' purified from ribosomes of *B. megaterium* under (1) nondenaturing or (2) denaturing conditions (see text) was analysed by two methods of polyacrylamide gel electrophoresis (see legend to Figure 4.2). Each gel received 15µg of 'nondenatured' protein and 3µg of 'denatured' protein.

(a) 0.1% SDS/polyacrylamide gel electrophoresis.

(b) 8M-urea/polyacrylamide gel electrophoresis at pH 4.5.
Figure 4.5

Net binding of $[^{35}S]$thiostrepton (pmol/pmol cores) vs. pmoles thiostrepton-binding protein.
Stimulation of the binding of thiostrepton to core-particles by

*B. megaterium* 'thiostrepton-binding protein'

Assay mixtures containing 25 pmol 2M-LiCl core-particles (from *B. megaterium* wild-type) and zero to 300 pmol 'thiostrepton-binding protein' in RS buffer (total volume 85µl) were incubated for 5 minutes at 0°C and then 5 minutes at 20°C. [³⁵S]thiostrepton (50 pmol in 5µl 50% (v/v) DMSO solution) was added and incubation continued at 20°C for 10 minutes. The amount of drug bound by the core-particles was then measured using the 'Norit adsorption' technique (see Chapter 6). Results are expressed as pmol drug bound per pmol core-particles, from which this value for core-particles in the absence of added protein (0.05) has been subtracted.

O — O thiostrepton-binding protein prepared under non-denaturing conditions (section 4.4).

● — ● thiostrepton-binding protein prepared in 6M-urea (section 4.3).
4.5 The Immunological Identification of the Thiostrepton-Binding Protein

Since it had been shown that a single ribosomal protein (L11) was necessary for the binding of thiostrepton by ribosomes of *E. coli* (Highland et al., 1975b), the relationship between this protein and the (major) thiostrepton-binding protein purified from *B. megaterium* was investigated. This was achieved by the use of immunological techniques. All the experiments in this section were carried out by Dr. Georg Stöffler and co-workers at the Max Planck Institut für Molekulare Genetik, Berlin-Dahlem, Germany, to whom I am greatly indebted.

(a) Immunochemical methods

Antisera to *E. coli* protein L11 were prepared and characterised as already described (Stöffler & Wittmann, 1971a, b; Stöffler, 1974). Antiserum raised against the thiostrepton-binding protein of *B. megaterium* was prepared as described elsewhere (Cundliffe et al., 1979).

Double-immunodiffusion was performed according to Ouchterlony (1968) with modifications as necessary for the analysis of ribosomal proteins (Stöffler & Wittmann, 1971a, b; Stöffler, 1974), while modified immuno-electrophoresis was performed on cellulose acetate strips as previously reported (Zubke et al., 1977).

(b) Identification of the thiostrepton-binding protein

The antiserum raised against the *B. megaterium* thiostrepton-binding protein was first examined by double-immunodiffusion with *E. coli* protein L11 and total ribosomal protein prepared from both ribosomes and subunits of this organism. Complete cross-reaction occurred between protein L11, TP70 and TP50, while no precipitin band was formed with *E. coli* TP30 (Figure 4.6(a)). These preliminary results suggested that the thiostrepton-binding protein of *B. megaterium* was serologically related to *E. coli* ribosomal protein L11. The thiostrepton-binding protein from *B. megaterium* was
Figure 4.6
Legend to Figure 4.6

Identification of the 'thiostrepton-binding protein' from *B. megaterium* (protein BM-L11) by double immunodiffusion

(a) Reaction of anti-BM-L11 with ribosomal protein from *E. coli*

The centre well contained 260μl anti-BM-L11 γ-globulin. The peripheral wells contained (1) 400μg *E. coli* TP70; (2) 280μg *E. coli* TP50; (3) 5μg *E. coli* L11; (4) Blank; (5) 120μg *E. coli* TP30; (6) 200μg *E. coli* TP30; (7), (8) Blank.

(b) Reaction of protein BM-L11 with antisera raised against purified ribosomal proteins from the 50S subunit of *E. coli*

The centre well contained 1μg protein BM-L11. The peripheral wells contained (1) anti-*E. coli* L7, γ-globulin (90μl serum-equivalents);

(2) anti-*E. coli* L8, γ-globulin (300μl serum-equivalents);

(3) anti-*E. coli* L9, γ-globulin (300μl serum-equivalents);

(4) anti-*E. coli* L10 (60μl serum);

(5) anti-*E. coli* L11 (0.2 A<sub>280</sub> units specific antibody purified over an *E. coli* L11 affinity column);

(6) anti-*E. coli* L12, γ-globulin (90μl serum equivalents).

The negative results for the antisera raised against the remaining 28 proteins from the *E. coli* 50S ribosomal subunit are not presented.

(c) Reaction of anti-BM-L11 with single ribosomal proteins from the 50S subunit of *E. coli*

The centre well contained anti-BM-L11, γ-globulin (1750 serum-equivalents). The peripheral wells contained (1) 2μg *E. coli* L10;

(2) 2μg *E. coli* L11; (3) 2μg *E. coli* L12; (4) 3μg *E. coli* L13; (5) 2μg *E. coli* L14; (6) 3μg *E. coli* L15; (7) 2μg *E. coli* L16; (8) 2μg *E. coli* L18.

/contd.
Legend to Figure 4.6 (contd.)

With the exception of L8, L17 and L31 the remaining proteins of the E.coli 50S ribosomal subunit were similarly tested (data not given).

(d) **Incomplete cross-reaction between E.coli protein L11 and B.megaterium protein BM-L11**

The centre well contained anti-E.coli L11 (0.9 $A_{280}$ units specific antibody purified as in (b)). The peripheral wells contained (1) 3µg BM-L11; (2) 10µg E.coli L11; (3) 3µg BM-L11.

(e) **Incomplete cross-reaction between E.coli protein L11 and B.megaterium protein BM-L11**

The centre well contained anti-BM-L11, γ-globulin (1750µl serum-equivalents). The peripheral wells contained (1) 2µg E.coli L11; (2) 8µg BM-L11; (3) 2µg E.coli L11.

Double immunodiffusion assays were performed in Petri dishes containing 1.5% (w/v) agarose in 19mM-sodium barbital buffer (pH 8.6), 0.75M-LiCl and 0.2% (w/v) Cialit (ASID Institut, Munich). Immunodiffusion occurred at 4°C for approximately 48 hours. Photographs were taken without staining.
therefore examined by double-immunodiffusion with monospecific antisera raised against each of the 34 proteins of the E.coli 50S ribosomal subunit. Only with an antiserum to E.coli protein L11 was a precipitin band formed; none of the other antisera gave any precipitation (Figure 4.6(b); most negative results omitted). When the converse experiment was performed using antiserum raised against the purified thiostrepton-binding protein from B.megaterium, out of all the proteins of the E.coli 50S ribosomal subunit only L11 was precipitated (Figure 4.6(c); most negative results not shown). These results demonstrated unequivocally that E.coli protein L11 and the thiostrepton-binding protein from B.megaterium ribosomes are homologous. The latter protein was therefore designated protein BM-L11.

However, further comparison of the two proteins by double-immunodiffusion revealed that they were not identical. Thus while anti-E.coli L11 formed precipitin bands with both E.coli protein L11 and protein BM-L11 of B.megaterium (Figure 4.6(d), cross-reaction with the latter protein was weaker. The formation of spurs between the precipitin bands indicated that although the two proteins are serologically related they are not identical. A similar result was obtained from the converse experiment using anti-BM-L11 (Figure 4.6(e). This conclusion was strengthened by data obtained using the particularly sensitive technique of modified immunoelectrophoresis (Figure 4.7). In these experiments the antiserum raised against protein BM-L11 formed single strong precipitin bands with TP70 from both B.megaterium (wild-type) and E.coli ribosomes. While in the latter case the precipitin band had the electrophoretic mobility expected for protein L11, protein L11 and protein BM-L11 clearly migrated differently during electrophoresis. The converse experiment (using anti-E.coli L11) yielded qualitatively identical results (data not shown).

The relatedness of the two proteins was therefore established using antisera raised against both protein L11 and protein BM-L11. The former
Figure 4.7
Legend to Figure 4.7

Identification of protein BM-L11 by modified immunoelectrophoresis

50µg TP30 from E. coli (1), 50µg TP50 from E. coli (2), 25µg TP70 from B. megaterium (3) and 50µg TP70 from E. coli (4) were applied to a cellulose acetate strip. Electrophoresis was for 80 minutes at 350V (3.8mA) in buffer containing 0.1M-Tris, 0.1M-Bicine, 0.05M-β-mercaptoethanol and 8M-urea (pH 8.8). Strip (a) was stained directly but strip (b) was soaked in antiserum raised against protein BM-L11 prior to staining.
antiserum was monospecific and reacted exclusively with purified *E. coli*
protein L11 (Stöfler & Wittmann, 1971a, b; Stöfler, 1974). Although
the monospecificity of the antiserum raised against protein EM-L11 could not
be so thoroughly demonstrated in the absence of individual purified
proteins from the *B. megaterium* ribosome, since all the results obtained
using both antisera were in total agreement it was deduced that anti-
EM-L11 was also monospecific. The 'satellite' bands observed during
modified immunoelectrophoresis (Figure 4.7) do not change this conclusion.
They were formed specifically with antisera raised against protein L11 or
protein EM-L11 which both reacted with a single ribosomal protein (viz.
L11 or EM-L11), indicating that the satellites were serologically related
to proteins L11 and EM-L11. The satellite bands probably represent modified
species of the two proteins. Since both proteins L11 and EM-L11 are heavily
methylated (Alix & Hayes, 1974; Chang & Chang, 1975; Dognin & Wittmann-
Liebold, 1977; Cannon & Cundliffe, 1979) it is possible that the satellites
are differently-methylated variants of the proteins. Such a difference
would not be expected to abolish completely the antigenicity of the proteins
and in fact a non-methylated form of protein L11 (Colson & Smith, 1977) is
precipitated by anti-*E. coli* L11 (G. Stöfler, unpublished data).

The immunological evidence presented in this section also attests to
the high purity of the protein EM-L11 preparations used during the present
study. Thus antisera raised against such preparations were monospecific,
a conclusion reached from examination of the antisera by both double
immunodiffusion and the more sensitive method of modified immunoelectro-
phoresis.

(c) **Attempted identification of the 'minor binding protein'**

Although the minor binding protein isolated above (section 4.3)
showed somewhat different solubility properties compared with protein
EM-L11 (section 4.3(f), the former was considered most likely to be a variant form of the latter. While such a variant could represent a differently-methylated form of protein EM-L11 (see above) or have resulted from carbamylation during purification, neither hypothesis would be compatible with the more basic nature of the minor binding protein (Figure 4.2(a). However, the smaller size of the minor binding protein (Figure 4.2(b) may indicate that it was derived from protein EM-L11 by proteolysis.

When examined by modified immunoelectrophoresis, the minor binding protein formed a single band, which was of the same intensity whether or not the cellulose acetate strip was soaked in anti-EM-L11 prior to staining (data not shown). It was therefore concluded that no cross-reaction had occurred but that the antiserum had failed to leach out the minor binding protein (in the absence of cross-reaction) due to the solubility properties of the latter. However, since firm conclusions cannot be drawn from negative immunochemical data (see eg. Wittmann et al., 1975), an antiserum was raised against the minor binding protein in order to attempt further characterisation. Preliminary evidence (G. Stöffler, personal communication) suggests that this antiserum is not monospecific and that one of the proteins it precipitates from B. megaterium TP70 has the same electrophoretic mobility as protein EM-L11. This result is clearly compatible with the presence of protein EM-L11 in the preparation of the minor binding protein (see also Figure 4.2(a). Thus characterisation of the minor binding protein will require a purer preparation and an antiserum raised against it that is demonstrably monospecific.

4.6 The Preparation of Protein L11 from E.coli

Ribosomal protein L11 from E.coli was prepared for purposes of comparison with B.megaterium protein EM-L11, using two methods—
(a) **Preparation under denaturing conditions**

Protein L11 prepared in this manner was the generous gift of Dr. G. Stöffler and was isolated in phosphate buffer (pH 6.5) containing 6M-urea, by a procedure described fully elsewhere (Wienen et al., 1979).

(b) **Preparation under nondenaturing conditions**

Protein L11 was prepared at 4°C in buffers containing sodium acetate (all adjusted with acetic acid to pH 5.6). The method used differed from that described in section 4.4 only in the following respects.

Ribosomes (300mg) obtained from frozen cells of strain MRE 600 (grown at the MRE, Porton) were extracted according to Dijk & Littlechild (1979). They were resuspended at 3mg/ml in buffer containing 10mM-HEPES-NaOH (pH 7.0), 10mM-MgCl₂, 1.0M-LiCl. After 5 hours at 0°C the core-particles thus formed were removed as already described (section 3.4(a). The split-proteins (100ml) were diluted with an equal volume of 5mM-HEPES-NaOH(pH 7.0) and dialysed against 3 x 1 l of 10mM-sodium acetate. They were then chromatographed on CM-cellulose as described above (data not shown). It was found that the ability to restore the binding of [³⁵S]thiostrepton to core-particles was not associated with fractions containing a single protein. 'Active' fractions were therefore pooled, concentrated to 1.5ml by dialysis against 100mM-sodium acetate containing 20% (w/v) PEG 20,000 and rechromatographed over Sephadex G-100. This was performed as described above (section 4.3(e) but replacing CM-buffer with 100mM-sodium acetate (pH 5.6). 'Activity' was recovered in fractions judged to contain a single protein; these fractions were pooled, concentrated as above (this section) and finally dialysed at 2°C against 2 x 500 volumes RS buffer. Storage was at -70°C and the protein concentration estimated as before (section 4.3(e).

Electrophoretic comparison of this preparation of protein L11 with protein EM-L11 (isolated under similar conditions) showed that both proteins
Comparison of proteins L11 \textit{(E. coli)} and BM-L11 \textit{(B. megaterium)} by polyacrylamide gel electrophoresis

\textit{B. megaterium} protein BM-L11 (1) and \textit{E. coli} protein L11 (2) were examined by polyacrylamide gel electrophoresis in the presence of 8M-urea (a) or 0.1\% (w/v) SDS (b) as described in the legend to Figure 4.2. Each gel received 5\mu g protein BM-L11 and 20\mu g protein L11, both of which were prepared by non-denaturing procedures (see text). Molecular weight markers were chymotrypsin (24,500); lysozyme (14,500); RNase A (13,700).
behaved similarly in the two gel systems used (Figure 4.8). However, protein L11 was observed to have a lower molecular weight than protein HM-L11 (as judged from SDS/polyacrylamide gels; Figure 4.8(b)). This is consistent with the above conclusion that the two proteins are structurally related but not identical.

Figure 4.8(a) revealed that the preparation of L11 contained small but significant quantities of a second protein, the nature of which is not known.
CHAPTER 5: SYNTHESIS OF POLYPHENYLALANINE BY UNFRACTIONATED AND
FRACTIONATED CELL EXTRACTS FROM BACILLUS MEGATERIUM KM

5.1 Introduction

Since much early research into protein synthesis centred on
eukaryotic tissues, it was not until about 1960 that cell-free protein
synthesis was described in systems derived from cells of E.coli
(Lamborg and Zamecnik, 1960; Tissières et al., 1960; Matthaei
& Nirenberg, 1961). These systems were all dependent upon endogenous
messenger RNA but it was soon found that they would translate the synthetic
polyribonucleotide polyuridylic acid (poly U) under appropriate conditions,
synthesising polyphenylalanine (Nirenberg & Matthaei, 1961). This
provided a model system in which polypeptide chain elongation occurs, but
since poly U contains no known initiation or termination codons, neither
initiation nor termination occur by the normal means (Chapter 1). In order
to promote initiation in the absence of the correct codons higher
concentrations of magnesium ions are used than in systems where natural
mRNA is translated, while release of nascent polyphenylalanine does not
occur (Gilbert, 1963).

During the present investigation a poly U-dependent polypeptide-
synthesising system was used to localise within cells of the B.megaterium
mutants described in Chapter 2 the mechanism of resistance to thioestrepton.
Techniques have now been developed for observing poly U-dependent protein
synthesis in cell-free extracts from a variety of microorganisms other
than E.coli, including Bacillus subtilis (eg. Takeda & Lipmann, 1966)
and B.megaterium (Deutscher et al., 1968). However, the procedure
employed in the present study was based on one developed for the examination
of protein synthesis in cell-free extracts of wheat-germ (Davies &
Kaesberg, 1973), which yielded highly active systems for polyphenylalanine
synthesis when used with extracts from \textit{B. megaterium} KM.

5.2 Methods

The synthesis of polyphenylalanine was measured by studying the incorporation of \(^{14}\text{C}\)phenylalanine into hot trichloroacetic acid-precipitable material. S-30 fraction (in 50\(\mu\)l TMA\(\beta\) buffer), crude ribosomes (110 'nominal' pmol in 5 to 10\(\mu\)l RS buffer) plus S-100 fraction (in TMA\(\beta\) buffer, to give 50\(\mu\)l final volume), or high-salt-washed ribosomes (50 pmol in 20\(\mu\)l RS buffer) plus S-100* fraction (0.4 to 0.7 A\(_{260}\) units in 30\(\mu\)l RS buffer) were mixed with 50\(\mu\)l assay cocktail containing: 40mM-HEPES-KOH (pH 7.5), 100mM-potassium acetate, 17mM-magnesium acetate, 19 amino acids (excluding phenylalanine) each at 0.075mM, 5mM-ATP, 0.75mM-GTP, 10mM-PEP, 10 units pyruvate kinase, 50\(\mu\)g poly U, 100\(\mu\)g \textit{E. coli} unfractionated tRNA, L-[U-\(^{14}\text{C}\)]phenylalanine (0.5\(\mu\)Ci, 513mCi/mmole unless stated otherwise). The final ionic conditions were therefore as follows: 20mM-HEPES-KOH (pH 7.5), 5mM-Tris-HCl (pH 7.6), 13.5mM-Mg\(_{2+}\), 25mM-NH\(_4\)\(^+\), 50mM-KCl. Incubation was at 37\(^\circ\)C. Samples each of 5 or 10\(\mu\)l were taken into 10\% (w/v) trichloroacetic acid (TCA) and heated at 90\(^\circ\)C for 30 minutes. Precipitates were collected on glass-fibre discs (Whatman GF/A), washed three times with 4ml 5\% (w/v) TCA, dried and their radioactivity estimated by liquid-scintillation spectrometry. When incorporation of \(^{14}\text{C}\)phenylalanine into TCA precipitates was rapid, the standard Amersham product was suitably diluted with non-radioactive phenylalanine such that in subsequent experiments incorporation never exceeded 50\% of the total input of radioactivity.

The effect of thioestrepton in this system was tested by the addition of 0.5 to 2.0 \(\mu\)g thioestrepton (in 2 to 5\(\mu\)l 25\% (v/v) IMSO solution) to the S-30 fraction or to ribosomes (crude or high-salt-washed) for 5 minutes at 20\(^\circ\)C, prior to addition of the other assay components. IMSO proved to be
slightly inhibitory and was therefore included in all controls.

Precipitation by TCA allows the recovery only of oligophenylalanine containing about four or more residues; \((\text{phe})_3\) and \((\text{phe})_2\) remain essentially unprecipitated while phenylalanine itself is TCA-soluble (Pestka et al., 1969). Thus even when the efficiency of incorporation of \(^{14}\text{C}\text{phenylalanine}\) into acid-precipitable material is low, it still reflects the synthesis of oligopeptides containing at least four residues. Conversely, when few moles of \(^{14}\text{C}\text{phenylalanine}\) are incorporated per mol ribosomes it may be inferred that not all the ribosomes in the incubation mixture are active in polyphenylalanine synthesis.

5.3 Synthesis of Polyphenylalanine by S-30 Fractions from Strains of

\underline{\text{B. megaterium}}

S-30 fractions prepared from both the wild-type and the thiostrepton-resistant strains of \underline{\text{B. megaterium}} were found to be quite active in the synthesis of polyphenylalanine (Figure 5.1). Synthesis was entirely dependent upon the addition of poly U (data not presented), indicating only a low level of 'endogenous' synthetic activity in the extracts. For purposes of comparison the amount of polyphenylalanine synthesised per 'nominal' pmol ribosomes (see section 3.5(a) over a given time was determined. Such comparison revealed considerable variation in the activity of extracts from the same or different strains although synthesis typically fell between 2 and 10 pmol \(^{14}\text{C}\text{phenylalanine}\) incorporated per nominal pmol ribosomes after 40 minutes (at 37°C). Synthesis of polyphenylalanine was generally most efficient around a nominal ribosome concentration (NRC) of 1μM, suggesting that components in the assay cocktail were limiting to synthesis in more concentrated S-30 fractions (data not given).

When the effect of thiostrepton upon the synthesis of polyphenylalanine
Synthesis of polyphenylalanine by S-30 fractions

The ability of S-30 fractions prepared from B. megaterium wild-type (w.t.) and thioestrepton-resistant strains MJ1 and PD1 to synthesise polyphenylalanine was assayed as described in section 3.2, in the presence (●) or absence (○) of 12μM-thioestrepton. The 'nominal' ribosome concentrations (see section 3.5(a) in the reaction mixtures were 1.0μM (wild-type), 0.9μM (mutant MJ1) and 3.3μM (mutant PD1). L-[U-14C]-phenylalanine was used at a specific radioactivity of 486mCi/mmol (960 cpm/pmol) in mixtures containing wild-type S-30 fraction and 173 mCi/mmol (340 cpm/pmol) in mixtures containing S-30 fractions from the two mutant strains. The values plotted represent synthesis of polyphenylalanine in samples of 5μl, from which the background (80 cpm) has been subtracted.
by S-30 fractions from different strains of *B. megaterium* was examined, the drug was present in at least a three-fold molar excess over ribosomes in the incubation mixture. Under such conditions synthesis by S-30 fractions from wild-type *B. megaterium* was completely inhibited whereas synthesis in such extracts from the thio- strepton-resistant mutants was in every case tolerant of the drug (Figure 5.1; results for mutants MJ3, MJ5, PD5 and PD18 omitted). It was therefore concluded that in all these cases resistance to thio- strepton resulted from intracellular change(s) and was not attributable to alteration in the permeability of the cell surface to the antibiotic. Since it has already been shown that ribosomes from mutant PD1 are altered (Dixon, 1976), the data presented in Figure 5.1 are consistent with this conclusion.

5.4 Synthesis of Polyphenylalanine by Crude Ribosomes and S-100 Fraction

S-30 fractions were separated into crude ribosomes and S-100 fraction (see section 3.3(a) in order to attempt further localisation of the resistance to thio- strepton observed in extracts of the mutant strains. In preliminary experiments it was found that the crude ribosomal fraction retained some activity when examined alone for the ability to synthesise polyphenylalanine, but a stimulation of at least three-fold was observed by supplementing such material with the S-100 fraction (Figure 5.2), which was itself inactive. Optimal synthetic activity (typically 6 to 12 pmol [\(^{14}\text{C}\)]phenylalanine incorporated per nominal pmol ribosomes in 40 minutes) in systems recombined from crude ribosomal and S-100 fractions of *B. megaterium* wild-type was observed at a NRC of about 1\(\mu\text{M}\) (data not given). Optimal synthetic activity using components from the mutant strains was also observed at this NRC but was somewhat lower (2 to 9 pmol [\(^{14}\text{C}\)]phenylalanine incorporated per nominal pmol ribosomes in 40 minutes).

The effect of thio- strepton in systems recombined from mutant and
Figure 5.2

The figure shows the time course of $^{14}C$ radioactivity (c.p.m. $\times 10^{-3}$) over time (min). The graph illustrates the increase in radioactivity with time, with three distinct curves, each representing a different condition or treatment. The x-axis represents time in minutes (0 to 40), and the y-axis represents the $^{14}C$ radioactivity on a logarithmic scale (0 to 15). The data points are connected by lines to show the trend over time.
Legend to Figure 5.2

Synthesis of polyphenylalanine by subcellular fractions from wild-type B. megaterium

Crude ribosomes and S-100 fraction prepared from B. megaterium wild-type were assayed alone or in combination for the ability to synthesise polyphenylalanine. Assays containing 45μl S-100 fraction plus 5μl RS buffer (●), 5μl crude ribosomes plus 45μl TMAβ buffer (□), or 5μl crude ribosomes plus 45μl S-100 fraction (○) were supplemented with 50μl assay cocktail and incubated as described (section 5.2). Crude ribosomes (where present) were at a final nominal ribosome concentration of 1.1μM and L-[U-14C]phenylalanine was used at a specific radioactivity of 190mCi/mmol (380 cpm/pmol). The values plotted represent synthetic activity in 5μl samples of each reaction mixture, from which the background (80 cpm) has been subtracted.
Figure 5.3

Radioactivity (c.p.m. x 10^{-3}) vs. time (min)

- w.t. R
  - w.t. S100
- w.t. R
  - PD 1 S100
- PD 1 R
  - w.t. S100
- PD 1 R
  - PD 1 S100
Legend to Figure 5.2

Synthesis of polyphenylalanine by combinations of ribosomes and S-100 fractions

Crude ribosomes (in 5μl RS buffer) and S-100 fraction (in 45μl TMAβ buffer) from wild-type B. megaterium and mutant PD1 were recombined in homologous or heterologous manner as denoted in the Figure. Crude ribosomes were present at 1.1μM (nominal) throughout and synthesis of polyphenylalanine in the presence (●) or absence (O) of 12μM-thiostrepton was determined as described (section 5.2). L-[U-14C]phenylalanine was used at a specific radioactivity of 173mCi/mmol (340 cpm/pmol) and values plotted represent synthesis occurring in 5μl samples of reaction mixtures, from which a background of 80 cpm has been subtracted.

