STUDIES ON THE PURIFICATION AND CHARACTERIZATION OF RIBITOL DEHYDROGENASE MESSENGER RNA FROM KLEBSIELLA AEROGENES

by

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ABSTRACT

Previous work on ribitol dehydrogenase (RDH) system in Klebsiella aerogenes and Escherichia coli has provided many strains that hyperproduce this enzyme in amounts up to 30% of the soluble protein of the cell. These were selected in chemostats under the simple constraint of growing the organisms on a poor substrate, xylitol.

Studies of the kinetics of enzyme production and of the binding of ^{125}I-labelled specific anti-RDH antibodies to polysomes bearing nascent RDH polypeptides, have shown that the mutant RDH-mRNA is extremely stable in vivo in one, and possibly more, of these super-producing strains. This shows, for the first time, that evolution to a highly stable bacterial message has occurred under selective pressure.

I have purified the RDH-mRNA by using a double antibody/polysome precipitation, and translated it in a crude E. coli cell-free system containing ^{35}S-Met. By far the major product of in vitro translation (and apparently the only one) is a polypeptide which co-migrates electrophoretically to exactly the same position as wildtype RDH on SDS-polyacrylamide gels. A 2-dimensional electrophoretic fingerprint of the methionine peptides produced after thermolysin digestion confirms that the in vitro product is ribitol dehydrogenase. This is the first intact messenger RNA coding for a metabolic protein to be purified from bacterial cell-extracts, and the first intact mRNA isolated from bacterial polysomes.

Additionally, by extensive studies on bacterial polysomes, evidence is presented for the existence of a novel 'general mRNA shut down mechanism' which appears to be triggered at a particular cell denisty of culture, while expressing its full effect within 20 minutes.

ACKNOWLEDGEMENTS

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All the work described herein is my own except where otherwise stated. This dissertation has not been submitted, in whole or in part, for any other degree at any other University.

SYMBOLS AND ABBREVIATIONS

A	Absorbancy
Ab	Antibody
Ag	Antigen
A.R.	Activity Ratio/Analytical Reagent
BSA	Bovine Serum Albumin
CM	Carboxymethyl
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
Ig	Immunoglobulin
Km	Michaelis Constant
Log	Logarithms/Logarithmic
NAD	Nicotinamide adenine dinucleotide
O.D.	Optical density
PBS	Phosphate buffered saline
Rib	Ribitol
RDH	Ribitol dehydrogenase
SDS	Sodium dodecyl sulphate
Sp.Act./S.A.	Specific activity
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet
XDH	Xylitol dehydrogenase
Xyl	Xylitol

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CHAPTER I

INTRODUCTION

A. Ribitol Dehydrogenase : A System for Experimental Enzyme Evolution

The primary and tertiary structures of serine proteases suggest that they have evolved from a common ancestor (Hartley, 1970). Although they show a great deal of difference in primary structure, the overall conformation is conserved to a great extent. One or two amino acid changes in the substrate-binding site are needed to account for profound changes in the side-chain specificity, yet 60% of the sequences are different, and the differences occur mostly in the surface away from the active site, so that 90% of the surface residues differ. Even in the hydrophobic interior there are clusters of side chains that differ radically without affecting the architecture of the polypeptide in the least (Hartley et al., 1976).

In order to explain how these changes might have come about, Hartley and associates (Hartley,1966; Hartley et al.,1972) postulated the scheme that: divergent evolution of an enzyme family from a common ancestor must involve gene duplication as the first step, followed by mutation of one copy to a 'silent gene' which produces an inactive product that cannot fold correctly. Natural selection will conserve the sequence of the viable copy but the progeny of the silent gene will become heterogeneous in the population by both mutation and recombination. A reversion of the original lesion or a compensating mutation may restore at any time the capacity of the protein to fold correctly and mutations accumulated in the 'silent' phase will then be expressed. Natural selection

may refine the structure thereafter either back to the old specificity, whereby isoenzymes would result, or to a new specificity, establishing an enzyme family. However, firm evidence for this hypothesis is likely to arise only from model experiments in which the sequence changes in an enzyme are investigated during the process of evolution towards a new substrate specificity.

An elegant model was devised by studying the ribitol dehydrogenase of Klebsiella aerogenes during continuous culture on xylitol in chemostats (Rigby et al., 1974). This organism grows well on ribitol or D-arabitol as sole carbon and energy source, which are relatively abundant in nature, but not on xylitol or L-arabitol. Specific inducible dehydrogenases exist for ribitol and D-arabitol but not for the other pentitols (Mortlock et al., 1965). Pentitol metabolism pathways in K.aerogenes are shown in Fig.1.1. Mutants constitutive for ribitol dehydrogenase grow poorly on xylitol by using a side-specificity of this enzyme to convert xylitol to D-xylulose (Wu et al.,1968). At physiological concentrations of NAD (1 mM), the apparent Km for ribitol is about 20 mM and kcat is $300S^{-1}$, whereas for xylitol Km (app.) is 1.2 M and kcat is $36S^{-1}$ (Burleigh et al.,1974). Hence the ribitol dehydrogenase (RDH) acts as a very poor xylitol dehydrogenase (XDH) and its activity limits the growth rate of the organism on xylitol.

When the RDH-constitutive mutants of K.aerogenes are grown on xylitol in a chemostat the steady-state biomass is well below that found for growth on ribitol, and there is a high concentration of residual xylitol in the effluent. The system can be used to monitor evolutionary steps that lead to adaptation to the new

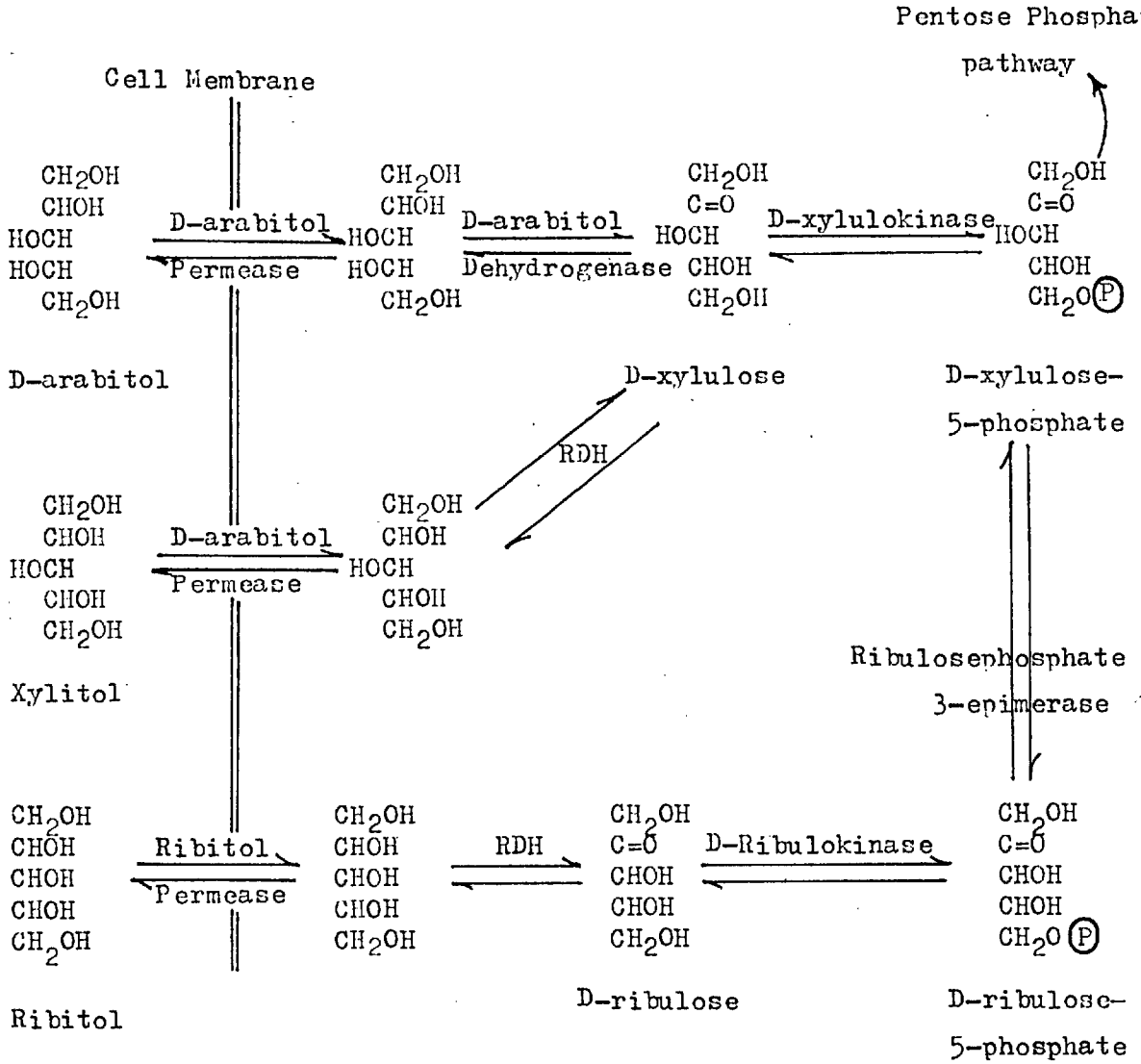


FIG. 1.1. Pentitol metabolism in Klebsiella aerogenes.

carbon source, since faster growing mutants take over from the ancestral population and such events are signalled by a rise in biomass and a fall in the effluent xylitol concentration (Rigby et al., 1974).

B. Production of RDH high synthesis mutants and improved XDH mutants

Extensive continuous culture studies by Rigby (Rigby et al., 1974) and later by Dothie (1974), between 1969-1974 in Cambridge, showed that RDH enzyme superproduction was an invariable response to the selective pressure to grow on xylitol either under conditions of mild mutagenesis (using ultraviolet light or nitrosoguanidine) or by spontaneous mutations. Initial takeover events occurred spontaneously after about 50-150 generations (1-3 weeks), all the mutants investigated proved to have enzymes with unaltered specificities but with 3-5 times higher specific activities in the extracts. Having purified and investigated the kinetics of the RDH-A enzyme of the original RDH-constitutive K.aerogenes A mutant, (Burleigh et al, 1974), these events were classified as an increase in the concentration of RDH-A from about 1% of the total protein in the extract of the ancestral strain A to about 5% in the first stage 'evolvants'. These initial takeovers were succeeded by second takeovers after a similar number of generations, and these proved to contain enzyme of unchanged specificity amounting to about 20% of the total protein in the extract. During the above investigations chemostats containing about 10^{11} organisms for over 3000 generations were screened and over 20 takeover events were detected, all of which appeared to be 'superproducers' rather than 'specificity mutants'. If the latter could arise by single point mutations one would expect them to

occur at frequencies greater than 10^{-10}. On this basis it was concluded that a minimum of two base substitutions was necessary to produce an efficient enzyme with better specificity for xylitol (Rigby et al., 1974).

The basis of RDH superproduction was also investigated by Rigby et al., (1974). The original constitutive strain A produces RDH as over 1% of its total protein in the extract, so they suspected that 'up promoter' mutations or gene-duplication to be the answer. They analysed wild-type and three superproducers (A1, A11 and A211) by 'gene-dosage' (i.e. the frequency of RDH^+ organisms mutating to RDH^-) and by 'segregation' experiments and concluded that strain A1 was not gene-duplicated whereas strain A11 was. Strain 211 appeared to have three copies of the gene for RDH-A (Rigby et al., 1974).

Lack of a well-characterized genetic system for K.aerogenes limited the depth in which this evolutionary model could be analysed. Therefore, the genes for pentitol metabolism were incorporated into the chromosome of E.coli K12 (Rigby et al., 1976). This was achieved by isolating strains of K.aerogenes that were sensitive to the transducing coliphage PICM clr 100, which confers chloramphenicol resistance on its host. Lysates induced from such lysogens contain generalised transducing particles; thus by infecting E.coli K12 with PICM clr 100 lysate an E.coli K12 construct (EA) capable of growth on both ribitol and D-arabitol was isolated. The K.aerogenes pentitol catabolic genes inserted into the chromosome of E.coli K12, mapped at about 40 minutes (Rigby et al, 1976). Having integrated the K.aerogenes RDH gene into E.coli, its evolution under the same selective pressures as in K.aerogenes in chemostats was followed. The preliminary experiments by Gething (unpublished)

showed that under the conditions of mild mutagenesis or spontaneous mutations, enzyme superproduction was the sole response. At first, the strain grows weakly on xylitol in a chemostat although it contains RDH-A as about 4% of its total soluble protein, but soon reaches a higher steady state biomass. Perhaps such events may involve improvements of a xylitol transport system.

The conclusion that improved xylitol dehydrogenases require multiple amino acid changes was apparently strengthened by studies of strains subjected to more powerful NG (N-methyl-N'-nitro-N-nitrosoguanidine) mutagenesis. A culture subjected to severe NG mutagenesis was screened in a chemostat and samples from this run were inoculated into two further chemostats. Each run resulted in the isolation of a separate mutant with improved specificity for xylitol. In a separate experiment, powerful NG mutagenesis gave yet another specificity mutant, strain G. The properties of these strains were shown in Hartley et al., (1976). One of these enzymes (RDH-D) is particularly stable, and has been purified and examined in detail (Dothie, 1974). Its kinetics resemble that of RDH-B (a 'specificity mutant' obtained by Wu et al., (1968) after powerful NG mutagenesis) in that the increased xylitol activity is exhibited in better binding of NAD^+ and of xylitol, and in increased turnover rate. Moreover, as with RDH-B the increased activity towards xylitol is not at the expense of the activity towards ribitol (Burleigh et al, 1974).

Having completed the amino acid sequence of RDH-A (Morris et al., 1974; Moore, C.H., Taylor, S.S., Smith, M.J. and Hartley, B.S., unpublished evidence), considerable effort was spent in identifying the mutations in RDH-D. It was hoped that these might be revealed by peptide mapping, but in fact a rather

exhaustive sequence study was necessary. A change of Ala-196 in RDH-A to Pro-196 in RDH-D was detected relatively easily, but over 90% of the rest of the D sequence were screened without finding any other change. This result together with the results of later chemostat studies forced this group to conclude that improved xylitol dehydrogenases can arise by single point mutations (Hartley et al.,1976).

In June 1975, during analysis of the spontaneous evolvants of a chemostat run, where I had intended to regenerate very high-RDH-synthesis mutants from a partially segregated RDH superproducer strain (E.coli K12, EA1111) which originally was evolved in a chemostat run by M.J. Gething, I accidentally discovered the first spontaneous 'improved xylitol dehydrogenase evolvants' (see Results, Chapter III). Several isolates were tested for the RDH specific activity and for the activity ratio, all had significantly increased xylitol/ribitol activity ratios. This result was later confirmed by Dr. Dothie. Further spontaneous takeovers of the same chemostat run showed still greater improvements in XDH activity.

