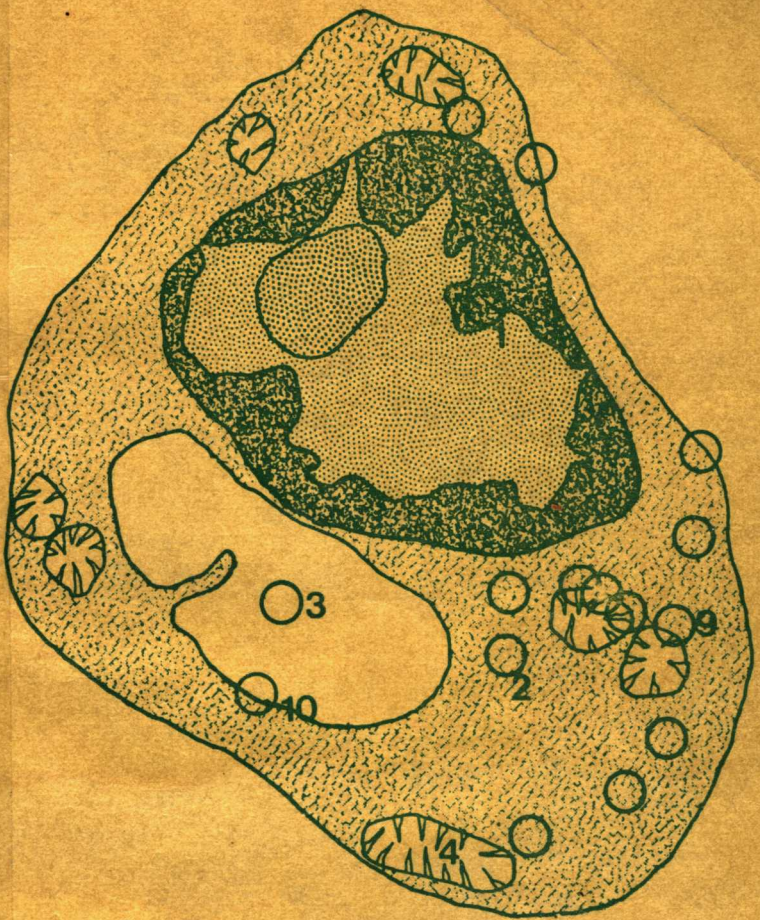

Volume 3

TECHNIQUES IN PROTEIN BIOSYNTHESIS

edited by **P. N. Campbell** *and* **J. R. Sargent**

TECHNIQUES *in* PROTEIN BIOSYNTHESIS

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Preface

Since embarking on the present series in 1964 very many spectacular advances have taken place in protein biosynthesis. Not only do we now have a very much better understanding of the basic mechanisms, but the study of protein biosynthesis in a wide variety of micro-organisms and tissues from animals and plants has contributed significantly to our appreciation of wider problems in cell biology. Indeed, we have reached the point where in many cases the investigation of protein biosynthesis has become but one tactical approach to a specific biological problem rather than an end in itself. This is not to deny, however, that many aspects of protein biosynthesis remain to be unravelled.

With the deepening and widening of our understanding of protein biosynthesis we believe that our initial reasons for embarking on this series are, if anything, more valid today than they were ten years ago. We are still very much concerned with helping those who are new entrants in the field and especially those who have not been able to learn the methodology of protein biosynthesis at first hand from experienced workers. Thus as in previous volumes we have invited as authors scientists who are active workers in their respective fields.

Our approach is based on the belief that techniques can be discussed intelligibly rather than presented as mere lists of manipulations. For this reason we have encouraged authors wherever possible to discuss techniques against a theoretical background in an attempt to illustrate how particular techniques and strategies have contributed to our present day understanding of protein biosynthesis.

Once more it is our pleasant task to thank the authors who have contributed to the present volume for their co-operation and enthusiasm. We also thank the various authors and publishers who have given permission to reproduce original figures.