R = ribosomes
Legend to Figure 5.4

Synthesis of polyphenylalanine by combinations of ribosomes and S-100 fractions

Crude ribosomes (in 8μl RS buffer) and S-100 fraction (in 42μl TMAβ buffer) from wild-type B.megaterium and mutant MJ1 were recombined in homologous or heterologous manner as denoted in the Figure. Crude ribosomes were present at 1.1μM (nominal) throughout and synthesis of polyphenylalanine in the presence (●) or absence (○) of 12μM-thiostrepton was determined as described (section 5.2). L-[U-14C]phenylalanine was used at a specific radioactivity of 513mCi/mmol (1090 cpm/pmol) and values plotted represent synthesis occurring in 5μl samples of reaction mixtures, from which a background of 80 cpm has been subtracted.

③ = ribosomes
wild-type components (in both homologous and heterologous combinations) was next examined. Results presented for mutant PD1 (Figure 5.3) and mutant MJ1 (Figure 5.4) show that sensitivity or resistance of polyphenylalanine synthesis to thiostrepton in heterologous systems was determined by the source of the ribosomes employed, regardless of the origin of the S-100 fraction. These results confirmed the conclusion that mutant PD1 has altered ribosomes and supported the contention that thiostrepton-resistance in mutant MJ1 is also a ribosome-based phenomenon. That strains PD5, PD18, MJ3 and MJ5 are also resistant to the drug by virtue of ribosomal alterations was suggested from the results of similar experiments (data not presented). Accordingly, certain strains were chosen for further study of the nature of such alterations. The results of these investigations will be presented in the following Chapters.

5.5 Synthesis of Polyphenylalanine by High-Salt-Washed Ribosomes

High-salt-washed ribosomes and S-100 fraction (see section 3.3) from strains of B. megaterium were inactive when assayed individually for ability to synthesise polyphenylalanine, but good activity was obtained when the two fractions were suitably recombined (data not presented). The optimum input of S-100 was determined for each preparation used during the study and was found to be 0.4 to 0.7 A230 units per incubation (100μl, containing 50pmol ribosomes). By the use of high-salt-washed ribosomes, such recombined systems provided a more clearly-defined means of examining polyphenylalanine synthesis.

When ribosomes and S-100 fraction from wild-type B. megaterium and mutant MJ1 were recombined in homologous and heterologous fashion, the thiostrepton phenotype of polyphenylalanine synthesis was again dependent on the source of the ribosomes (Figure 5.5), confirming the previous results obtained using crude ribosomes. However, the use of high-salt-
Legend to Figure 5.5

Synthesis of polyphenylalanine by combinations of ribosomes and S-100* fractions

Salt-washed ribosomes (50 pmol in 20μl RS buffer) from B. megaterium wild-type or mutant strain MJ1 were incubated at 20°C for 10 minutes with thiostrepton (0.5μg in 5μl 25% (v/v) DMSO solution) or DMSO solution alone. Polyphenylalanine synthesis was measured as described already (section 5.2) in all four possible combinations after addition of S-100* fraction from either the wild-type (0.7 A230 units in 30μl RS buffer) or the mutant strain (0.4 A230 units in 30μl RS buffer). L-[U-14C]phenylalanine was used at a specific radioactivity of 513mCi/mmol (1090 cpm/pmol) and the values plotted represent polyphenylalanine synthesis in 10μl samples of reaction mixtures, from which a background of 120 cpm has been subtracted.

○ untreated

● plus thiostrepton

R = ribosomes
washed ribosomes enabled more accurate comparison to be made between the activity of ribosomes from the mutant and wild-type strains. This revealed that synthesis by ribosomes from the wild-type was consistently twice as efficient (2 to 6 pmol [$^{14}$C]phenylalanine incorporated per pmol ribosomes in 40 minutes) as synthesis by ribosomes from mutant MJ1 (eg. Figure 5.5) in the absence of thiostrepton. This effect again was independent of the source or batch of S-100* used and reflected the relative growth-rates of the two strains (Table 2.2). The basis of this difference in activity was subsequently investigated and forms an integral part of this dissertation.

It should be noted that the efficiency of synthesis using high-salt-washed ribosomes was improved by reducing the quantity of ribosomes in the incubation mixture. The standard input used (50 pmol) represents a compromise between efficiency of synthesis and total incorporation.

5.6 Inhibition of Polyphenylalanine Synthesis as a Function of Thiostrepton Concentration

Using the more clearly-defined system for measuring poly U-dependent protein synthesis just described, the effect of varying the thiostrepton concentration on synthesis by ribosomes from wild-type B. megaterium and mutant MJ1 was compared (Figure 5.6). The minimum concentration of thiostrepton required for complete inhibition of polyphenylalanine synthesis by ribosomes from mutant MJ1 was only between 10 and 20 times the concentration needed to abolish synthesis by ribosomes from the wild-type. This difference in sensitivity is surprisingly less than that observed in vivo (section 2.5) and was smaller still when considering (albeit on a percentage basis) incomplete inhibition of activity.

Figure 5.6 shows that ribosomes from mutant MJ1 are still able to interact with thiostrepton to an appreciable extent. This is in contrast
to ribosomes from *S. azureus* (the organism that produces thiostrepton) which synthesise polyphenylalanine at an uninhibited rate in the presence of thiostrepton at the limit (88μg/ml) of its aqueous solubility (E.Cundliffe, personal communication). The interaction of ribosomes from mutant MJ1 with $^{35}$Sthiostrepton was subsequently investigated using equilibrium dialysis (Chapter 6).

The shape of the curve obtained for the inhibition of activity by wild-type ribosomes is curious. While it is suggestive of a requirement for the binding of more than one thiostrepton molecule per ribosome in order to achieve complete inhibition, such a hypothesis can obviously not be made on the basis of this data alone.
Figure 5.6

Percentage activity remaining

Final concentration of thiostrepton (μg/ml)

w.t.

MJ 1
Legend to Figure 5.6

Sensitivity to thio strepton of poly phenylalanine synthesis

Ribosomes (50 pmol in 20μl RS buffer) from wild-type B. megaterium (□) or mutant MJ1 (○) were incubated for 10 minutes at 20°C with thio strepton (0.05 to 5.0μg, in 5μl 25% (v/v) DMSO solution) or with 25% (v/v) DMSO alone. S-100* fraction from the wild-type strain (0.7 A230 units in 25μl RS buffer) was added in all cases and then polyphenylalanine synthesis measured as described (section 5.2). The initial rate of synthesis (which was constant) was determined and for each strain the rates obtained in the presence of thio strepton were expressed as percentages of the control (uninhibited) rate. Values from two separate experiments are plotted. In the absence of thio strepton, synthesis of polyphenylalanine during the first 30 minutes was as follows:

Ribosomes from B. megaterium wild-type: 2.5 pmol [14C]phenylalanine incorporated per pmol ribosomes.

Ribosomes from B. megaterium mutant MJ1: 1.5 pmol [14C]phenylalanine incorporated per pmol ribosomes.
CHAPTER 6: THE BINDING OF \([^{35}\text{S}]\text{THIOSTREPTON}\) TO RIBOSOMES FROM STRAINS
OF \textit{BACILLUS MEGATERIUM}

6.1 Introduction

Widespread use has been made of radiolabelled antibiotics in studies of ribosome structure and function (see e.g. Vazquez, 1979), most obviously in the study of the ribosomal binding sites of antibiotics and in particular of the components involved at these sites. In the present study, measurement of the binding of radiolabelled thiostrepton to ribosomes from \textit{B. megaterium} was used to characterise further ribosomes from the thiostrepton-resistant strains examined above. This approach has already been used successfully to demonstrate that ribosomes from mutants FD1 and FD14 are unable to bind thioestrepton with high affinity (Dixon, 1976).

Radioactive atoms may be incorporated into antibiotic molecules by a number of methods. In the case of thioestrepton, biosynthesis in the presence of a radioactive precursor has proved the most useful approach. Thus several methods have been developed for the production of \([^{35}\text{S}]\text{thioestrepton}\) by fermentation of \textit{Streptomyces azureus} (the producing organism) in media containing \([^{35}\text{S}]\text{sulphate}\) (Sopori & Lengyel, 1972; Dixon et al., 1975; Highland et al., 1975a). Although the growth requirements and antibiotic production of \textit{S. azureus} have been studied in detail (Pagano et al., 1956; Platt & Frazier, 1961) it is not clear whether conditions optimal for thioestrepton production are the most suitable for biosynthesis of \([^{35}\text{S}]\text{thioestrepton}\) of high specific radioactivity.

Sulphur-35 is a useful label since the thioestrepton molecule contains six sulphur atoms (Figure 1.2). However, attempts to produce thioestrepton containing more stable radionuclides by tritium exchange or biosynthesis in the presence of \([^{14}\text{C}]\text{amino acids}\) failed to yield radiolabelled material.
with biological activity (Dixon, 1976).

For use in the present study, \([^{35}S]\\text{thiostrepton}\) was prepared by a modification of the procedure used by Dixon (1976). I am indebted to Dr. E. Cundliffe for preparing most of this material.

6.2 Preparation and Characterisation of \([^{35}S]\\text{thiostrepton}\)

(a) Fermentative production of \([^{35}S]\\text{thiostrepton}\)

\textit{Streptomyces azureus} was grown at 30°C on DYM agar until copious sporulation occurred. The spores were taken up in 5ml sterile water, inoculated into 50ml Fermentor medium and incubated at 30°C with orbital shaking. After 18 hours, carrier-free \([^{35}S]\\text{sulphate}\) (5 to 10mCi) was added and incubation continued for 8 days. The cells and medium were then adjusted to pH 8.5 (with KOH) and extracted three times each with 100ml of ethylene dichloride by vigorous shaking at 30°C for 12 hours. The pooled organic phases were filtered through sintered glass and then dried down by rotary evaporation at 35°C under reduced pressure. A large flask was used such that the residue was spread over the maximum area. This residue was washed three times in petroleum ether (boiling point range 60 to 80°C), which removed much of the yellow colour. The dry residue was redissolved in ethylene dichloride (20ml).

Such preparations contained a variable and often large proportion of radiolabelled material other than \([^{35}S]\\text{thiostrepton}\), some of which bound non-specifically to ribosomes. The following purification step was therefore routinely adopted. Approximately 1g of DE-52 cellulose was added to the solution of \([^{35}S]\\text{thiostrepton}\) (in 20ml ethylene dichloride) and stirred at ambient temperature for about 5 hours. The resin was pelleted by centrifugation for 5 minutes at 5,000 rpm and the supernatant dried down by rotary evaporation as before. The residue was finally taken up in DMSO (1 to 10ml) and diluted with an equal volume of water before use.
Preparations were stored at -20°C.

(b) Characterisation of $[^{35}\text{S}]$thiostrepton

(i) Binding by ribosomes: Preparations of $[^{35}\text{S}]$thiostrepton were analysed for their ability to bind to ribosomes using the gel filtration or Norit adsorption techniques (see below). The proportion of the total radioactivity bindable by ribosomes (present in large excess) was routinely found to be around 95%, of which all but an insignificant fraction could be competed away by pre-treatment of the ribosomes with sufficient authentic, non-radioactive thiostrepton (data not given).

(ii) Chromatographic analysis: $[^{35}\text{S}]$thiostrepton was analysed by ascending chromatography on silica-gel thin-layer plates ('Kieselgel', Merck) using a 9:1 chloroform : methanol mixture as the eluent. Material containing $^{35}\text{S}$ was located by autoradiography and its mobility compared with that of authentic, non-radioactive thiostrepton. Greater than 95% of both the radioactive and the non-radioactive material comigrated (Figure 6.1).

(iii) Determination of specific radioactivity: Ideally, the specific radioactivity of a radiolabelled antibiotic is determined by isotope dilution with unlabelled antibiotic, which must obviously be of high purity. During the present study, thiostrepton of guaranteed high chemical purity was not available and attempts to determine the specific radioactivity of $[^{35}\text{S}]$thiostrepton by isotope dilution yielded equivocal results. The specific radioactivity was therefore determined by plotting a curve for the binding of $[^{35}\text{S}]$thiostrepton to 70S ribosomes from *E. coli* (to which the drug binds very tightly) assuming a 1:1 binding stoichiometry as demonstrated by others (Sopori & Lengyel, 1972; Highland et al., 1975a). The specific radioactivity
Figure 6.1

- $R_f = 1.00$
- $R_f = 0.62$
- $R_f = 0.00$
Legend to Figure 6.1

Analysis of $[^{35}\text{S}]$thiostrepton by thin-layer chromatography

Authentic non-radioactive thiostrepton ($5\mu\text{g}$) and a sample of $[^{35}\text{S}]$thiostrepton (approximately $10^4$ cpm) were dried down together at $20^\circ\text{C}$ onto a silica gel thin-layer plate and developed in the ascending mode using chloroform:methanol 9:1. The authentic thiostrepton was detected under ultra-violet light by its ability to quench the fluorescence of a phosphor in the silica gel (a) and radioactive components were located by autoradiography (b). Such analysis revealed that the majority (greater than 95%) of both radioactive and fluorescence-quenching material comigrated with an $R_f$ of 0.62 (●) and only traces of impurities (○) could be detected.
Figure 6.2

35S radioactivity (c.p.m. x 10^-3)

pmol ribosomes added

pmol ribosomes added
Legend to Figure 6.2

Determination of the specific radioactivity of a [\(^{35}\text{S}\)]thiostrepton preparation

The binding of [\(^{35}\text{S}\)]thiostrepton to \(E.\text{coli}\) 70S ribosomes (4 to 30 pmol) was measured by gel filtration using BioGel A-1.5m as described in section 6.3(a). A binding curve was plotted and from the slope of the line obtained the specific radioactivity (here equivalent to 410 cpm/ pmol) was determined by assuming a 1:1 stoichiometry of drug binding (see text).
was thus given by the slope of the line obtained (Figure 6.2). The \( ^{35}S \)thiostrepton preparations employed during this study originally gave between 200 and 500 cpm/pmol and were used through not more than two half-lives (approximately six months).

6.3 Measurement of the Binding of \( ^{35}S \)thiostrepton to Ribosomal Particles

Throughout all the experiments described in this Chapter, salt-washed ribosomes have been used unless stated otherwise. Such ribosomes are substantially free of supernatant factors, peptidyl-tRNA and mRNA, although the latter two entities would in any case not be expected to influence the binding of thiostrepton (Contreras & Vazquez, 1977).

The binding of \( ^{35}S \)thiostrepton to ribosomes has generally been expressed as pmol \( ^{35}S \)thiostrepton bound per pmol ribosomes (ie. the 'binding ratio') and was examined as follows:–

(a) Gel filtration

Ribosomal particles (25 to 50 pmol) in 50\( \mu l \) RS buffer were incubated with 5\( \mu l \) \( ^{35}S \)thiostrepton (35 to 100 pmol in 50\% (v/v) DMSO solution for 10 minutes at 20°C. The incubation mixture was then applied to a column (7.0 x 0.55cm) of Sepharose 6B or BioGel A-1.5m equilibrated with RS buffer and eluted with the same. Fractions (120\( \mu l \)) were collected and their content of radioactivity determined by liquid-scintillation spectrometry. Ribosomes are excluded by such columns, whereas free \( ^{35}S \)thiostrepton is included and thus separated from that bound by ribosomes (Figure 6.3). The binding of \( ^{35}S \)thiostrepton to ribosomes was therefore determined from the net content of radioactivity in the column void volume (ie. less small background levels obtained from control determinations lacking ribosomes).

(b) Norit adsorption

All assays employing this method were performed in duplicate and the
Legend to Figure 6.3

Measurement by gel filtration of the binding of $[^{35}\text{S}]$thiostrepton to ribosomes

Ribosomes (50 pmol in 50µl RS buffer) from wild-type B.megaterium (O) or 50µl RS buffer alone (●) were incubated for 10 minutes at 20°C with $[^{35}\text{S}]$thiostrepton (70 pmol in 5µl 50% (v/v) DMSO solution).

Incubation mixtures were then loaded onto small columns (7.0 x 0.55cm) of Sepharose 6B and eluted with RS buffer. Fractions (120µl) were collected and the radioactivity present in each estimated by liquid-scintillation spectrometry. Radioactivity eluted in the void volume of the column (indicated by broken lines) only in the presence (O) of ribosomes. Binding of $[^{35}\text{S}]$thiostrepton to the ribosomes was calculated from the amount of radioactivity thus eluted, correcting for the traces of material eluted in the void volume in the absence (●) of ribosomes.
mean results presented. Ribosomal particles (25 to 50 pmol in 85μl RS buffer) were incubated with \([^{35}\text{S}]\)thiostrepton as in (a). To this incubation was then added 10μl of a 5% (w/v) suspension of activated charcoal ('Norit', Sigma) in RS buffer. After thorough mixing followed by 5 minutes at 20°C the Norit was pelleted by centrifugation at 12,000 x g for 5 minutes. Thiostrepton is adsorbed to charcoal quantitatively under these conditions unless bound by a component in the incubation mixture. Thus estimation of \(^{35}\text{S}\)-radioactivity contained in a sample of the supernatant provides a measure of the binding of \([^{35}\text{S}]\)thiostrepton by the ribosomal particles. Values obtained were corrected for the small background obtained in control determinations (lacking ribosomes).

(c) \textit{Equilibrium dialysis}

Equilibrium dialysis was performed in an apparatus consisting of two circular plastic blocks each containing eight wells of volume 0.55ml, such that by clamping the blocks together face-to-face across a dialysis membrane eight cells were formed. Introduction (or removal) of material into either side of a cell was through a small hole normally sealed by a self-adhesive disc.

Ribosomal particles (50 pmol in 0.5ml RS buffer) were introduced into one half of a cell and \([^{35}\text{S}]\)thiostrepton (50 to 800 pmol in 0.5ml RS buffer) into the other. Only preparations of \([^{35}\text{S}]\)thiostrepton greater than 95% pure (see section 6.2) were used. The drug was allowed to equilibrate across the membrane (mol. wt. cut-off 6,500 daltons) while the apparatus was rotated (60 rpm) at 4°C. When complete equilibration had occurred in control cells (typically after 70 to 140 hours) samples (200μl) were removed from each side of every cell and their content of \(^{35}\text{S}\) radioactivity determined. The amount of \([^{35}\text{S}]\)thio-

strepton bound by the particles was estimated from the measured difference in the radioactive concentration across the membrane.
The dissociation constant for the binding of $[^{35}\text{S}]$thiostrepton to ribosomes was obtained by Scatchard analysis, using a fixed concentration of ribosomes and a range of concentrations of labelled drug. The dissociation constant ($K_d$) is related to the free drug concentration ($f$), the number of drug molecules bound per ribosome ($\bar{V}$) and the number of binding sites per ribosome ($n$) as follows:

$$\bar{V} = \frac{n.f}{K_d + f}$$

Thus if values of $\bar{V}/f$ are plotted against $\bar{V}$, the line obtained has a slope of $-1/K_d$ and crosses the $\bar{V}$-axis at a value of $n$. Values for $f$ were estimated from the radioactive concentration in that half of the cell lacking ribosomes.

Only the gel filtration and Norit adsorption techniques (i.e. (a) and (b) above) were used routinely to measure the binding of $[^{35}\text{S}]$thiostrepton to ribosomal particles and both yielded comparable results (data not shown). Use of these techniques indicated that ribosomes from the thiostrepton-resistant mutants of *B. megaterium* are apparently unable to bind thiostrepton (see below). However, these methods can only detect high-affinity binding of $[^{35}\text{S}]$thiostrepton to ribosomes and use of equilibrium dialysis, a much more sensitive technique, revealed in the case of mutant MJ1 that the ribosomes do bind $[^{35}\text{S}]$thiostrepton, albeit with low affinity.

6.4 The Binding of $[^{35}\text{S}]$thiostrepton to Ribosomes from Strains of *B. megaterium*

Thiostrepton binds to the *E. coli* 50S ribosomal subunit with 1:1 stoichiometry and is not bound at all by the 30S subunit (Sopori & Lengyel, 1972; Highland et al., 1975a; Cundiffe, 1976). Since
Table 6.1

The binding of $[^{35}\text{S}]$thiostrepton to ribosomes and ribosomal subunits from wild-type B.megaterium

<table>
<thead>
<tr>
<th>Ribosomal particles</th>
<th>pmol THS* bound/ pmol ribosomal particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>70S ribosomes</td>
<td>0.97</td>
</tr>
<tr>
<td>50S ribosomal subunits</td>
<td>0.85</td>
</tr>
<tr>
<td>30S ribosomal subunits</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* THS : $[^{35}\text{S}]$thiostrepton

The binding of $[^{35}\text{S}]$thiostrepton (50 pmol, 135 cpm/pmol) to 25 pmol ribosomes or ribosomal subunits from wild-type B.megaterium was determined using the Norit adsorption technique (section 6.3) in a final volume of 100μl RS buffer.
ribosomes from *B. megaterium* (wild-type) invariably bound approximately one pmol \[^{35}S\]thiostrepton per pmole independently of the preparation of labelled antibiotic (eg. Tables 6.1 to 6.3) a similar binding stoichiometry was indicated. Furthermore, measurement of the binding of \[^{35}S\]thiostrepton to purified ribosomal subunits from *B. megaterium* confirmed that only the large subunit could bind the drug (Table 6.1). In the following experiments it was therefore assumed that whenever binding of \[^{35}S\]thiostrepton was observed it was occurring upon 50S ribosomal subunits or particles derived from them.

Ribosomes from the thiostrepton-resistant mutants of *B. megaterium* were next examined for their ability to bind the drug. Table 6.2 shows that ribosomes from mutants MJ1 (line e), MJ5 (line h) and PD1 (line k) all bound \[^{35}S\]thiostrepton poorly by comparison with ribosomes from the wild-type (line a), reflecting their resistance to the drug *in vitro* (Chapter 5).

The effect of 1M-LiCl split-proteins on the binding of \[^{35}S\]thiostrepton by ribosomes was also examined. In wild-type *B. megaterium* this protein fraction contains EM-L11 (the thiostrepton-binding protein) and has been shown to restore the binding of thiostrepton to ribosomes from mutant PD1 (Dixon, 1976). Table 6.2 shows that ribosomes from mutants MJ1 (line f), MJ5 (line i) and mutant PD1 (line l) were stimulated to bind \[^{35}S\]thiostrepton quantitatively by addition of excess split-proteins from the wild-type. In control assays this split-fraction had little effect on ribosomes from the wild-type (line b). By comparison, 1M-LiCl split-proteins from mutants MJ1 and MJ5 did not stimulate drug-binding by the homologous ribosomes (lines g, j) nor could they compete away the binding of \[^{35}S\]thiostrepton to ribosomes from the wild-type although present in excess (lines c, d). Furthermore, heterologous combinations of ribosomes and split-proteins from the thiostrepton-
Table 6.2

The binding of $^{35}$S thiostrepton to ribosomes from wild-type and mutant strains of *B. megaterium*

<table>
<thead>
<tr>
<th>Source of ribosomes</th>
<th>Additions</th>
<th>pmol THS* bound per pmol ribosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. wild-type</td>
<td>—</td>
<td>0.95</td>
</tr>
<tr>
<td>b. wild-type</td>
<td>200 eq. w.t. 1M-LiCl splits*</td>
<td>1.00</td>
</tr>
<tr>
<td>c. wild-type</td>
<td>150 eq. MJ1 1M-LiCl splits</td>
<td>0.93</td>
</tr>
<tr>
<td>d. wild-type</td>
<td>110 eq. MJ5 1M-LiCl splits</td>
<td>0.89</td>
</tr>
<tr>
<td>e. mutant MJ1</td>
<td>—</td>
<td>0.02</td>
</tr>
<tr>
<td>f. mutant MJ1</td>
<td>200 eq. w.t. 1M-LiCl splits</td>
<td>0.89</td>
</tr>
<tr>
<td>g. mutant MJ1</td>
<td>150 eq. MJ1 1M-LiCl splits</td>
<td>0.05</td>
</tr>
<tr>
<td>h. mutant MJ5</td>
<td>—</td>
<td>0.03</td>
</tr>
<tr>
<td>i. mutant MJ5</td>
<td>100 eq. w.t. 1M-LiCl splits</td>
<td>0.99</td>
</tr>
<tr>
<td>j. mutant MJ5</td>
<td>110 eq. MJ5 1M-LiCl splits</td>
<td>0.05</td>
</tr>
<tr>
<td>k. mutant ED1</td>
<td>—</td>
<td>0.01</td>
</tr>
<tr>
<td>l. mutant ED1</td>
<td>200 eq. w.t. 1M-LiCl splits</td>
<td>1.02</td>
</tr>
</tbody>
</table>

* THS : $^{35}$S thiostrepton; eq. : pmol equivalent; w.t. : wild-type

Ribosomes (50 pmol in 50μl RS buffer) were incubated for 5 minutes at 0°C then 5 minutes at 20°C in the presence or absence of 100 to 200 pmol equivalents of 1M-LiCl split-proteins (splits) from the sources indicated. The binding of $^{35}$S thiostrepton (60 pmol, 280 cpm/pmol) was then determined in each case by gel filtration using Sepharose 6B (see text). No apparent binding of $^{35}$S thiostrepton was observed in the absence of ribosomes or the presence alone of any preparation of 1M-LiCl split-proteins.
Table 6.3
The effect of protein BM-L11 upon the binding of \([^{35}S]\)thiostrepton by ribosomes from strains of B. megaterium

<table>
<thead>
<tr>
<th>Source of ribosomes</th>
<th>Additions</th>
<th>pmol THS* bound per pmol ribosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td></td>
<td>0.97</td>
</tr>
<tr>
<td>wild-type</td>
<td>100 pmol BM-L11</td>
<td>1.06</td>
</tr>
<tr>
<td>mutant MJ1</td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>mutant MJ1</td>
<td>100 pmol BM-L11</td>
<td>1.18</td>
</tr>
<tr>
<td>mutant MJ5</td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>mutant MJ5</td>
<td>100 pmol BM-L11</td>
<td>1.06</td>
</tr>
<tr>
<td>mutant PD1</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>mutant PD1</td>
<td>100 pmol BM-L11</td>
<td>1.03</td>
</tr>
</tbody>
</table>

* THS: \([^{35}S]\)thiostrepton

Ribosomes (25 pmol in RS buffer) were incubated in the presence or absence of protein BM-L11 for 5 minutes at 0°C then 5 minutes at 20°C (total volume 85μl). The binding of \([^{35}S]\)thiostrepton (50 pmol, 370 cpm/pmol) was then determined using the Norit adsorption technique (see text). Protein BM-L11 was prepared under 'denaturing' conditions and did not bind \([^{35}S]\)thiostrepton in assays containing the protein alone.
resistant strains were unable to support the binding of $[^{35}\text{S}]$thiostrepton (data not presented), suggesting that the mutants share a similar deficiency.