The unexpected observation of spontaneous mutations to improved xylitol dehydrogenases in the E.coli K12 EA prompted further investigation of K.aerogenes by Dr. J.M. Dothie. The original arginine auxotroph XI of Wu et al, (1968), strain 'A', was investigated during six chemostat runs on xylitol at low dilution rates ($D = 0.06\ hr^{-1}$) lasting 1-2 months each (Hartley et al.,1976). In five of these there was no change in the XDH/RDH activity ratio throughout the run, and in two cases that were investigated spontaneous takeovers by superproducers had occurred. However, during the sixth run strains were isolated from the chemostat wherein the XDH/RDH activity ratio had

increased from 0.04 to 0.14 or 0.67. The latter finally took over completely. Separate chemostat runs of these 'improved' strains gave superproducers in each case. Moreover in a run at normal dilution rates ($\sim$ 0.2 hr^{-1}) three consecutive takeovers by 'specificity mutants' were observed with XDH/RDH of 0.12, 0.47 and 1.00 respectively. Thus, out of a total of about twelve spontaneous takeover events, five specificity mutants of K.aerogenes had evolved in response to the selective pressure for growth on xylitol. Further chemostat studies with E.coli K12 EA gave rise to five 'specificity mutants' of RDH-A versus four 'superproducers' in the nine takeover events. Hence we were now in a position to study the stepwise changes in sequence that are responsible for these spontaneous evolutionary events. However, two important conclusions of Rigby et al. (1974) had to be withdrawn: that improved xylitol dehydrogenases do not arise spontaneously, also the argument that single amino acid changes cannot provide a significant increase in specificity of RDH for xylitol.

Reiner (1975) discovered natural stains of E.coli C which grow on ribitol and D-arabitol. The genes corresponding to the enzymes involved in this metabolism were found to be closely linked and map at about the same position (40 min) in E.coli C as in the E.coli K12 rbt^+, dal^+, construct (Rigby et al, 1976). However, Reiner was unable to find any natural E.coli B or E.coli K12 strains that grew on these pentitols, nor does E.coli K12 contain DNA homologous to the pentitol operons. The genes for pentitol metabolism in K.aerogenes are closely clustered in the following order :-

dalK–dalD–dalC–rbtB–dalB–rbtC–rbtD–rbtK

where B and C are control genes (possibly repressor and operator

respectively) for the ribitol (rbt) or D-arabitol (dal) pathways (Charnetzky and Mortlock, 1974). rbt D codes for ribitol dehydrogenase and dal K for xylulokinase. Therefore, it is not surprising that the whole of this region could be transduced with coliphage P1 into E.coli K12.

Both Reiner (1975) and Hartley et al (1976) suspected that E.coli C may have gained its pentitol genes by interspecies gene transfer from a Klebsiella-like organism, and that this may have been a relatively recent evolutionary event. To test this hypothesis the sequence homology in the respective ribitol dehydrogenases was undertaken at Imperial College. First superproducers of RDH from Reiner's E.coli C strain were evolved by growth on xylitol in a chemostat. Further growth yielded spontaneous takeover by an improved xylitol dehydrogenase (Hartley et al., 1976). E.coli C RDH was purified from the RDH-superproducer strain. Preliminary sequence results showed that there are only four differences in 85 residues screened so far : indicating a sequence identity of about 95% with Klebsiella enzyme (Hartley et al., 1976).

Continuous culture studies so far had shown that RDH enzyme superproduction (apparently as a result of gene multiplication) is a frequent response to the selective pressure for growth on xylitol. Gene duplication would be the necessary first step in the evolution of an enzyme family. It was argued (Hartley, 1966; Rigby et al., 1974) that the second step might be the accumulation of mutations in one of these copies once the selective pressure for growth on the new foodstuff was removed. This implied that the rate of elimination of the second copy of the gene is low, while Koch (1972) had produced theoretical arguments that this may be so (Hartley et al., 1976). In order to test these

hypotheses K.aerogenes strain A11, which contains two copies of the rbt D gene (Rigby et al., 1974), was grown on non-selective carbon sources for a large number of generations with repeated UV mutagenesis. If multiple mutations could thus accumulate in one of the copies of the RDH gene, a greatly improved xylitol dehydrogenase might then emerge when the population was switched back to growth on xylitol. But the results of these experiments showed the opposite to these expectations. With either ribitol or glucose as the first substrate, strains with an increased RDH specific activity took over the chemostats where growth was switched back to xylitol. These were speculated to be gene-tripled stains that would arise from A11 by unequal crossover (Hartley et al., 1976). Glucose and ribitol catabolite repress (Pastan et al., 1974) the synthesis of RDH, so the selective pressure to eliminate a second constitutive gene might be negligible. Catabolite repression is, however, much less with inositol as sole carbon source (Hartley et al., 1976), and here they found evidence for rapid selection of a single-gene strain during the growth on inositol. Furthermore, not one xylitol specificity evolvant was detected during any of these experiments (Hartley et al., 1976).

It was realized that a specialised lambda transducing phage carrying the ribitol operon ($\lambda \frac{rbt}{}$) would be extremely useful, both so as to allow very severe in vitro mutagenesis of the ribitol genes (and hence the isolation of a much improved xylitol dehydrogenase) as well as enabling the genetic manipulation and detailed investigation of the various mutant operons which caused very high synthesis of the RDH. The construction of the $\lambda \frac{rbt}{}$ phage was achieved by the transduction of the ribitol genes into a bacterial strain containing a deletion

for the λ attachment site. Lysogenisation of this strain by a suitable λ yielded a population of lysogens with λ integrated at the many secondary attachment sites dispersed over the E.coli chromosome. Subsequent induction yielded a lysate containing a variety of specialized transducing phage. Infection of this lysate into E.coli allowed the screening for the λ rbt phage. This isolate was found to confer on wild-type E.coli the ability to grow on ribitol but not on D-arabitol (Hartley et al., 1976). Such lysogens grow very weakly on xylitol, but can be evolved to better growth in chemostats.

My own work on ribitol dehydrogenase messenger RNA began in February 1975. Our group's interest in purifying this messenger was mainly from the point of view of having an additional probe to investigate enzyme evolution. Of particular interest to the group was the knowledge of the size and the amount of RDH-mRNA transcript from the wild-type and various high-synthesis mutant strains. We were also hoping to investigate possible tandem gene duplication by purified mRNA-DNA hybridization. In addition, it was realized that having purified a bacterial messenger RNA it can always be used to explore other interesting questions. There were certain clear advantages in dealing with purification of RDH-mRNA. In some of the high synthesis mutants, RDH enzyme is present up to about 30% of the total soluble protein of the organisms, this suggested that a high population of the total pulse-labelled RNA may be represented by RDH-mRNA. Additionally. the complete amino acid sequence of RDH was already determined.

C. Prokaryotic Messenger RNA

Most of our present knowledge of the mechanisms of gene expression comes from the in vivo and in vitro studies of the processes of RNA transcription and translation in microorganisms. A thorough critical review of this subject, not surprisingly,

requires a tremendous effort. However, I have felt justified in briefly summarizing progress and some unresolved problems of RNA synthesis and prokaryotic mRNA metabolism within the limitations of my own work. No attempt will be made to be all-inclusive or historical, rather it is hoped to provide a useful guide to the key references. Gene expression requires the transfer of genetic information from DNA into RNA molecules, the direct templates for protein synthesis. Transcription of genetic sequences is mediated by DNA-dependent RNA polymerase, the enzyme which catalyzes the initiation, elongation and termination of polyribonucleotide chains, and uses ribonucleoside triphosphates as substrates. The synthetic reaction shows an absolute requirement for a divalent metal ion and usually requires the presence of DNA or a polydeoxyribonucleotide to serve as a template in the reaction.

By the early 1960's, the speculations that had previously linked DNA, RNA and protein synthesis were rapidly replaced by firm experimental evidence. Prior to this time there had been a widespread belief that RNA probably functioned as a template for protein synthesis. This idea was fortified by the results of Nirenberg and Matthaei (1961) and Lengyel, Speyer and Ochoa (1961), who showed that natural and synthetic polyribonucleotides specifically direct amino acid incorporation into polypeptide chains in bacterial cell-free extracts. In that same year, Jacob and Monod (1961) formulated their ideas of the DNA operon and messenger RNA, which were soon given experimental support by the DNA-RNA hybridization method of Hall and Spiegelman (1961). Earlier experiments by Volkin and Astrachan (1956) had shown that T_2 bacteriophage infection of E.coli resulted in the appearance of RNA with base ratios similar to those of T_2 DNA. As an

extension of this observation, Hall and Spiegelman (1961) convincingly demonstrated by hybridization analysis the presence of T_2 RNA species complementary in base sequence to T_2 DNA in T_2-infected cells. This result not only substantiated the idea of the existence of natural mRNAs, but also provided indirect evidence that DNA must be able to serve as a template for RNA synthesis in vivo. Soon, independently, Hurwitz, Weiss, Stevens and their associates described an enzyme, RNA polymerase, which catalyzes formation of an RNA in the DNA-dependent reaction that its base composition, as well as the nearest neighbor base frequencies, was almost identical to that of the DNA primer, with uridine substituted for thymidine. Today, voluminous evidence exists indicating that the action of RNA polymerase is primarily responsible for the synthesis of cytoplasmic RNA species. One of the major questions remaining, however, is that of the precise cellular mechanisms that regulate the action of this enzyme. A 900-page volume published recently by Cold Spring Harbor Laboratory (ed. Losick R. and Chamberlin, M., 1976) has been devoted entirely to work on DNA-dependent RNA polymerase, which reflects the importance of this enzyme.

The bacterial RNA polymerases are large molecules (molecular weights between 400,000 and 500,000) having complex subunit structures. Two enzymatically active forms of the enzyme are currently known; these have been designated RNA polymerase holoenzyme and core polymerase. The latter form lacks a single subunit present in the former and is altered in its binding to DNA and its ability to initiate RNA chains. Bacterial RNA polymerases from different species appear to be closely related in subunit structure (Burgess, 1976). The E.coli enzyme has four major subunits, designated β', β, σ and α (Burgess 1969;

Berg, Barrett and Chamberlin 1971). Holoenzyme has the subunit composition $\alpha_2 \beta \beta' \sigma$ and can be resolved into two components: a catalytic component core enzyme with four subunits ($\beta' \beta \alpha_2$) and a fifth dissociable subunit, sigma (σ), responsible for the selection of specific sites for chain initiation (Burgess, 1971; Travers and Burgess, 1969; Berg and Chamberlin, 1970). The best current estimates of the molecular weight values from acrylamide gels after denaturation with SDS are β' = 160,000, β = 150,000, σ = 86,000 and α = 40,000 (Chamberlin 1976; Burgess 1976). The assignment of specific functions to the various subunits is somewhat tentative, particularly due to the lack of knowledge about the primary and three dimensional structure of the enzyme. Current evidence suggests that the β' subunit is involved in template-binding and that the β subunit contains at least part of the catalytic centre, but the function of the α subunit is unknown (Krakow et al., 1976; Zillig et al., 1976).

Presence of DNA-dependent RNA polymerase in all bacterial species, together with its sensitivity to drugs that inhibit bacterial transcription, indicates that it is the enzyme responsible for the major part of RNA synthesis in the bacterial cell. A newly reported bacterial RNA polymerase, the dna G gene product, which is not related to the classic enzyme, seems to be involved in initiation of DNA synthesis for certain replicons (Kornberg, 1976), however, this enzyme is not known to play a role in general transcription. In addition several bacteriophages induce RNA polymerases specified by the phage genome that are unrelated to the bacterial enzyme (Bautz, 1976). A variety of other forms of RNA polymerase have been reported

and in some cases, named: Polymerase II found in stationary cells (Chao and Speyer,1973); holoenzyme II found in small amounts and consisting of core enzyme and a polypeptide termed σ' (Fukuda, Iwakura and Ishihama, 1974); a series of activities in crude extracts with different sedimentation properties and template activities (Snyder,1973; Travers and Buckland,1973; Pitale and Jayaraman,1975); and polymerase III, able to prime DNA synthesis on M13 but not ΦX DNA (Wickner and Kornberg, 1974). In general, these have not been well characterized or shown to be biologically relevant, and in most cases they appear to be complexes between holoenzyme or core polymerase and other cell components (Burgess, 1976).

Although bacterial RNA polymerase holoenzyme can selectively read some transcription units in vitro, studies of the regulation of gene expression in vivo have made it clear that additional factors are required for synthesis of certain transcripts. Indeed, genetic studies of bacterial and bacteriophage systems have suggested that both positive and negative control factors are involved in transcription of certain genetic regions (Epstein and Beckwith 1968; Calendar 1970). Biochemical support was provided by demonstration that E.coli RNA polymerase cannot read the late transcription units from T_2 DNA (Khesin et al., 1963) and with the characterization of sigma subunit as a removable component of RNA polymerase essential for the utilization of certain promoter sites (Bautz et al., 1969). The search has been going on for altered forms of RNA polymerase or adjunct factors that could alter the selective properties of the holoenzyme and allow it to read other genetic regions of the DNA template. Activation of new transcriptional specificities in prokaryotic

systems has been found to occur through a variety of mechanisms, including modification of bacterial RNA polymerase leading to altered recognition of promoter or terminator signals on the DNA template (Losick and Pero, 1976; Zillig et al., 1976), de novo synthesis of new RNA polymerases having different transcriptional specificities (Bautz, 1976), and synthesis of regulatory proteins that interact with the DNA template to alter utilization of promoter or terminator signals by RNA polymerase (Gilbert, 1976; Roberts, 1976).

Although alterations of the transcription apparatus by alterations in RNA polymerase may well take place in most cells, it is well documented only in bacteriophage-infected systems (Chamberlin, 1976). Even here substantial uncertainty remains (Petrusek et al., 1976; Losick and Pero, 1976). Known alterations involve either modification of existing subunits of host RNA polymerase or introduction of new subunits into the enzyme. Different alterations may lead to quite different functional outcomes: the specificity for template site selection may be altered (Losick and Pero, 1976), or the enzyme may respond differently to termination signals on DNA (Franklin and Yanofsky, 1976; Roberts, 1976). Some alterations that lead to loss of site selection specificity may function to suppress host transcription in the infected cell (Stevens, 1976; Zillig et al., 1976). New phage-specified polypeptides become associated with bacterial RNA polymerase after infection with most phages, including T_3, T_4, T_5, T_7, λ , SP01 and SP82 (Losick and Pero, 1976). However, only in the case of the SP01- and SP82-modified enzymes is there evidence that the altered enzyme has an altered transcriptional specificity <u>in vitro</u> (Whitely et al., 1976; Petrusek et al., 1976; Losick and Pero, 1976). The T_4-altered

enzyme loses site selection specificity but does not gain a new specificity; hence it simply resembles the core polymerase (Stevens, 1974; Losick and Pero, 1976).