P. N. Campbell
J. R. Sargent

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CHAPTER 1

The Use of Antibiotics and other Inhibitors in Studies of Bacterial Protein Synthesis

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I. INTRODUCTION

Recent advances in studies of the synthesis, structure and function of macromolecules have made it possible to define more precisely the sites of action of many of the antibiotics that are such highly specific inhibitors of cell growth and division in sensitive organisms. Antibiotics exert a highly selective action on certain biochemical processes and may block a single step in a complex sequence of events. Many are inhibitors of

protein synthesis and act upon ribosomes—ribonucleoprotein complexes upon which proteins are synthesized—and in addition many of these antibiotics show selective toxicity towards either prokaryotic (e.g. bacteria) or eukaryotic (e.g. plant and animal) cells. In this chapter we will focus on the ribosomes themselves and consider how antibiotics have contributed towards our present knowledge of ribosome function. The mechanism of protein synthesis is reviewed only briefly since this topic is covered in detail elsewhere (Lengyel and Söll, 1969; Lucas-Lenard and Lipmann, 1971) and we do not intend to describe the modes of action of a wide range of antibiotics (for detailed treatment see Weisblum and Davies, 1968; Pestka, 1971; Cundliffe, 1972a). We will consider how antibiotics have been used as biochemical tools to aid in studies of protein synthesis and discuss cell mutations which involve changes in ribosome structure and which confer resistance to particular antibiotics. With a knowledge of both the mode of action of an antibiotic at the molecular level and the components involved, we are able to study the complex functional relationships which exist between the constituents which make up the protein-synthesizing system.

A. The Mechanism of Protein Synthesis

During protein synthesis, the various amino acids are joined in peptide linkage in a highly specific sequence and since protein molecules may be very large the problem of establishing the desired sequence is central to an understanding of the mechanism of protein synthesis.

Primary sequences of proteins are encoded in the nucleotide sequence of cellular DNA and this genetic information is transferred from one cell generation to the next by accurate replication of DNA. When this information is to be expressed it is first *transcribed* into the nucleotide sequence of messenger RNA (mRNA) molecules which subsequently associate with ribosomes to direct the synthesis of specific polypeptides in a process termed *translation*.

As will be discussed later in the chapter, ribosomes are exceedingly complex particles (radius 200Å) composed of multiple species of RNA and protein. Ribosomes from bacteria can be distinguished from those of higher organisms on the basis of sedimentation coefficient (s value); hence the practice of referring to "70s" ribosomes of prokaryotes and "80s" ribosomes of eukaryotes. Although these nominal s values hide a multiplicity of sedimentation coefficients, this convention is particularly useful since many antibiotics distinguish between the two types of ribosome. Ribosomes are formed from two subunits of unequal size which combine to form the monomeric ribosome particle and, in practice,

several ribosomes may sequentially attach to and simultaneously translate a given mRNA strand. Such *polyribosomes* are the functional units of protein synthesis. When not functioning, ribosomes may be found within the cell as subunits (50s and 30s in bacteria, 60s and 40s in higher organisms) or as free monomeric ribosomes. When required for protein synthesis, ribosomal subunits associate through the agency of mRNA and other components (see below).

During protein synthesis individual amino acids are delivered to the ribosome by special adaptor molecules (transfer RNA); for each amino acid there is at least one specific tRNA molecule and the given amino acids are attached to the correct tRNA by specific enzymes in reactions involving ATP.

$$\mathrm{R.CH.NH_3^+.CO.O^- + ATP + tRNA} \xrightarrow[\text{synthetase}]{\text{aminoacyl-tRNA}} \mathrm{R.CH.NH_3^+.CO - tRNA + P_i - P_i}$$

$$\mathrm{P_i - P_i} \xrightarrow{\text{pyrophosphatase}} \mathrm{P_i + P_i}$$

Each tRNA carrying its specific amino acid is selected at the ribosome by mRNA, the selection being determined by triplets of nucleotides (codons) in the mRNA. Since mRNA possesses four types of nucleotides, this provides for ample combination of triplets (64) to code for all the amino acids (20) which are incorporated into peptide linkage. Codons are recognized by complementary sequences (anti-codons) in tRNA and codon:anticodon recognition really determines the specificity of protein synthesis. Using synthetic polynucleotides, or triplets of known nucleotide sequence, the genetic code has been completely elucidated. Thus, a triplet codon UUU selects tRNA specific for phenylalanine and AAA codes for lysine. Messenger RNA molecules are translated in sequence starting from the 5′ terminus, there being a codon (AUG) to specify the start of a polypeptide sequence and others (UAA, UAG, UGA) to specify its termination. Some mRNA molecules are polycistronic and carry the information in linear sequence for more than one polypeptide.