In a further series of experiments the ability of purified protein BM-L11 to mimic the effect of wild-type split-proteins was examined. Addition of the protein to ribosomes from mutants MJ1, MJ5 and PD1 restored their ability to bind $[^{35}\text{S}]$thiostrepton with 1:1 stoichiometry in each case, while the protein had little effect on ribosomes from the wild-type (Table 6.3). Similar results (data not shown) were also obtained using all the other thiostrepton-resistant mutants of E. megaterium (i.e. strains MJ3, PD5, PD14 and PD18). Apparent binding of $[^{35}\text{S}]$thiostrepton by protein BM-L11 alone was never observed.

It was therefore concluded that in all the above mutant strains protein BM-L11 was either altered or absent from the ribosomes as isolated by the standard procedure. Thus the wild-type protein could displace or replace the mutant form on such ribosomes, enabling them to bind thiostrepton. In this context it is noted that protein L11 of E. coli, the serological homologue of protein BM-L11 (Chapter 4), has been shown to undergo exchange in vitro between ribosome-bound and free forms (Robertson et al., 1977; Subramanian & van Duin, 1977). Failure of excess split-proteins from the mutant strains to reduce the binding of $[^{35}\text{S}]$thiostrepton by wild-type ribosomes (Table 6.3) is not, however, conclusive evidence for the absence of a defective form of protein BM-L11 from ribosomes of the mutants. Such a variant may not have been removed by 1M-LiCl or have been unable to exchange significantly with the wild-type copy.

In another experiment the ability of ribosomes in S-30 fractions to bind $[^{35}\text{S}]$thiostrepton was examined. Although S-30 fractions have been
Table 6.4

Binding of $[^{35}S]$thiostrepton to ribosomes in S-30 fractions prepared from *B. megaterium*

<table>
<thead>
<tr>
<th>Source of S-30 fraction</th>
<th>Additions</th>
<th>pmol THS bound per pmol ribosomes (nominal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>—</td>
<td>0.91</td>
</tr>
<tr>
<td>wild-type</td>
<td>200 pmol EM-L11</td>
<td>0.87</td>
</tr>
<tr>
<td>mutant MJ1</td>
<td>—</td>
<td>0.15</td>
</tr>
<tr>
<td>mutant MJ1</td>
<td>200 pmol EM-L11</td>
<td>0.89</td>
</tr>
</tbody>
</table>

*THS: $[^{35}S]$thiostrepton

Samples of S-30 fractions containing a 'nominal' 50 pmol ribosomes (see section 3.5(a) were incubated in the presence or absence of protein EM-L11 (200 pmol) in a total volume of 50μl RS buffer. After 5 minutes at 0°C followed by 5 minutes at 20°C, 10μl $[^{35}S]$thiostrepton (100 pmol in 50% (v/v) DMSO solution, 250 cpm/pmol) was added and incubation continued for 10 minutes at 20°C. The binding of $[^{35}S]$thiostrepton to ribosomes was then measured by gel filtration using BioGel A-15m (section 6.3).
Table 6.5
Comparison of the effect of ribosomal proteins on the binding of
[^35S]thiostrepton to ribosomal particles from B.megaterium

<table>
<thead>
<tr>
<th>Ribosomal particles</th>
<th>Additions</th>
<th>pmol THS* bound per pmol ribosomal particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>mutant MJ1 ribosomes</td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>mutant MJ1 ribosomes</td>
<td>100 eq. w.t.*</td>
<td>1.23</td>
</tr>
<tr>
<td>mutant MJ1 ribosomes</td>
<td>100 pmol BM-L11</td>
<td>1.18</td>
</tr>
<tr>
<td>mutant MJ1 ribosomes</td>
<td>100 pmol L11</td>
<td>1.06</td>
</tr>
<tr>
<td>mutant MJ1 ribosomes</td>
<td>350 pmol lysozyme</td>
<td>0.01</td>
</tr>
<tr>
<td>w.t. 2M-LiCl core-particles</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>w.t. 2M-LiCl core-particles</td>
<td>100 eq. w.t.</td>
<td>1.19</td>
</tr>
<tr>
<td>w.t. 2M-LiCl core-particles</td>
<td>100 pmol BM-L11</td>
<td>1.07</td>
</tr>
<tr>
<td>w.t. 2M-LiCl core-particles</td>
<td>100 pmol L11</td>
<td>1.05</td>
</tr>
</tbody>
</table>

* THS : [^35S]thiostrepton; eq. : pmol equivalent; w.t. : wild-type

Ribosomes (from mutant MJ1) or wild-type 2M-LiCl core-particles (25 pmol in a total volume of 85µl RS buffer) were incubated alone or in the presence of one of the following: 100 pmol equivalents of wild-type 1M-LiCl split-proteins (splits), 100 pmol B.megaterium protein BM-L11, 100 pmol E.coli protein L11, 350 pmol lysozyme. Incubation was for 5 minutes at 0°C followed by 5 minutes at 20°C. The binding of [^35S]thiostrepton (50 pmol, 370 cpm/pm) was then determined using the Norit adsorption technique (see text). In control assays containing splits or purified proteins alone, no apparent binding of [^35S]thiostrepton was observed.
subjected to much less manipulation than high-salt-washed ribosomes, the poor binding of $^{35}$S-thiostrepton by ribosomes in an S-30 fraction from mutant MJ1 was again improved to wild-type levels by addition of protein BM-L11 (Table 6.4). Thus the effect of protein BM-L11 on salt-washed ribosomes from mutant MJ1 was apparently not an artefact associated with their preparation.

In a final experiment the relatedness of protein BM-L11 (B. megaterium) and protein L11 (E. coli) was further probed. Table 6.5 shows that both proteins were as effective as wild-type 1M-LiCl split-proteins in restoring the binding of $^{35}$S-thiostrepton either to 2M-LiCl core-particles from wild-type B. megaterium or to 70S ribosomes from mutant MJ1. The failure of lysozyme (a basic protein of similar size to proteins L11 and BM-L11) to mimic this effect serves to emphasise its specificity. Accordingly it was concluded that although E. coli protein L11 and B. megaterium protein BM-L11 are not structurally identical (Chapter 4) they apparently show functional similarity.

Ribosomes from these mutant strains were subjected to further investigation using electrophoretic and immunological techniques (Chapters 7 and 8).

6.5 The Binding of $^{35}$S-thiostrepton by Ribosomes from B. megaterium

Mutant MJ1

Ribosomes from the mutant strains of B. megaterium did not bind $^{35}$S-thiostrepton as assayed by gel filtration or Norit adsorption and yet their activity could still be inhibited by the drug (Chapter 5). This suggested that the interaction between thiostrepton and such ribosomes was too weak to be detected in these ways. Accordingly the ability of
ribosomes from mutant MJ1 to bind $[^{35}\text{S}]$thiostrepton was examined by equilibrium dialysis. This technique is particularly sensitive, since it measures the relative accumulation of ligand across a dialysis membrane resulting from interaction with a macromolecule present only on one side, thus not requiring disruption of the equilibrium between bound and free ligand for the purpose of measuring ligand binding.

In a preliminary experiment where both ribosomes and free $[^{35}\text{S}]$thiostrepton were at a concentration of 0.1μM, ribosomes from mutant MJ1 bound the drug with a stoichiometry of 0.6 molecules per ribosome. By comparison, under similar conditions ribosomes from wild-type B.megaterium bound one drug molecule each, while ribosomes from S.azureus (the organism which produces thiostrepton) showed no significant interaction with thiostrepton at these concentrations.

In order to quantify the observed binding of $[^{35}\text{S}]$thiostrepton to ribosomes from the mutant, the dissociation constant for such binding was determined (see section 6.3(c)). A similar experiment could not be performed using ribosomes from the wild-type since they bind thiostrepton so tightly. Results with ribosomes from mutant MJ1 (Figure 6.4) showed a dissociation constant for the binding of $[^{35}\text{S}]$thiostrepton of $2 \times 10^{-7}$M, which is of the same order of magnitude as the dissociation constants for the binding of a number of antibiotics (eg. chloramphenicol, dihydrostreptomycin) to E.coli ribosomes (see Cundliffe, 1979b). This estimate should by virtue of the experimental design be free from any error due to systematic loss of either ribosomes or $[^{35}\text{S}]$thiostrepton (Nimmo et al., 1977). However, the reliability of the value obtained for the number of drug-binding sites per ribosome (1.5) is less certain, particularly since a binding stoichiometry of greater than 1:1 was never observed. It was therefore concluded that ribosomes from mutant MJ1 bind thiostrepton with considerable affinity, although with far less affinity
Figure 6.4

$K_d = 2 \times 10^{-7} \text{ M}$

$[\text{THS}] \text{ concentration of free } ^{35}\text{S}\text{thiostrepton}$

$\frac{[\text{SH}]^{\text{L}}}{[\text{A}]}$
Legend to Figure 6.4

Measurement of the dissociation constant for the binding of [35S]thiostrepton to ribosomes from B. megaterium strain MJ1

The binding of [35S]thiostrepton to ribosomes from mutant MJ1 was measured by equilibrium dialysis (see section 6.3). Each cell contained 50 pmol ribosomes (in 0.5ml RS buffer) on one side of the membrane and 0.5ml RS buffer containing (initially) 5 to 80μl [35S]thiostrepton (50 to 800 pmol, 120 cpm/μmol in 50% (v/v) DMSO solution) on the opposite side. After complete equilibration of the drug across the membrane, the free [35S]thiostrepton concentration and the number of [35S]thiostrepton molecules bound per ribosome (▽) were calculated for each cell. Results from two separate experiments were combined in a single Scatchard plot and the line of best fit (obtained by the least squares method of linear regression) was calculated. From the slope of this line (-5.1μM⁻¹) a dissociation constant (K_d) of 2 x 10⁻⁷M was indicated.
than ribosomes from the wild-type strain.

6.6 **Attempted Measurement of Binding of \[^{35}\text{S}]\text{thiostrepton to Protein EM-L11**}

At the present time, *E. coli* protein L15 is the only ribosomal protein that has been shown to bind an antibiotic (erythromycin) in isolation from the ribosomes (Teraoka & Nierhaus, 1978). Although protein EM-L11 has been referred to as the 'thiostrepton-binding' protein, there is no evidence to suggest that the protein actually binds thiostrepton off (or on) the ribosome. Thus binding of \[^{35}\text{S}]\text{thiostrepton to the purified protein was never detected either by the Norit adsorption technique (see above) or by gel filtration using Sephadex G-25 (data not given). However, in order to examine more rigorously the possibility of an interaction between the protein and the drug, equilibrium dialysis was used.}

Protein EM-L11 (600 pmol in 0.5ml RS buffer) was dialysed against 0.5ml RS buffer containing 20μl \[^{35}\text{S}]\text{thiostrepton (200 pmol, in 50% (v/v) DMSO solution) in the above-described apparatus (section 6.3(c). After complete equilibration of the drug across the membrane (which excluded the protein) the distribution of \[^{35}\text{S}]\text{thiostrepton was determined. This repeatedly revealed equal concentrations of \[^{35}\text{S}]\text{thiostrepton in both halves of the cell (eg. Table 11.4). Conversely, others have shown that under somewhat similar conditions E. coli protein L15 binds approximately 0.5 mol \[^{14}\text{C}]\text{erythromycin per mol protein and a dissociation constant for such binding of 2 x 10^{-5}M was measured (Teraoka & Nierhaus, 1978). Thus inability to detect the binding of \[^{35}\text{S}]\text{thiostrepton to protein EM-L11 by this means strongly suggests that the protein cannot by itself interact with the drug.**}
6.7 Stability of the Interaction between Protein EM-L11 and Ribosomes from Mutant MJ1

Protein EM-L11 can clearly stimulate the binding of $[^{35}\text{S}]$thiostrepton to ribosomes from the thiostrepton-resistant mutants. However, this does not reveal whether the protein is correctly integrated into such ribosomes, particularly since the presence of $[^{35}\text{S}]$thiostrepton in the above experiments could have stabilised the interaction. In addition, during this study ribosomes from the mutants were reconstituted with protein EM-L11 at low temperatures (0°C and 20°C) and low ionic strength (Highland et al., 1975b). However, partial reconstitution of active ribosomal particles may best be achieved by heating the components at high monovalent cation concentrations (Traub et al., 1971; Maglott & Staehelin, 1971). It was therefore pertinent to establish how well protein EM-L11 was bound to the mutant ribosomes after reconstitution.

Ribosomes from mutant MJ1 were reconstituted with protein EM-L11 under the standard conditions and then subjected to two cycles of high-salt-washing (section 3.3(b). The ability of such particles to bind $[^{35}\text{S}]$thiostrepton with high affinity was used as a probe for the presence of protein EM-L11. Table 6.6 shows that (relative to ribosomes from the wild-type) no loss of ability to bind $[^{35}\text{S}]$thiostrepton occurred when the reconstituted particles were thus treated. It was therefore concluded that protein EM-L11 is not readily lost from the mutant ribosomes after reconstitution and again inferred that such loss does not occur during their preparation (see also section 6.4).

Since it will be shown that protein EM-L11 can induce functional changes in ribosomes from mutant MJ1 (Chapters 9 and 10) under the reconstitution conditions used, it is concluded that protein EM-L11 is correctly assembled into ribosomes from the mutant in vitro.
Table 6.6

Stability of the interaction between protein BM-L11 and ribosomes from mutant MJ1

<table>
<thead>
<tr>
<th>Source of ribosomes</th>
<th>Additions</th>
<th>pmol THS* bound per pmol ribosomes prior to washing</th>
<th>pmol ribosomes after washing</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. megaterium wild-type</td>
<td>—</td>
<td>0.90</td>
<td>0.81</td>
</tr>
<tr>
<td>B. megaterium mutant MJ1</td>
<td>BM-L11</td>
<td>0.93</td>
<td>0.82</td>
</tr>
</tbody>
</table>

* THS: [35S]thio strepton

Ribosomes (2.25 nmol) from B. megaterium wild-type or mutant MJ1 were incubated in the presence or absence of 5.0 nmol protein BM-L11 for 5 minutes at 0°C and then 5 minutes at 20°C in 150µl RS buffer. A small sample (containing 75 pmol ribosomes) was removed from each incubation and the ability of the ribosomes to bind [35S]thio strepton (first column) was determined by gel filtration using BioGel A-1.5m (see section 6.3). The remainder was in each case layered over 8ml HS buffer containing 20% (w/v) sucrose and centrifuged at 48,000 rpm and 2°C for 7½ hours in the Beckman Ti75 rotor. The resulting pellets were resuspended in 8ml of the same buffer, recovered by centrifugation as before and finally resuspended in 150µl RS buffer. The ability of ribosomes (50 pmol) in samples of the resuspended pellets to bind [35S]thio strepton was determined as before (second column).
CHAPTER 7: ELECTROPHORETIC ANALYSIS OF RIBOSOMAL PROTEINS

7.1 Introduction

Analysis of prokaryotic ribosomal proteins by gel electrophoresis has proved a particularly useful technique. Thus the small size of such proteins (9,000 to 35,000 daltons in E. coli, excluding protein S1) means that mutations in individual proteins may appreciably change their electrophoretic mobility. In early studies separation of ribosomal proteins was achieved by one-dimensional electrophoresis using starch gels (Waller & Harris, 1961; Waller, 1964), although polyacrylamide was quickly adopted since by altering the percentage of acrylamide or the ratio of acrylamide to N,N'-methylenebisacrylamide the properties of gels could easily be changed.

However, electrophoresis in one dimension proved inadequate for detailed analyses in view of the large number of ribosomal proteins in bacteria (52 in E. coli), many of which have similar properties. In E. coli a large proportion are of similar size (Wittmann, 1974) while most have isoelectric points of pH 10 or above (Wittmann & Wittmann-Liebold, 1974). With the development of a two-dimensional (2-D) procedure (Kaltschmidt & Wittmann, 1970a, b) much better separation of ribosomal proteins was obtained. By this means the full complement of proteins in the E. coli ribosome could be identified, although some proteins were not fully resolved unless the two ribosomal subunits were separately examined (Kaltschmidt & Wittmann, 1970b). Resulting from such analyses a nomenclature for the ribosomal proteins of E. coli was derived (Wittmann et al., 1971).

The resolving power of the Kaltschmidt & Wittmann system results from the different pH and gel properties in the two dimensions. Later methods have also incorporated the discovery of Shapiro et al (1967) that in the
presence of SDS the mobility of a protein is primarily governed by its molecular weight. Despite the similar size of many ribosomal proteins, the use of SDS in the second dimension can complement appropriate first dimension conditions in producing a 2-D gel system of high resolution (Martini & Gould, 1971; Hultin & Sjöqvist, 1972; Mets & Bogorad, 1974).

In the present study two methods of 2-D polyacrylamide gel electrophoresis were used to compare ribosomal proteins from mutant and wild-type strains of \textit{B. megaterium}. Electrophoresis by the method of Kaltschmidt & Wittmann (1970a, b) incorporated the modifications of Howard & Traut (1973, 1974), while electrophoresis according to Mets & Bogorad (1974) was performed with only slight modification. This latter technique is particularly sensitive to mutational changes in proteins since at the pH of the first dimension (5.0) all ionisable amino acid side-chains are fully charged.

Although both methods of 2-D gel electrophoresis used in this study were modifications of the original published procedures, they will henceforth be referred to by means of the original authors (viz. Kaltschmidt & Wittmann; Mets & Bogorad).

7.2 Materials and Methods

(a) Materials

The following compounds were used during polyacrylamide gel electrophoresis:

- Acrylamide
- \textit{N}, \textit{N}'-methylenebisacrylamide (MBA)
- \textit{N}, \textit{N}, \textit{N}', \textit{N}'-tetramethylethylenediamine (TEMED)

\textbf{BDH Ltd., U.K.}  
\textbf{Kodak, London}
Bromophenol blue
Diaminoethanetetra-acetic acid (EDTA),
   disodium salt  Fisons Ltd., U.K.
Sodium dodecyl sulphate (SDS)
Urea
Bis[2-hydroxyethyl]imino tris[hydroxymethyl]methane (Bis-Tris)
2[N-morpholino]ethane sulphonlic acid (MES)
Basic fuschin

(b) Ribosomal proteins

Proteins from 70S ribosomes (TP70) or purified ribosomal subunits (TP50, TP30) were prepared as already described (section 3.4). Dried acetone precipitates were taken up in 8M-urea/10mM-DTT and analysed within two weeks of their preparation. Storage was at -20°C.

Before electrophoresis, bromophenol blue (0.004% (w/v) final concentration) or basic fuschin (0.01% (w/v) final concentration) were added to samples of protein as tracker dyes (see below).

(c) Composition of gels and buffers for electrophoresis according to Kaltschmidt & Wittmann

(i) First dimension

Separation gel (pH 8.5) : 36.0g urea
   4.0g acrylamide
   0.1g MBA
   0.8g Na₂ EDTA
   3.2g boric acid
   4.86g Tris-base
   water to 100ml

Samples of the gel solution were de-gassed and polymerised by addition of 1µl TEMED and 2µl of a fresh 10% (w/v) solution of
ammonium persulphate per ml of solution.

Running buffer (pH 8.2):  
1.2g Na₂ EDTA  
(both reservoirs)  
2.4g boric acid  
3.625 Tris-base  
water to 1 l

Conditions: 'Basic' proteins (ie. pK greater than 8.5) were examined by electrophoresis at 300V anode-to-cathode for 10½ hours at 4°C, using basic fuschin as the tracker dye. 'Acidic' proteins (ie. pK less than 8.5) were analysed using the reverse polarity and electrophoresed at 150V for 4½ hours at 4°C, with bromophenol blue as the tracker dye. In both cases the voltage was held constant and each gel drew a current of 3 to 5mA.

(ii) Second dimension

Proteins in the first dimension gel were electrophoresed in the second dimension without prior dialysis against the second dimension running buffer.

Separation gel (pH 4.5):  
360g urea  
180g acrylamide  
2.5g MBA  
53ml glacial acetic acid  
2.7g KOH  
water to 1 l

Samples of the gel solution were de-gassed and polymerised by addition of 3μl TEMED and 30μl of a fresh, 10% (w/v) solution of ammonium persulphate per ml of solution.
Running buffer (pH 4.0): 14g glycine
(both reservoirs) 1.5ml glacial acetic acid
water to 1 l

Conditions: Electrophoresis was at 120V (constant) for 20 hours at 4°C. Each gel drew 50mA under these conditions. Electrophoresis was anode-to-cathode and gels were overlayed with a little 10% (v/v) glycerol solution containing 0.01% (w/v) basic fuschin as a tracker dye. Both 'acidic' and 'basic' proteins migrate towards the cathode under these conditions.

(d) Composition of gels and buffers for electrophoresis according to Mets and Bogorad

(i) First dimension
Separation gel (pH 5.0): 48g urea
4.0g acrylamide
0.1g MBA
1.192g Bis-Tris
water and acetic acid to 100ml and pH 5.0

De-gassed gel solution was polymerised by addition (per ml)
of 5µl TEmED and 5µl of fresh, 10% (w/v) ammonium persulphate solution.

Running buffer (pH 4.0): 2.09g Bis-Tris
(upper reservoir) water and acetic acid to 1 l and pH 4.0

Running buffer (pH 4.5): 17.5g potassium acetate
(lower reservoir) water and acetic acid to 1 l and pH 4.5
Conditions: Electrophoresis was conducted at 80V (constant) for 12 hours at 4°C, each gel drawing about 3mA. The polarity was anode-to-cathode and basic fuschin was used as the tracker dye.

(ii) Second dimension

Separation gel (pH 6.75):  
100g acrylamide  
2.5g MBA  
30g Bis-Tris  
water and HCl to 1 l and  
pH 6.75

De-gassed gel solution was polymerised by addition (per ml) of 1µl TEMED and 2µl of fresh, 10% (w/v) ammonium persulphate solution.

Running buffer (pH 6.5):  
13.7g MES  
(upper reservoir)  
14.6g Bis-Tris  
1.0g SDS  
1ml 1M-DTT  
water to 1 l

Running buffer (pH 6.75):  
5.85g Bis-Tris  
(lower reservoir)  
water and HCl to 1 l and  
pH 6.75

Conditions: Electrophoresis was at 50V (constant) for 6 hours at ambient temperature (cathode-to-anode). Gels were overlayed with a little 10% (v/v) glycerol containing 0.004% (w/v) bromophenol blue as a tracker dye.

(e) Procedure for 2-D gel electrophoresis

Electrophoresis using both gel systems was performed in the same
manner. The polymerising first dimension gel solution was poured to a depth of 12cm in siliconised glass tubes (13cm x 0.4cm), which had been temporarily sealed at the base. The solution was overlayed with water-saturated butanol to ensure a flat gel surface and to hasten polymerisation. When set, the gel surfaces were drained, the gel tubes placed in a standard disc-gel apparatus (Davis, 1964) and each reservoir filled with 500ml buffer. Protein samples (in 50 to 100μl 8M-urea/10mM-DTT) containing tracker dye were layered under the buffer onto the gel surfaces before commencing electrophoresis. Protein was quantitated by measurements of optical density at 230nm and the following quantities loaded: TP30, 0.2 A230 units (≈40μg); TP50, 0.4 A230 units (≈80μg); TP70, 0.3 to 0.6 A230 units (≈60 to 120μg).

When electrophoresis in the first dimension was complete, gels were recovered from the tubes by forcing water between the glass and the gel. Each first dimension gel was then embedded in the second dimension gel as illustrated in Figure 7.1. The former was sandwiched between two glass plates (separated by perspex spacers 1.5mm thick) just below a cut-out section in the rear plate. The whole assembly was clamped together and sealed along the sides and base using plastic tubing (1.8mm diameter). The polymerising second dimension gel solution (approximately 20ml) was poured between the plates such that the first dimension gel was just covered. The gel solution was then overlayed with water-saturated butanol and when polymerisation was complete the tubing was removed.

The apparatus used for electrophoresis was similar to that described by Reid & Bieleski (1968). The gel assembly was clamped vertically to the apparatus, its lower edge resting in the lower reservoir and the cut-out section in the rear glass plate allowing access to the top of the gel by buffer in the upper reservoir. Leakage of buffer between the apparatus and the gel assembly was prevented by inclusion of a rubber mat. Each
Preparation of the second dimension gel during two-dimensional polyacrylamide gel electrophoresis

The first dimension (1st-D) disc gel was embedded in the second dimension (2nd-D) slab gel according to the procedure described in the text.
reservoir contained 350ml of buffer (see above).

After electrophoresis the gel slabs were stained using Coomassie brilliant blue G250 as already described (section 3.7) and finally destained prior to photography.

Analysis of protein samples using the Kaltschmidt and Wittmann systems required the use of two gels (see above) in order to examine all the proteins present. For purposes of presentation, photographs of such pairs of gels (containing the acidic and basic proteins) were spliced together along their common edge. Weakly-staining protein spots, lost during photographic reproduction of gels, have been added where applicable (broken outlines).