Bacterial RNA polymerase carries out several distinct kinds of polynucleotide synthesis and a variety of reactions that represent steps in the overall pathway of polynucleotide synthesis (Chamberlin, 1976). These are (1) template directed formation of complementary polyribonucleotides, (2) template-directed formation of homopolymers or repeating polymers (reiteration), and (3) unprimed synthesis of polyribonucleotides. When a helical DNA is employed as template, the product is free RNA, and the template DNA is left unaltered after synthesis. The RNA product is precisely complementary to the region of the DNA employed as template and is antiparallel in sequence. In most cases, due to the restriction imposed by the existence of sites on the DNA required for initiation and termination of RNA chains, only certain regions of the DNA template are transcribed. Because of its biological significance, it is the study of this "selective transcription" that is of primary concern to those interested in transcription (Chamberlin, 1974 and 1976).

The DNA-directed synthesis of RNA by RNA polymerase may be broken down into a number of substeps (Burgess, 1971; Chamberlin, 1974). Each of these steps is itself complex, and the overall process is referred to as the transcription cycle (Chamberlin, 1976). The commonly accepted steps for the reading of transcription units which require only RNA polymerase holoenzyme for selective transcription are: (1) template site selection and activation, in which the RNA polymerase holoenzyme attaches to the DNA template, locates a specific site at which chain initiation can occur, and assumes an active conformation (Chamberlin, 1976b);

(2) RNA chain initiation, in which the enzyme catalyzes the coupling of ATP or GTP with a second ribonucleoside triphosphate to eliminate inorganic pyrophosphate and generate a dinucleoside tetraphosphate of the structure ppp PupX ; this moiety remains tightly bound to the RNA polymerase-DNA complex (Krakow et al., 1976). (3) RNA chain elongation, in which successive nucleoside monophosphate residues are added from substrate nucleoside triphosphates to the initial dinucleoside tetraphosphate at its 3'-OH terminus in order to elongate the nascent RNA chain (Krakow et al, 1976). (4) RNA chain termination and enzyme release, in which the newly formed RNA chain and RNA polymerase are released from the template DNA (Roberts, 1976). The process of promoter site selection is a key point at which cellular regulation of transcription takes place, and there are a number of reviews of the subject (Burgess, 1971; Chamberlin, 1971, 1974b, 1976& 1976b; Losick, 1972; Bautz, 1972; von Hippel and McGhee, 1972). In addition, regulation at the step of site selection involves the participation of both repressors and positive control factors, and the interactions involved have also been extensively reviewed (Gilbert et al., 1974; Bourgeois and Pfahl 1976; Gilbert, 1976).

In prokaryotic cells, gene transcription and subsequent translation to protein appear to occur coordinately (Stent, 1966; Zubay and Chambers, 1969; Morse et al., 1969). Electron microscope visualization has confirmed the coupling of these processes, showing protein synthesis occurring on polyribosomes attached to messenger RNA transcripts which are still in association with the template DNA (Miller et al., 1970). No one had up to now purified from bacterial cell extracts an intact

messenger RNA coding for a bacterial enzyme or metabolic protein, nor had anyone isolated an intact messenger RNA from bacterial polysomes. This probably reflects the inherent instability of such mRNAs and polysomes, which is essential for rapid metabolic control of the level of enzymes in these cells. Biochemical studies have shown that prokaryotic mRNA is labile, with an average half-life of less than 3 minutes (Gros et al., 1961; Kepes, 1963; Nakada and Magasanik, 1964; Schechter et al., 1965; Leine, 1965). Previous work on purified bacterial mRNA has therefore been largely with mRNA fragments transcribed *in vitro* from DNA of specialized phages, ϕ80 p*trp* phages (Rose et al., 1973) and λ p*lac*5 phage (Majors, 1975), or with material isolated from cell extracts that has proved to be extensively degraded (Musso et al., 1974; Squires et al., 1976).

More stable species of mRNAs have been described, for example for certain T_7 phage proteins (Summers, 1970), for penicillinase production (Harris and Sabath, 1964) and sporulation (Aronson and Rosas de Valle, 1964) in *Bacillus cereus*. Recently a group of outer membrane proteins in *E.coli* has been identified which appear to be synthesized from mRNA species with half-lives of 5.5 - 11.5 min. (Hirashima et al., 1973; Lee and Inouye, 1974). The mRNA for one of these proteins (lipoprotein) has been highly purified by taking advantages of the following characteristics: the relative stability of mRNA, the size of the mRNA (7S RNA), and the amount of the mRNA was also assumed to be abundant (Hirashima et al., 1974; Wang et al., 1976). The mRNA for the lipoprotein was purified from the lysate by phenol extraction, Nacl fractionation, gel filtration on Sephadex G-100, 5-20% sucrose density gradient centrifugation, and gel filtration on Sephadex G-200 (wang et al., 1976). The mRNA thus purified has

been found to be biologically active, and the product in a cell-free system directed by the mRNA has been identified to be the lipoprotein by immunological assay and by peptide mapping (Hirashima et al., 1974). Another outer membrane protein in E.coli minicells which lack DNA has been shown to be coded by a very stable mRNA with an estimated half-life of 40-80 min. (Levy, 1975). Degradative proteins such as alkaline phosphatase along with a number of amino acid and sugar-binding proteins also appear to have relatively stable mRNAs; none of these proteins are found in the cytoplasm, but they occur in the space between the cytoplasmic membrane and the outer membrane - so called "the periplasmic space or periplasm" (Malamy and Horecker, 1964; Brockman and Heppel, 1968; Heppel, 1971). Recently Ø80 transducing phages have been isolated carrying the structural gene for alkaline phosphatase (Brickman and Beckwith, 1975). These phages now provide DNA preparations highly enriched for the alkaline phosphatase structural gene. These DNA preparations and the in vitro transcribed RNA from the same DNA which has been purified by phenol extraction have been used as templates in a cell-free system to demonstrate the synthesis of the active enzyme, alkaline phosphatase. The preliminary results indicated that the stability of alkaline phosphatase mRNA in the S30 system varies from one preparation of the S30 fraction to another; with some preparations, the synthesis of the enzyme proceeded linearly for as long as 70 minutes (Inouye et al., 1977). None of these reports give any information about the reason for the relative stability of the mRNA, or about the location of the synthesis of the mRNA and the corresponding protein, or as to whether special components (such as membrane fraction) are necessary for the expression of genes coding for proteins destined to be exported

from inside the cell.

In bacterial cells, and following infection by a DNA-containing bacteriophage, it appears that synthesis of specific proteins is regulated largely, if not exclusively, by synthesis of the cognate mRNA. There is excellent correlation between the amounts of mRNA corresponding to the E.coli lac (Lewin, 1974; Reznikoff, 1972; Contesse et al., 1970; Varmus et al., 1970), ara (Engelsberg and Wilcox, 1974; Schleif, 1971), gal (Miller et al., 1971), and trp (Imamoto et al., 1965a; Imamoto et al.,1965b; Morse et al., 1969; Bertrand et al., 1975) operons and the rates of synthesis of the corresponding proteins. Likewise, following infection by the DNA phage T_4, there is sequential synthesis of a number of T_4-specific proteins. With one minor exception, synthesis of an inactive form of lysozyme mRNA soon after infection (Kasai and Bautz, 1969), the rate of synthesis of each of these proteins is proportional to the amount of the corresponding mRNA (Salser et al., 1967; Salser et al., 1970; Gold and Schweiger, 1970; Wilhelm and Haselkorn, 1971; Black and Gold, 1971; Sauerbier and Hercules, 1973; Lewin, 1974).

The abundances and rates of synthesis of different RNA species in bacteria vary greatly. Apart from the obvious contribution of differing stabilities of ribosomal, transfer, and messenger RNA, and the fact that a particular mRNA species from an undifferentiated bacterial cell is relatively much less abundant than a mRNA from a specialized enkaryotic cell, vastly different rates of initiation also play a determining role. For example, in rapidly growing E.coli at $30^{o}C$, the polymerization of ribosomal and tRNA chains is initiated approximately every 1 to 2 seconds at each chromosomal site (Roberts, 1964; Geiduschek, 1969). Yet under similar growth conditions, the maximum

achievable induced rate of initiation of the tryptophan operon is one chain approximately every 2½ minutes at 30°C (Imamoto, 1968; Baker and Yanafsky, 1968). The average rate of polymerization of initiated chains of ribosomal and trp (tryptophan operon) RNA probably differs by no more than a factor of two, 15 to 26 nucleotides per second (Geiduschek and Haselkorn, 1969; Imamoto, 1968; Baker and Yanofsky, 1968; Manor et al., 1969; Mangiarotti et al., 1968; Winslow and Lazzarini, 1969; Zimmermann and Levinthal, 1967; Bremer and Yuan, 1968). Thus the relative rate of synthesis of these two RNA species mainly reflects their relative frequency of initiation (their relative abundance is further determined by the instability of mRNA). Moreover, there is at least one other site in the tryptophan operon of Salmonella typhimurium and E.coli (Margolin and Bauerle, 1966; Bauerle and Margolin, 1967; Morse and Yanofsky, 1969) at which the synthesis of the operator-distal part of the trp operon message coding for the two tryptophon synthetase subunits and indole glycerophosphate synthetase can be initiated constitutively (with respect to tryptophan). In S.typhimurium the level of expression determined by this site is approximately 50-fold lower than at the principal trp promotor.

Messenger RNA degradation in bacteria proceeds from 5'- to the 3'-end. The end which is made first is digested first. Break down this way will not lead to the synthesis of incomplete chains. On the contrary, if breakdown were 3' to 5', then the ends of many messengers would be destroyed before ribosomes had translated their sequences. There is ample support for an inactivating event occurring at the 5' end of mRNA: cessation of enzyme synthesis has the same polarity as translation of

the lac (kepes and Beguin, 1966) and trp (ito and Imamoto, 1966) messages following re-repression. Repression of the trp operon by addition of an excess of tryptophon 1.5 min after the commencement of derepression in the presence of an excess of ^{3}H-uridine blocks further initiation by polymerase molecules, limiting transcription to a single wave produced by those polymerases which had started during the short period of derepression. The results of hybridization of this polycistronic mRNA isolated at various times during derepression and after re-repression to DNAs from transducing phages carrying different regions of the trp operon demonstrated a sequential accumulation of ^{3}H-uridine in newly synthesized RNA hybridizable to the E, the D, and then the BA gene regions (gene order being, O-E-D-C-B-A). This pattern reflects sequential transcription of these regions as the wave of polymerase molecules travels down the operon. The subsequent disappearance of this labelled mRNA reflects its rapid degradation. Degradation of the operator-proximal E messenger begins while the wave of polymerase transcribes the next distal (D) gene; D messenger is then degraded while the more distal BA messenger is being synthesized. Thus, degradation proceeds from the 5'- to the 3'-end of the nascent messenger, lagging behind the polymerase by only about 2 min. The average length of the trp messenger, under these conditions, is therefore only about 30% of the full operon length, $\sim$ 6700 nucleotide length (Morse et al., 1969). An interesting result is that starvation for an amino acid increases the lifetime of trp mRNA by about twofold (Morse et al., 1969; Morikawa and Imamoto, 1969), in both RC^{rel} and RC^{str} strains (Edlin and Broda, 1968).

The enzymic mechanism by which individual mRNA molecules are broken down in vivo has not yet been clarified, though several

ribonuclease activities have been identified in E.coli : RNAse I (Spahr and Hollingworth, 1961; Neu and Heppel, 1964; Gesteland, 1966) is an endonuclease, but since this enzyme is inhibited by Mg^{++}, is non-specific, is located in the periplasmic space, and is a dispensible function, it is highly unlikely to be required for mRNA degradation. Polynucleotide phosphorylase (Ochoa, 1957; Littauer and Kornberg, 1957; Reiner, 1969) is Mg^{++} dependent but it is inhibited by high concentrations of K^{+}, because it is a dispensible enzyme and it is an exonuclease which can digest RNA in the 3' to 5' direction, it is unlikely to play an important role in mRNA degradation in vivo. RNAse II (Spahr and Schlessinger, 1963; Spahr, 1964; Singer and Tolbert, 1965; Castles and Singer, 1969; Gorelic and Apirion, 1971; Venkov et al., 1971; Weatherford et al., 1972; Simon and Apirion, 1972), is an exonuclease working in the 3' to 5' direction. It could play a role in the processing or degradation of several different RNA species; it occurs on ribosomes, is Mg^{++}, K^{+}, and NH_4^{+} activated and inhibited by Na^{+}, ATP and DNA. This enzyme by itself is also unlikely to be responsible for mRNA degradation in vivo. RNAse II also copurifies with an RNAse activity (RNAse II-endo) capable of cleaving the 17S precursor to 16S rRNA (Altman and Robertson, 1973). RNAse III (Robertson et al., 1968; Westphal and Crouch, 1975), is an endonuclease, uses helical RNA as substrate, requires Mg^{++} and Mn^{++}, has an absolute requirement for NH_4^{+}, Na^{+}, and K^{+} and occurs on ribosomes. It cleaves adenovirus mRNA and mammalian 28S and 18S ribosomal RNA. Fragmentation is not random, but in each case a specific collection of products is generated. Cleavage by RNAse III abolishes the capability of adenovirus mRNA to direct cell-free synthesis of virus polypeptides. This enzyme may be involved as an endonuclease in mRNA metabolism

(Altman & Robertson, 1973). RNAse P (Altman and Smith, 1971; Robertson et al., 1972) is an endonuclease involved in stable RNA (probably just tRNA) tailoring, also occurs on ribosomes and has similar cation requirement to RNAse III. Another endonuclease which could in principle play a role in mRNA degradation is RNAse IV (Gesteland and Spahr, 1969; Min Jou et al., 1969). In vitro, this enzyme cleaves phage RNA specifically, but little is known about its in vivo function; it does not occur on ribosomes. A new RNAse activity, named RNAse V, was found in cell free extracts of E.coli (Kuwano et al., 1969; Bothwell and Apirion, 1971; Holmes and Singer, 1971; Mangiarotti et al., 1971). This is the most probable enzyme responsible for mRNA degradation in vivo. This activity requires ribosomes, G and T factors, tRNA, K^+ or NH_4^+, Mg^{++}, GTP, and a sulfhydryl compound to degrade poly U, poly A, T_4 phage mRNA, or E.coli mRNA. RNAse V is specific for mRNA, it does not attack ribosomal RNA. It is inhibited by antibiotics that decrease breakdown of mRNA in vivo, such as chloramphenicol and streptomycin, and by such agents as 5'- β, γ -methylene-guanosine triphosphate, and fusidic acid, which inhibit ribosome-dependent GTPase and translocation of ribosomes along mRNA. The evidence suggests that RNAse V is either an integral part of the ribosome or is tightly associated with it, and that it selectively degrades mRNA in intact cells. There has been a certain amount of confusion as to whether RNAse V is a separate activity or an artifact arising from a combination of other activities already described; furthermore, it has been suggested that RNAse II could act together with RNAse P or with RNAse III or possibly RNAse IV in various stages of mRNA metabolism (Altman and Robertson, 1973). Neither of these claims seem justified to me; evidence will be presented later in this

work that none of the activities described above other than RNAse V could play a major role in mRNA degradation in vivo.