Ribosomes have distinct binding sites for both mRNA and tRNA. The smaller ribosomal subunit binds mRNA and provides a site for codon:anticodon interaction between mRNA and amino acyl-tRNA. The larger subunit aids this binding and in addition binds a tRNA molecule carrying the growing polypeptide chain which is built up sequentially commencing from the NH_2-terminus.

We can now consider how the polypeptide chain is synthesized—a process which falls into three phases; initiation, elongation and termination.

1. Initiation

Bacterial cells contain two species of tRNA capable of accepting methionine. One complex, met-$tRNA_F^{met}$ can be formulated to produce F-met-$tRNA_F^{met}$; the other complex, met-$tRNA_M^{met}$ cannot be formulated. F-met-$tRNA_F^{met}$ recognizes an AUG codon at the start of an mRNA cistron (or possibly a GUG codon) and is thus concerned with peptide chain initiation, whereas met-$tRNA_M^{met}$ recognizes AUG codons internally within mRNA cistrons. It follows, therefore, that all bacterial proteins should commence with formyl-methionine at their NH_2-terminus; subsequently the formyl group or formyl-methionine is cleaved from the nascent polypeptide chain. The actual initiation signal in mRNA, possibly including a ribosome-recognition sequence, is undoubtedly far more complex than a single AUG or GUG codon.

Translation of mRNA starts by attachment of a 30s ribosomal subunit to the initiation sequence of mRNA. The complex now attracts a molecule of F-met-$tRNA_F^{met}$ by base pairing between the AUG codon of mRNA and the tRNA anticodon. Next a 50s ribosomal subunit binds and the initiation complex is formed. The overall process requires three protein initiation factors designated IF1, IF2 and IF3 and also GTP which undergoes hydrolysis.

2. Elongation

Aminoacyl-tRNA corresponding to the second mRNA codon is now bound to the initiation complex. A protein elongation factor (EFT) is involved and again the reaction requires GTP with its concomitant hydrolysis. At this stage the ribosome carries two adjacent aminoacyl-tRNA molecules bound in response to the first two translatable mRNA codons. Since F-met-$tRNA_F^{met}$ interacts initially with a site on 30s subunits this tRNA presumably moves to a second site before another aminoacyl-tRNA can be bound. This second site is thought to be on the larger ribosomal subunit and is called the P site. The second aminoacyl-tRNA binds into the so-called A site of the ribosome which contains both the codon recognition region of the 30s subunit and also part of the 50s subunit.

The ribosome carrying two adjacent aminoacyl-tRNA molecules is ready to form the first peptide bond. This reaction is catalysed by an enzymic activity (peptidyl transferase) associated with the larger ribosomal subunit. This "enzyme" is a catalytic centre within the complex structure of the ribosome and to date peptidyl transferase has not been obtained in a soluble form. The peptide bond is formed

between the carboxyl group of the initiator amino acid and the amino group of the second. The reaction involves transfer of F-met from tRNA in the P site on to aminoacyl-tRNA in the A site which now carries dipeptidyl-tRNA. Before the next peptide bond can form, the dipeptidyl-tRNA must be moved to the P site before another aminoacyl-tRNA can bind into the A site. This and subsequent aminoacyl-tRNA molecules are bound in reactions involving EFT and