7.3 Electrophoretic Examination of Ribosomal Proteins from Wild-Type B. megaterium

(a) Analyses of TP70

Electrophoretic analysis of TP70 from wild-type B. megaterium yielded a reproducible pattern of stained protein spots using either method described, although the relative intensities of particular spots often varied. Figure 7.2 compares typical results and includes for reference published data using E.coli TP70.

Using the system of Mets & Bogorad the array of spots obtained was remarkably similar to that published for E.coli (Figure 7.2a, b). By comparison this was not the case when the Kaltschmidt & Wittmann technique was used (Figure 7.2c, d). Such lack of similarity was first noted by others (Geisser et al., 1973) when comparing the ribosomal proteins of E.coli with those of several Bacilli.

Although up to 54 proteins should (in theory) be visible upon analysis of E.coli TP70, neither electrophoretic technique reveals the full number here (e.g., Figure 7.2b, d). Some proteins stain poorly, while
Legend to Figure 7.2

Analysis of ribosomal proteins by 2D-polyacrylamide gel electrophoresis

Ribosomal proteins were analysed using the techniques of Mets & Bogorad (a, b) and Kaltschmidt & Wittmann (c, d) as described in the text. The polarity of electrophoresis in both the first (horizontal) and second (vertical) dimensions is indicated.

(a) E.coli MRE600 TP70: taken from Kyriakopoulos & Subramanian (1977)

(b) B.megaterium KM (wild-type) TP70 (0.3 $A_{230}$ units)

(c) E.coli B TP70: taken from Kaltschmidt & Wittmann (1970b).

(d) B.megaterium KM (wild-type) TP70 (0.3 $A_{230}$ units)

With the exception of (a) and (c) in this Figure, all other photographs in Figures 7.2 to 7.7 are of gels run by the present author.
Figure 7.3
Legend to Figure 7.3

Analysis of ribosomal proteins by 2-D polyacrylamide gel electrophoresis

Ribosomal proteins from *B. megaterium* wild-type were examined using the Mets & Bogorad gel system:

(a) TP30 (0.2 $A_{230}$ units)

(b) TP50 (0.4 $A_{230}$ units)

(c) Diagram representing the sum of the protein spots visible in (a) and (b)

- O : protein present in TP30
- ● : protein present in TP50
- ⊗ : protein contributed by both TP30 and TP50

(d) TP70 (0.3 $A_{230}$ units)
others may share similar mobilities in both dimensions and might not be clearly resolved. Such 'overlaps' are particularly noticeable using the Mets & Bogorad system. In the present study the number of proteins visible on 2-D gels agreed closely with the numbers routinely observed by others on gels containing E.coli TP70 (Kaltschmidt & Wittmann, 1970b; Kyriakopoulos & Subramanian, 1977). This suggested the absence of serious deficiencies in the protein extraction procedure or the execution of the two electrophoretic techniques.

(b) Examination of TP30 and TP50

Only the technique of Mets & Bogorad was used to examine proteins from purified ribosomal subunits. Close agreement was again noted between the numbers of spots obtained and those expected upon similar analysis of E.coli TP30 and TP50 (Subramanian, 1974; Kyriakopoulos & Subramanian, 1977). Typical results are given in Figure 7.3(a, b) which also shows that superimposition of the patterns of spots observed upon analysis of TP30 and TP50 yielded a pattern (Figure 7.3c) closely similar to that obtained using TP70 (Figure 7.3d). This revealed at least seven overlapping spots in gels containing the latter.

7.4 Comparison of Ribosomal Proteins from Wild-Type and Mutant Strains of B.megaterium

(a) Analysis using the Mets & Bogorad system

Comparison of TP70 preparations from ribosomes of the wild-type and mutant strains of B.megaterium yielded almost identical arrays of spots. However, gels containing TP70 prepared from the mutant strains (Figure 7.4b, e, f; data for PD14 omitted) lacked a single spot present on gels containing wild-type TP70 (Figure 7.4a), while no additional spots were apparent. Any extra spots could have indicated a mutationally altered
Legend to Figure 7.4

Analysis of ribosomal proteins by 2-D polyacrylamide gel electrophoresis

Ribosomal proteins from wild-type and mutant strains of *B. megaterium* were examined using the technique of Mets & Bogorad (see text):-

(a) wild-type TP70 (0.3 \( A_{230} \) units)

(b) mutant MJ1 TP70 (0.3 \( A_{230} \) units)

(c) wild-type TP50 (0.4 \( A_{230} \) units)

(d) mutant MJ1 TP50 (0.3 \( A_{230} \) units)

(e) mutant PD1 TP70 (0.35 \( A_{230} \) units)

(f) mutant MJ5 TP70 (0.3 \( A_{230} \) units)

(g) mutant MJ5 TP70 (0.3 \( A_{230} \) units) plus protein EM-L11 (14\( \mu \)g)

(h) protein EM-L11 (8\( \mu \)g)

Protein EM-L11 was prepared under denaturing conditions and was dissolved in 8M-urea/10mM-DTT solution. The position of the protein on gels containing TP70 or TP50 is indicated (\( \bullet \)).
Figure 7.5

First dimension:

- w.t. SP

Second dimension:

- MJ1 SP
- w.t. TP70
Legend to Figure 7.5

Analysis of 1M-LiCl split-proteins by 2-D polyacrylamide gel electrophoresis

Ribosomal proteins from mutant and wild-type strains of B. megaterium were compared using the technique of Mets & Bogorad (see text):

(a) wild-type 1M-LiCl split-proteins (300 pmol equivalents)

(b) mutant MJ1 1M-LiCl split-proteins (300 pmol equivalents)

(c) wild-type TP70 (0.3 A_{230} units)

The preparations of 1M-LiCl split-proteins were supplemented with urea to give a final concentration of approximately 6M. After addition of basic fuchsin (0.01% (w/v) final concentration) electrophoresis was performed as described above. The position of protein BM-L11 is indicated (✓).
protein with changed electrophoretic mobility. Comparison of TP50 preparations from the wild-type and mutant MJ1 (Figure 7.4c, d) again revealed the same, single difference, indicating that the protein seemingly missing from ribosomes of the mutants was a component of the large subunit. Similar analysis of TP30 from the two strains yielded no obvious differences (data not presented).

The electrophoretic mobility of purified protein BM-L11 was identical with that of the missing protein spot (Figure 7.4h). In addition, when preparations of TP70 from the mutants were supplemented with the purified protein, the missing spot reappeared upon electrophoresis (Figure 7.4g; data for mutants MJ1 and PD1 not given). It was therefore concluded that protein BM-L11 was absent from the ribosomes of the mutant strains or else was altered such that it co-migrated with another protein.

In a final experiment it was demonstrated that protein BM-L11 was also undetectable in the 1M-LiCl split-proteins from mutant MJ1. Figure 7.5 reveals that this was the only apparent difference between such split-protein preparations from the wild-type (a) and mutant (b) strains.

(b) Analysis using the Kaltschmidt & Wittmann system

Comparison of TP70 from mutants MJ1, PD1 and PD14 with that from the wild-type strain again revealed only a single difference (Figure 7.6, data for PD14 omitted). Thus gels containing TP70 from these mutants lacked the densely-staining spot corresponding to protein BM-L11 (Figure 7.6d) although when such preparations were supplemented with the purified protein this spot reappeared upon electrophoresis (data not shown).

The results presented here and in section (a) clearly demonstrate that protein BM-L11 must have considerably altered properties in all these mutant strains. The same conclusion is also supported by the
Figure 7.6

First dimension

w.t.  a

MJ1  b

PD1  c

BM-L11  d
Legend to Figure 7.6

Analysis of ribosomal proteins by 2-D polyacrylamide gel electrophoresis

Ribosomal proteins from wild-type and mutant strains of *B. megaterium* were analysed using the technique of Kaltschmidt & Wittmann (see text):

(a) wild-type TP70 (0.3 $A_{230}$ units in each polarity)

(b) mutant MJ1 TP70 (0.3 $A_{230}$ units in each polarity)

(c) mutant PD1 TP70 (0.45 $A_{230}$ units anode-to-cathode; 0.26 $A_{230}$ units cathode-to-anode)

(d) protein BM-L11 (13µg in each polarity)

Protein BM-L11 was prepared under denaturing conditions and for the purpose of the analysis shown in (d) was dissolved in 8M-urea/10mM-DTT solution. It's position in gels containing TP70 is indicated ( ).
results of electrophoresis of mutant TP70 in cellulose acetate gels (using three buffer systems), when the protein was similarly undetectable (G. Stöffler, personal communication). These data are therefore consistent with the inability of ribosomes from the mutant strains to bind \[^{35}S\text{thio}^\ast\text{strepton}\] with high affinity and also with the ability of protein EM-L11 to reverse this deficiency. However, absence of any form of EM-L11 from the ribosomes of the mutant strains is not implicit in the above results since a variant of the protein may have comigrated with another ribosomal protein during electrophoresis. This possibility was considered less likely by the agreement of the results obtained using the different experimental techniques, but was not discounted until the completion of an immunological examination (Chapter 8).
CHAPTER 8: IMMUNOLOGICAL PROPERTIES OF RIBOSOMAL PROTEINS FROM THE THIOSTREPTON-RESISTANT MUTANTS OF BACILLUS MEGATERIUM

In an attempt to resolve the question of whether ribosomes from the mutant strains of B. megaterium lack protein BM-L11, an immunological examination was undertaken. I am once again most grateful to Dr. Georg Stöffler and co-workers at the Max Planck Institut für Molekulare Genetik, Berlin-Dahlem, Germany, who performed these experiments.

8.1 Materials and Methods

Total ribosomal protein (TP70) was prepared as described above (section 3.4) and details of the immunological techniques used, including the preparation and properties of antisera raised against protein BM-L11 can be found in section 4.5. The data presented in this latter section demonstrate the monospecificity of the antisera.

Total cellular protein from the mutant strains of B. megaterium was prepared by acetic acid extraction. Cells grown in rich medium were harvested in late logarithmic phase and resuspended in 100mM-MgCl₂ solution at approximately 500mg wet weight/ml. After mixing with two volumes of cold glacial acetic acid the suspension was kept at 0°C for one hour, with frequent agitation. The cell debris was removed by centrifugation for 10 minutes at 5000 rpm and the supernatant diluted twenty-fold with water before lyophilisation.

8.2 Analysis of Ribosomal Proteins by Double Immunodiffusion

In section 4.5 it was shown that antisera elicited against protein L11 from E.coli or protein BM-L11 from B. megaterium each cross-reacted exclusively with a single protein when used to examine TP70 from either organism, demonstrating that the two proteins are homologous. Thus, when
TP70 from wild-type *B. megaterium* was examined by double immunodiffusion with antiserum raised against *E. coli* protein L11, a single precipitin band was formed (data not shown). Conversely, TP70 from mutants MJ1, FD1 and FD14 did not cross-react with this antiserum when similarly examined. From this it was inferred that protein EM-L11 is altered in the mutant strains since cross-reacting material related to *E. coli* protein L11 was undetectable in their ribosomes. Further, the inability of the antiserum to precipitate any material present in TP70 from the mutants suggested that protein EM-L11 may be absent from their ribosomes. This possibility was more rigorously tested using antiserum elicited against protein EM-L11, which reacted more strongly with the homologous antigen in *B. megaterium* ribosomes. Although TP70 from wild-type *B. megaterium* reacted strongly with anti-EM-L11 (Figure 8.1a), TP70 from mutants FD1, FD14, MJ1 and MJ5 (Figure 8.1, b to e) gave no cross-reaction over a range of antigen concentrations. These data therefore provide stronger evidence for the contention that ribosomes from the four mutant strains lack protein EM-L11.

Having failed to identify cross-reacting material (related to protein EM-L11) in ribosomes from the mutant strains using antiserum raised against either protein L11 or protein EM-L11, total cellular protein was examined. When an acetic acid extract of mutant FD1 was analysed by double immunodiffusion with antiserum raised against protein EM-L11, cross-reacting material was found (Figure 8.1f). However, only partial serological identity of this material with protein EM-L11 was indicated by the formation of a spur by TP70 from the wild-type strain over the protein from the mutant. Similar cross-reacting material was additionally detected in the S-100 fraction of mutant FD1 and also in acetic acid extracts of mutants MJ1 and FD14 (data not shown). The nature of this material is not yet known.
Figure 8.1
Legend to Figure 8.1

Analysis of ribosomal proteins by double immunodiffusion

These experiments were performed as described in the legend to Figure 4.6.

(a) Reaction of TP70 from wild-type B. megaterium with anti-BM-L11

The centre well contained 260μl anti-B. megaterium protein BM-L11. The peripheral wells contained TP70 from B. megaterium wild-type:

1. 100μg TP70;
2. 150μg TP70;
3. 200μg TP70.

(b) to (e) inclusive: Failure of TP70 from mutant strains of B. megaterium to react with anti-BM-L11

All the centre wells were as in (a). In each case the peripheral wells contained: (1) 100μg TP70; (2) 150μg TP70; (3) 200μg TP70.

The origin of the TP70 preparations was as follows: (b) mutant PD1; (c) mutant PD14; (d) mutant MJ1; (e) mutant MJ5.

(f) Reaction of anti-BM-L11 with material present in total cellular protein from B. megaterium mutant PD1

The centre well was as in (a). The peripheral wells contained:

1. 100μg TP70 from wild-type B. megaterium;
2. 600μg total cellular protein from mutant PD1 (see text).
The ability to detect cross-reacting material in the total cellular protein of the mutant strains reinforces the above conclusion that ribosomes isolated from the mutants are devoid of protein BM-L11. In the absence of such positive immunological data it is not possible to demonstrate unambiguously the absence of a protein from ribosomes. For example, Wittmann et al. (1975) found that TP70 from four mutant strains of *E. coli* gave no cross-reaction with two antisera elicited against protein S20, but when tested with two more similar antisera a weak cross-reaction was observed. This emphasises the danger of basing conclusions on negative immunological results. However, since in the present study material related to protein BM-L11 could be detected in the total cellular protein of the mutant strains, this suggests that had such material been present on ribosomes from these strains then it would also have been detected.

In addition, the above data support the contention that in strains MJ1, PD1 and PD14 the gene for protein BM-L11 is not totally deleted. Thus some form of protein BM-L11 is apparently synthesised in these mutant strains but probably not assembled into their ribosomes.

### 8.3 Examination of Ribosomal Proteins by Modified Immunelectrophoresis

Ribosomal proteins from the wild-type and mutant strains of *B. megaterium* were analysed using modified immunelectrophoresis in an attempt to demonstrate more convincingly that ribosomes from the mutants are devoid of any form of protein BM-L11. This technique is based on the binding of specific antibody to antigen immobilised on a cellulose acetate strip and not only on immunoprecipitation. Thus when examining strong antigens the method has been shown to be about ten times more sensitive than Ouchterlony double immunodiffusion. Even weak antigens such as fragments of ribosomal proteins have been detected using modified immunelectrophoresis (R. Ehrlich and G. Stöffler, personal
Legend to Figure 8.2

Analysis of ribosomal proteins by modified immunoelectrophoresis

Ribosomal proteins were applied to the cellulose acetate strips as follows:

(a) TP70 from *B. megaterium* wild-type, 25μg (tracks 1 and 7) and 12.5μg (tracks 2 and 8); TP70 from *B. megaterium* mutant PD1, 12.5μg (track 3), 25μg (track 4), 37.5μg (track 5) and 50μg (track 6);

(b) TP70 from *B. megaterium* mutant MJ5, 37.5μg (track 1), 25μg (track 2), 12.5μg (track 3); TP70 from *B. megaterium* wild-type, 37.5μg (track 4), 25μg (track 5), 12.5μg (track 6).

Electrophoresis in both cases was for 100 minutes at 250V in buffer containing 0.12M-Bis-Tris¹, 0.12M-MES², 0.05M-β-mercaptoethanol, 8M-urea (pH 6.8). Each strip was rinsed twice in phosphate-buffered saline before soaking overnight in antiserum raised against protein BM-L11. In the case of strip (a) this was an unfractionated γ-globulin preparation, whereas strip (b) was soaked in a specific antibody purified by affinity chromatography (see text). The strips were stained in Coomassie brilliant blue R250 prior to photography.

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¹ Bis-Tris; [bis-(2-hydroxyethyl)imino-tris-(hydroxymethyl) methane]
² MES ; [2-(N-morpholino)ethanesulphonic acid]
communication) although such fragments do not form precipitin bands during double immunodiffusion.

When TP70 from wild-type B. megaterium was examined by modified immunoelectrophoresis using antiserum elicited against protein BM-L11, a single, strong band and a weaker satellite band (see section 4.5) were again observed (Figure 8.2). However, similar examination of TP70 from mutants PD1 (Figure 8.2a, tracks 3,4,5,6), MJ1 and PD14 (data not presented) revealed no precipitin bands, even though the proteins were assessed over a range of concentrations. It was concluded from these data that only the remotest possibility remained of there being material related to protein BM-L11 in the ribosomes from the three mutant strains.

The ribosomal proteins of mutant MJ5 were also examined by modified immunoelectrophoresis (Figure 8.2b). However, the antibody used in these experiments was not the crude γ-globulin preparation used above (Figure 8.2a) but a specific antibody. This had been purified by affinity chromatography over a column on which the ribosomal protein BS-L11 from B. subtilis (i.e. homologous with E. coli protein L11) was immobilised. Use of such specific antibody preparations renders modified immunoelectrophoresis even more sensitive to weak antigens (G. Stöffler, personal communication). Nevertheless, when TP70 from mutant MJ5 was analysed by this means using the specific antibody against protein BM-L11, no precipitin bands were observed. This result demonstrated that ribosomes from mutant MJ5 almost certainly contain no form of protein BM-L11.

8.4 Implications of the Absence of Protein BM-L11 from Ribosomes of the Mutant Strains

As already discussed (section 8.2) it can be difficult to demonstrate
unequivocally the complete absence of a protein from ribosomes. However, in view of the total agreement of results from several different immunochemical and electrophoretic analyses (here and in Chapter 7) and the ability to detect cross-reacting material related to protein EM-L11 in total cellular protein from the mutants, it was concluded that ribosomes from the mutant strains do indeed lack any variant or fragment of protein EM-L11. Further, had such material ever been present in these ribosomes the immunological techniques used would have been expected to detect residual traces. Thus it was also considered most unlikely that some altered form of protein EM-L11 had been lost from the ribosomes during their preparation.

A number of conclusions previously reached in the absence of these arguments may now be re-examined. Thus the hypothesis that protein EM-L11 is able to restore the tight binding of $[^{35}\text{S}]$thiostrepton to ribosomes from the mutants by exchange with some ribosome-bound form of the protein is clearly refuted by the above data. In addition it may now be concluded that the weak interaction of $[^{35}\text{S}]$thiostrepton with ribosomes from mutant MJ1 (section 6.5) occurs in the absence of any form of the 'thiostrepton-binding' protein. Similar interaction of thiostrepton with ribosomes from the other mutant strains may also be inferred from their sensitivity to the drug in vitro (Chapter 5) albeit at high concentrations. From this it may be concluded that components of the \textit{B. megaterium} ribosome other than protein EM-L11 contribute towards its ability to interact with thiostrepton. This conclusion has also been reached using \textit{E. coli} ribosomes, since the binding of $[^{35}\text{S}]$thiostrepton to core-particles lacking protein L11 can be detected by equilibrium dialysis (Bernabeu et al., 1976) while functions exhibited by such particles may be inhibited by the drug (Schrier & Müller, 1975). In this
context it was noted that when ribosomal core-particles from several organisms were treated with an enzyme present in *Streptomyces azureus*, specific methylation of a single residue in the 23S RNA abolished their ability to bind $[^{35}\text{S}]{\text{thiostrepton}}$ when subsequently reconstituted with the split-proteins (Cundliffe & Thompson, 1979; J. Thompson & E. Cundliffe, personal communication). Thus the hypothesis that 23S rRNA alone may be sufficient to account for the interaction of $[^{35}\text{S}]{\text{thiostrepton}}$ with ribosomes from mutant MJ1 was examined, together with the possibility that other ribosomal proteins are involved at the thiostrepton binding site (Chapter 11).

The absence of protein BM-L11 from ribosomes of the mutant strains implies that it can play no indispensable role in protein synthesis, although such roles have been suggested for the homologous protein (L11) in the *E.coli* ribosome (Nierhaus & Montejo, 1973; Schrier & Müller, 1975). This implication is further substantiated by results not yet presented concerning the biological role of protein BM-L11 (Chapter 9).

Similar results to some of those reported in the preceding Chapters have been obtained concurrently using certain thiostrepton-resistant mutants of *B.subtilis* (Goldthwaite & Smith, 1972). These mutants contain ribosomes that do not bind $[^{35}\text{S}]{\text{thiostrepton}}$ (Vince et al., 1976) and synthesis of polyphenylalanine by cell-free extracts of such strains shows marked tolerance to the antibiotic (Pestka et al., 1976). A detailed electrophoretic and immunological analysis of the *B.subtilis* mutants (Wienen et al., 1979) has revealed that they also lack a protein (BS-L11) immunologically related to *E.coli* protein L11. These mutant strains do, however, differ in a number of respects from the mutant strains of *B.megaterium* described in this study (see eg. Smith et
al., 1978), most notably in their ability to revert readily to the thiostrepton-sensitive phenotype. In six such spontaneously-derived revertant strains the protein BS-L11 has been shown to recur (Wienen et al., 1979).
CHAPTER 9: THE ROLE OF PROTEIN BM-L11 IN POLYPEPTIDE CHAIN ELONGATION

9.1 Introduction

Although there have been no previous studies of the biological role of B. megaterium protein BM-L11, possible functions may be inferred from studies of E. coli protein L11, with which protein BM-L11 is homologous (Chapter 4). Protein L11 from E. coli is one of the most extensively studied ribosomal proteins. It binds to 23S ribosomal RNA when prepared under non-denaturing conditions (Littlechild et al., 1977) and has been cross-linked to proteins L7, L12 (Expert-Bezançon et al., 1976) and to proteins L2, L4 and L14 (Kenny & Traut, 1979). Protein L11 is also the most heavily methylated protein in ribosomes from E. coli (Alix & Hayes, 1974; Chang & Chang, 1975) as has been shown to be the case for protein BM-L11 in B. megaterium ribosomes (Cannon & Cundliffe, 1979), although no functional significance has yet been attached to these observations.

The function of E. coli protein L11 has proved difficult to establish, although several experimental approaches have been used in attempts to demonstrate its biological role(s). When a number of proteins were examined for their ability to restore peptidyl transferase activity to inactive ribosomal core-particles, both proteins L11 (Nierhaus & Montejo, 1973; Dietrich et al., 1974) and L16 (Dietrich et al., 1974; Moore et al., 1975) were implicated. Evidence also that antibodies raised against both these proteins could inhibit peptide chain termination in vitro (Tate et al., 1975) supported these results, since the hydrolysis event of the latter process involves the ribosomal peptidyl transferase activity (Caskey et al., 1971). Conversely, other reports have indicated that ribosomal core-particles may possess peptidyl transferase activity in the absence of protein L11 (Ballesta & Vazquez, 1974; Howard & Gordon, 1974) and core-particles largely depleted of the protein are still active.
in release factor-mediated peptide chain termination (Armstrong & Tate, 1978). However, additional evidence supports a role for protein L16 at the peptidyl transferase centre (Bernabeu et al., 1977; Dohme & Fahnestock, 1979). Thus, some of the above conflicting results may be reconciled by the observation that protein L11 apparently aids integration of protein L16 into the ribosome during \textit{in vitro} reconstitution (Alix et al., 1979).

Protein L11 has also been implicated in the GTP hydrolysis carried out by ribosomes in the presence of elongation factor EF G (Schrier & Müller, 1975) and the protein is labelled by photoaffinity analogues of either GDP (Maasen & Müller, 1974) or GTP (Maasen & Müller, 1978) when these are bound to the ribosome in the presence of the elongation factor. Protein L11 has even been proposed to be the ribosomal GTPase itself (Schrier & Müller, 1975). Furthermore, the protein is the \textit{relC} gene product and appears to play a role in the synthesis of guanosine tetra- and penta-phosphates in response to the stringent factor (Parker et al., 1976; see also Chapter 10). Both EF G-dependent GTP hydrolysis and the formation of these regulatory nucleotides are very sensitive to thiostrepton, which is bound tightly by ribosomes only in the presence of protein L11 (Highland et al., 1975b).

There have also been suggestions that protein L11 (in conjunction with other proteins) may be particularly involved in the association of ribosomal subunits, either during the initiation of polypeptide synthesis (Naaktgeboren et al., 1976b) or under a variety of \textit{in vitro} conditions (Kazemie, 1975). Certainly, the results of immune electron microscopy and other topographical studies (review: Stöffler & Wittmann, 1977) have suggested that protein L11 is located in that region of the ribosome where interaction with initiation factor IF 2 elongation factors and release factors occurs.

Concerning the biological role of protein EM-L11, it was noted in
Chapter 5 that ribosomes from mutant MJ1 are significantly less active in the synthesis of polyphenylalanine (in response to poly U) when compared with ribosomes from *B. megaterium* wild-type. Such activities also correlated well with the relative growth-rates of the two strains. Since ribosomes from mutant MJ1 completely lack protein EM-L11 (Chapter 8), this suggested a strategy for determining the function(s) of the protein. Thus ribosomes from the mutant strain, alone or supplemented with protein EM-L11, have been compared *in vitro* with ribosomes from the wild-type. Various partial reactions of polypeptide chain elongation were examined in order to look for a function in which protein EM-L11 could be implicated.

Most of the experiments described above involved the production *in vitro* of protein-deficient ribosomal particles from *E. coli* followed by examination of the effects of reconstitution with some of the missing proteins. The experiments presented in this Chapter are at present unique, since no such manipulation of ribosomes *in vitro* was required because mutant MJ1 yields naturally-occurring protein-deficient ribosomes. Thus the experiments to be described are clearly free from any putative artefacts associated with the above techniques.