Mechanism of protein synthesis at the level of messenger RNA tranalation has been covered in several recent reviews and books (Lucas-Lenard and Lipmann, 1971; Bosch, 1972; Haselkorn and Rothman-Denes, 1973; Nomura et al., 1974; Lewin, 1974; Lodish, 1976; Weissback and Ochoa, 1968), for this reason I do not intend to discuss this in any detail. However, I shall refer to certain aspects of it.

In both prokaryotic and eukaryotic cells, initiation of synthesis of all polypeptides is mediated by a specific methionyl-tRNA, generally called $tRNA_f^{met}$. This corresponds to an AUG codon or, rarely, a GUG or UUG codon (Ganem et al., 1973) in the mRNA. For instance, both α and β-hemoglobin chains (Hausman et al., 1970; Jackson and Hunter, 1970; Wilson and Dintzis, 1970) and all three genes encoded by the RNA phages (Adams and Capecchi, 1966; Webster et al., 1966; Lodish, 1968) are initiated with a methionyl-tRNA. Insertion of methionine into internal positions of polypeptides is mediated by another isoaccepting species, $tRNA_m^{met}$, which also utilizes an AUG codon. In most bacteria and in chloroplasts and mitochordria (Smith and Marcker, 1968; Galper and Darnell, 1969; Burkard et al., 1969) a formyl residue is added to the amino group of the methionine after the latter is esterified to $tRNA_f^{met}$. This N-formylmethionyl-$tRNA_f^{met}$ is essential for function of the initiator tRNA. In the cytoplasm of enkaryotic cells, by contrast, the initiator Met-$tRNA_f^{met}$ is not formylated, although, curiously, the eukaryotic Met-$tRNA_f^{met}$ can be formylated by the bacterial enzyme.

In E.coli initiation proceeds by the orderly addition of the mRNA and the initiator tRNA to the small, 30 S, ribosome

subunit, followed by the addition of the large, 50 S, ribosome subunit. This results in formation of the complete "initiation complex", and addition of the second aminoacyl-tRNA can then occur, utilizing the well-established elongation factors EFTu, EFTs, and EFG. Binding of the initiator fMet-$tRNA_f^{met}$ requires the participation of at least two specific initiation factors, IF-1 and IF-2. GTP is also required in this process. Binding of natural mRNA to the small ribosome subunit requires at least one other factor, IF-3. However, translation of artificial mRNAs containing an AUG codon, such as $AUGU_n$, does not require IF-3. IF-3 also acts as a ribosome dissociation factor in that it binds to free 30 S ribosomes and prevents their binding to a 50 S subunit, forming an inactive, mRNA-free, 70 S ribosome.

Another important issue is the search for identification of the sequence or the structure in mRNA which is recognized as a site for initiation of protein synthesis. Clearly not every AUG triplet could function (as this would result in synthesis of some nonsense polypeptides of various lengths), nor could a few nucleotides on either side of the AUG be the primary signal. For example, the same sequence GCAUGGC is found both at the beginning and in the middle of the R17 coat gene (Adams et al, 1969; Steitz, 1969). One possibility is that any AUG codon could potentially function as initiators, but that the conformation of the RNA is such that most of these sequences are prevented from doing so (Lodish, 1976). For instance, the above AUG sequence in the middle of the phage coat gene and many other noninitiating AUG sequences are apparently found in double-stranded regions (Min Jou et al, 1972). Supporting this concept is the finding that mild denaturation of f_2 RNA with formaldehyde greatly increases the rate of chain initiation of two of the three "normal" f_2 proteins

and also allows ribosomes to initiate synthesis at three or more additional, illicit sites (Lodish, 1971; Lodish, 1970). Another possibility, not mutually exclusive with the one above, is that a specific sequence of nucleotides and/or certain secondary structure(s) near the AUG codon is required. However, there is no clear answer to this very important question at present. Because of the obvious relation of the specific ribosome attachment sites to the efficiency of translation process, this problem will be further discussed in Chapter XI.

Perhaps the best examples of translational modulation of protein synthesis occur during infection of E.coli by the RNA bacteriophages $Q\beta$, f_2, R17 and MS2. This area has been reviewed extensively in the past few years (Lodish, 1976; Zinder, 1975; Weissmann et al., 1973; Hindley, 1973; Kozak and Nathans, 1972). Each of these phage RNAs (3300-4000 bases) encodes three proteins: a major coat protein, a phage coded subunit of RNA synthetase, and an A or maturation protein - a minor but essential component of the virion (Horiuchi et al., 1966; Hussin, 1966). The three genes are arranged in the order 5' end-A-coat-synthetase-3' end (Jeppesen et al., 1970). Both in the infected cell and in cell-free extracts, the phage coat protein is made in the largest amounts (Nathans et al, 1966; Vinuela et al., 1967; Fromageot and Zinder, 1968; Nathans et al., 1969). When intact phage RNA is used, ribosomes will bind primarily to the initiation site for coat protein synthesis (Steitz, 1969; Hindley and Staples, 1969; Gupta et al., 1970). Apparently, the ribosome attachment sites for the other two genes are intrinsically capable of initiating protein synthesis but are buried within the RNA structure (perhaps being in double-stranded regions) or are in

some other way prevented by the RNA structure from functioning (Gupta et al., 1970; Lodish, 1969; Steitz, 1975; Lodish, 1975).

Many bacterial and bacteriophage mRNAs, like RNA phage RNAs, are polycistronic and contain several signals for initiation and termination of polypeptide synthesis. Recent work indicates that both ribosome subunits fall off the phage f_2 mRNA at the termination codon at the end of the coat gene, different ribosomes initiate at the adjacent start signal (Webster and Zinder, 1969; Kaempfer 1971; Martin and Webster, 1975). Experiments with the translation initiation inhibitor, kasugamycin, suggested that a similar situation exists for the E.coli trp operon (Zalkin et al., 1974). It is of interest to determine whether each of the genes on a polycistronic mRNA initiates protein synthesis at the same rate, but two complications make experiments in this area difficult. First, translation of most bacterial mRNAs begins while the RNA is still being transcribed from DNA. Second, degradation of the 5' end of mRNA can begin while the 3' end of the molecule has not yet been synthesized. Since segments of an mRNA encoding different proteins can have different half-lives, the determination of the level of mRNA encoding a particular protein is not easy. In the E.coli trp operon, equimolar amounts of at least three of the five polypeptides are made. The regions of trp mRNA encoding the three genes are of course synthesized in equimolar amounts and have only slightly different functional half-lives. Hence, the rate of polypeptide chain initiation on each of these three segments is about the same (Forchhammer et al., 1972; Morse et al., 1968; Baker and Yanofsky, 1972). A similar conclusion obtains for the lac operon. About 3.5 - 5 times more of the operator-proximal z gene product, β-galactosidase,

is made relative to the distal a gene product transacetylase (Zabin and Fowler, 1970). However, the half-life of transacetylase-mRNA segment at 37°C is half that of z mRNA (Blundell et al., 1972; Kennell and Bicknell, 1973; Blundell and Kennell, 1974). Hence, the rate of initiation of translation of the two genes differs by no more than twofold. By contrast, mRNAs encoding the bacteriophage λ late proteins are synthesized in equimolar amounts and have the same functional half-lives, but the yield of protein per mRNA segment differs by as much as 870-fold (Herskowitz and Singer, 1970; Murialdo and Siminovitch,1972; Ray and Pearson, 1974; Ray and Pearson, 1975). Presumably, this is due to differences in the rates of ribosome attachment and chain initiation.

CHAPTER II

Materials and Methods

1. Chemicals

Unless otherwise indicated, A.R. grade chemicals, or the purest grade available, were used throughout. The following reagents were obtained through Sigma (St. Louis, Mo., U.S.A): Sodium-heparin (Grade I), Freud Adjuvants (Difco), Chloramphenicol, Rifampicin, 5-iodouracil, Lysozyme (Grade 1, 3 x recrystallized), Brij 58 (Polyoxethylenecetylether; Atlas Chemical Co., New Jersey, U.S.A), and Sucrose (Grade 1; Crystalline, RNase activity none, suitable for density gradient studies with RNA). Deoxyribonuclease 1 (Bovine pancreas, electrophoretically purified from RNase activity) was purchased from Cambrian-Worthington, Cambrian Chemicals Ltd., Suffolk House, George St., Croydon, U.K. SDS (low UV absorption such as BDH specially purified) was used. All of the radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Buckinghamshire, U.K.

2. Bacterial Strains

All Klebsiella aerogenes strains used are derivatives of strain 1033 (Neidhart and Magasanik 1956).

Strain A is prototrophic derivative of the arg and gua auxotroph supplied in 1970 to P.W.J. Rigby by Prof. E.C.C. Lin of the Department of Microbiology and Molecular Genetics, Harvard Medical School.

All Escherichia coli K-12 strains are derivatives of auxotrophic strain CA64 supplied to P.E.J. Rigby by Prof. S. Brenner of M.R.C. Laboratory of Molecular Biology, Cambridge, U.K.

All strains, with the exception of the ones described in the results, were supplied by Dr. J.M. Dothie.

Stocks of these strains are maintained in stabs at room temperature, or in 20% glycerol at -20^{o}, and for day to day use on plates at $4^{o}C$.

Strain			
Lab. Collection No.	Other Coll. No.	Genotype	Origin/Reference
K. aerogenes FG5	1033	arg, gua, inducible rbt	From Harvard (Neidhart & Magasanik, 1956)
K. aerogenes 1A	5P14; XI	arg, gua, rbtC101	From Harvard (Wu et al., 1968)
K. aerogenes 2A	XI arg$^-$	arg, rbtC101	Spontaneous from 1A (Rigby et al., 1976)
K. aerogenes A	3A; FG9	rbtC101	Spontaneous from 2A (Rigby et al., 1976)
K. aerogenes A1	3A1	rbtC101, rbtDS	Spontaneous from A (Rigby et al., 1974)
K. aerogenes A11	3A11; FG101	rbtC101, $(rbtDS)_2$	Spontaneous from A1 (Rigby et al., 1974)
K. aerogenes A111	3A111	rbtC101, $(rbtDS)_3$	Spontaneous from A11 (Hartley et al., 1976)
K. aerogenes A112	3A112	rbtC101, $(rbtDS)_3$	Spontaneous from A11 (Hartley et al., 1976)
K. aerogenes A114	3A114	rbtC101, $(rbtDS)_3$	Spontaneous from A11 (Hartley et al., 1976)
K. aerogenes D	AN2	rbtC101, $(rbtD11)_2$	Spontaneous from A (J.M. Dothie, 1974)
K. aerogenes E	AN2'	rbtC101, rbtD13S	NG from A (J.M. Dothie, 1974)
K. aerogenes F	AN2"	rbtC101, rbtD14	NG from A (J.M. Dothie, 1974)
K. aerogenes AN3		rbtC101, rbtDS	NG from A (J.M. Dothie, 1974)

Strain

Lab. Collection No.	Other Coll. No.	Genotype	Origin/Reference
E. coli K-12 CA388		gal, str, tsx	(Rigby et al., 1976)
E. coli K-12 EA	MJ1000	gal, str, rbtC101, $rbtD^+$, $dalD^+$, tsx	Constructed by transfer of genes from K. aerogenes using P1CMclr100 transducing-phage (Rigby et al., 1976).
E. Coli K-12 EA1	MJ1001	gal, str, rbtC101, $rbtD^+S$, $dalD^+$, tsx	Spontaneous from EA (M.J. Gething, Cambridge, unpublished).
E. coli K-12 EA11	MJ1002	gal, str, rbtC101, $rbtD^+S$, $dalD^+$, tsx	Spontaneous from EA1 (M.J. Gething, unpublished)
E. coli K-12 EA111	MJ1003	gal, str, rbtC101, $rbtD^+S$, $dalD^+$, tsx	Spontaneous from EA11 (M.J. Gething, unpublished)
E. coli K-12 EA1111	MJ1004	gal, str, rbtC101, $rbtD^+S$, $dalD^+$, tsx	Spontaneous from EA111 (M.J. Gething, unpublished).
E. coli K-12 ED		rbtC101, $rbtD^+12$, etc.	Spontaneous from 'EA1111' by plate selection (see results)
E. coli K-12 EE		rbtC101, $rbtD^+10$, etc.	Spontaneous from 'EA1111' by chemostat selection (see results)
E. coli K-12 EF		rbtC101, $rbtD^+15$, etc.	Spontaneous from EE, by chemostat selection (see results)

rbtC101, constitutive for RDH synthesis. $rbtD^+10$-15 mutations in the structural gene for RDH to better XDH. $(rbtD)_{2-3}$ duplicate and triplicate copies of the structural gene for RDH. rbtDS, mutation resulting in synthesis of large amounts of RDH.

3. Media

(a) Liquid Media

M9 is: 5.8g Na_2HPO_4 ,3.0g KH_2PO_4, 0.5g NaCI, 1.0g NH_4CI, 0.12g $MgSO_4$; made up to 1 litre with distilled water. (The $Mg-SO_4$ is added after autoclaving as a sterile IM solution).

M9 is supplemented with the carbon source to a final concentration of 0.2% by addition of the required amount of a sterile 20% solution of pentitol. Casamino acids are sterilized with M9 , to a final concentration of 2.0% (w/v).

LB medium is , per litre: Bacto tryptone 10g, Bacto yeast extract 5g, NaCI 10g.

(b) Solid Media

Minimal plates are: M9 plus appropriate supplements solidified with 15g agar/litre.

TYE plates are: 15g agar, 8g NaCI, 10g Bacto Tryptone, 5g Yeast extract, 5mg Thymidine; in 1 litre of distilled water.

Stab agar is: 3g agar, 4g NaCI, 5g Bacto tryptone, 2.5g Yeast extract, 5mg Thymidine; in 500ml of distilled water.

All bacterial media are from Difco Laboratories.

