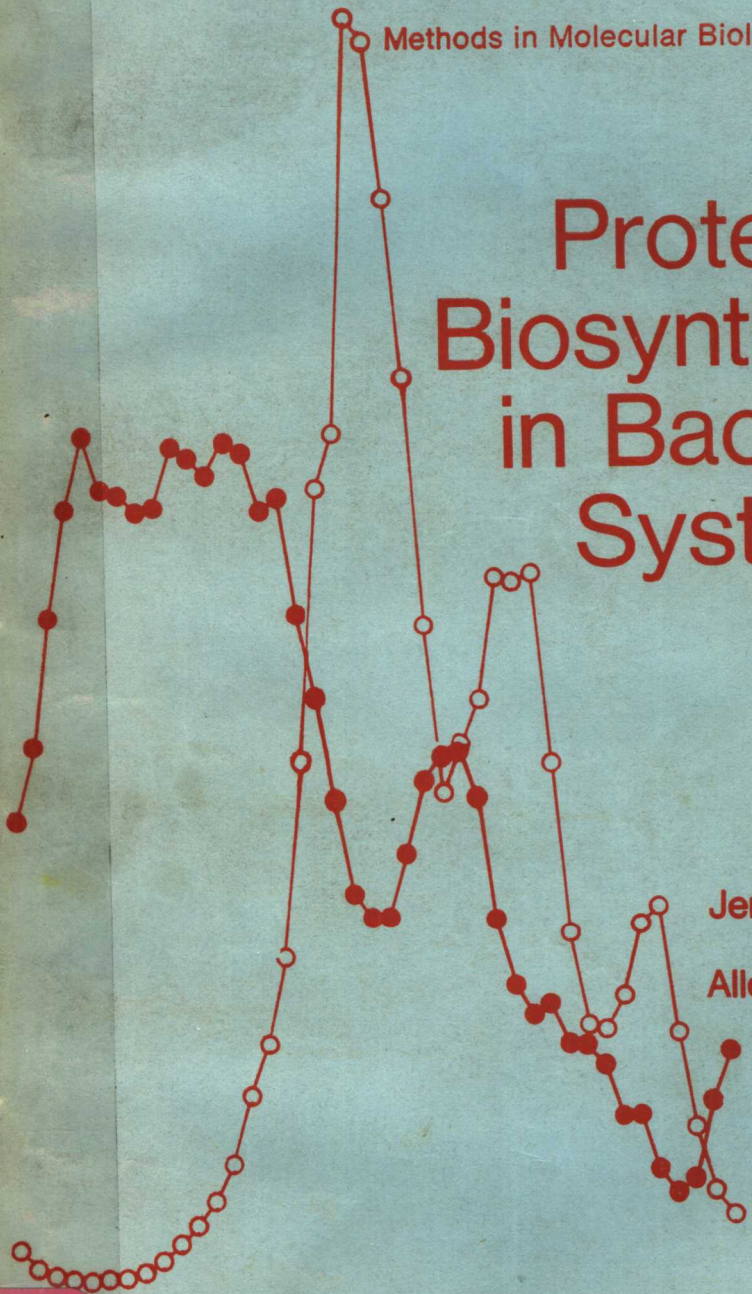
Methods in Molecular Biology Volume 1

Protein Biosynthesis in Bacterial Systems



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Washington, D.C.

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PREFACE

Every laboratory research worker has experienced the many frustrations associated with attempting to duplicate a procedure described in the conventional scientific literature. Rarely does a paper describe the experimental methods in a manner that allows one readily to reproduce them in his own laboratory. This problem has resulted in the appearance of a variety of books, manuals, etc., on "methods," "techniques," and "procedures." In many instances, however, the descriptions found in such books may be no easier to follow than those in the original literature; they may be "buried" in a volume of unrelated techniques, and often may be in a book with a rather high cost.

Methods in Molecular Biology represents an attempt to provide small, relatively inexpensive, topically organized volumes, which might be particularly beneficial to new workers in a field, to graduate students beginning a problem, to new technicians, etc.

The authors were asked to write descriptions of the methods used in a particular area as critically as possible, and whenever appropriate, to discuss such things as: why a particular approach was taken, why a particular reagent was used, what alternatives are feasible and acceptable, and what to do "if things go wrong," etc.

In treating the subject of *in vitro* protein biosynthesis in bacteria, this volume begins with a description of some of the "crude" (S-30) cell-free systems, first from *Escherichia coli*, and then examples of other systems: a gram-positive (*Bacillus subtilis*) and a specialized (halophilic) system. The case in which the *in vitro* synthesis is directed by DNA is followed by chapters concerned with various purified components of the protein-synthesizing machinery—ribosomes; initiation, elongation, and termination factors; aminoacyl-tRNA synthetases; tRNA; phage messenger RNA; ribosomal RNA. Volume 2 of the series will deal with systems other than bacteria.

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Chapter 1

THE S-30 SYSTEM FROM *Escherichia coli*

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I. INTRODUCTION

The S-30 system from Escherichi coli was first described in 1961 by Nirenberg and Matthaei [1]. It consists of a crude DNase-treated extract of E. coli, freed of cells and cell debris by centrifugation at 30,000 X g, containing most of the components necessary for polypeptide synthesis (ribosomes; tRNA; amino acid-activating enzymes; initiation, elongation, and termination factors; and so on) plus many other more-or-less ill-defined materials (S-30 extract). The extract is usually preincubated to deplete its content of endogenous messenger (iS-30 extract). It is supplemented with amino acids, ATP, an ATP-regenerating system, GTP, and a synthetic or natural mRNA. With adequate concentrations of Mg^{2+} and K^+ or NH_4^+ ions, such a system can carry out extensive incorporation of amino acids into polypeptides; it is capable of synthesizing complete specific proteins [2-5] and enzymes [6].