9.2 Materials and Methods

(a) The EF Tu-dependent binding of \(^{14}C\)phenylalanyl-tRNA to ribosomes
   (i) Preparation of \(^{14}C\)phenylalanyl-tRNA

\[^{14}C\]phenylalanyl-tRNA(\[^{14}C\]phe-tRNA) was obtained by charging *E. coli* unfractionated tRNA (Sigma) with \[^{14}C\]phenylalanine, using an S-100 fraction from *E. coli* MRE 600 as a source of phenylalanyl-tRNA synthetase and a procedure similar to that of Ravel & Shorey (1971). Incubations for charging contained (in 2.5ml) 50mg tRNA (equivalent
to 24 nmol tRNA\textsuperscript{PHE}, 40 nmol \[^{14}\text{C}]\text{phenylalanine} (513\text{mCi/mmol, 1090 cpm/nmol}), 5 \mu\text{mol ATP, 0.6 \mu mol CTP, 25 \mu mol PEP, 100 units pyruvate kinase, 700\mu l E.coli S-100 fraction (in RS buffer)} and buffer such that the final ionic conditions were 100mM-Tris-HCl (pH 7.4), 13.5mM-MgCl\textsubscript{2}, 60mM-NH\textsubscript{4}Cl, 3mM-\(\beta\)-mercaptoethanol. After 25 minutes at 37°C, the mixture was made 0.2M in sodium acetate by addition of 2M-sodium acetate (pH 5.0) and extracted by mixing with an equal volume of phenol (redistilled and saturated with 0.2M-sodium acetate pH 5.0). The two phases were clarified by centrifugation for 5 minutes at 5,000 rpm and the aqueous phase removed. The phenolic phase was re-extracted in a similar manner using 0.2M-sodium acetate (pH 5.0) and the two aqueous phases pooled. Potassium acetate (2M, pH 5.0) was added to a final concentration of 0.2M and 3 volumes of cold absolute ethanol were added. After mixing and then standing for 60 minutes at -20°C the RNA precipitate was recovered by low-speed centrifugation and taken up in 5ml water. After re-precipitation in a similar manner overnight, the pellet of RNA was dried \textit{in vacuo}, taken up in 500\mu l of 2mM-potassium acetate (pH 5.0) and stored at -70°C in aliquots. Analysis of the content of \(^{14}\text{C} \) radioactivity and the \(A_{260}\) of the solution revealed that typically 30 to 60% of the tRNA\textsuperscript{PHE} had been aminoaacylated.

(ii) \textbf{Quantitation of [EF Tu-GDP]}

The ability of guanine nucleotide bound by EF Tu to exchange readily with free GDP was used to measure the amount of active elongation factor in the preparation of [EF Tu-GDP] used throughout this work. Duplicate assays contained approximately 200 pmol [EF Tu-GDP] and 1.0 nmol \([^ {14}\text{C}]\text{GDP} \text{(293mCi/mmol)} \) in 100\mu l buffer of the following composition: 60mM-Tris-HCl(pH 7.4), 10mM-MgCl\textsubscript{2},
30mM-NH$_4$Cl, 30mM-KCl, 5mM-DTT. After incubation at 37°C for 15 minutes, the mixtures were filtered through Sartorius cellulose nitrate discs (0.45μm pore-size) and washed twice in buffer containing 10mM-Tris-HCl(pH 7.4), 10mM-MgCl$_2$, 10mM-NH$_4$Cl. The radioactivity bound to the dried discs was estimated by liquid-scintillation spectrometry and the mean value of the two assays determined. The amount of EF Tu present in the preparation was calculated using the relationship:

$$\text{pmol active EF Tu} = T + (C - T/p)$$

where $T$ = mean cpm bound.

$C$ = counting efficiency of [U-$^{14}$C]GDP (590 cpm/pmol).

$p$ = pmol [U-$^{14}$C]GDP added.

(iii) Measurement of the EF Tu-dependent binding of [$^{14}$C]phenyl-alanyl-tRNA to ribosomes

The complex [EF Tu·GTP] was prepared by incubating 60 pmol [EF Tu·GDP] with 5 μmol GTP, 0.4 μmol PEP and 1 unit pyruvate kinase for 10 minutes at 37°C in 25μl of buffer containing 40mM-HEPES-KOH (pH 7.6), 14mM-magnesium acetate and 300mM-potassium acetate. After cooling to 0°C, 25 pmol ribosomes (in 10μl RS buffer) and 10μg poly U (in 1μl water) were added together with 15 pmol [$^{14}$C]phe-tRNA (1090 cpm/pmol) in 5μl 2mM-sodium acetate). Thus the final incubation mixture (50μl) allowing for additions of thiostrepton or protein EM-L11 (see legend to Table 9.1) was of the following composition: 20mM-HEPES-KOH(pH 7.6), 3mM-Tris-HCl(pH 7.6), 7mM-magnesium acetate, 3mM-MgCl$_2$, 150mM-KCl and 15mM-NH$_4$Cl. After 2 minutes at 0°C the mixture was diluted with 3ml ice-cold 'wash buffer' (5mM-Tris-HCl(pH 7.6), 10mM-magnesium acetate, 160mM-potassium acetate), filtered through a Sartorius cellulose nitrate
disc (0.45μm pore-size) and washed twice using 3ml of wash buffer.
Radioactivity bound to dried filters was determined by liquid-
scintillation spectrometry and results are expressed as the means
of duplicate determinations, which usually agreed to within ± 2%.
In controls, similar reaction mixtures were not diluted with wash
buffer but instead were heated at 90°C for 30 minutes prior to
filtration through glass-fibre discs (Whatman GF/C). After
washing with 5% (w/v) TCA and drying, radioactivity bound to the
filters was determined as above. Failure to recover significant
amounts of radioactivity on such filters demonstrated that synthesis
of polyphenylalanine was not occurring and that the binding of
[^14C]phe-tRNA to ribosomes was being measured in the absence of
peptide bond formation (data not shown).

(b) Uncoupled hydrolysis of GTP by ribosomes and EF Tu
The method used was based upon one previously described (Ballesta,
1974). The complex [EF Tu-[γ-32P]GTP] was formed by incubating 150 pmol
[EF Tu·GDP] with 2 nmol [γ-32P]GTP (0.1 to 0.2 Ci/mmol), 200 nmol PEP
and 1 unit pyruvate kinase in 60μl buffer containing 60mM-Tris-HCl(pH 7.4),
10mM-MgCl₂, 30mM-NH₄Cl, 30mM-KCl and 5mM-DTT. After 10 minutes at 37°C,
ribosomes (37.5 pmol) and methanol (20% (v/v) final) were added and the
ionic conditions immediately adjusted to give (in 150μl total volume)
25mM-Tris-HCl (pH 7.4), 6mM-MgCl₂, 88mM-NH₄Cl, 12mM-KCl and 2mM-DTT.
Incubation was continued at 37°C and samples (20μl) taken at intervals
into 20μl ice-cold 1M-perchloric acid (PCA) to terminate hydrolysis.
Unhydrolysed, radioactive GTP was removed by addition of 200μl of a 5% (w/v)
Norit suspension (in water) followed by centrifugation at 12,000 x g
for 5 minutes to remove both the Norit and the macromolecular precipitate.
Radioactivity ([γ-32P]phosphate) in the supernatant was estimated by liquid-
scintillation spectrometry.

(c) **Assay of the peptidyl transferase reaction**

The 'fragment reaction' (Monro, 1971) was used to examine the peptidyl transferase activity of ribosomes. This activity is normally expressed only during polypeptide chain synthesis, requiring the presence of aminoacyl-tRNA bound codon-specifically into the ribosomal A-site and peptidyl-tRNA similarly present in the ribosomal P-site (see Chapter 1). However, in the presence of methanol (or ethanol) the requirements for ribosomal peptidyl transferase activity become less stringent and the normal substrates can be replaced by aminoacyl-oligomucleotide 'fragments' or analogues thereof (Monro & Marcker, 1967). The particular reaction used here involved the transfer of N-acetyl-[\(^3\)H]leucine from CACCA-(N-acetyl-[\(^3\)H]leucine) to puromycin (an analogue of the charged 3'-terminus of a tRNA molecule).

(i) **Preparation of the fragment**

*Escherichia coli* unfractionated tRNA was charged as described above except that L-[4,5-\(^3\)H]leucine (55 Ci/mmol) replaced L-[U-\(^14\)C]phenylalanine in the reaction mixture. The tRNA was recovered after phenol extraction by ethanol precipitation as already described (section (a)). The L-[4,5-\(^3\)H]leucyl-tRNA thus obtained was acetylated according to Haenni & Chapellville (1966). Reaction mixtures containing 20μg charged tRNA in 1ml of 0.2M-sodium acetate-acetic acid (pH 5.0) were incubated with 20μl of acetic anhydride at 0°C for 40 minutes. Two further 20μl portions of acetic anhydride were then added, with similar 40 minute incubations after each addition. The mixture was finally made 60% (v/v) in ethanol and the tRNA allowed to precipitate overnight at -20°C. After low-speed centrifugation, the pellet of tRNA was washed in absolute ethanol,
dried in vacuo and taken up in water.

The N-acetyl-[\(^{3}\)H]leucyl-tRNA was next digested for 30 minutes at 37°C with RNase T\(_1\). Reaction mixtures contained 4μg acetylated, charged tRNA and 20μg RNase T\(_1\) in 200μl buffer containing 0.25M-sodium acetate (pH 5.4 with acetic acid) and 2mM-disodium EDTA. After incubation the reaction mixture was diluted to 500μl with water, loaded as a narrow band 15cm long on Whatman No. 52 paper and electrophoresed for 2 hours cathode-to-anode at 2.5kV (ie. 50V/cm) using buffer containing 5%(v/v) acetic acid and 0.5%(v/v) pyridine (pH 3.5). The paper was dried and radioactivity contained in 1cm sections of a small strip removed from one edge was then determined by liquid-scintillation spectrometry. Two peaks of radioactivity were thus located migrating 4cm and 8cm towards the anode. These corresponded to CACCA-(N-acetyl-[\(^{3}\)H]leucine) and UACCA-(N-acetyl-[\(^{3}\)H]leucine) respectively (Monro, 1971). The two strips of paper containing these materials were excised from the electropherogram and eluted separately using 2ml of 0.1M-sodium acetate (pH 5.0 with acetic acid). After lyophilisation the eluted material was redissolved in 200 to 500μl water and stored at -70°C. All the results presented were obtained using CACCA-(N-acetyl[\(^{3}\)H]leucine).

(ii) Assay for the fragment reaction

This was performed according to Monro (1971) with modifications (Cox et al., 1976). All assays were performed at 0°C and each incubation contained 50 μmol ribosomes and 50μg puromycin hydrochloride in 100μl buffer (50mM-Tris-HCl(pH 7.6), 20mM-magnesium acetate, 400mM-KCl. Incubations were supplemented with methanol (50μl) and the reaction was started by addition of 1 μmol (33,000 cpm) of CACCA-(N-acetyl-[\(^{3}\)H]leucine). Termination of the reaction by addition of 200μl 0.25M-NaOH in saturated NaNO\(_3\).
solution was followed by incubation at 37°C for 10 minutes. This treatment eliminated high background values for extractable radioactivity (see below) due probably to the formation of N-acetyl-[\(^{3}\text{H}\)]leucine methyl ester (Cox et al., 1976). Radioactivity transferred to puromycin was estimated following partition of the reaction mixture into 2.5ml scintillation fluid prepared by dissolving 4g Butyl-PBD in a mixture of 800ml toluene, 200ml ethyl acetate and 0.4ml acetic acid. N-acetyl-[\(^{3}\text{H}\)]-puromycin enters the organic phase under these conditions and can therefore be estimated by liquid-scintillation spectrometry of the final mixture (Cox et al., 1976).

(d) EF G-dependent binding of guanine nucleotides to ribosomes

The binding of \([8-^{3}\text{H}]\text{GTP}\) to ribosomes was measured as the formation of \([\text{ribosome-EF G}\text{-guanine nucleotide}]\) complexes in the presence of fusidic acid (Bodley et al., 1970b). Reaction mixtures contained 25 pmol ribosomes in 20μl RS buffer together with protein EM-L11 or thiostrepton (see legend to Table 9.2) to give a total volume of 30μl. To this was added 30μl CB buffer containing 30 pmol EF G, 60 pmol \([8-^{3}\text{H}]\text{GTP}\) (10Ci/mmol) and sodium fusidate (2mM final concentration). The composition of CB buffer was 10mM-Tris-\(\text{HCl}\)(pH 7.6), 20mM-magnesium acetate, 20mM-NH\(_4\)Cl, 1mM-DTT. After 10 minutes at 0°C the mixture was diluted with 3ml CB buffer containing 0.15mM-sodium fusidate and filtered through a Sartorius cellulose nitrate disc (0.45μm pore-size), which was then washed twice with 3ml CB buffer containing 0.15mM-sodium fusidate. Finally, the filters were dried and bound radioactivity estimated by liquid-scintillation spectrometry. Results are expressed as the means of duplicate determinations, which usually agreed to within ± 2%.

Binding to ribosomes of \([8-^{3}\text{H}]\beta, \gamma\text{-imido guanosine 5'-triphosphate}
(GMPNP; 9.4 Ci/mmol) was determined in a similar manner except that sodium fusidate was omitted.

(e) Uncoupled hydrolysis of GTP by ribosomes and EF G

Reaction mixtures (150μl) contained 37.5 pmol 70S ribosomes or 50 to 150 pmol 50S ribosomal subunits (see Figure legends) together with 7.5 nmol[γ-32P]GTP (0.1 to 0.2 Ci/mmol) and 75 pmol EF G, in buffer containing 15mM-Tris-HCl(pH 7.6), 10mM-MgCl2, 80mM-NH4Cl and 1mM-DTT. During incubation at 37°C, samples (20μl) were taken at intervals into 20μl 1M-PCA and processed with Norit as above (section (c) in order to determine the extent of release of [32P]phosphate.

(f) Analysis of the association of ribosomal subunits

The stability of 70S ribosomes to dissociation over a range of Mg2+ concentrations was examined by sucrose density-gradient ultracentrifugation. Ribosomes at a concentration of 10 A260 units/ml (0.22μm) in buffer containing 10mM-Tris-HCl(pH 7.6), 15mM-MgCl2, 100mM-KCl, 3mM-β-mercaptoethanol were warmed for 50 minutes at 37°C in order to promote the association of the ribosomal subunits. Portions were then removed and dialysed overnight at 2°C against 1000 volumes of buffers containing 10mM-Tris-HCl(pH 7.6), 100mM-KCl, 3mM-β-mercaptoethanol and magnesium chloride concentrations of between 5 and 10mM. Samples (approximately 100μl) of each dialysed portion (ie. containing 1.0 A260 unit) were then layered over 5ml 5 to 20% (w/v) sucrose density-gradients made up in buffer identical with the particular dialysis buffer used. After centrifugation for 110 minutes at 2°C and 40,000 rpm in the Beckman SW50.1 rotor, the gradients were pumped through an ISCO UA-5 analyser and absorbance was monitored continuously at 254nm.

Ribosomes from E.coli were used as markers to determine the sedimentation positions of 70S, 50S and 30S particles under these
conditions. The position of 70S particles was deduced from the major peak of absorbance seen when such ribosomes were analysed using density-gradients prepared in buffer containing 10mM-Tris-HCl(pH 7.6), 15mM-MgCl₂, 100mM-KCl, 3mM-β-mercaptoethanol. The use of gradients made up in buffer containing 10mM-Tris-HCl(pH 7.6), 5mM-MgCl₂, 100mM-NaCl, 3mM-β-mercaptoethanol, enabled the positions of 30S and 50S particles to be determined.

(g) Measurement of [³⁵S]thiostrepton-binding by ribosomes

The binding of [³⁵S]thiostrepton to ribosomes under the particular conditions employed for assay of the fragment reaction and the EF Tu-dependent uncoupled hydrolysis of GTP was measured as follows. Standard reaction mixtures were set up as described above, except for the omission of CACCA-(N-acetyl[³H]leucine) from fragment reaction mixtures and the replacement of [γ-³²P]GTP by non-radioactive GTP in GTP hydrolysis mixtures. Each mixture was then supplemented with 10μl[³⁵S]thiostrepton (100 pmol in 50% (v/v) IMSO solution) and incubated for 5 minutes at 0°C (fragment reaction mixtures) or 37°C (GTPase reaction mixtures). After addition of 15μl of a 5% (w/v) suspension of Norit (in RS buffer) followed by centrifugation for 5 minutes at 12,000 x g, the binding of [³⁵S]thiostrepton to ribosomes in samples (150μl) of the supernatants was determined by liquid-scintillation spectrometry.

9.3 The Effect of Protein BM-L11 on the Synthesis of Polyphenylalanine by Ribosomes from Mutant MJ1

As already discussed, ribosomes from thiostrepton-resistant mutant MJ1 were found to be less efficient than those of wild-type B. megaterium in the poly U-dependent synthesis of polyphenylalanine, regardless of whether S-100* fraction from the wild-type (Figure 9.1) or the mutant strain (Figure 9.2) was used. However, reconstitution of ribosomes from
Figure 9.1

Wild-type

Mutant MJ1

Plus or minus BM-L11

Plus or minus BM-L11

Plus BM-L11 and THS

Plus THS

Untreated

Radioactivity (cpm x 10^-3)

Time (min)
Legend to Figure 9.1

**Effect of protein EM-L11 on cell-free synthesis of polyphenylalanine**

Ribosomes (50 pmol in 20μl RS buffer) were incubated with either protein EM-L11 (200 pmol in 5μl RS buffer) or with buffer alone for 5 minutes at 0°C followed by 5 minutes at 20°C. Subsequent incubation for 5 minutes at 20°C with either thioestrepton (0.5μg in 5μl 25% (v/v) DMSO solution) or 25% (v/v) DMSO alone was followed by addition of 20μl S-100 fraction from *B. megaterium* wild-type (0.6 A230 units in RS buffer) and 50μl assay cocktail (see section 5.2). The incorporation of [14C]phenylalanine (513mCi/mmol; 1090 cpm/pmol) into hot TCA-precipitable material was determined during incubation at 37°C as described above (section 5.2). Values plotted represent incorporation in 10μl samples of reaction mixtures.

- □ untreated
- ■ plus thioestrepton
- ○ plus protein EM-L11 (prepared under denaturing conditions)
- ● plus protein EM-L11 (as above) and thioestrepton

**THS**: thioestrepton
Legend to Figure 9.2

Effect of protein EM-L11 on cell-free synthesis of polyphenylalanine

This experiment was performed exactly as described in the legend to Figure 9.1, with the exception that S-100\(^*\) fraction from \textit{B. megaterium} strain MJ1 (0.4 \(A_{230}\) units in 20\(\mu\)l RS buffer) was used.

- □ untreated
- ■ plus thiostrepton
- ○ plus protein EM-L11 (prepared under denaturing conditions)
- ● plus protein EM-L11 (as above) and thiostrepton

THS : thiostrepton
mutant MJ1 with purified protein BM-L11 restored their activity to wild-type levels while rendering synthesis fully sensitive to thiostrepton. In contrast, ribosomes from the wild-type were unaffected by addition of the protein. These results suggested that protein BM-L11 is involved in at least one step during polypeptide chain elongation without being obligatory at any stage. This conclusion was also reached from comparable experiments using ribosomes from mutant FD1, which behaved similarly to those from mutant MJ1 (data not presented).

Even when synthesis of polyphenylalanine was examined using S-30 fractions from the wild-type and mutant strains, such synthesis by the latter could be rendered more efficient (but thiostrepton-sensitive) by the addition of protein BM-L11 (Figure 9.3). Similar treatment of wild-type S-30 fractions had no effect on their activity. These results show that the effect of protein BM-L11 on the activity of washed ribosomes (Figures 9.1 and 9.2) is apparently not an artefact resulting from their mode of preparation, while supporting previous data (Table 6.4) also indicating that protein BM-L11 can interact with ribosomes in S-30 fractions from the mutant.

Various partial reactions of polypeptide chain elongation were next examined, in an attempt to determine if any were stimulated on ribosomes from mutant MJ1 by the presence of protein BM-L11. As these ribosomes were about half as active as those from the wild-type in vitro (Figures 9.1 and 9.2) it was not expected that any ribosomal function would be totally defective. Since it was conceivable that partial impairment of reactions occurring only once on each participating ribosome might escape detection, assay systems in which ribosomes function catalytically rather than stoichiometrically were chosen where possible. It was hoped by this means to amplify any small differences in activity between ribosomes from the wild-type and the mutant, or between those of
Figure 9.3

- **wild-type**
- **mutant MJ 1**
- **plus or minus BM-L11**
- **plus BM-L11**
- **plus or minus BM-L11 plus THS**
- **plus THS**
- **plus BM-L11 and THS**

Radioactivity (c.p.m. x 10^-3) vs time (min)
Legend to Figure 9.3

Effect of protein EM-L11 on the cell-free synthesis of polyphenylalanine using S-30 fractions

Protein EM-L11 (100 pmol in 15μl RS buffer) or RS buffer alone were mixed with 5μl S-30 fraction and incubated for 5 minutes at 0°C followed by 5 minutes at 20°C. Thiostrepton (0.5μg in 5μl 10% (v/v) DMSO solution) or 10% (v/v) DMSO alone were added and incubation continued at 20°C for 5 minutes. Each mixture was supplemented with 25μl assay cocktail (see section 5.2) and incorporation of [14C]phenylalanine (513mCi/mmol; 1090 cpm/pmol) into polyphenylalanine determined as above (section 5.2) during incubation at 37°C. Values plotted represent incorporation in 5μl samples of reaction mixtures. The final nominal ribosome concentration was 0.47 mM (assay mixtures containing wild-type S-30 fraction) or 0.39 mM (assay mixtures containing mutant MJ1 S-30 fraction).

☐ untreated

■ plus thiostrepton

○ plus protein EM-L11 ('nondenatured' preparation)

● plus protein EM-L11 (as above) and thiostrepton

THS : thiostrepton
the mutant in the presence or absence of protein EM-L11.

9.4 Investigation of Functions Associated with Elongation Factor EF Tu

The heterologous system described above (section 9.2(a) was used to measure the EF Tu-dependent binding of \[^{14}\text{C}]\phe-tRNA to ribosomes of \textit{B.\megaterium} in the presence of poly U. Such binding occurred at 20 to 25\% of the level observed using ribosomes from \textit{E.\coli} (ie. in a completely homologous system). Ribosomes from both wild-type \textit{B.\megaterium} and mutant MJ1 behaved similarly in this assay but the binding of \[^{14}\text{C}]\phe-tRNA to the latter was significantly more resistant to thiostrepton (Table 9.1). Reconstitution of ribosomes from mutant MJ1 with protein EM-L11 caused only slight stimulation of \[^{14}\text{C}]\phe-tRNA binding, although their resistance to thiostrepton was abolished. No effect of the protein on the activity of ribosomes from the wild-type was observed using this assay.

For the purpose of examining whether protein EM-L11 may play a role in the GTPase activity associated with EF Tu, the uncoupled hydrolysis of GTP in the presence of ribosomes, EF Tu and 20\% (v/v) methanol was studied. In this reaction ribosomes and EF Tu act catalytically in the hydrolysis of GTP, a process normally tightly coupled to the binding of aminoacyl-tRNA into the ribosomal A-site and therefore occurring stoichiometrically (Ballesta, 1974). By choosing to assay this uncoupled GTPase function of ribosomes and EF Tu, it was hoped to render detection of any small differences between ribosomes from \textit{B.\megaterium} wild-type and mutant MJ1 more likely. In fact ribosomes from these strains were almost indistinguishable in this assay (Figure 9.4) and their activity was unaltered by addition of protein EM-L11. However, as previously noted (Ballesta, 1974) thiostrepton did not inhibit GTP hydrolysis in this system. Thus the effect of protein EM-L11 on the thiostrepton phenotype
<table>
<thead>
<tr>
<th>Additions to ribosomes</th>
<th>EF Tu-dependent binding of $[^{14}C]$phe-tRNA (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wild-type ribosomes</td>
</tr>
<tr>
<td>None</td>
<td>0.77</td>
</tr>
<tr>
<td>thiostreton</td>
<td>0.05</td>
</tr>
<tr>
<td>protein EM-L11*</td>
<td>0.80</td>
</tr>
<tr>
<td>protein EM-L11* and thiostreton</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* prepared under 'denaturing' conditions

Ribosomes (25 pmol in 10µl RS buffer) were incubated with either protein EM-L11 (100 pmol in 5µl RS buffer) or buffer alone, followed by thiostreton (0.2µg in 4µl 25% (v/v) DMSO solution) or 25% (v/v) DMSO alone as described in the legend to Figure 9.1. After cooling to 0°C, [EF Tu·GTP], poly U and $[^{14}C]$phe-tRNA were added and binding of the latter to ribosomes was measured as described in section 9.2(a). The results are presented minus the binding which occurred in the absence of EF Tu. This was 0.15 pmol $[^{14}C]$phe-tRNA per 25 pmol ribosomes from either wild-type or mutant MJ1. Enzymic binding was entirely poly U-dependent (data not shown).

Under the above conditions, ribosomes isolated from *E. coli* (MRE 600) bound 2.62 pmol $[^{14}C]$phe-tRNA in an EF Tu-dependent manner.
Figure 9.4

mutant MJ1

wild-type

pmol [γ-32p]GTP hydrolyzed/pmol ribosomes

pmol

0 2 4 6 8 10

0 2 4 6 8 10

time (min)
Uncoupled hydrolysis of GTP by ribosomes and EF Tu

Ribosomes (37.5 pmol in 15µl RS buffer) were incubated either with protein EM-L11 (150 pmol in 7.5µl RS buffer) or with buffer alone, followed by thiostrepton (1.5µg in 7.5µl 25% (v/v) DMSO solution) or 25% (v/v) DMSO alone, as described in the legend to Figure 9.1. The reaction was started by addition of the ribosomes to the remaining components of the assay (see section 9.2(b)).