4. Culture Conditions

Plates are incubated at 37°C for the required period.

Liquid cultures are grown either in 250ml - 2000ml conical flasks or in 50ml bubbler tubes immersed in a water bath at 37° with vigorous aeration being provided from the laboratory compressed air supply via cotton wool and membrane filters and a glass manifold.

5. Preparation of Cell Extracts

(a) Lysozyme - Triton

The procedure used was developed by P.W.J Rigby (J.M. Dothie, personal communication). Ten ml of a bacterial culture grown in a 20ml bubble tube is centrifuged in a refrigerated Sorvall SS-34 rotor at 5000 rpm, for 3 min. The pellet is resuspended in 8ml of 0.1M Tris-HCI, pH 8.0, containing 5mM EDTA. To this is added 0.08ml of 10mg/ml lysozyme solution, in the above buffer. The suspension is incubated at 37°C for 20 min. 0.1ml of a 1:1 mixture of 0.1M Mg SO_4 and 0.1M Tris-HCI pH 8.0 containing 5% (w/v) Triton X-100 is added and 5 min further incubation is carried out. The lysate is then centrifuged at 12,000 rpm (Sorvall SS-34) for 10 min and the supernatant is retained and stored at -20°C.

(b) Ultrasonication

The procedure used is by J.M. Dothie (personal communication).

A 200ml solution is centrifuged at 3000 g for 10 min, washed in M9 once, spun and resuspended in 5ml of 0.1 M potassium phosphate buffer pH 7.0. The suspension is cooled in ice and subjected to ultrasonication for five 1 min periods with 2 min cooling in between each period using a Dawe Instrument Soniprobe at power setting 4 tuned to the particular sample to give a current of about 5 amps. The sonicate is then centrifuged at 15,000 g for 20 min, and the supernatant is decanted and stored at -20°C.

When preparing extracts of strains containing unstable RDH e.g. K. aerogenes strain B, NAD^+ at 1mM is added to the suspension prior to sonication. When assaying extracts of such bacteria, the assay mixture must contain

bovine serum albumin at 0.1mg/ml to prevent denaturation of the RDH by dilution into a solution of low protein concentration.

6. RDH Enzyme Assay (Dothie, 1974)

RDH activity is measured in 0.1M potassium phosphate, pH 7.0 by following the increase in OD_{340}nm occuring on reduction of 0.83mM NAD^+ by 50mM ribitol or 0.5M xylitol assuming OD_{340}nm of 6.25 = 1mM NADH.

Cuvettes containing 1ml 0.3M potassium phosphate pH 7.0 + 0.25ml 10mM NAD^+, + 0.15ml 1M ribitol or 1.5ml 1M xylitol + water to 3ml, are incubated at 28°C and when equilibrated the enzyme (ca 10µl) is added using a Hamilton syringe. After thorough mixing the OD_{340}nm is followed in a Gilford 2000 Recording Spectrophotometer with Unicam monochromator thermostated at 28°C. Traces are optimum at a chart speed of 2"/min with a full scale deflection of 0.1, and a rate of change of OD_{340}nm of about 0.02/min.

7. Protein Estimation

(a) Wang and Smith Method

The Lowry procedure has been modified for use in the presence of Triton X-100 by the addition of 10% sodium dodecyl sulphate (Wang and Smith, 1975).

Procedure To a 0.2ml sample containing 10-200µg of protein is added 1ml of alkaline copper solution (250mg/l of the disodium cupric salt of EDTA, 2% (w/v) Na_2CO_3, 0.1N NaOH). The mixture is shaken briefly on a vortex mixer and allowed to remain at room temperature for 15 min. One ml of 10% (w/v) SDS is added and immediately the contents of each tube are mixed thoroughly on a

vortex mixer. Finally, 0.1ml of Folin-Ciocalteau phenol reagent (Harleco Co. Ltd. diluted 1:1 with H_2O) is introduced and mixed as above. The samples are permitted to stand for 30 min prior to spectrophotometric analysis at 500nm.

The addition of 10% SDS is the crucial step in this modified procedure, since it prevents formation of the precipitate which forms when phenol reagent is introduced into samples containing Triton X-100. Fig. 2.1 shows the linearity of colour formation with concentration of BSA in the range of 50 - 500µg/ml.

(b) Lowry Method

The modified Lowry method of Miller (1959) was employed. To 1ml of neutral protein solution containing between 0.04 and 0.2mg protein is added 1ml of 10% (w/v) Na_2CO_3 in 0.5N NaOH containing 0.05% $CuSO_4$ and 0.1% potassium tartarate, mixed just prior to use. The mixture is allowed to stand for 10 min and 3.0ml of a 1 to 11 dilution of Folin-Ciocalteau reagent is added. After shaking and incubation at 50^{o} for 10 min, or at room temperature for 30 min, the OD at 560nm is determined in a spectrophotometer. For each set of protein estimations carried out, a standard curve is contructed using a solution of bovine serum albumin.

8. Distillation Methods for Phenol and m-Cresol

Phenol and m-cresol used for RNA extraction must be freshly distilled and free from oxidation products. The distillation methods are illustrated by labelled diagrams, (Fig. 2.2 and 2.3). Distilled products could be stored under an atmosphere of nitrogen at $4^{o}C$ for 2 - 3 weeks.

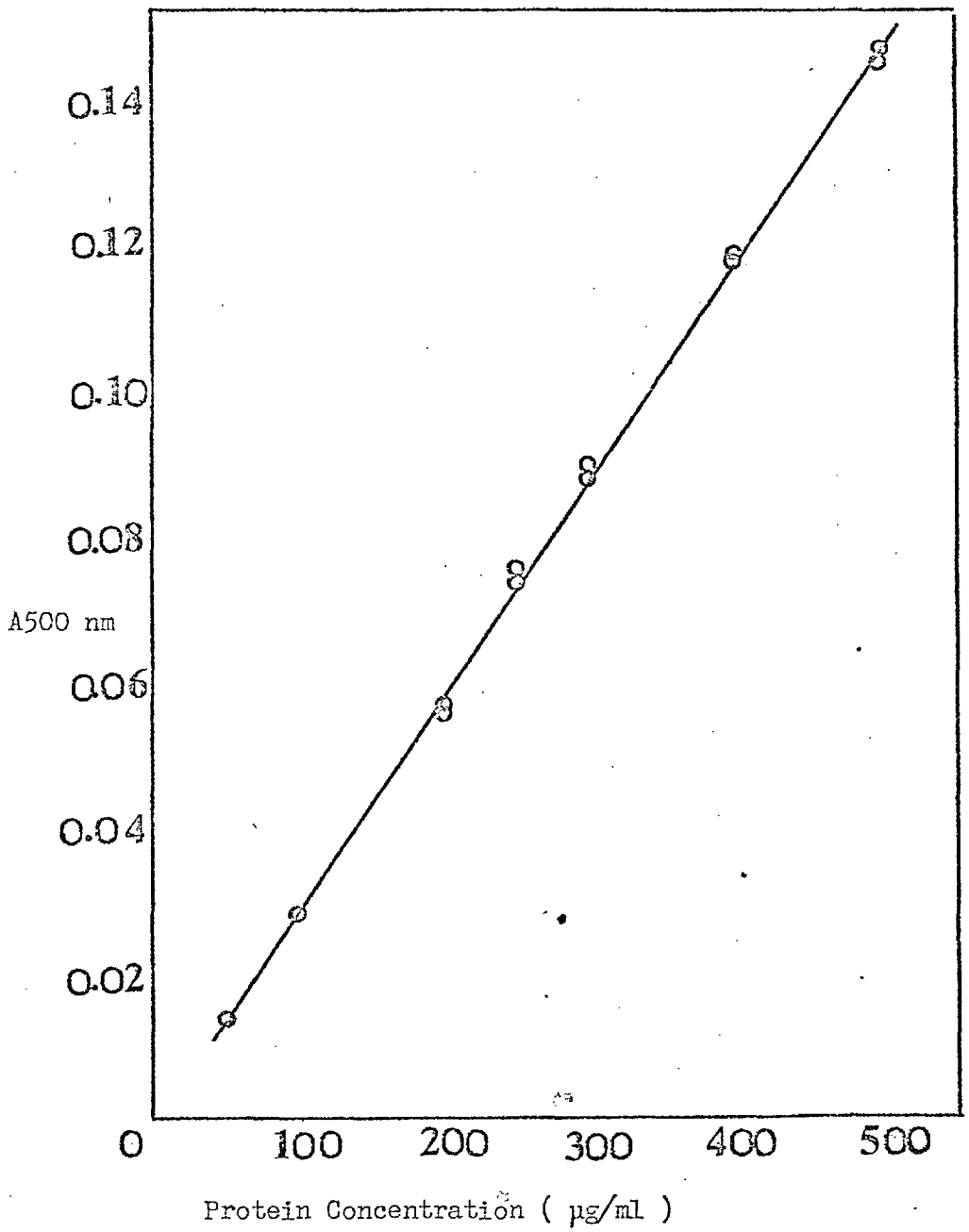


FIG. 2.1. Absorbance of chromagen produced by varying amounts of bovine serum albumin. Protein samples were in 0.2 ml of 0.1% Triton X-100 (v/v). Values were averaged from two experiments.

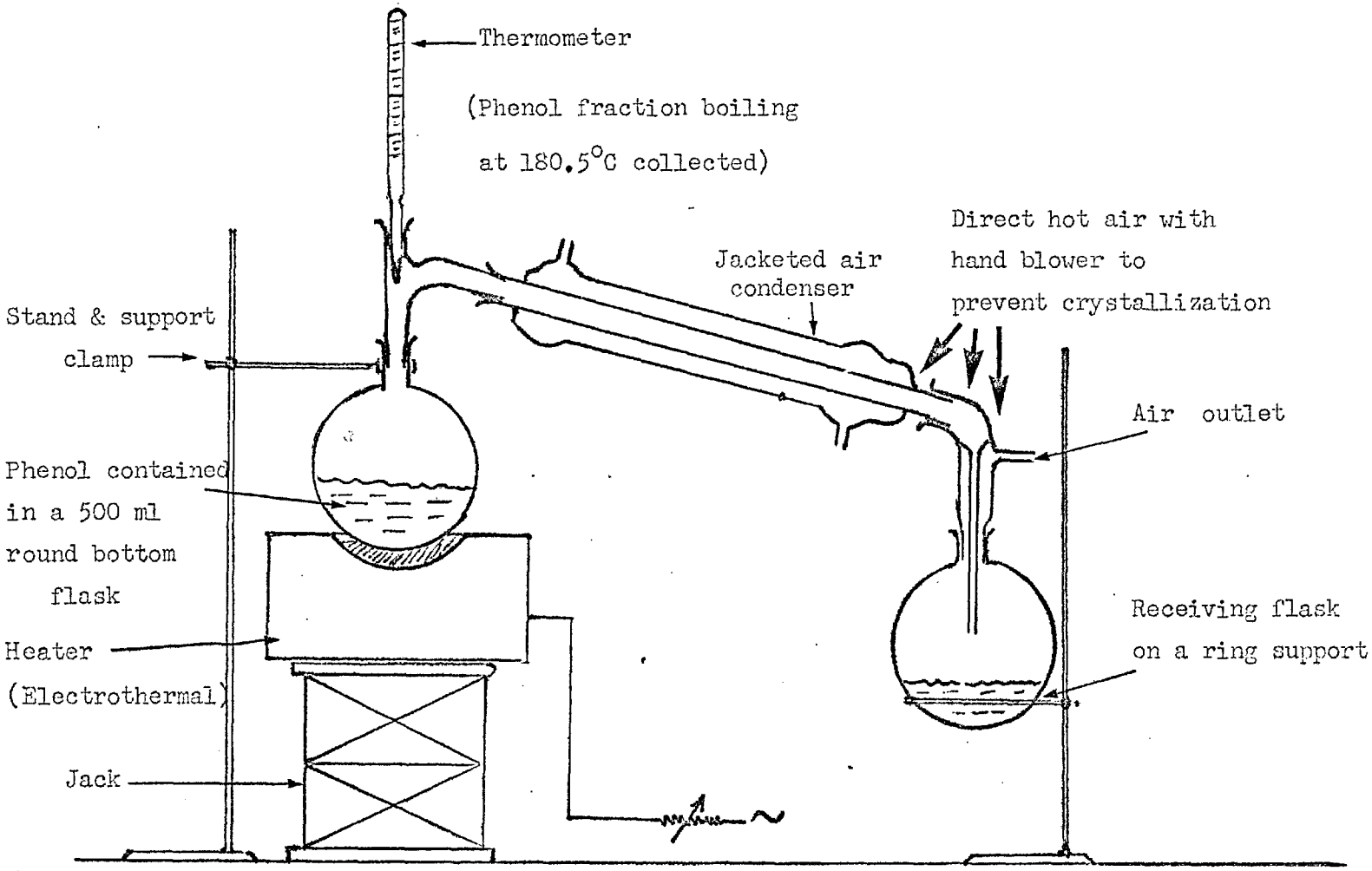


FIG. 2.2. Schematic of the apparatus used for distillation of phenol.