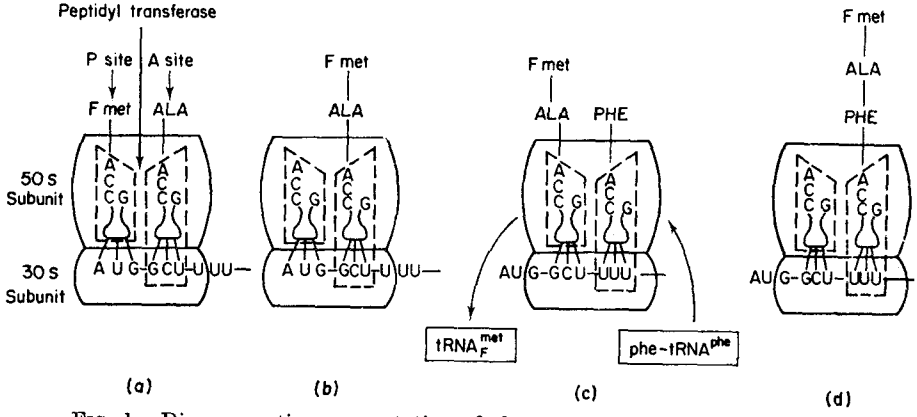

FIG. 1. Diagrammatic representation of the two site model for peptide bond formation on ribosomes, illustrating the stepwise growth of a polypeptide chain. (a) F-met-tRNA is attached at the ribosomal P site and alanyl-tRNA is bound at the adjacent A site; (b) a peptide bond has been formed by the action of peptidyl transferase. The resultant dipeptidyl-tRNA is bound at the ribosomal A site and the deacylated initiator tRNA is associated with the P site; (c) dipeptidyl-tRNA has been translocated from the ribosomal A site to the P site. The mRNA has moved across the ribosome and phenylalanyl-tRNA has been bound at the ribosomal A site as directed by the relevant codon. The deacylated initiator tRNA has been ejected from the P site; (d) a further peptide bond has formed and the resultant tripeptidyl-tRNA is associated with the ribosomal A site.

GTP as described above. Movement of peptidyl-tRNA from the A site to the P site ("translocation") involves an additional protein elongation factor (EFG) and GTP, the latter being cleaved to GDP and inorganic phosphate. Translocation also involves displacement of deacylated tRNA from site P with simultaneous movement of the ribosome along mRNA. The sequence of events is illustrated in Fig. 1.

A peptide bond can normally form only if both the P and A sites of the ribosome are occupied. At peptide bond formation the P site is occupied by either F-met-tRNA$_F^{met}$ (initial peptide bond-forming reaction) or by peptidyl-tRNA. In either case the A site is occupied by aminoacyl-tRNA. When the peptide bond forms, peptidyl-tRNA is bound at the A site prior to translocation, although this site is poorly

characterized for peptidyl-tRNA binding. The peptide does, however, appear to be associated mainly with the larger ribosomal subunit. Since codon:anticodon recognition is a property of the smaller subunit, the A site is presumably overlapping between the two ribosomal subunits.

The protein chain grows from its NH_2-terminal end by the addition of amino acids. As mRNA is translated its 5′ end extrudes from the ribosome and is free to link up with further ribosomal subunits thereby forming initiation complexes. Each mRNA can thus be translated simultaneously by several ribosomes each one carrying a growing polypeptide chain at a different stage of completion. These complexes are the polyribosomes whose size will depend upon the length of the mRNA undergoing translation, a given ribosome occupying approximately 70 nucleotides of the mRNA. In rapidly growing bacterial cells polyribosomes represent over 80% of the total ribosome population; the remaining ribosomes being present as subunits and free monomers.

3. *Termination*

Normal release of completed protein chains from ribosomes is coded for in the nucleotide sequence of natural mRNA. Three codons—UAG, UGA and UAA—have been implicated in release as have three proteins —release factors RF1, RF2 and RF3. RF1 and RF2 recognize the release codons of which UAA is probably the most important. RF3 plays a stimulatory role in the release reaction without participating in codon recognition. Termination involves release of the completed polypeptide *via* the hydrolysis of the bond between the peptide and its associated tRNA. The hydrolysis step is not yet fully characterized but appears to involve a modified action of peptidyl transferase. When the completed polypeptide is released, the ribosome leaves the mRNA and, since re-initiation involves ribosomal subunits separately, released ribosome monomers must dissociate prior to reutilization. The mechanism of this dissociation step is presently controversial but appears to involve a dissociation factor which may be identical with initiation factor IF3.

II. THE PUROMYCIN REACTION AND ITS RELEVANCE TO PROTEIN SYNTHESIS

The central reaction in protein synthesis is the formation of the peptide bond as catalysed on ribosomes by peptidyl transferase. Elucidation of the mechanism of peptidyl transfer has been aided by use of the drug puromycin—a unique inhibitor of protein synthesis. Puromycin reacts with the carboxy-terminus of the growing polypeptide