- O plus protein EM-L11 (prepared under denaturing conditions)
- ■ plus thiostrepton
- □ no additions of protein EM-L11 or drug

The reaction was entirely dependent on the presence of EF Tu (data not given), but a low rate of hydrolysis was observed by EF Tu alone when ribosomes were omitted (▲).
of ribosomes from the mutant could not be used to demonstrate their interaction with the added protein. To show that ribosomes from MJ1, reconstituted with protein BM-L11, did not lose the protein in the presence of methanol, their ability to bind [\(^{35}\)S]thiostrepton under these conditions was assessed using the Norit adsorption technique (see section 9.2(g). By this means the reconstituted particles were observed to bind [\(^{35}\)S]thiostrepton quantitatively (data not shown), indicating that protein BM-L11 remained bound to the ribosome under the particular assay conditions employed. It was therefore concluded that protein BM-L11 was not required for either of the EF Tu-dependent functions assayed.

9.5 Peptidyl Transferase Activity

Ribosomes from wild-type \textit{B. megaterium} and mutant MJ1 were equally efficient in the catalysis of N-acetyl-\([\(^3\)H]leucyl\) puromycin formation (Figure 9.5) during the fragment reaction described above (section 9.2(c). Activity in both cases was inhibited by sparsomycin, a specific inhibitor of peptide bond formation (Goldberg & Mitsugi, 1967), but not by thiostrepton. In addition, the presence of protein BM-L11 did not influence the activity of ribosomes from either source. By use of the Norit adsorption technique, the binding of [\(^{35}\)S]thiostrepton to ribosomes from mutant MJ1, reconstituted with protein BM-L11, was measured under the assay conditions employed and shown to occur quantitatively (data not presented). Thus inability of protein BM-L11 to influence the activity of ribosomes from mutant MJ1 in this system could not be attributed to failure of the protein to interact with the ribosomes. From these results it was concluded that there was no apparent effect of protein BM-L11 upon the ability of ribosomes to catalyse peptide bond formation.
Figure 9.5

- Wild-type
  - ± BM-L11 or THS
  - + sparsomycin

- Mutant MJ 1
  - ± BM-L11 or THS
  - + sparsomycin

N-acetyl-[3H]leucyl puromycin formed (c.p.m. x 10^3)

Time (mins)
Legend to Figure 9.5

Peptidyl transferase activity of ribosomes

Ribosomes (50 pmol in 20 μl RS buffer) were incubated with protein EM-L11 (100 pmol in 5 μl RS buffer) or with buffer alone as in the legend to Figure 9.1. Ribosomes which had not received protein EM-L11 were then incubated for 5 minutes at 20°C with sparsomycin (10 μg in 5 μl 25% (v/v) DMSO), thioestrepton (2.5 μg in 5 μl 25% (v/v) DMSO) or 25% (v/v) DMSO alone, while 25% (v/v) DMSO was added to ribosomes which had received the protein. After cooling to 0°C, peptidyl transferase activity was measured as described above (section 9.2(c)). Each point in this Figure was obtained from a separate incubation.

■ ribosomes plus thioestrepton

▲ ribosomes plus sparsomycin

○ ribosomes plus protein EM-L11 (prepared under 'nondenaturing' conditions)

□ ribosomes alone

THS : thioestrepton
9.6 Examination of Functions Dependent upon Elongation Factor EF G

Elongation factor EF G normally functions together with GTP during the translocation reaction of protein synthesis, in the course of which the GTP is hydrolysed to GDP and inorganic phosphate. Translocation can be assayed directly in vitro by the use of puromycin, which reacts with peptidyl-tRNA only when the latter occupies the ribosomal P-site after translocation has occurred (Haenni & Lucas-Lenard, 1968). However, since translocation of peptidyl-tRNA in this assay can occur only once per active ribosome it was deemed unlikely that partial impairment of this function would be detectable. Thus the interactions of ribosomes, EF G and guanine nucleotides were studied as an alternative probe of translocation.

The binding of EF G and guanine nucleotides to 70S ribosomes from both wild-type B. megaterium and mutant MJ1 was first examined, using two different assay systems. Initially the formation of complexes between ribosomes, EF G and \( [\text{8-}^3\text{H}]\text{GTP} \) in the presence of fusidic acid was studied. This antibiotic allows each active ribosome to hydrolyse one molecule of GTP before stabilising complexes of the form [ribosome·EF G·GDP] (Brot et al., 1971). After preliminary experiments had revealed that EF G was not limiting to activity under the chosen assay conditions (data not given), the formation of such complexes by ribosomes from the wild-type and mutant MJ1 was examined. Both types of ribosome showed similar activity in this assay except that thioestrepton was much more inhibitory with ribosomes from the wild-type (Table 9.2). Activity of ribosomes from either source remained unchanged by addition of protein BM-L11, although this treatment restored thioestrepton-sensitivity to ribosomes of the mutant.

Similar observations to these were made when the EF G-dependent binding to ribosomes of GMPPNP (a non-hydrolysable GTP analogue) was examined. These experiments were performed in the absence of fusidic
Table 9.2

The formation of \([\text{ribosome\textcdot}\text{EF G}[^{3}\text{H}]{\text{GDP}}]\) complexes in the presence of

fusidic acid

<table>
<thead>
<tr>
<th>Additions to ribosomes</th>
<th>pmol [ribosome\textcdot}\text{EF G}[^{3}\text{H}]{\text{GDP}}] complex formed per 100 pmol ribosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wild-type ribosomes</td>
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<tr>
<td>none</td>
<td>29.9</td>
</tr>
<tr>
<td>thiostrepton</td>
<td>1.2</td>
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<tr>
<td>protein EM-L11*</td>
<td>30.1</td>
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<tr>
<td>protein EM-L11* and thiostrepton</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* prepared under denaturing conditions

Ribosomes (25 pmol in 10\(\mu\)l RS buffer) were incubated either with protein EM-L11 (100 pmol in 5\(\mu\)l RS buffer) or with buffer alone, followed by thiostrepton (0.25\(\mu\)g in 5\(\mu\)l 25\% (v/v) DMSO solution) or 25\% (v/v) DMSO alone, as described in the legend to Figure 9.1. After addition of other assay components as a cocktail, the extent of formation of \([\text{ribosome\textcdot}\text{EF G}[^{3}\text{H}]{\text{GDP}}]\) complexes was determined (see section 9.2(d)). Such complex formation was entirely dependent on the presence of both ribosomes and EF G (data not shown).
Table 9.3

<table>
<thead>
<tr>
<th>Additions to ribosomes</th>
<th>pmol $[^3]H$GMPPNP bound per 100 pmol ribosomes</th>
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<td></td>
<td>wild-type ribosomes</td>
</tr>
<tr>
<td>none</td>
<td>13.2</td>
</tr>
<tr>
<td>thioestrepton</td>
<td>3.6</td>
</tr>
<tr>
<td>protein EM-L11*</td>
<td>13.6</td>
</tr>
<tr>
<td>protein EM-L11* and thioestrepton</td>
<td>4.4</td>
</tr>
</tbody>
</table>

* prepared under denaturing conditions

Ribosomes (25 pmoles) were incubated with or without protein EM-L11 (100 pmoles) and then in the presence or absence of thioestrepton (0.125μg), as described in the legend to Table 9.2. After addition of $[^3]H$GMPPNP and EF G, binding of $[^3]H$GMPPNP to ribosomes was determined (see section 9.2(d) and, in controls, was found to be entirely dependent upon the presence of EF G (data not given).
acid and again EF G was shown not to be limiting to activity in this assay (data not given). Although the activity of ribosomes in this assay was somewhat lower, ribosomes from both the wild-type and mutant MJ1 bound GMPPNP to similar extents (Table 9.3). However, binding by ribosomes from the mutant was considerably less sensitive to thiostrepton. This difference was eliminated by reconstitution of ribosomes from mutant MJ1 with protein BM-L11, while their overall ability to bind GMPPNP remained almost unchanged. Similar treatment of ribosomes from the wild-type produced no significant effect.

From the above data it was tentatively concluded that protein BM-L11 is not important for the EF G-dependent binding of guanine nucleotides to ribosomes. However, since both the above assays measured a ribosomal function that occurs stoichiometrically, they suffer from the same drawback discussed in relation to the direct measurement of translocation. Thus the catalytic hydrolysis of GTP by EF G and ribosomes, uncoupled from the translocation reaction with which it is normally associated, was also examined. Using components from E.coli this reaction is supported by either 70S ribosomes or 50S ribosomal subunits alone, although the latter are stimulated by the presence of 30S ribosomal subunits (Nishizuka & Lipmann, 1966b). It can be seen from Figures 9.6 and 9.7 respectively that 70S ribosomes or 50S subunits from mutant MJ1 were considerably impaired in their ability to catalyse the EF G-dependent uncoupled hydrolysis of GTP, when compared with equivalent ribosomal particles from wild-type B.megaterium. The 70S ribosomes and 50S subunits from the mutant strain also showed much more resistance to thiostrepton in this assay. However, reconstitution of such ribosomes and subunits with protein BM-L11 stimulated their activity to wild-type levels in either case, while conferring full sensitivity to thiostrepton. The specificity of this effect was shown by the use of 1M-LiCl split-proteins. Thus
Figure 9.6

Wild-type

Mutant MJ 1

Pmol [γ-32P]CTP hydrolyzed/ Pmol ribosomes

Time (min)

200

150

100

50

0
Legend to Figure 9.6

Effect of protein EM-L11 on the uncoupled hydrolysis of GTP by 70S ribosomes and EF G

Ribosomes (37.5 pmol in 15μl RS buffer) were incubated either with protein EM-L11 (150 pmol in 7.5μl RS buffer) or with buffer alone, followed by thiostrepton (0.75μg in 7.5μl 25% (v/v) DMSO solution) or 25% (v/v) DMSO alone, as described in the legend to Figure 9.1. The reaction was started by addition of the ribosomes to the other components of the assay (see section 9.2(e)). Hydrolysis was entirely dependent upon both ribosomes and EF G (data not given).

- O ribosomes plus protein EM-L11 (prepared under denaturing conditions)
- ■ ribosomes plus thiostrepton
- ● ribosomes plus protein EM-L11 (as above) and thiostrepton
- □ ribosomes alone

THS : thiostrepton
Legend to Figure 9.7

Effect of protein BM-L11 on the uncoupled hydrolysis of GTP by 50S ribosomal subunits and EF G

50S ribosomal subunits (50 pmol in 40μL RS buffer) were incubated either with protein BM-L11 (100 pmol in 5μL RS buffer) or with buffer alone, followed by thiostrepton (0.375μg in 7.5μL 25% (v/v) IMSO solution) or 25% (v/v) IMSO alone, as described in the legend to Figure 9.1. The reaction was started by addition of the subunits to the other components of the assay (see section 9.2(e)). Hydrolysis was entirely dependent upon both 50S ribosomal subunits and EF G (data not given).

- ribosomes plus protein BM-L11 (prepared under 'nondenaturing' conditions)
- ribosomes plus thiostrepton
- ribosomes plus protein BM-L11 (as above) and thiostrepton
- ribosomes alone

THS : thiostrepton
mutant MJ1 split-proteins had no effect on the activity of ribosomes from mutant MJ1 in this assay, while split-proteins from the wild-type closely mimicked the effect of purified protein EM-L11 (data not presented).

The above results therefore demonstrate a direct effect of protein EM-L11 upon EF G-dependent hydrolysis of GTP by ribosomes from B. megaterium. Since experiments using both 70S ribosomes and 50S ribosomal subunits yielded similar results, any indirect effect of protein EM-L11 via promotion of ribosomal subunit association may be discounted. In fact, addition of 30S subunits from wild-type B. megaterium to 50S subunits from either wild-type or mutant produced a two-fold stimulation of GTPase activity in both cases (Figure 9.8). This suggested that 50S subunits from the two strains were equally able to associate with 30S subunits. However, 30S-50S couples containing MJ1 50S subunits were considerably less active than those involving wild-type 50S subunits, unless first supplemented with protein EM-L11 (Figure 9.8). Thus the lower activity in this assay of 50S subunits from mutant MJ1 paralleled that observed both with intact 70S ribosomes and 30S-50S couples. These results confirmed that the effect of protein EM-L11 was indeed not mediated by some putative effect on the association of ribosomal subunits, while their consistency affirmed that the protein was required for maximal activity in this assay.

It may be noted from Figure 9.6 that a thioestrepton-insensitive GTPase was evident in some of these experiments. The nature of this activity is unclear since it was manifested only when ribosomes and EF G were present together and not by either preparation alone. However, this activity was diminished by the conversion of 70S ribosomes to 50S ribosomal subunits (compare Figures 9.6 and 9.7) and was not subsequently recovered on the re-addition of 30S subunits (data not shown).
Legend to Figure 9.8

Effect of 30S ribosomal subunits on uncoupled GTP hydrolysis by 50S ribosomal subunits and EF-G

50S ribosomal subunits (150 pmol in 30μl RS buffer) were incubated either with protein BM-L11 (230 pmol in 2.5μl RS buffer) or with buffer alone for 5 minutes at 0°C followed by 5 minutes at 20°C. Some incubation mixtures were subsequently supplemented with 30S ribosomal subunits from wild-type B. megaterium (300 pmol in 15μl RS buffer), prior to addition of the other components of the assay (see section 9.2 (e)).

- □ 50S subunits alone
- ■ 50S plus 30S subunits
- ○ 50S subunits plus protein BM-L11 (prepared under non-denaturing conditions)
- ● 50S subunits plus protein BM-L11 (as above) plus 30S subunits
- ▲ 30S subunits alone
The results presented so far in this Chapter demonstrate that protein BM-L11 clearly has a role in the GTP hydrolysis catalysed by ribosomes and EF G, but apparently not in any of the other reactions studied. However, stimulation by protein BM-L11 of any other reaction assayed in which ribosomes functioned stoichiometrically without appearing to require the protein cannot be eliminated for the reasons already discussed. Such reactions include the EF Tu-dependent binding to ribosomes of [\(^{14}\text{C}\)]phe-tRNA and the EF G-dependent binding to ribosomes of guanine nucleotides. Since the rate of peptidyl transferase measured during the fragment reaction may be limited by the binding of the substrates, it too may fall into this category. However, there is no reason to suppose, from the above data, that protein BM-L11 plays a role in any of these reactions.

Although the GTPase activities dependent upon EF Tu and EF G appear to involve a single ribosomal site (Cundliffe, 1971; Modolell et al., 1971), these two activities are clearly dissimilar. Thus only the EF Tu-dependent activity is rendered insensitive to thioestrepton by methanol (Ballesta & Vazquez, 1972b) and EF Tu (but not EF G) can hydrolyse GTP in the absence of ribosomes, if kirromycin (Wolf et al., 1974) or ribosomal proteins L7 and L12 are present (Donner et al., 1978). The data presented in this study also indicate that the GTPase activities of these elongation factors differ, since protein BM-L11 is seemingly involved in the uncoupled hydrolysis of GTP catalysed by only one of the two.

9.7 Association of Ribosomal Subunits

Protein BM-L11 has been shown to promote at least one step during polypeptide chain elongation on B. megaterium ribosomes. Although in E. coli dissociation and association of ribosomal subunits is not thought to occur during this process, a role for protein L11 (in conjunction
Figure 9.9
Legend to Figure 9.9

Lack of effect of protein EM-L11 on the stability of 70S ribosomes from mutant MJ1 to dissociation at low magnesium ion concentrations

Ribosomes from *B. megaterium* mutant MJ1 (650 pmol in 70μl RS buffer) were incubated for 5 minutes at 0°C and then 5 minutes at 20°C in the presence or absence of 1.2 nmol protein EM-L11 (prepared under 'nondenaturing' conditions). The mixtures were then each diluted (to give a ribosome concentration of 0.22μM) using buffer containing 15mM-MgCl₂ and warmed at 37°C to promote the association of ribosomal subunits (see section 9.2(f)). Samples of each mixture were dialysed into buffers containing 5.0 to 10.0 mM-MgCl₂ and analysed by sucrose density-gradient ultracentrifugation as described in section 9.2(f). The sedimentation positions marked were determined using ribosomes from *E. coli* (see text).

1.a to 1.e : ribosomes alone
2.a to 2.e : ribosomes plus protein EM-L11

Samples from each batch of dialysed ribosomes were examined for their ability to bind [³⁵S]thiostrepton, using the 'Norit adsorption' technique (data not given). This confirmed that protein EM-L11 had remained bound to the mutant ribosomes analysed on gradients 2 (a) to 2 (e).
with other proteins) in the association of ribosomal subunits has been proposed (Kazemie, 1975). It was therefore decided to examine the stability to dissociation of 70S ribosomes from wild-type *B. megaterium* and mutant MJ1 over a range of Mg\(^{2+}\) concentrations (from 5 to 10mM). This was assessed as described above (section 9.2(f) by sucrose density-gradient ultracentrifugation. At each particular Mg\(^{2+}\) concentration analysis of ribosomes from the two strains revealed a closely similar mixture of subunits, loose couples (approximately 60S) and tight 70S couples (see Hapke & Noll, 1976), while in both cases decreasing magnesium concentration promoted a net dissociation of 70S ribosomes (data not shown). When ribosomes from mutant MJ1, alone or supplemented with protein BM-L11 were compared by this procedure (Figure 9.9), no significant effect of the protein on the distribution of particles in the gradients was observed at any Mg\(^{2+}\) concentration. It was therefore concluded that ribosomal subunits from *B. megaterium* wild-type and mutant MJ1 do not notably differ in their ability to associate or dissociate in response to changes in the ionic environment. Such a conclusion is consistent with data presented above (Figure 9.8) showing that 30S ribosomal subunits were capable of stimulating EF G-dependent uncoupled GTPase activity by 50S subunits from either strain to similar extents.

9.8 The Ability of *E. coli* Ribosomal Protein L11 to Mimic the Effects of *B. megaterium* Protein EM-L11 on the Activity *in vitro* of Ribosomes from Mutant MJ1

Results presented earlier in this dissertation have demonstrated that *E. coli* protein L11 and *B. megaterium* protein EM-L11 are serologically related although not identical (Chapter 4). Nevertheless, *E. coli* protein L11 can promote the binding of \(^{35}\)Sthiostrepton to both wild-type
Figure 9.10

Wild type and mutant MJ1, with and without L11 or THS treatment, show different radioactivity profiles over time. The graph plots radioactivity (c.p.m. x 10^3) against time (min) from 0 to 60 minutes.
Legend to Figure 9.10

Effect of protein L11 on cell-free synthesis of polyphenylalanine

This experiment was performed exactly as described in the legend to Figure 9.1 with the substitution of \textit{E. coli} protein L11 for \textit{B. megaterium} protein BM-L11. Protein L11 was prepared under denaturing conditions.

- untreated
- plus thiostrepton
- plus \textit{E. coli} protein L11
- plus \textit{E. coli} protein L11 and thiostrepton

THS : thiostrepton
Figure 9.11

[Graph showing the hydrolysis of \([\gamma^32P]GTP\) in wild-type and mutant MJ1. The x-axis represents time (min) and the y-axis represents pmol \([\gamma^32P]GTP\) hydrolysed/pmol ribosomes. There are different conditions shown: plus or minus L11, plus L11, plus THS, plus L11 and THS, untreated.]
**Legend to Figure 9.11**

**Effect of protein L11 on the uncoupled hydrolysis of GTP by ribosomes and EF G**

This experiment was performed exactly as described in the legend to Figure 9.6, substituting *E. coli* protein L11 for *B. megaterium* protein EM-L11. Protein L11 was prepared under denaturing conditions.

- □ untreated
- ■ plus thioestrepton
- ○ plus *E. coli* protein L11
- ● plus *E. coli* protein L11 and thioestrepton

**THS : thioestrepton**
Figure 9.12

- pmol of [γ-32P]GTP hydrolysed/pmol 50S ribosomal subunits

Wild-type and mutant MJ1 treated with different conditions:
- plus or minus L11
- plus THS
- plus L11 and THS
- untreated

Graph showing time (min) on the x-axis and pmol [γ-32P]GTP hydrolysed/pmol 50S ribosomal subunits on the y-axis.
Legend to Figure 9.12

Effect of protein L11 on the uncoupled hydrolysis of GTP by 50S ribosomal subunits and EF G

This experiment was performed exactly as described in the legend to Figure 9.7, with the substitution of *E.* coli protein L11 for *B.* megaterium protein EM-L11. Protein L11 was prepared under denaturing conditions.

- □ untreated
- ■ plus thiostrepton
- ○ plus *E.* coli protein L11
- ■ plus *E.* coli protein L11 and thiostrepton

THS: thiostrepton
B. megaterium 2M-LiCl core-particles and to ribosomes from mutant MJ1 (Table 6.5), indicating that the two proteins must be quite similar. Thus inferences concerning the role of protein L11 in the E. coli ribosome may justifiably be made from observations of the function(s) of protein BM-L11 in ribosomes from B. megaterium.

However, in order to substantiate such inferences, the ability of E. coli protein L11 to stimulate the activity of ribosomes from mutant MJ1 was examined in each of the assay systems where protein BM-L11 had been shown to have an effect.

As seen from Figure 9.10, protein L11 was perfectly able to restore the efficiency of polyphenylalanine synthesis by ribosomes from mutant MJ1 to the level observed using ribosomes from the wild-type. At the same time the protein abolished the resistance of such ribosomes to thioseptone, but had no effect on polyphenylalanine synthesis by ribosomes from the wild-type. These results were therefore in complete agreement with those using the homologous protein BM-L11 (Figure 9.1). Furthermore, protein L11 was also able to stimulate the EF G-dependent uncoupled hydrolysis of GTP by either 70S ribosomes (Figure 9.11) or 50S ribosomal subunits (Figure 9.12) from mutant MJ1, while abolishing their resistance to thioseptone. Again, no effect of the protein was noted in control experiments using components from B. megaterium wild-type. Thus E. coli protein L11 was able to mimic closely the effects of B. megaterium protein BM-L11 in all three cases; the specificity of this phenomenon was revealed by the complete inability of E. coli ribosomal proteins L7, L10 and L12 to influence GTPase activity by 50S ribosomal subunits from mutant MJ1 in the above assay (data not shown). It was therefore concluded that in addition to showing a serological homology, E. coli protein L11 and B. megaterium protein BM-L11 are functionally related.
In conclusion, it is proposed that protein BM-L11 has a role in the EF G-dependent GTPase activity of the *B. megaterium* ribosome that normally occurs during the translocation event of peptide chain elongation. This does not imply that the protein is without function during other steps of protein synthesis. Since protein BM-L11 is related serologically and functionally to *E. coli* ribosomal protein L11, a role for the latter protein in the hydrolysis (as opposed to the binding) of GTP in the presence of ribosomes and EF G is also strongly suggested. Such a role has already been proposed by others (Schrier & Müller, 1975; Maassen & Müller, 1978) using two quite different experimental approaches from that described in this study. Although core-particles lacking protein L11 showed some GTPase activity in the presence of EF G (see Table 1 of Schrier & Müller, 1975) these authors have proposed that protein L11 may be the *E. coli* ribosomal GTPase. The present study has shown, however, that protein BM-L11 cannot be the *B. megaterium* ribosomal GTPase *per se*. Thus unless the residual GTPase activity of ribosomes from mutant MJ1 represents a catalytic site unique to *Bacillus*, *E. coli* protein L11 may also be dispensable for GTP hydrolysis and any other essential ribosomal function.
CHAPTER 10: THE ROLE OF PROTEIN EM-L11 IN THE STRINGENT CONTROL OF RNA SYNTHESIS

10.1 Introduction

In stringent strains of bacteria the synthesis of stable RNA is coupled to the rate of protein synthesis. Amino acid starvation of such strains results in the appearance (Cashel, 1969) of the regulatory nucleotides guanosine 5'-diphosphate, 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate, 3'-diphosphate (pppGpp). The interaction of these guanosine polyphosphates with the RNA polymerase (Travers, 1976; Van Ooyen & Gruber, 1976) specifically inhibits the transcription of genes coding for rRNA (Lazzarini & Dahlberg, 1971) and evidently also ribosomal proteins (Dennis & Nomura, 1974) and tRNA (Primakoff & Berg, 1970; Ikemura & Dahlberg, 1973). In response to starvation for an essential amino acid, synthesis of ppGpp and pppGpp occurs on the ribosome by means of pyrophosphate transfer from ATP to the 3'-OH of GDP or GTP respectively (Haseltine et al., 1972; Sy & Lipmann, 1973). This is catalysed by the stringent factor (Haseltine et al., 1972), an enzyme which is loosely attached to ribosomes (Block & Haseltine, 1975) and which is activated following codon-specific binding of deacylated tRNA into the ribosomal A-site (Pedersen et al., 1973; Haseltine & Block, 1973). The stringent factor is the product of the relA gene (Stent & Brenner, 1961).

Bacterial strains which do not exhibit the stringent response are termed 'relaxed'. In E.coli at least three types of mutation, of which two have been characterised, lead to the relaxed phenotype. Thus relA mutations result in the production of defective stringent factor (Block & Haseltine, 1973), whereas relC strains contain mutations in the structural gene (rplK) for ribosomal protein L11 (Parker et al., 1976).
In consequence, the ribosomes of \textit{relC} mutants are impaired in the ability to synthesise ppGpp and pppGpp but such strains contain normal stringent factor (Parker et al., 1976; Friesen et al., 1974). This involvement of protein L11 in the stringent response is also supported by evidence from certain thiostrepton-resistant mutants of \textit{B. subtilis} (Goldthwaite & Smith, 1972). These mutants have the relaxed (\textit{rel}⁻) phenotype (Smith et al., 1978) and their ribosomes appear to lack a protein (BS-L11) immunologically related to \textit{E. coli} protein L11 (Wienen et al., 1979). Ribosomes from these mutants support the synthesis of the regulatory nucleotides \textit{in vitro} at only 10% of the level observed using ribosomes from the wild-type strain, but spontaneous reversion to thiostrepton sensitivity results in the reappearance of protein BS-L11 and concurrent recovery of the \textit{rel}⁺ phenotype both \textit{in vivo} and \textit{in vitro}.