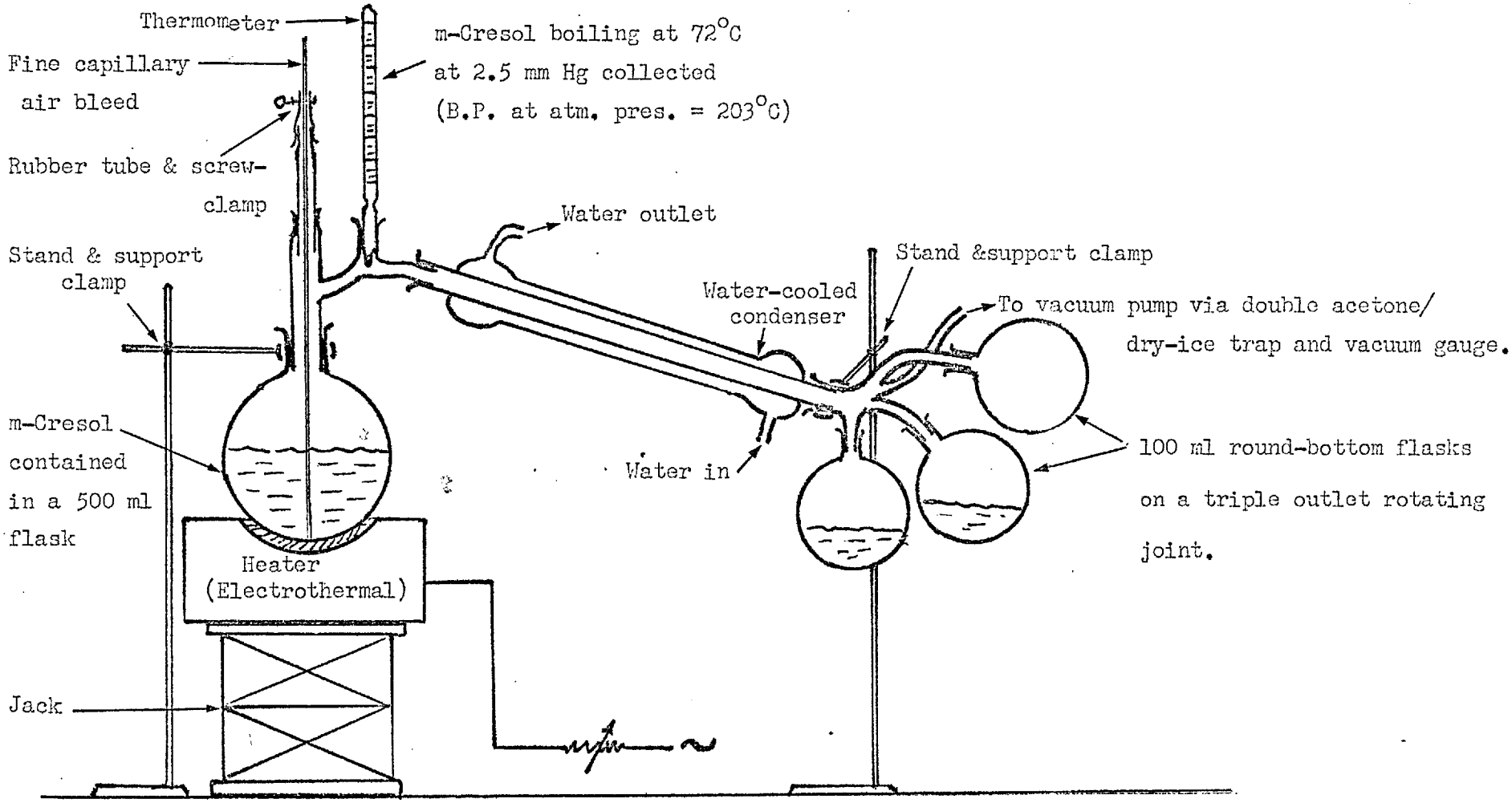


FIG. 2.3. Schematic of the apparatus used for distillation of m-cresol under reduced pressure.

9. Preparation of Pulse-labelled Bacterial RNA

The procedure for rapid cell lysis is adopted from Rogerson (1975), while RNA extraction is based on the procedure of Kirby (1968), with some minor modifications.

A growing bacterial culture at an appropriate cell density is labelled for usually 1 minute with 2μCi/ml of 5,6 - ^{3}H uridine, and if needed chased for about 30 seconds with excess (10mM) cold UTP before quick lysis. The pulse-labelled culture (10ml) is mixed rapidly with an equal quantity of a solution consisting of 10mM Tris-HCl pH 7.2, 10mM EDTA, 2% SDS, which had been prewarmed in a 60^{o}C bath. The mixture is stirred quickly to bring up the temperature to 52 - 55^{o}C and held at that temperature range for 10 - 15 seconds(i). This treatment lyses the bacterial cells as judged by a clearing of the solution and a noticeable increase in viscosity. The lysate is quickly cooled to room temperature(ii).

1.2g solid sodium 4- amino salicylate (6% w/v) is added(iii), shaken to dissolve, then an equal volume (20ml) of phenol-cresol mixture(iv) is added (phenol-cresol mixture: 50g of freshly distilled phenol; 7ml of freshly distilled m-cresol; 0.05g of 8-hydroxyquinoline; and 6ml of aqueous 0.1M NaCl(v)). The mixture is stirred on a wrist-action shaker for 20 minutes at 20^{o}C and then centrifuged at 12000g (10000 rpm on a Sorval SS-34 rotor) for 10 min at 10^{o}C. As much of the viscous aqueous layer was removed as could be removed without contamination by the interface and phenol layer, and added to 0.5 volumes of phenol-cresol mixture. The NaCl concentration is adjusted to 0.5M(vi) by adding 0.3g solid NaCl to each 1ml of aqueous phase and the mixture is kept on the shaker with gentle agitation. The phenol layer, interface and the remaining aqueous layer, are extracted for 7 min with 0.5

volumes of 0.1M Tris-HCl buffer pH 9.0 containing 0.5% SDS, and centrifuged as before. The aqueous phase, together with 0.5 volumes phenol-cresol mixture, are added to the first extract on the shaker, the NaCl concentration is adjusted to 0.5M and the mixture is then shaken at 20°C for 10 min and centrifuged at 8000g (about 8250 rpm) for 16 minutes at 5°C(vii). The aqueous phase is re-extracted once more with 0.5 volumes of phenol-cresol, centrifuged as before, and the aqueous phase mixed with 2 volumes of cold ethanol-m-cresol (9:1, v/v). The solution is allowed to stand at 2°C for 1hr. The precipitate is centrifuged off and extracted twice with cold 3M sodium acetate, pH 6.0 (25ml each time), or, according to a later paper (Salaman and Kirby, 1968), dissolved in 0.1M sodium acetate pH 6.0, then ribosomal RNA and rapidly labelled RNA are precipitated with 4M NaCl. This removes DNA, glycogen, tRNA, and other impurities. The RNA is centrifuged off each time at 8000g for 15 minutes at 5°C, and is washed once with a cold mixture of water (25ml), NaCl (1g), and ethanol(75ml), once with ethanol-water (3:1), twice with ethanol, and then dried in a vacuum desicator over $CaCl_2$.

Foot Notes:

(i) Heating the extract to more than 55°C during the first step results in degradation of DNA, as judged by the disappearance of the DNA peak, normally seen by electrophoresis, and the appearance of a new, diffuse peak centered near 16 S RNA (Rogerson, 1975).

(ii) Cooling the lysate to much below room temperature will precipitate SDS.

(iii) p-Aminosalicylate has metal complexing properties; it helps to release all nucleic acids into aqueous phase during phenol extraction (Kirby, 1957).

If the RNA precipitate is not washed thoroughly, p-aminosalicylate

will remain as a major contaminant. It absorbs very strongly at 260nm and in excess quantities it interferes with RNA mobility on gels. Its presence is indicated by a very diffuse peak running faster than RNA on gels, and by its characteristic UV absorption spectrum shown in Fig. 2.4. Other relevant UV spectra are also presented for comparison, Figs. 2.5 and 2.6. It may be convenient to somewhat reduce the amount of this compound added during RNA extraction.

(iv) m-Cresol is added to the phenol and serves two purposes: (1) the mixture can be cooled to 5^{o} without the phenol crystallizing, and (2) the phenol-cresol mixture is a better deproteinizing agent than phenol alone (Kirby, 1968).

(v) Sodium chloride is present to maintain ionic strength required to prevent loss of secondary structure of the RNA in the presence of m-cresol.

(vi) The two stage extraction is essential for the preparation of stable RNA. The first stage leaves DNA at the interface, but, as some protein is solubilized into aqueous phase, a second extraction with 0.5M NaCl solution is necessary to remove this protein into the phenol phase (Kirby, 1965).

(vii) It is important to carry out the centrifugations after the second phenol extraction at $5^{o}C$ in order to remove most of SDS by precipitation, while preventing phenol from crystallizing below $5^{o}C$.

10. RNA Gels

(a) Purification of Acrylamide and Bis-Acrylamide

Dissolve 70g acrylamide in 1 litre chloroform at $50^{o}C$. Filter hot without suction. Crystallize at $-20^{o}C$. Wash with cold

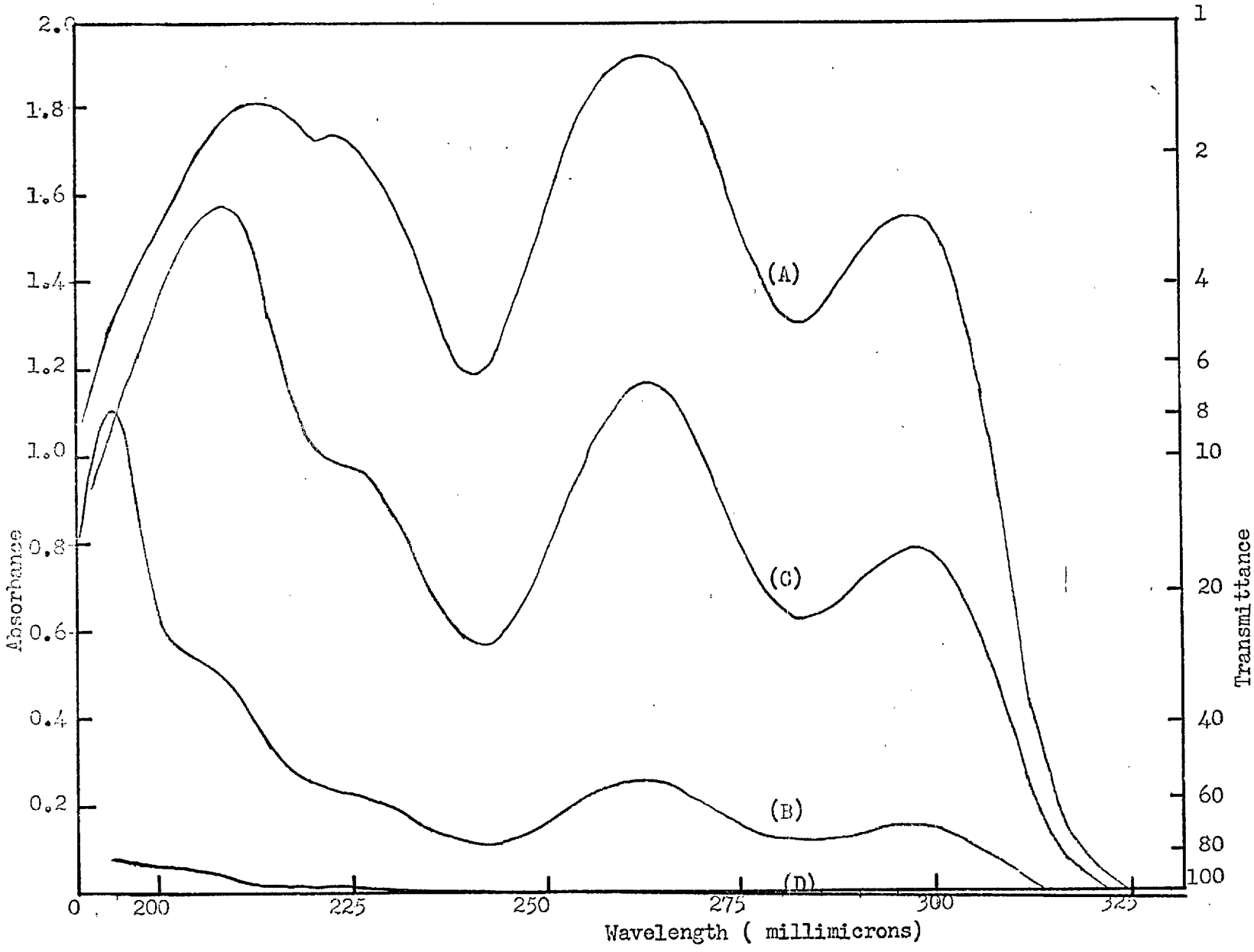


FIG. 2.4.

U.V. absorbance spectra: (A), "impurity"(in an RNA precipitate extracted with 30 ml of /M NaCl) diluted with H20, 1:10; (B),"impurity" diluted 1:100; (C), 4-amino-salysilic acid-sodium salt, very dilute solution about $1:10^6$; (D), H20 or 1% SDS solution(base line).

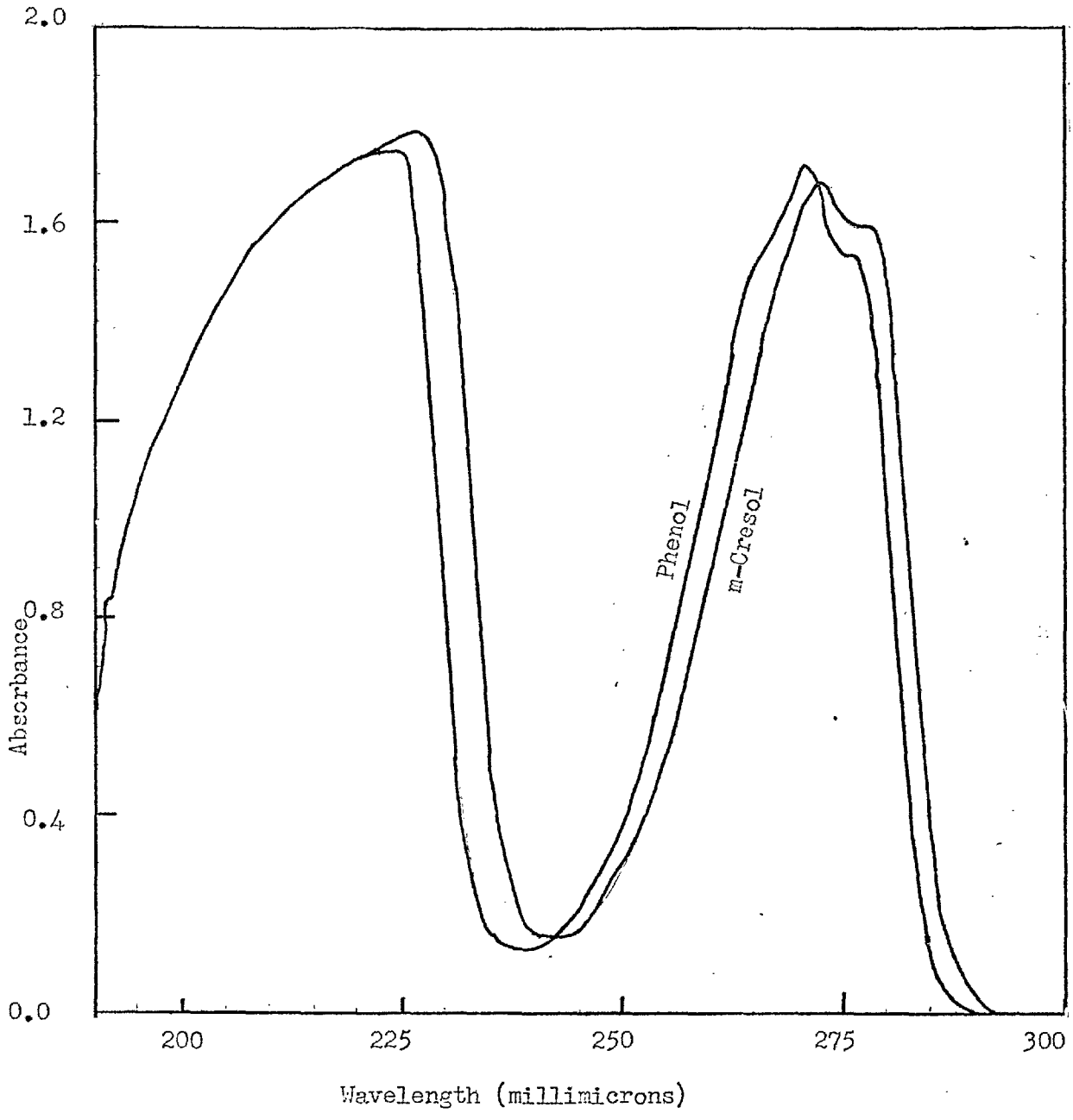


FIG. 2.5. Ultraviolet absorbance spectra of phenol and m-Cresol in H_2O, 1:10,000 dilution. Spectrophotometer: Unicam SP. 800.