Despite the advent of more purified and better defined systems for amino acid incorporation, the S-30 system is still preferred in many experiments because of its simplicity of

preparation, stability, and high activity. Moreover, when directed with natural messenger, it fairly reproduces most of the steps of protein synthesis in the whole cell: physiological initiation with N-formylmethionyl-tRNA (fMet-tRNA), ribosomal subunits, and initiation factors; elongation with extensive formation of polysomes; physiological termination with release of the completed polypeptide chain; and recycling of ribosomes for new rounds of synthesis. Thus it affords an in vitro polypeptide-synthesizing system suitable for the study of any of these steps.

In this chapter procedures are given for growing cells, preparing the S-30 extract, setting up the amino acid-incorporating system, determining the amount of synthesized product, and analyzing the system in sucrose density gradients. As a complement to the S-30 system, the preparation of cell-free extracts under conditions mild enough to preserve the cell polysomes is also discussed.

II. BACTERIAL STRAINS

Escherichia coli B and K12 have been the most commonly used strains for the preparation of S-30 extracts. However, in principle, any strain should be suitable. Strains deficient in RNase I, such as MRE 600 [7] or Q13 (also deficient in polynucleotide phosphorylase [8]), are becoming increasingly popular and their use is recommended. In our experience, comparing strains S26 (an RNase I^+, K12 strain [9]) and MRE 600, MRE 600

formed polysomes in an S-30 system directed with R17 RNA that were more stable under prolonged incubations than those formed by S26. Still, the activity of both S-30 systems, measured as the total number of amino acids incorporated per ribosome, was about the same.

III. GROWTH OF CELLS

Normally, bacteria are grown at 37°C in a rich medium such as nutrient broth or L medium [10]: 10 g Bacto-tryptone, 1 g yeast extract (both Difco products), and 5 g NaCl in 1 liter of water, to which glucose is added (to 0.2%) after autoclaving (4 ml of 50% glucose per liter of medium). Growth is conducted in large Erlenmeyer flasks (up to 1.5 liters of medium in a 6-liter flask) on an appropriate shaking incubator, or in a large carboy or fermentor under forced vigorous aeration. Air should be filtered by passing it through flasks filled with cotton wool and injected into the medium through three or four tubes terminated in fritted-glass plaques to disperse it into fine bubbles. Since excessive foaming may occur under these conditions, the use of an appropriate sterile antiform is indicated (minimal amounts should be used).

To start growth the preheated medium is inoculated with 1/100 vol of a fresh stationary phase culture (a few-days-old culture can also be used if it has been kept refrigerated). The cells are grown to midlog phase, that is, to approximately 1×10^9 cells per milliter (equivalent to an A_{490} of 0.45 in a

Lumetron colorimeter, or 140 Klett units at 490 nm in a Klett-Summerson colorimeter). Growth is terminated by rapidly cooling the cells; pour the culture onto crushed ice or add about one-fifth the weight of the culture of ice to the flask and agitate. Cells are collected by centrifugation in the cold at 10,000 X g for 10 min or in a continuous-flow centrifuge (Sharples). The yield is approximately 2 g of packed cells per liter of culture. Lodish [11] recently reported that cell yields as high as 13-20 g per liter can be obtained by using a more concentrated medium and letting growth proceed up to saturation. Extracts from these cells also appear to be very active.

Immediately after they are harvested, the cells are washed at 0°C by resuspension in approximately 25 ml of standard buffer [10 mM tris-HCl (pH 7.8), 60 mM NH_4Cl, 10 mM magnesium acetate, 6 mM 2-mercaptoethanol; see Section IV] per liter of original culture, and collected by centrifugation at 20,000 X g for 10 min. Unless the cells are going to be used immediately, they should be quickly frozen. This can be conveniently done by immersing the centrifugue tube (stainless steel or polypropylene) in a dry ice-acetone mixture. The frozen pellet can be quickly weighed and stored wrapped in plastic or aluminum foil. Cells can be stored at -70°C or in liquid nitrogen for weeks (and possibly months) and still yield extracts as active as those prepared from fresh cells. However, storage at -20°C for

a substantial period of time results in extracts with decreased activity when assayed with natural messenger. Temperature of storage may be less critical if the extracts are used with synthetic messengers such as poly U.

IV. PREPARATION OF S-30 EXTRACTS

Grinding with alumina, under the conditions described by Nirenberg and Matthaei in 1961 [1], is still the method most widely used to break cells. Many variants of their procedure have been published [2-4, 11-14]. The one described here has been used repeatedly in our laboratory and consistently yields extracts of satisfactory activity and stability. Other alternative methods, such as the French pressure cell, can also be employed successfully [12-15]. Sonication, however, is not recommended. Milder methods,which preserve the polysomes of the intact cell, and use of hydrolytic enzymes to attack the cell wall and detergents to lyse cells, are discussed later (Section VIII). Generally, they are not adequate for preparing amino acid-incorporating systems directed by exogenous messengers.

All the operations are done between 0 and 4°C. Grinding with alumina is usually done in a cold room with a chilled mortar and pestle. Alternatively, it can be done in the laboratory if the mortar is placed in a larger container and surrounded with well-packed crushed ice (this alternative should not be followed when small mortars are used since it is difficult to avoid some ice chips falling into the mixture during grinding).