Since ribosomes prepared from \textit{B. megaterium} mutant MJ1 lack completely a protein (BM-L11) also homologous with \textit{E. coli} protein L11 (Chapter 8), the control of RNA synthesis in this mutant was examined. Having demonstrated that the strain has the \textit{rel}⁻ phenotype, the ability of its ribosomes to support guanosine polyphosphate production \textit{in vivo} and \textit{in vitro} was studied.

10.2 Methods

(a) Measurement of RNA and protein synthesis \textit{in vivo}

Synthesis of RNA was measured as the incorporation of \([5-\text{³H}]\)uridine into trichloroacetic acid (TCA)-precipitable material. For this purpose the \([5-\text{³H}]\)uridine obtained from the Radiochemical Centre, Amersham (28Ci/mmol) was diluted with non-radioactive uridine to give a specific activity of 8.75mCi/mmol. Bacterial cells were grown at 37°C in 10ml of M9 medium containing 0.2% (w/v) glucose and 0.2% (w/v) casamino acids. With cultures in exponential growth at about 2 x 10⁷ cells/ml (section
2.4), experiments were started by the addition of \([5-\text{H}]\)uridine at a radioactive concentration of 2\(\mu\)Ci/ml. Samples (0.5ml) were periodically removed into 1.0ml ice-cold 10\%(w/v) TCA solution and mixed well. Precipitates were filtered through glass-fibre discs (Whatman GF/C) and washed twice with 3ml of cold, 5\%(w/v) TCA solution. Radioactivity bound to the dried filters was determined by liquid-scintillation spectrometry.

Synthesis of protein was measured similarly in parallel cultures (10ml each) labelled with 50\(\mu\)Ci of L-[4, 5-\text{H}]leucine (55 Ci/mmol). TCA precipitates were heated at 90\(^\circ\)C for 30 minutes prior to filtration.

Sodium pseudomomate (100\(\mu\)g/ml final concentration) was added to certain cultures as indicated in the Figure legends.

(b) Measurement of ppGpp and pppGpp production in vivo

Bacterial cells were grown at 37\(^\circ\)C in low phosphate medium (see section 2.2). When cultures (2.5ml) in exponential growth had reached a density of approximately 1.5 \(\times\) 10\(^7\) cells/ml, carrier-free \([\text{\textsuperscript{32}}\text{P}]\)phosphate was added at a radioactive concentration of 105\(\mu\)Ci/ml. At suitable times culture samples of 50\(\mu\)l were placed on ice and mixed with an equal volume of ice-cold 2M-formic acid. After centrifugation at 12,000 \(\times\) g for 1 minute, 10\(\mu\)l samples of the supernatants were spotted out onto polyethyleneimine(PEI)-cellulose MN300-coated thin-layer chromatography plates (Camlab, U.K.). These had been soaked in water for at least 30 minutes and dried before use. The chromatograms were developed in 1.5M-KH\(_2\)PO\(_4\) (pH 3.4) and the location of the various nucleotides determined by autoradiography overnight. The \(R_f\) values obtained for the nucleotides were similar to those observed by Cashel (1969). Radiolabelled spots corresponding to nucleotides were cut out and their \(\text{\textsuperscript{32}}\text{P}\) content determined by liquid-scintillation spectrometry.
In some experiments, sodium pseudomonate (100 μg/ml final concentration) was added to cultures (see legend to Figure 10.3).

(c) Synthesis of ppGpp and pppGpp in vitro

Synthesis of the guanosine polyphosphates in vitro was measured in a homologous system using partially-purified stringent factor from B. megaterium wild-type, which was prepared using the procedure of Block & Haseltine (1975) with slight modification. Ribosomes (approximately 0.5 g) were isolated from freshly-grown cells by centrifugation through RS buffer containing 40% (w/v) sucrose and then washed once through RS buffer (containing 10 mM- Tris-HCl (pH 7.6), 30 mM-MgCl₂, 100 mM-NH₄Cl, 3 mM-β-mercaptoethanol) supplemented with 20% (w/v) sucrose (see section 3.3(b)). The supernatant from this latter step (25 ml) was brought to 50% saturation with ammonium sulphate by the addition of 6.13 g (NH₄)₂SO₄ and 30 μl 1M-NaOH, then stirred at 4°C for 15 minutes. The precipitate was recovered by centrifugation for 10 minutes at 18,000 rpm and 4°C in the Beckman JA-21 rotor and taken up in 5.0 ml HS buffer. Ammonium sulphate (0.57 g) was added to bring the solution to 20% saturation and the protein precipitate recovered after stirring as above. The latter was re-dissolved in HS buffer (0.5 ml) and then dialysed at 0°C against 2 x 500 volumes of buffer containing 5 mM-Tris-HCl (pH 7.6), 5 mM-MgCl₂, 0.5 M-NH₄Cl, 3 mM-β-mercaptoethanol. The preparation was finally frozen rapidly in aliquots and stored at -70°C.

Reaction mixtures for the measurement of guanosine polyphosphate production contained (in 50 μl total volume) 25 pmol ribosomes (in 10 μl RS buffer), 25 μg poly U, 50 μg E. coli unfractionated tRNA, 4 mM-GTP, 1.5 mM-[γ³²P]ATP (300 to 350 cpm/pmol) and 16.5 μl crude stringent factor (dissolved in buffer as above). The final ionic conditions, allowing for additions of protein EM-L11 and thiostrepton (see legend to Figure
were 20mM-Tris-HCl(pH 7.8), 4.6mM-MgCl$_2$, 15.4mM-magnesium acetate, 180mM-NH$_4$Cl, 2mM-β-mercaptoethanol. Reaction mixtures were incubated for 1 hour at 37°C, then transferred to ice and mixed thoroughly with an equal volume of ice-cold solution containing 2M-formic acid and 10% (w/v) TCA. After centrifugation for 1 minute at 12,000 x $g$, 5μl samples of the supernatants were spotted onto PEI-cellulose plates which were developed and analysed as already described (section (b)). In such assays the major product was pppGpp and combined production of both regulatory nucleotides was typically 30 to 80 pmol/μmol ribosomes.

10.3 Synthesis of Protein and RNA in vivo

Pseudomonic acid, a specific inhibitor of isoleucyl-tRNA synthetase (Hughes & Mellows, 1978), is a potent inhibitor of protein synthesis in vivo as cells are effectively starved for isoleucine. In stringent strains this leads to inhibition of RNA synthesis, mediated by the production of ppGpp and pppGpp, whereas in otherwise isogenic relaxed strains the antibiotic has no effect on the synthesis of RNA (Hughes & Mellows, 1978). Use of pseudomonic acid therefore affords a particularly convenient way of investigating the coupling of the synthesis of RNA and protein by the stringent control mechanism.

The effect of pseudomonic acid on protein and RNA synthesis by E.coli strains MRE 600 (rel$^+$) and prm-1 (relA) was first examined. The latter strain was used merely because it contained a relA mutation and not because it carried prm-1. As shown in Figure 10.1(a), addition of pseudomonic acid (100μg/ml) to cultures of E.coli MRE 600 led to immediate cessation of protein synthesis and in duplicate cultures RNA synthesis was greatly reduced, demonstrating stringent control of RNA synthesis. However, although the same concentration of pseudomonic acid similarly inhibited protein synthesis in cultures of the relaxed strain (Figure
Figure 10.1

(a) Incorporation of \(^3\)Hleucine and (b) incorporation of \(^3\)Huridine over time.

- **Incorporation of \(^3\)Hleucine (c.p.m. x 10\(^{-3}\))**
  - **Incorporation of \(^3\)Huridine (c.p.m. x 10\(^{-3}\))**

*Time (min)*
Legend to Figure 10.1

The effect of pseudomonic acid on the synthesis of protein and RNA by strains of E. coli

Synthesis of protein and RNA was measured as the incorporation of \([4,5-^3H]\)leucine and \([8-^3H]\)uridine respectively into TCA precipitates over a 40 minute time course (see section 10.2). Sodium pseudonolate (100\(\mu\)g/ml) was added to some cultures (●) at the time indicated by the arrow. In control cultures (○) growth remained exponential during the course of the experiment (data not shown).

(a) *E. coli* MRE 600 (rel⁺)

(b) *E. coli* prn-1 (relA)

Legend to Figure 10.2

The effect of pseudomonic acid on the synthesis of protein and RNA by strains of *B. megaterium*

This experiment was carried out as described in the legend to Figure 10.1.

(a) *B. megaterium* KM wild-type

(b) *B. megaterium* KM mutant MJ1
Figure 10.2

incorporation of $[^3H]$-leucine (c.p.m. x 10^-3)

incorporation of $[^3H]$-uridine (c.p.m. x 10^-3)

time (min)

$0$ $20$ $40$

$0$ $20$ $40$
10.1(b), synthesis of RNA was unaffected by the antibiotic. These results therefore confirmed those obtained by Hughes & Mellows (1978) and provided data with which to compare results from similar experiments using wild-type and mutant strains of \textit{B. megaterium}.

The effect of pseudomonic acid on protein synthesis by \textit{B. megaterium} wild-type can be seen in Figure 10.2(a). At the chosen concentration the antibiotic quickly caused complete inhibition of protein synthesis while in parallel cultures synthesis of RNA was rapidly curtailed. These results were thus analogous to those observed using the stringent strain of \textit{E. coli} (MRE 600). Although pseudomonic acid at the same concentration produced a similar inhibitory effect on protein synthesis by mutant MJ1 (Figure 10.2(b), in cultures of this strain synthesis of RNA was not affected at all. These results suggested that mutant MJ1 was phenotypically relaxed.

10.4 Production of ppGpp and pppGpp in vivo

When cultures of \textit{B. megaterium} wild-type or mutant MJ1 were grown in the presence of \textsuperscript{32}P phosphate and were examined for production of ppGpp and pppGpp, neither nucleotide could be detected (Figure 10.3a, b). Following treatment with pseudomonic acid, the wild-type showed rapid accumulation of both regulatory nucleotides to high levels (Figure 10.3a, c). In cultures of mutant MJ1, however, only trace quantities of pppGpp could be detected after addition of the drug (Figure 10.3b, c). It was therefore concluded that mutant MJ1 was unable to synthesise ppGpp or pppGpp in response to amino acid starvation and that this deficiency was the likely reason for the lack of coupling of RNA synthesis to protein synthesis observed above.
Figure 10.3

**(a)** Untreated
- ATP—
- GTP—
- ppGpp—
- pppGpp—

5 15 25 35 50

**b** Untreated
- ATP—
- GTP—
- ppGpp—
- pppGpp—

5 15 25 35 50

ψ = pseudomononic acid

Time (min): 0 10 20 30 40
The effect of pseudomonic acid on the intracellular levels of ppGpp and pppGpp in *B. megaterium* wild-type and mutant MJ1

The synthesis of guanosine polyphosphates was observed in cultures of *B. megaterium* wild-type (a) and mutant MJ1 (b) by thin-layer chromatography of extracted nucleotides, which were subsequently located by autoradiography (see section 10.2). Experiments were performed in the presence or absence of pseudomonic acid, added at time 10 minutes where applicable (see Figure).

The radioactivity contained in the spots corresponding to ppGpp and pppGpp was determined (see section 10.2) and values obtained for pseudomonic acid-treated cultures are presented (c). In the absence of pseudomonic acid the levels of each nucleotide in either strain remained constant at the baseline level (see 5 minute time-point; data omitted for clarity).

ppGpp (□) and pppGpp (○) in *B. megaterium* wild-type

ppGpp (■) and pppGpp (●) in *B. megaterium* mutant MJ1
10.5 Production of ppGpp and pppGpp in vitro

Comparison of the ability of ribosomes from \textit{B. megaterium} wild-type and mutant MJ1 to support the synthesis of guanosine polyphosphates \textit{in vitro} revealed, in the latter case, a deficiency sufficient to account for the relaxed phenotype of the organism. As shown in Figure 10.4, ribosomes from the wild-type were active in the synthesis of (p)ppGpp (track 1), whereas those of mutant MJ1 were completely inactive (track 4). However, if ribosomes from the mutant were first supplemented with protein BM-L11, they showed full activity in this system (track 5) although the protein itself was inactive (track 7). Similar treatment of ribosomes from the wild-type with protein BM-L11 (track 2) had little or no effect on their activity. Synthesis of the two nucleotides by either wild-type ribosomes or by ribosomes from mutant MJ1 supplemented with protein BM-L11 was totally inhibited by thiostrepton (tracks 3 and 6 respectively), indicating that synthesis was ribosome-based. Control experiments showed that neither stringent factor (track 8) nor wild-type ribosomes (track 9) alone could support the synthesis of ppGpp or pppGpp.

It was therefore concluded that the relaxed phenotype of \textit{B. megaterium} strain MJ1 can be directly attributed to the absence of protein BM-L11 from the ribosomes. This conclusion is not simply based on the inability of such ribosomes to support the synthesis of ppGpp or pppGpp \textit{in vivo} and \textit{in vitro}. Thus the direct effect of the purified protein BM-L11 in restoring to ribosomes of the mutant the ability to support synthesis of these regulatory nucleotides clearly indicates that the presence of protein BM-L11 is an obligatory requirement for this process. By analogy \textit{E. coli} ribosomal protein L11 would also be expected to be necessary for the synthesis of (p)ppGpp on the ribosome, as was first suggested by analysis of the \textit{relC} strains (Parker et al., 1976).

The data presented above may also reveal more concerning the nature
Figure 10.4

ATP-  GTP-  ppGpp-  ppGpp-  origin-
Legend to Figure 10.4

Synthesis of guanosine polyphosphates by ribosomes from B. megaterium

wild-type and mutant MJ1 in vitro

Assay conditions were as described in section 10.2. Additionally, ribosomes were first incubated with protein EM-L11 (100 µmol in 5 µl RS buffer) or with RS buffer alone (5 µl) for 5 minutes at 0°C followed by 5 minutes at 20°C. Finally, thiostrepton (1 µg in 2 µl 25% (v/v) DMSO solution) was added to some ribosomes for 5 minutes at 20°C prior to their addition to reaction mixtures. All other ribosomes received 25% (v/v) DMSO alone.

Key:

1: Ribosomes from B. megaterium wild-type
2: Wild-type ribosomes plus protein EM-L11
3: Wild-type ribosomes plus thiostrepton
4: Ribosomes from B. megaterium strain MJ1
5: Mutant MJ1 ribosomes plus protein EM-L11
6: Mutant MJ1 ribosomes plus protein EM-L11 and then thiostrepton
7: Protein EM-L11 in the absence of ribosomes
8: Ribosomes omitted; stringent factor present
9: Wild-type ribosomes; stringent factor omitted
10: Both ribosomes and stringent factor omitted

Stringent factor was present in incubations 1 to 8 and protein EM-L11 was prepared under 'nondenaturing' conditions (section 4.4).
of mutant MJ1. Thus although relC mutants are (by definition) relaxed, ribosomes from relC strains of E.coli show considerable activity (20 to 35% as compared with wild-type) in the in vitro synthesis of guanosine polyphosphates (Parker et al., 1976), presumably due to residual activity of their variants of protein L11. This is in direct contrast to the above observation that ribosomes from mutant MJ1 of B.megaterium do not support any synthesis of such compounds in vitro. Furthermore, the observation that guanosine polyphosphates are not produced in intact cells of strain MJ1 supports the contention that ribosomes of this mutant do not possess any form of BM-L11 in vivo and that absence of the protein from isolated ribosomes does not merely reflect the loss of such material during their preparation.

10.6 The Inability of Protein L11 from E.coli to Stimulate Guanosine Polyphosphate Synthesis on Ribosomes from Mutant MJ1

Since a functional relationship between E.coli protein L11 and B.megaterium protein BM-L11 had already been established (see Chapter 9), it was expected that addition of protein L11 (as opposed to protein BM-L11) to ribosomes of the mutant would similarly enable them to support the synthesis of guanosine polyphosphates. However, when the effect of protein L11 on the behaviour of ribosomes from the mutant was examined using the above in vitro assay, such reconstituted particles remained inactive in the synthesis of the regulatory nucleotides (data not shown). This result was obtained regardless of whether protein L11 was isolated under denaturing or non-denaturing conditions (see section 4.6) and so apparently did not reflect the mode of preparation of the protein.

Failure of protein L11 to substitute for protein BM-L11 in this system does not, however, refute the above conclusions that the two
proteins are related functionally, but merely fails to provide additional evidence for such an argument. In these experiments only B. megaterium stringent factor was used. Thus, inability of E. coli protein L11 to promote the synthesis of guanosine polyphosphates on ribosomes from mutant MJ1 may simply reflect incompatibility with the stringent factor used. Since stringent factor prepared from E. coli is active on ribosomes from B. subtilis (Smith et al., 1978), such a preparation may be active on ribosomes from B. megaterium in the presence of protein BM-L11 or protein L11.
The binding of thiostrepton to *B. megaterium* ribosomes was further investigated by studying the ability of $[^{35}S]$thiostrepton to interact with rRNA-protein complexes and sub-ribosomal particles. In the former case the work described stemmed from observations (see below) originally made by Dr. J. Thompson, to whom I am indebted for the data presented in Table 11.1. Certain experiments described in this Chapter were similar to others performed concurrently by Dr. Thompson using components from the *E. coli* ribosome.

11.1 Materials and Methods

All experiments described in this Chapter which employed purified *B. megaterium* protein EM-L11 used material prepared under non-denaturing conditions (section 4.4). However, the purified *E. coli* protein L11 used in some experiments had been isolated in solutions containing 6M-urea (section 4.6(a)).

The binding of $[^{35}S]$thiostrepton to rRNA-protein complexes was measured using methods similar to those already described for the estimation of binding of $[^{35}S]$thiostrepton to ribosomal particles:

(a) Norit adsorption

Incubations contained rRNA (20 to 30 pmol) and ribosomal proteins (generally 50 to 200 pmol; see legends to Tables and Figures) in 85μl M$^{1.5}$ buffer (10mM-Tris-HCl (pH 7.6), 1.5mM-MgCl$_2$, 50mM-NH$_4$Cl, 3mM-β-mercaptoethanol). After addition of 50 pmol $[^{35}S]$thiostrepton (in 5μl 50% (v/v) DMSO solution) the mixture was kept at 20°C for 20 minutes, when 10μl of a 5% (w/v) suspension of activated charcoal (Norit) in M$^{1.5}$ buffer was added with thorough mixing. Unless bound by components in
the incubation, $[^{35}\text{S}]$thiostrepton is adsorbed by Norit under these conditions. The Norit was pelleted after 5 minutes at 20°C by centrifugation at 12,000 x g for 5 minutes. Binding of $[^{35}\text{S}]$thiostrepton to any RNA-protein complex present was then determined as above (section 6.3) by liquid-scintillation spectrometry of samples from the supernatants.

(b) Gel filtration

Incubations were prepared as in (a) except that the total volume was 50μl. After incubation, the mixture was applied to a column (7 x 0.55cm) of BioGel A-0.5m equilibrated with M$^1.5$ buffer and eluted using the same. Fractions (120μl) were collected and the binding of $[^{35}\text{S}]$thiostrepton to components eluted in the column void volume was determined as in section 6.3 (a). Under these conditions both 16S and 23S rRNA (but not 5S rRNA) are voided by the column and thus separated from free ribosomal proteins and unbound $[^{35}\text{S}]$thiostrepton.

11.2 Interaction of $[^{35}\text{S}]$thiostrepton with rRNA-Protein Complexes

(a) Reconstitution of a high-affinity thiostrepton-binding site

High-affinity binding of thiostrepton to ribosomes from E.coli requires the presence of protein L11, which alone can restore such binding of the drug to 4M-LiCl core-particles (from E.coli) containing only 23S RNA and seven proteins (Highland et al., 1975b). Similarly, B.megaterium protein BM-L11 is essential for tight binding of thiostrepton to ribosomes from this organism (Chapters 4 and 6) and is also able to restore high-affinity drug-binding to ribosomal core-particles lacking many ribosomal proteins (Table 4.2). The minimum requirement for reconstitution of the high-affinity thiostrepton binding site in both organisms was therefore examined.

Initial experiments (performed by Dr. J. Thompson) revealed that
addition of 1M-LiCl split-proteins from wild-type *B. megaterium* to RNA70 from the same strain created a thioestrepton binding site present on neither preparation alone (data not given). Subsequently, purified protein EM-L11 alone was found to give to *B. megaterium* RNA70 the ability to bind \[^{35}\text{S}]\text{thioestrepton}\) with high affinity (Table 11.1). Fractionation of the RNA70 into its three components revealed that this binding site was created exclusively on 23S RNA. Since thioestrepton is unusual in binding so tightly to ribosomes, the ability to detect binding of the drug to RNA-protein complexes by the Norit adsorption technique (Table 11.1) or by gel filtration (eg. Table 11.2) affirmed that the physiological binding site was being examined. These methods yielded a stoichiometry of drug binding approaching 1:1, a value which was subsequently confirmed by the use of equilibrium dialysis (see below).

Use in similar experiments of 23S RNA and protein L11 both from *E. coli* or heterologous combinations of 23S RNA and protein (EM-)L11 from *E. coli* and *B. megaterium* in each case created a tight binding site for thioestrepton (Table 11.1). The latter observation once more demonstrated the homology between these two proteins and also implied that the 23S RNA from both organisms shares regions of similar sequence or structural homology.

The results presented in Table 11.1 were obtained using the Norit adsorption technique. However, more direct evidence for the binding of \[^{35}\text{S}]\text{thioestrepton}\) to a complex of 23S RNA and protein EM-L11 came from gel filtration experiments (eg. Table 11.2) and in particular from the results of sucrose density-gradient centrifugation. Thus Figure 11.1 shows that in the presence (a) but not the absence (b) of protein EM-L11, a sharp peak of \[^{35}\text{S}]\text{thioestrepton}\) co-sedimented with *B. megaterium* 23S RNA.

From these results it was concluded that in both *E. coli* and *B. megaterium* the minimal requirement for a high-affinity thioestrepton
Table 11.1

Binding of $[^{35}\text{S}]$thiostrepton to RNA-protein complexes measured by 'Norit' adsorption

<table>
<thead>
<tr>
<th>RNA input</th>
<th>Additions</th>
<th>pmol THS* bound per pmol RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. megaterium RNA70</em></td>
<td>BM-L11</td>
<td>0.61</td>
</tr>
<tr>
<td>*B. megaterium 23S RNA</td>
<td>BM-L11</td>
<td>0.70</td>
</tr>
<tr>
<td>*B. megaterium 16S RNA</td>
<td>BM-L11</td>
<td>0.00</td>
</tr>
<tr>
<td>*B. megaterium 5S RNA</td>
<td>BM-L11</td>
<td>0.00</td>
</tr>
<tr>
<td>*E. coli 23S RNA</td>
<td>L11</td>
<td>0.77</td>
</tr>
<tr>
<td>*E. coli 23S RNA</td>
<td>BM-L11</td>
<td>0.80</td>
</tr>
<tr>
<td>*B. megaterium 23S RNA</td>
<td>L11</td>
<td>0.86</td>
</tr>
<tr>
<td>*B. megaterium 23S RNA</td>
<td>BM-L11</td>
<td>0.70</td>
</tr>
</tbody>
</table>

* THS : $[^{35}\text{S}]$thiostrepton; RNA70 : total ribosomal RNA.

RNA70 from *B. megaterium* (wild-type) or fractionated ribosomal RNA components (23S, 16S or 5S) from *B. megaterium* or *E. coli* (25 pmol in each case) were incubated with 100 pmol *B. megaterium* protein BM-L11 or *E. coli* protein L11 and $[^{35}\text{S}]$thiostrepton (50 pmol in 5μl 50% (v/v) DMSO solution, 150 cpm/μmol) in 90μl M$^{1.5}$ buffer (see section 9.2). After 20 minutes at 20°C the binding of $[^{35}\text{S}]$thiostrepton to RNA-protein complexes was determined by 'Norit adsorption' (section 11.1). Control experiments (data not presented) established that none of the RNA or protein preparations used here bound $[^{35}\text{S}]$thiostrepton to any detectable level. Binding was calculated as pmol $[^{35}\text{S}]$thiostrepton bound per pmol RNA input.

I am indebted to Dr. J. Thompson for allowing me to present this data.
Figure 11.1

(a) 16S 23S

(b) 16S 23S

Fraction no.

A_{254}

[35S]thiostrepton c.p.m.
Legend to Figure 11.1

**Sucrose density-gradient analysis of the binding of \(^{35}\text{S}\)thiostrepton to the 23S rRNA-protein EM-L11 complex**

\(M^{1.5}\) buffer (125\(\mu\)l) containing *B. megaterium* RNA70 (130 pmol) in the presence (a) or absence (b) of protein EM-L11 (500 pmol) was supplemented with 375 pmol \(^{35}\text{S}\)thiostrepton (190 cpm/pmole, in 37.5\(\mu\)l 50\%(v/v) DMSO solution) and incubated for 10 minutes at 0°C. After thorough mixing with 50\(\mu\)l of a 5\%(w/v) suspension of Norit in \(M^{1.5}\) buffer, each incubation was centrifuged for 5 minutes at 12,000 x g. Samples (70\(\mu\)l) of the supernatants were layered over 5ml 5 to 20\%(w/v) sucrose density-gradients made up in \(M^{1.5}\) buffer and centrifuged for 4 hours at 40,000 rpm and 2°C in the Beckman SW50.1 rotor. The gradients were then pumped through an ISCO UA-5 analyser and the absorbance of components contained therein was monitored continuously at 254nm (—). Fractions (0.125ml) were collected and their content of \(^{35}\text{S}\)thiostrepton determined by liquid-scintillation spectrometry (●).
binding site was a complex between protein (BM-)L11 and 23S rRNA.