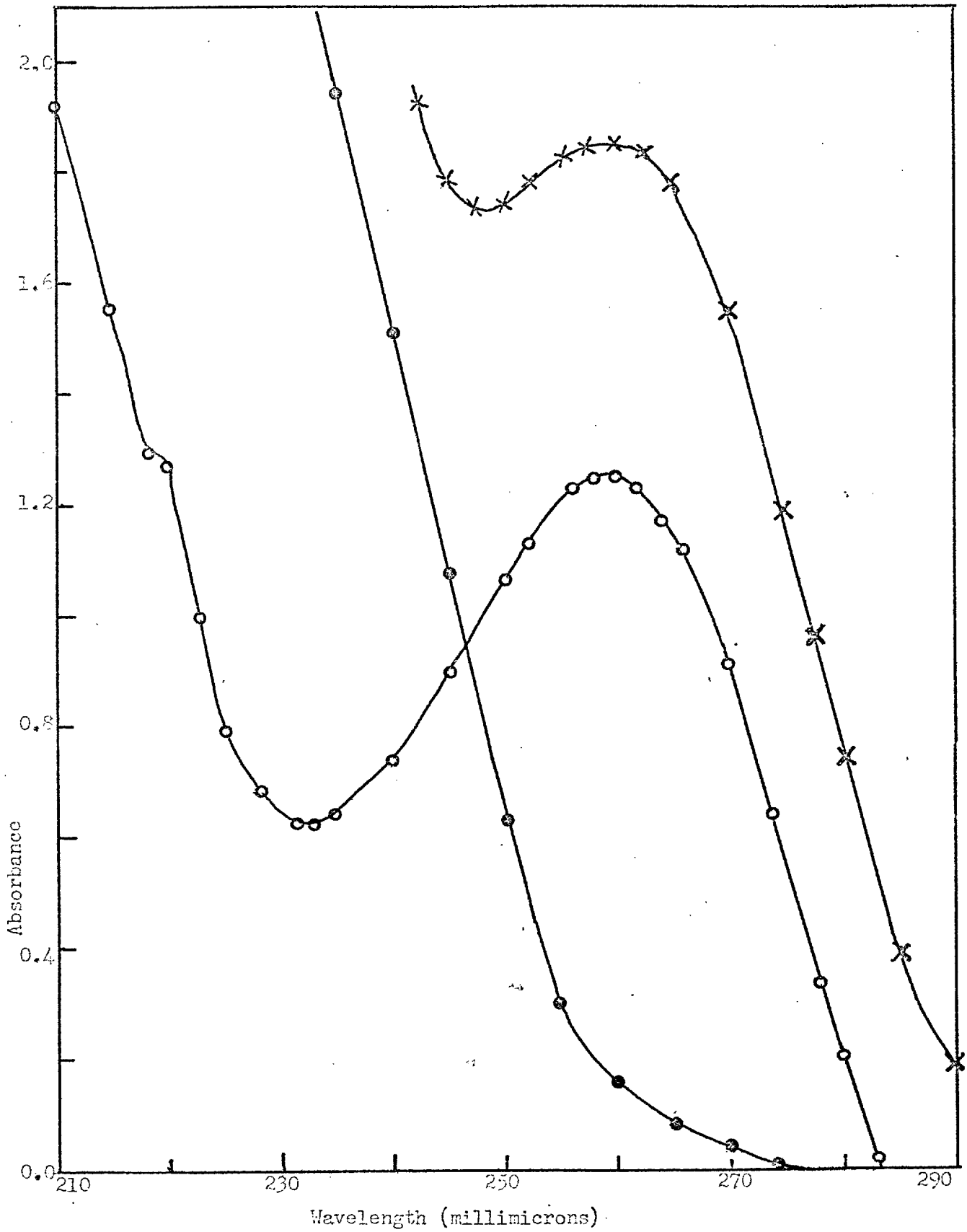


FIG. 2.6. U.V. absorbance spectra: ● , polyacrylamide gel; O, RNA in polyacrylamide gel; X, impurity (4-aminosalysilic acid) in polyacrylamide gel.

chloroform and dry under reduced pressure.

Dissolve 10g bisacrylamide in 1 litre acetone at 40 - 50°C. Filter hot as above. Crystallize at -20°C. Wash with cold acetone and dry.

(b) Stock buffer, (Loening, 1967)

		Final Running Concentration
Tris	21.7gm	36mM
Sodium dihydrogen phosphate, $2H_2O$	23.4gm	30mM
di-sodium EDTA, $2H_2O$	1.85gm	1mM
Water to total volume of	1000ml	

pH should be 7.6 to 7.8 at room temperature. A higher sodium phosphate concentration can be used to give a lower pH.

(c) Running buffer is 200ml of the stock buffer + 20ml of 10% (w/v) sodium dodecyl sulphate (Low UV absorption, such as BDH specially purified grade), diluted with H_2O to 1 litre.

(d) 4% Gels

4ml stock buffer

2ml 40% acrylamide + 0.4% bisacrylamide in stock buffer (stored at 4°C)

12ml distilled water

Degas the above mixture, then add:

2ml freshly dissolved ammonium persulphate, 1.5% (w/v)

20µl N,N,N,N, tetramethylethylene diamine

Mix the solutions and pipette immediately into siliconed gel tubes which are sealed with Parafilm at the bottom. The gels are immediately layered with butanol. Butanol can later be pipetted off and the top of the gels washed with running buffer. Before sample application the gels are run for ½ hr at constant 50 volts in running buffer.

(e) Application of Sample

Nucleic acids are dissolved in running buffer, containing 10% sterile and 'RNAse-free' sucrose, or glycerol.The sample (10 - 30µl, at 1mg/ml) is applied to the gel and over layered carefully with the running buffer. A trace of bromophenol blue as tracker dye may be added.

(f) Electrophoresis

The positive electrode is connected to the reservoir in contact with the bottom of the gel, and the negative to the top. Electrophoresis is carried out at constant 50v for 1.5hr, for 5mm diameter x 9cm length disc gels, in 10cm long siliconed tubes.

(g) UV Scanning

Gels are scanned inside the silicon tubes at 260nm, using Gilford Instrument Spectrophotometer 240 with Gilford Linear Transport and Venture Servoscribe Potentiometric Recorder RE 541. 20. spectrophotometer switched to UV or Vis. Set slit window at 0.2mm, recorder, at 60mm/min. Ratio switch set at 0.25, follow the Operation Instructions "Recorder Caliberation" written on the instrument. Slit is then set to an arbitrary value of 0.9, Read/Blank switch to Read. The base line is set to zero with the lowest reading point on gel, by turning Absorbance knob. The gels are scanned at 1cm/min.

(h) Radioisotope Determination

For determination of the isotope distribution, the gel is first eased out of the tube by injecting distilled water carefully with a syringe, between the gel and the tube at both ends, and applying pressure and suction with a rubber teat. The gel is placed without stretching on to a wet Whatman filter paper which is taped to the cutting surface of the gel Slicer (Mickle Lab. Eng. Co., Gomshall, Surrey, UK). The gel is frozen and stuck

firmly to the paper by covering it with crushed dry-ice for about 5 minutes. One end is gradually exposed and tried for firmness. If the gel is too hard, it will be too brittle, difficult to cut, and easy to crack and lose. If it is too soft, it will be sticky and it will adhere to the cutting blade. The gels are sliced by slowly lowering the blade. Each 1mm thick slice is picked with a blade, placed in a glass counting vial and dried overnight at room temperature. Each gel is digested in the capped vial with 0.25ml of a mixture, made of 99 parts 100 volume cold H_2O_2 to one part ammonia (A.R., sp. gravity 0.88), and incubated at 37^oC overnight, or longer if necessary. 5ml of 2-methoxyethanol(AR) is added to each vial, shaken, then 10ml of scintillation cocktail (Toluene 1 litre, Butyl-PBD 6g, Naphthalene 50g) added, mixed thoroughly and counted.

11. Sterile and RNAse-free technique

RNA, polysomes and *in vitro* protein synthesizing systems are very sensitive to contaminants. They lose activity in the presence of traces of ribonuclease, heavy metal ions, or if stored without quick cooling.

In order to minimize the possibility of RNAse contamination, all plastic or glassware is soaked in deionized, distilled water containing detergent, washed thoroughly with detergent, rinsed with distilled water, and soaked in HCl solution overnight. Badly contaminated glassware is either soaked in chromic acid or in alcoholic KOH, (50g KOH in 100ml H_2O + 900ml 96% ethanol). The acid-washed containers and pipettes are rinsed thoroughly with deionized distilled water, then sterilized by autoclaving for 45 - 60 min, or baking at 180^oC for 3 - 4 hrs.

All buffers and sucrose solutions with which polysomes, RNA, or protein synthetic systems come in contact are prepared in sterile deionized, distilled water, then autoclaved for the required length of time. Sterilized acid washed, thin rubber gloves are worn whenever there is a possibility of touching and thereby contaminating one's preprations during experiments.

According to Palacious et al., (1972), all reactions with polysomes are carried out in sterile plastic tubes, and all solutions are handled with sterile plastic pipettes (apparently glass has destabilizing effect on polysomes). Suspensions of DEAE and CM-cellulose and Sepharose in equilibration buffers are sterilized by autoclaving. Dialysis tubings are boiled in 5% $NaHCO_3$ containing 0.1mM EDTA, washed and boiled seveal times in sterile, distilled water and autoclaved

The perspex sucrose gradient former is made RNAse-free by successive washings with detergent, SDS solution, H_2O, dilute HCl and sterile H_2O. It withstands alcoholic KOH and 6M HCl. For storage it is rinsed with ethanol-sterile distilled H_2O, then wrapped with Clingfilm and aluminium foil.

12. Protein Synthesis In Vitro (Method 1. I.C.)

(a) Growth of Cells

The bacteria (E. coli K-12, strain MRE600) were grown at 37°C in an Imperial College Biochemistry Department Pilot Plant fermenter in a rich medium as follows:

Ingredients	% W/V	Weight
Peptone (oxoid)	0.50	300g
Yeast extract paste (oxoid)	0.71	426g
Sodium chloride	0.20	120g
Potassium di-hydrogen phosphate	0.26	156g
Di-sodium hydrogen phosphate	0.42	252g

Ingredients	% W/V	Weight
Polyglycol P-2000 (antifoam)	0.01v/v	6ml

+ 45 litres distilled water stirred at 70°C. The ingredients were added in the above order at 2 min intervals, stirred for 15 min, pH adjusted to 6.9 - 7.1 with sodium hydroxide/sulphuric acid and the volume was made up to 55 litres with distilled water. After sterilization, 2 l sterile glucose solution, equivalent to 1% (660g), was added to this medium.

To start culture growth, the medium at 37°C was inoculated with 2000ml of a fresh stationary phase culture of E. coli MRE600 cells. (Agitator shaft speed, 367rpm; air flow rate 30 l/min; pressure 15 p.s.i.) Subsequent minimal additions of antifoam were made as required. Cell denisty was estimated by monitoring oxygen tension and by Nephelometer readings, using the data of an identical pilot culture presented in Table 2.1 and Fig. 2.7. The culture was harvested at cell density of 3.5×10^8 by quick transfer of the culture into a cooling container and passing it through a Sharples Continuous Flow centrifuge at a fast flow rate, 3 l/min. The cell paste was resuspended in 110ml of chilled buffer I (0.01M Tris-HCl pH 7.8; 0.01M Mg acetate; 0.06M KCl; and 0.006M 2-mercaptoethanol), and the cells recovered by centrifugation at 3000 x g at 4°C. The washed cell pellet was quick frozen inside a polypropylene centrifuge bottle in dry ice-acetone mixture.

(b) Preparation of the S30 Extract

The frozen cell paste (23.2g) is transferred to a clean and sterile mortar and kept at -20°C for 1 - 2 hr, and then ground with 46.4g of levigated alumina, Sigma, which has been treated with HCl and washed free of acid before use, and 35ml of buffer mixture 1. Half of the alumina is added at first and ground for 5 min, then the rest of alumina is added and ground for further 5 - 10 min. A few

TABLE 2.1.

Experimental Data of an *E. coli* MRE600 Pilot Culture (EC 51) Grown in a Fermenter

Time (hours)	pH	Bacterial Dry Wt.	Neph. Reading	Cell count (saline)	% O_2 Tension	Reduction in % O_2 (Uptake)
Pre inoc.	6.9	0.065	51.5	-	21.0	0
Post inoc.						
0	6.9	0.140	63	3.5×10^7	21.0	0
0.5	6.9	0.179	112	5.27×10^7	20.99	0.01
1	6.9	0.132	144	1.01×10^8	20.93	0.07
1.5	6.8	0.138	243	2.53×10^8	20.80	0.20
2.0	6.5	0.200	495	5.38×10^8	20.52	0.48
2.25	-	-	-	-	20.28	0.72
2.5	6.4	0.236	990	1.72×10^9	19.94	1.06
3.0	6.05	0.266	1732	3.48×10^9	19.37	1.63
3.5	5.9	0.239	2340	5.63×10^9	19.17	1.83
4.0	5.65	0.267	2835	7.2×10^9	19.43	1.57
4.5	5.5	0.272	3600	8.0×10^9	19.75	1.25

Cell count by hemocytometer, Thoma grid by Hawksley, in physiological saline.