(b) Properties of the 23S RNA-BM-L11-[\(^{35}\)S]thiostrepton complex

When ribosomal RNA and protein BM-L11 both prepared by different methods were examined for their ability to support the binding of \([^{35}\text{S}]\)thiostrepton, in neither case did the isolation procedure significantly affect the results obtained. Thus RNA prepared from ribosomes by the standard method of phenol extraction (section 3.5(b) was of similar activity to that isolated by treatment of ribosomes with acetic acid (data not presented) or with lithium chloride plus urea (J. Thompson, personal communication). Similarly, *B. megaterium* protein BM-L11 prepared under either denaturing or non-denaturing conditions (Chapter 4) was effective in promoting the binding of \([^{35}\text{S}]\)thiostrepton to *B. megaterium* RNA70, when present in a three-to four-fold molar excess over RNA (Figure 11.2, cf. Figure 4.5). In addition, although *E. coli* protein L11 has been reported to bind to RNA only when prepared under non-denaturing conditions (Littlechild et al., 1977), the 'denatured' preparation (section 4.6) used in these experiments was active (Table 11.1). It was therefore concluded that the mode of preparation of either RNA or protein was not crucial for their ability to create a thiostrepton binding site. However, since the presence of thiostrepton may stabilise interaction between the RNA and the protein (see below), any normal requirements for a particular conformational state of the protein (or RNA) may be overriden.

Routinely, conditions of 1.5mM-Mg\(^{2+}\), 50mM-NH\(_4\)\(^+\) and 20°C were used when examining the formation of the 23S RNA-protein BM-L11-[\(^{35}\text{S}\)]thiostrepton complex. However, when formation of the complex over a range of Mg\(^{2+}\) (1 to 20mM) and NH\(_4\)\(^+\) (5 to 250mM) concentrations and over a range of temperatures (0 to 40°C) was studied, little variation in the amount of \([^{35}\text{S}]\)thiostrepton bound was observed (J. Thompson, personal communication).
Figure 11.2

![Graph showing net binding of [35S]thiostrepton (pmol/pmol RNA 70) against pmol protein BM-L11.](image-url)
The binding of $[^{35}\text{S}]$thiostrepton to the 23S rRNA-protein EM-L11 complex

Assay mixtures (50µl) containing 30 pmol E. megaterium RNA70, 50 pmol $[^{35}\text{S}]$thiostrepton (in 5µl 50% (v/v) DMSO solution, 120 cpm/pmol) and zero to 200 pmol protein EM-L11, in M$^{1.5}$ buffer (section 9.1), were incubated for 20 minutes at 20°C. The binding of $[^{35}\text{S}]$thiostrepton to the 23S rRNA-protein EM-L11 complex was then determined by gel filtration as described above (section 9.1). Protein EM-L11 prepared under nondenaturing (○) and denaturing conditions (□) was compared. Values were corrected for the background obtained in the presence of RNA alone (0.06).
The binding of \(^{35}\text{S}\)thiostrepton to RNA-protein complexes was next examined using the gel filtration method described earlier (section 11.1). This method was generally found to yield slightly higher values for the stoichiometry of \(^{35}\text{S}\)thiostrepton-binding to RNA-protein complexes than the rapid Norit adsorption technique (data not given). Again it was observed that RNA from the small ribosomal subunit was unable to support the creation of the high-affinity thiostrepton binding site, while RNA50 from either wild-type \textit{B. megaterium} or mutant MJ1 was competent in the assay. Both protein BM-L11 alone and 1M-LiCl split-proteins from \textit{B. megaterium} (wild-type) could stimulate binding of \(^{35}\text{S}\)thiostrepton to RNA50, although the latter were generally more active. Thus although 23S RNA and protein BM-L11 are sufficient to create the high-affinity drug binding site, the presence of other proteins may stabilise the primary interaction.

Table 11.2 also shows that split-proteins from mutant MJ1 had little effect on the capacity of RNA50 from wild-type \textit{B. megaterium} to support the binding of \(^{35}\text{S}\)thiostrepton, demonstrating the specificity of the requirement for protein BM-L11 in these experiments. Furthermore, this split-fraction from the mutant strain could not prevent purified protein BM-L11 from acting in the system.

RNA50 preparations from either \textit{B. megaterium} wild-type or mutant MJ1 were similarly capable of giving rise to a high-affinity thiostrepton binding site in the above experiments. This demonstrates that the absence of protein BM-L11 from ribosomes of mutant MJ1 (Chapter 8) does not reflect alteration of the 23S RNA in this strain and is consistent with the ability of the protein to restore tight binding of thiostrepton to the intact ribosomes (Chapter 6).

The stability of the interaction between 23S RNA and protein BM-L11 in the absence of thiostrepton was next examined. The complex was
<table>
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<th>Source of RNA</th>
<th>Additions</th>
<th>Binding ratio</th>
</tr>
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<tbody>
<tr>
<td>w.t. RNA30*</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>w.t. RNA30</td>
<td>BM-L11</td>
<td>0.05</td>
</tr>
<tr>
<td>w.t. RNA30</td>
<td>w.t. 1M-LiCl split-proteins</td>
<td></td>
</tr>
<tr>
<td>w.t. RNA50*</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>w.t. RNA50</td>
<td>BM-L11</td>
<td>0.69</td>
</tr>
<tr>
<td>w.t. RNA50</td>
<td>w.t. 1M-LiCl split-proteins</td>
<td>0.88</td>
</tr>
<tr>
<td>w.t. RNA50</td>
<td>MJ1 1M-LiCl split-proteins</td>
<td>0.21</td>
</tr>
<tr>
<td>w.t. RNA50</td>
<td>MJ1 1M-LiCl split-proteins +BM-L11</td>
<td>0.82</td>
</tr>
<tr>
<td>MJ1 RNA50</td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>MJ1 RNA50</td>
<td>BM-L11</td>
<td>0.80</td>
</tr>
<tr>
<td>MJ1 RNA50</td>
<td>w.t. 1M-LiCl split-proteins</td>
<td>0.96</td>
</tr>
</tbody>
</table>

* RNA30 : RNA extracted from 30S ribosomal subunits
RNA50 : RNA extracted from 50S ribosomal subunits
Legend to Table 11.2

Binding of [35S]thiostrepton to RNA-protein complexes measured by gel filtration

Assays were performed in modified RS buffer containing 3.0mM-MgCl₂. Incubations (55µl total volume) contained 20 pmol ribosomal RNA (RNA30 or RNA50), 50 pmol [35S]thiostrepton (150 cpm/pmol, in 5µl 50% (v/v) DMSO solution) and where indicated 200 pmol protein BM-L11 and/or 1M-LiCl split-proteins (50 pmol equivalents). Preparations of both rRNA and split-proteins were obtained from B. megaterium wild-type or mutant MJ1 as denoted. The binding of [35S]thiostrepton to components in the incubation mixtures was measured after 20 minutes at 20°C by gel filtration over BioGel A-0.5m (see section 9.2). In control assays none of the protein preparations alone gave rise to apparent drug binding.
Figure 11.3

![Graph showing fraction no. vs. 35S radioactivity (c.p.m. \( \times 10^{-3} \))](image)
Legend to Figure 11.3

Gel filtration of the complex between B. megaterium 23S rRNA and protein EM-L11

RNA70 from B. megaterium (120 pmol) was incubated in the presence (O) or absence (□) of protein EM-L11 (200 pmol) for 10 minutes at 20°C in 50µl modified RS buffer containing 4mM-MgCl₂. Incubation mixtures were then loaded onto a column of BioGel A-0.5m equilibrated with M^1.5 buffer (section 9.1) and eluted with the same, collecting 90µl fractions. To each fraction was added 50 pmol [³⁵S]thiostrepton (151 cpm/pmoll, in 5µl 50% (v/v) DMSO solution) and after 5 minutes at 20°C the binding of the drug to components in the fractions was measured as above following the addition of 10µl of a 5% (w/v) suspension of 'Norit' in M^1.5 buffer. Values (net of 400 cpm background) are plotted in each case. In control experiments 50µl of modified RS buffer (4mM-MgCl₂) containing 200 pmol protein EM-L11 were eluted as described. Each fraction was supplemented with RNA70 (20 pmol) together with [³⁵S]thiostrepton (50 pmol) and incubated at 20°C for 20 minutes, before determination of thiostrepton-binding as above (○). This experiment demonstrated the ability of the column to separate the protein from the RNA-protein complex (O).

The apparent stoichiometry of [³⁵S]thiostrepton-binding to the RNA-protein complex thus isolated was calculated to be 0.63 pmol [³⁵S]thiostrepton per pmol RNA70. This is lower than the 'binding ratio' obtained when the complex was assayed before gel filtration using the 'Norit' adsorption technique (0.72).
formed in the absence of the drug and subjected to gel filtration such that free protein BM-L11 would have been separated from the RNA. When column fractions were supplemented with \[^{35}S\]thiostrepton and binding of the latter to components present measured, drug-binding 'activity' eluted at the column void volume ahead of the free protein (Figure 11.3). It was therefore concluded that the RNA-protein complex was stable to gel filtration and that little dissociation had occurred.

(c) Protection of 23S RNA from nuclease digestion by the binding of protein BM-L11

Treatment of RNA70 from \(B.\) \(megaterium\) with RNase A substantially reduced its ability to support the binding of \[^{35}S\]\(\)thiostrepton when protein BM-L11 was subsequently added, while treatment with RNase \(T_1\) almost abolished this ability. These results were obtained regardless of whether \[^{35}S\]\(\)thiostrepton was present (Table 11.3A) or absent (data not given) during nuclease treatment. However, when the 23S RNA-protein BM-L11 complex was pre-formed (Table 11.3B) and in particular when \[^{35}S\]\(\)thiostrepton was also present (C), protection of the ability of the RNA to support the binding of thiostrepton was observed. Using RNase A, such protection of the RNA in the presence of protein BM-L11 and thiostrepton was almost 100% (compare Table 11.3C with D), but less when the RNA and protein alone were subjected to the ribonuclease (B). This suggests that the presence of thiostrepton stabilises the RNA-protein complex. Greater protection of the drug-binding capability of the RNA-protein complex to nuclease digestion in the presence of thiostrepton was not, however, observed using RNase \(T_1\).

Use of both nucleases together was much more effective than either alone in reducing the capacity of the RNA-protein complex to bind \[^{35}S\]\(\)thiostrepton (B), clearly revealing the stabilising effect of the
<table>
<thead>
<tr>
<th></th>
<th>Order of additions</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>first</td>
<td>second</td>
<td>third</td>
</tr>
<tr>
<td>A.</td>
<td>RNA70 + [(^{35})S]thiostrepton</td>
<td>0.5μg RNase A</td>
<td>EM-L11</td>
</tr>
<tr>
<td></td>
<td>RNA70 + [(^{35})S]thiostrepton</td>
<td>5.0μg RNase A</td>
<td>EM-L11</td>
</tr>
<tr>
<td></td>
<td>RNA70 + [(^{35})S]thiostrepton</td>
<td>100 units RNase T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>EM-L11</td>
</tr>
<tr>
<td></td>
<td>RNA70 + [(^{35})S]thiostrepton</td>
<td>0.5μg RNase A + 100 units RNase T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>EM-L11</td>
</tr>
<tr>
<td>B.</td>
<td>RNA70 + EM-L11</td>
<td>0.5μg RNase A</td>
<td>[(^{35})S]thiostrepton</td>
</tr>
<tr>
<td></td>
<td>RNA70 + EM-L11</td>
<td>5.0μg RNase A</td>
<td>[(^{35})S]thiostrepton</td>
</tr>
<tr>
<td></td>
<td>RNA70 + EM-L11</td>
<td>100 units RNase T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>[(^{35})S]thiostrepton</td>
</tr>
<tr>
<td></td>
<td>RNA70 + EM-L11</td>
<td>0.5μg RNase A + 100 units RNase T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>[(^{35})S]thiostrepton</td>
</tr>
<tr>
<td>C.</td>
<td>RNA70 + EM-L11 + [(^{35})S]thiostrepton</td>
<td>0.5μg RNase A</td>
<td>———</td>
</tr>
<tr>
<td></td>
<td>RNA70 + EM-L11 + [(^{35})S]thiostrepton</td>
<td>5.0μg RNase A</td>
<td>———</td>
</tr>
<tr>
<td></td>
<td>RNA70 + EM-L11 + [(^{35})S]thiostrepton</td>
<td>100 units RNase T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>———</td>
</tr>
<tr>
<td></td>
<td>RNA70 + EM-L11 + [(^{35})S]thiostrepton</td>
<td>0.5μg RNase A + 100 units RNase T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>———</td>
</tr>
<tr>
<td>D.</td>
<td>RNA70 + [(^{35})S]thiostrepton</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td></td>
<td>EM-L11 + [(^{35})S]thiostrepton</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td></td>
<td>RNA70 + EM-L11 + [(^{35})S]thiostrepton</td>
<td>———</td>
<td>———</td>
</tr>
</tbody>
</table>
Legend to Table 11.3

Effect of ribonuclease on the ability of RNA70 to form a complex with protein BM-L11 and thioestrepton

RNA70 from *B. megaterium* (30 pmol in 70μl 10mM-Tris-HCl(pH 7.6), 50mM-NH₄Cl) was incubated for 10 minutes at 20°C with (A) 50 pmol [³⁵S]thioestrepton (150 cpm/pmol, in 5μl 50% (v/v) DMSO solution), (B) 200 pmol protein BM-L11 (in 10μl RS buffer), or (C) both [³⁵S]thioestrepton and protein BM-L11. Assay mixtures were then supplemented with 5μl RS buffer containing RNase A (0.5 or 5.0μg) and/or RNase T₁ (100 units) as indicated and incubated for 20 minutes at 30°C. Following digestion protein BM-L11 was added to (A) and [³⁵S]thioestrepton to (B) and incubation continued for 10 minutes at 20°C. All assay mixtures (each now 90μl total volume) finally received 10μl of a 5% (w/v) suspension of Norit in M⁻¹.⁵ buffer and the binding of [³⁵S]thioestrepton to RNA-protein complexes was determined as above (section 9.1). Incubations treated similarly in which ribonuclease solution was replaced by RS buffer alone (D) are provided for comparison.

The values obtained when RNA alone was incubated with ribonuclease and then protein BM-L11 and [³⁵S]thioestrepton added third were closely similar to those obtained in (A) (data not presented).
drug (compare B with C). This much greater sensitivity of the complex to the combined action of the enzymes may possibly imply the presence of secondary structure in the 23S RNA at the binding site of protein BM-L11.

Similar results to some of these have been obtained using E. coli 23S RNA and either E. coli protein L11 or B. megaterium protein BM-L11 (Thompson et al., 1979). However, the substantial resistance of the ability of B. megaterium RNA70 to give rise to thiostrepton-binding after treatment alone with RNase A (Table 11.3 A) was not observed using E. coli 23S RNA.

Treatment of the complex formed between E. coli 23S RNA, protein BM-L11 and $[^{35}\text{S}]$thiostrepton with RNase T$_1$ allowed the isolation of a ternary complex involving only a fragment of the RNA. This was achieved both by gel filtration on Sephadex G-75 and by sucrose density-gradient ultracentrifugation (Thompson et al., 1979). The latter technique enabled the mean molecular weight of this complex to be estimated, giving a value of approximately 30,000. Since protein BM-L11 has a molecular weight of about 15,500 (Chapter 4) it was concluded that about 50 nucleotides of E. coli 23S RNA were protected from digestion and remained bound to protein BM-L11 in the presence of thiostrepton.

11.3 Binding of $[^{35}\text{S}]$thiostrepton to Sub-Ribosomal Particles and 23S rRNA

Equilibrium dialysis was used to compare the binding of $[^{35}\text{S}]$thio-
strepton to ribosomes, ribosomal RNA and ribosomal core-particles using the apparatus described above (section 6.3(c). Table 11.4 shows the results of three such experiments conducted under closely similar conditions. The first two (A, B) indicate that the 23S RNA-protein BM-L11 complex binds $[^{35}\text{S}]$thiostrepton tightly with 1:1 stoichiometry (line 4) as do ribosomes from wild-type B. megaterium (line 1). However,
<table>
<thead>
<tr>
<th>Ribosomal particle or rRNA input</th>
<th>Additions</th>
<th>Binding ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 1. wild-type ribosomes</td>
<td>—</td>
<td>1.09</td>
</tr>
<tr>
<td>2. mutant MJ1 ribosomes</td>
<td>—</td>
<td>0.58</td>
</tr>
<tr>
<td>B. 3. wild-type RNA70</td>
<td>—</td>
<td>0.19</td>
</tr>
<tr>
<td>4. wild-type RNA70</td>
<td>EM-L11</td>
<td>1.03</td>
</tr>
<tr>
<td>5.</td>
<td>EM-L11</td>
<td>(0.00)*</td>
</tr>
<tr>
<td>C. 6. wild-type 1M-LiCl core-particles</td>
<td>—</td>
<td>0.05</td>
</tr>
<tr>
<td>7. MJ1 1M-LiCl core-particles</td>
<td>—</td>
<td>0.09</td>
</tr>
<tr>
<td>8. MJ1 1M-LiCl core-particles</td>
<td>MJ1 1M-LiCl split-proteins</td>
<td>0.46</td>
</tr>
<tr>
<td>9.</td>
<td>MJ1 1M-LiCl split-proteins</td>
<td>(0.05)*</td>
</tr>
</tbody>
</table>

* apparent binding ratio (see legend)
Measurement by equilibrium dialysis of the binding of $[^{35}\text{S}]$thiostrepton to ribosomal particles and ribosomal components from B. megaterium

Equilibrium dialysis was performed in the apparatus described previously (section 6.3). Each cell contained (in one half) 50 pmol ribosomes (from the wild-type or mutant MJ1) in 0.5ml RS buffer (A), 50 pmol RNA70 in 0.5ml M$^{1.5}$ buffer (B) or 40 pmol 1M-LiCl core-particles in 0.5ml RS buffer (C). In addition, protein BM-L11 (275 pmol) or 1M-LiCl split-proteins from mutant MJ1 (300 pmol equivalents) were present where indicated. The other half of each cell contained (initially) 0.4μm $[^{35}\text{S}]$thiostrepton (130 cpm/pmol) in similar buffer (0.5ml). After complete equilibration of the drug across the membrane (approximately 140 hours at 4°C) radioactivity in samples from both sides of each cell was determined. The binding ratio was then calculated as above (section 6.3) and is expressed as pmol $[^{35}\text{S}]$thiostrepton bound per pmol ribosomal particles or rRNA. When only protein was present (lines 5 and 9) the 'apparent' binding ratio was determined as if the RNA70 or core-particles had not been omitted.
weak interaction between $[^{35}\text{S}]$thio strepton and the RNA alone was also observed (line 3) although it was apparently not sufficient to account for the binding observed using ribosomes from mutant MJ1 (line 2). In the third experiment (C) the binding of $[^{35}\text{S}]$thio strepton to 1M-LiCl core-particles from both wild-type B. megaterium and mutant MJ1 was examined, revealing surprisingly little interaction in either case (lines 6 and 7) considering the result obtained using RNA alone (line 3). However, reconstitution of the core-particles from mutant MJ1 with 1M-LiCl split-proteins from the same strain resulted in recovery of their ability to interact with $[^{35}\text{S}]$thio strepton at a level similar to that shown by the intact 70S ribosomes (line 2). It was therefore concluded that one or more components of the 1M-LiCl split-fraction from the mutant strain were involved in the ability of ribosomes from the latter to interact weakly with thio strepton (see section 6.5).

More rigorous examination of the ribosomal components involved in low-affinity binding of thio strepton to ribosomal particles lacking the 'thio strepton-binding' protein will require the determination of dissociation constants for interaction of the drug with isolated ribosomal components and subribosomal particles. To date, such a detailed approach has only been used to examine the possibility that isolated 23S RNA can bind thio strepton weakly. Thus when the binding of $[^{35}\text{S}]$thio strepton (at a range of concentrations) to E. coli 23S RNA (at a fixed concentration) was examined by equilibrium dialysis, Scatchard analysis of the data (Figure 11.4) gave a value of $4 \times 10^{-7}$M for the dissociation constant (see section 6.3(c) for method). This value is similar to that obtained for the binding of $[^{35}\text{S}]$thio strepton to ribosomes from mutant MJ1 ($2 \times 10^{-7}$M; section 6.5). However, the significance of the number of drug-binding sites indicated (approximately 0.5 per molecule of 23S RNA) is unclear, possibly reflecting loss of competent RNA due to degradation during the dialysis.
Figure 11.4

\[ K_d = 4 \times 10^{-7} M \]

\[ \frac{[\text{THS}]}{[\text{SHL}]} / \Delta \]

[THS]: concentration of free \(^{35}\text{S}\text{thiostrepton} \]
Measurement of the dissociation constant for the binding of \[^{35}S\]thiostrepton to \textit{E. coli} 23S rRNA

The binding of \[^{35}S\]thiostrepton to \textit{E. coli} 23S rRNA was studied by equilibrium dialysis using the apparatus described above (section 6.3). Each cell contained 100 pmol \textit{E. coli} 23S rRNA (in 0.5m M\(^{1.5}\) buffer) on one side of the membrane and 0.5ml of the same buffer containing (initially) 20 to 100\(\mu\)l \[^{35}S\]thiostrepton (0.2 to 1.0 mmol in 50\% (v/v) DMSO solution, 120 cpm/pmol) on the second side. After complete equilibration of the drug across the membrane (approximately 140 hours at 4\(\degree\)C), the free \[^{35}S\]thiostrepton concentration and the number of drug molecules bound per molecule of 23S rRNA (\(\nabla\)) were calculated. From a Scatchard plot of this data (\(\bullet\)), a dissociation constant of 0.4\(\mu\)M was determined from the slope of the line obtained by linear regression of the points (using the least squares method). In a control experiment each cell contained 235 pmol RNA70 from \textit{S. azureus} in place of \textit{E. coli} 23S rRNA. This was prepared from 70S ribosomes of \textit{S. azureus} (a kind gift from Dr. J. Thompson) as described above (section 3.4 (c)). The experimental conditions were otherwise the same as those just described and data is presented as before (\(\bullet\)).
period (140 hours). Comparison of *E. coli* 23S RNA with *S. azureus* RNA demonstrated that the observed interaction is specific. Ribosomes from *S. azureus* do not detectably interact with thiostrepton (*E. Cundliffe*, personal communication; also section 6.5) and the ribosomal RNA similarly showed little ability to bind the drug (Figure 11.4).

Accordingly it was concluded that although protein L11 (or BM-L11) is vital for the tight binding of thiostrepton to ribosomes, the 23S RNA (in both *E. coli* and *B. megaterium*) and one or more other ribosomal proteins (in *B. megaterium*) apparently contribute to the interaction of ribosomal particles with the drug.

The binding of $[^{35}S]$thiostrepton to ribosomes from wild-type *B. megaterium* occurred with 1:1 stoichiometry under equilibrium dialysis conditions where 0.6 molecules of the drug were bound per ribosome from mutant MJ1 (Table 11.4A). It may thus be proposed that the low-affinity and high-affinity interactions of thiostrepton with ribosomes from *B. megaterium* occur at the same (or a closely-related) site. This contention is also supported by the observation that interaction of thiostrepton with ribosomes from both wild-type *B. megaterium* and mutant MJ1 affects the same functions (Chapter 9). Thus 23S rRNA may be considered of primary importance for the binding of thiostrepton to the bacterial ribosome although the affinity of the interaction may depend greatly on the presence of other ribosomal proteins. Such a hypothesis is consistent with the complete resistance of ribosomes from *S. azureus* to thiostrepton, which results from modification of the 23S rRNA by a specific methylase (*Cundliffe*, 1978; *Cundliffe* & *Thompson*, 1979).
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Properties of the Ribosomes of Bacterial Mutants Resistant to Thiopepactin

Abstract

A protein required for the binding of thiopeptactin to ribosomes from Bacillus megaterium has been purified and shown to be serologically homologous with Escherichia coli ribosomal protein L11. Addition of this B. megaterium protein (designated protein BM-L11) to 23S rRNA is sufficient to create a high-affinity thiopeptactin binding site present on neither protein nor RNA alone.

Examination in vitro of four independently-arising thiopeptactin-resistant mutants of B. megaterium revealed that drug resistance was in each case a property of the ribosomes, which were unable to bind $[^{35}S]$ thiopeptactin with high affinity. Addition of protein BM-L11 to such ribosomes restored their ability to bind the drug tightly. Weak interaction of $[^{35}S]$ thiopeptactin with ribosomes from one of the mutants (mutant MJ1) was detected using equilibrium dialysis ($K_d = 2 \times 10^{-7}$M).

Use of two-dimensional polyacrylamide gel electrophoresis suggested that ribosomes from the mutant strains lacked protein BM-L11, a conclusion verified by the results of immunological analyses. Such ribosomes showed only partial activity in protein synthesis in vitro when compared with ribosomes from the wild-type, but re-addition of protein BM-L11 restored the former to wild-type levels of activity. Examination of individual steps involved in polypeptide chain elongation demonstrated that ribosomes from mutant MJ1 were impaired in their ability to hydrolyse GTP in the presence of EF G. Reconstitution of the ribosomes with either B. megaterium protein BM-L11 or E. coli protein L11 reversed this deficiency, indicating a role for protein BM-L11 (and, by inference, E. coli protein L11) in EF G-dependent GTP hydrolysis and showing a functional relationship between the two proteins. However, protein BM-L11 is evidently not essential for ribosomal function.
Unlike the parental *E. coli* strain, mutant MJ1 is phenotypically relaxed. This can be directly attributed to lack of protein BM-Lll from the ribosomes. Thus ribosomes from mutant MJ1 do not synthesise \((p)ppGpp\) \textit{in vivo} during isoleucine starvation and can only produce these regulatory nucleotides \textit{in vitro} when supplemented with protein BM-Lll.