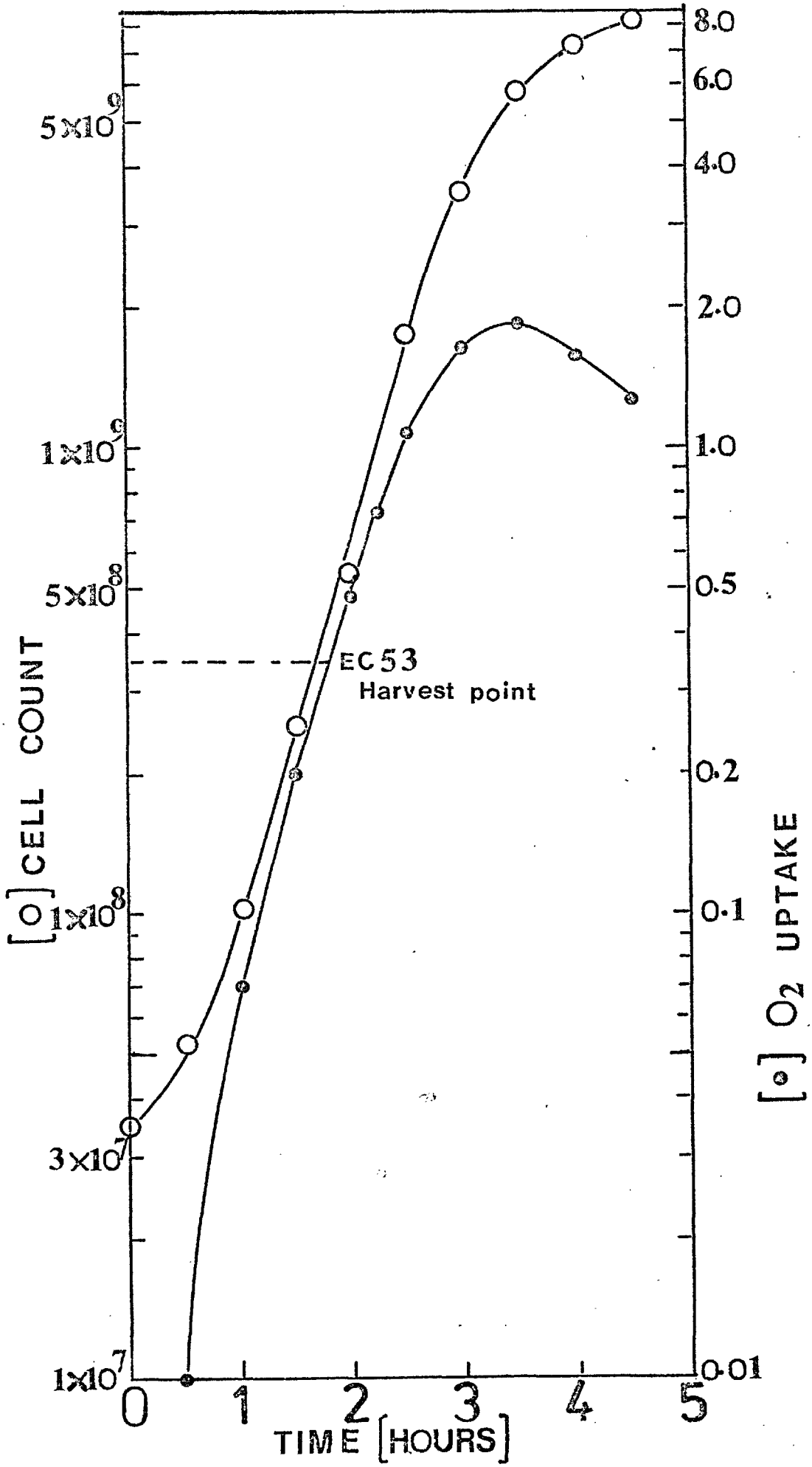


FIG. 2.7. Growth of Escherichia coli (MRE 600) monitored by cell count (hemocytometer) and O_2 uptake. Pilot plant culture: EC51.

ml of buffer is added and grinding continued. Total time of grinding is 45 min. A little buffer at a time is added, and in the last 15 min gradually more buffer is added in small amounts. Some buffer is left for rinsing the mortar and pestle. Gloves are worn during grinding, in order not to touch and contaminate the preparation. All the grinding is carried out at $4^{o}C$, with mortar resting in a container surrounded by ice. The suspension is transferred to a centrifuge tube and spun for 20 min at 15000rpm in an MSE Centrifuge at $2^{o}C$. The pellet is discarded, and deoxyribonuclease (electrophoretically purified, "RNAse-free") is added to the supernatant to a final concentration of 3µg/ml. The viscosity of the extract is reduced considerably in a few min. The extract is then centrifuged at 30,000 x g for 1 hr. About 20ml of the supernatant is carefully pipetted off, taking care not to disturb the precipitate and the suspension above it.

The supernatant is divided into 3.2ml portions, and frozen in acetone-dry ice mixture, and stored at $-20^{o}C$ until needed. To one of the 3.2ml S30 portions, 0.8ml of sterile 50% sucrose solution is added (10% sucrose, final). In order to destroy endogenous messenger RNA in S30 fraction, to the 4ml S30-sucrose solution is added 1ml of Buffer mixture II, and incubated at $35^{o}C$ for 40 min. Buffer mixture II contains components needed to make the composition of the S30 fraction as follows: Tris-HCl, pH 7.8, 80µmoles/ml; Mg acetate, 8µmoles/ml; KCl, 50µmoles/ml; 2-mercaptoethanol, 9µmoles/ml; mixture of 20 amino acids, 0.075µmoles/ml each; ATP, 2.5µmoles/ml; phosphoenolpyruvate, 2.5µmoles/ml; pyruvate kinase, 15µg/ml. After preincubation, the S30 preparation is dialysed overnight against 100 vol of buffer mixture III, changed once after 2½ hr.

Buffer mixture I: 0.01M Tris-HCl, pH 7.8; 0.01M Mg acetate; 0.06M KCl; 0.006M 2-mercaptoethanol.

Buffer mixture.III:Is the same as Buffer mixture I except that it also

contains 10% sucrose. Both Buffer mixtures I and III are prepared by diluting appropriate volumes of 10 x concentrated buffer, 50% sucrose and undiluted 2-mercaptoethanol.

Buffer Mixture II:

Tris-HCl, pH 7.8	0.36M
Mg acetate	0.0M
KCl	0.01M
2-mercaptoethanol	0.02M
Amino acids, each	0.375mM
ATP	2.5mM
Phosphoenolpyruvate	2.5mM

Pyruvate kinase, 75µg added separately from a 4mg/ml ammonium sulphate suspension.

A260nm of the dialysed S30 preparation is determined on a Unicam SP500 spectrophotometer. The S30 fraction is diluted with Buffer Mixture III to have an OD_{260} of 0.25 on 200 fold dilution.

(c) Incubation Mixture for In Vitro Protein Synthesis

The incubation mixture for amino acid incorporation contains in 1ml: S30 fraction, containing 1mg RNA (assuming 20 OD_{260} units of RNA = 1mg); Tris-HCl, pH 7.8, 100µmoles; GTP, 0.03µmoles; Mg acetate, 14µmoles (6 - 9µmoles for natural messengers and under physiological conditions); KCl, 50µmoles; 2-mercaptoethanol, 6µmoles; mixture of 19 amino acids, 0.2µmoles each; one ^{14}C-labelled amino acid, about 0.01µmole (about 0.05µCi); ATP, 1µmole; phosphoenolpyruvate, 7.5µmoles; PEP-kinase, 20µg; messenger RNA, 50µg.

In order to obtain proper concentration, components are added in various groups: (i) Salt buffer mixture, which contains the required amounts of Tris buffer, KCl, Mg acetate, mercaptoethanol, GTP; (ii) two different amino acid mixtures, one containing labelled Phe, the

other labelled Leu; (iii) a mixture of ATP, PEP, pyruvate kinase (energy generating system).

2-mercaptoethanol must be diluted just before each experiment since it is unstable; glutathione is more stable, therefore, is a better substitute.

PEP must be prepared fresh before each experiment.

Amino acid mixture must be neutralized.

ATP is best to be dissolved in 0.1M Tris-HCl buffer.

Incubation mixtures are prepared in sterile tubes and kept on ice while being prepared. Shake tubes well before incubation. Incubation mixtures in tubes are covered with Parafilm and incubated at 37°C for 30 min. The reaction is stopped by the addition of 0.4ml of 1 N NaOH containing 0.01M unlabelled Phe and Leu (to dilute the ^{14}C-amino acids present in the mixture). Tubes are incubated for further 5 min at 37°C and 15 min at room temperature. 5ml of 10% trichloroacetic acid (TCA) is added to each tube, whirlmixed, poured the contents onto glass fibre filters. The precipitates are washed twice with 5% TCA, 10ml each time, rinse tubes with the same solutions. The precipitate is washed again with the same amount of hot (nearly boiling) 5% TCA twice, and finally rinsed twice with distilled water. Each filter is placed in a scintillation vial, dried at 80 - 100°C in oven. 10ml of scintillation liquid (6g/l Butyl-PBD, 50g/l naphthalene in toluene) is added to each vial, and counted on a Beckman Scintillation Counter.

(d) Preparation of Amino Acid Mixture for Protein Synthesis

A mixture of all amino acids less Phe and Leu, 2μmoles/ml each, is prepared by dissolving the required amounts in neutral pH or distilled water, starting with tyrosine, as it is insoluble. Stir the tyrosine in portion of water and bring the pH down with 0.1M HCl gradually to 3 and stir for 1 hr, or at pH 2, stir for 20 min

to dissolve. Then neutralize the solution with 1M Tris-HCl pH 7.8, and dissolve the other amino acids by stirring. The pH is finally adjusted to 7.2 with Tris-HCl pH 7.8, and autoclaved. The amino acids, with their M.W. and solubilities in H_2O are listed below. Cystine is insoluble and is not required.

L-Amino Acids	M.W.	Solubility
Alanine	89.1	Soluble
Asparagine	132.1	Soluble
Aspartic acid	133.1	Soluble, very slow
Arginine-hydrochloride	210.7	Soluble
Cysteine, free base	121.2	Soluble
Cystine	240.3	Insoluble
Glutamic acid	147.1	Soluble, slow
Glutamine	146.1	Soluble
Glycine	75.1	Soluble
Histidine	191.7	Soluble
Isoleucine	131.2	Soluble
Leucine	131.2	Soluble
Lysine-monohydrochloride	182.7	Soluble
Methionine	149.2	Soluble
Phenylalanine	165.2	Soluble
Proline	115.1	Soluble
Hydroxyproline	131.0	Soluble
Serine	105.1	Soluble
Threonine	119.1	Soluble
Tryptophan	204.2	Soluble, very slow
Tyrosine	181.2	Insoluble
Valine	117.2	Soluble, slow

13. Protein Synthesis In Vitro (Method II, I.C.R.F.)

(a) Growth of Cells

The bacteria were grown at 25°C in a rich medium of concentrated nutrient broth containing 32g Bacto-tryptone, 20g yeast extract (both Difco products), 5g sodium chloride and 5ml of a 1M solution of sodium hydroxide were added to 1 litre of water, and after autoclaving, 4ml of a 50% glucose solution were added (giving a final glucose concentration of 0.2%). Growth was conducted in a 5 litre Erlenmeyer flask on a shaking incubator.

To start growth the preheated medium was inoculated with 100ml of a fresh stationary phase culture of E. coli MRE600 cells. The cells were grown into the subsequent stationary phase (OD_{550} = 7.0) overnight. The cells were collected by centrifugation in the cold at 10,000 x g for 10 minutes. Immediately after they were harvested, the cells were washed at 0°C by resuspension in 25ml buffer A (0.03M Tris pH 7.8, 0.06M KCl, 0.01M Mg acetate, 0.003M 2-mercaptoethanol), and the cells collected by centrifugation at 20,000 x g for 10 min. This washing procedure was then repeated.

Since the S30 could not be prepared immediately, the E. coli cells were frozen quickly by immersing the polypropylene centrifuge tubes in a dry ice-acetone mixture. The frozen pellet was wrapped in plastic and placed in a lunch box and stored at -70°C.

(b) Preparation of the S30 Extract

The S30 was prepared by the standard method of Dr. Harvey Lodish (Massachusetts Institute of Technology).

The frozen cell pellet was weighed and 10g of the 16g of cells obtained were placed in a chilled mortar and left in the cold room (+4°C) for 10 min, 25g of chilled levigated alumina were added and ground until a moderately viscous paste was obtained (about 10 min). 30ml buffer A was added in 10ml batches, mixing after each addition.

DNase (electrophoretically pure, ie RNase free) was added to 10μg per ml of buffer A and left standing at 0°C for 5 minutes. The cell debris and alumina were removed by centrifugation in the Sorval SS34 rotor for 10 min at 9,000rpm. The supernatant was then removed to another tube and centrifuged for 20 min at 16,000rpm, again in the SS34 rotor.

The clarified supernatant was removed and ATP (1/20th vol of a 0.05M solution), phosphoenol pyruvate (1/20th vol of a 0.07M solution) and pyruvate kinase (30μg/ml) were added. This mixture was incubated at 30°C for 1hr. This preincubated S-30 extract was dialysed overnight against two 1 litre changes of buffer A and frozen in 100μl aliquots under liquid nitrogen. The dialysis tubing used was cleaned by boiling in water, then in 0.1M $NaHCO_3$, twice more in water, once in 1mM EDTA (30 min each time) and finally wash with water.

(c) Incubation Mixture for In Vitro Protein Synthesis.

The components shown below are the standard ingredients of one 50μl incubation as recommended by H.F. Lodish:

Component	Volume Added	Final Concentration in 50μl Incubation
1M Hepes pH 7.0	2.5μl	50mM
2M NH_4Cl	2.5μl	100mM
0.1M Mg Acetate	1.5μl	3mM
1M 2-mercaptoethanol	0.5μl	10mM
0.05M ATP	2.0μl	2mM
0.07M PEP	3.0μl	4mM
0.01M GTP	1.5μl	0.3mM
AA-met	1.0μl	5mM of each A.A.
^{35}S-met	6.0μl	
E. coli S-30	15.0μl	See N.B. below
Water	4.5μl	
	40.0μl	

N.B. The E. coli S30 is suspended in buffer A (0.03M Tris pH 7.8; 0.06M KCl; 0.01M Mg acetate; 0.003M 2-mercaptoethanol). This therefore adds 9mM Tris, 18mM KCl and 3mM Mg acetate to the final concentration in a 50µl incubation. Hepes is a Biological buffer N-2-Hydroxyethylpeperazine-N'-2-ethanesulphonic acid (BDH).

The reaction mixtures were incubated at 37°C for 30 min (in the case of first two experiments, namely the MS2 RNA concentration curve and the ^{35}S-methionine concentration curve, the incubations were of 1 hour duration).

At the end of the incubation, 10µl of the reaction mixture was taken into 0.2ml 0.1M KOH and this was incubated for 20 min at 37°C to break the bonds between any tRNA molecules and amino acids not yet incorporated into protein. After this incubation time, the proteins synthesised in the cell free S30 system are precipitated with 3ml ice cold 10% TCA and standing on ice for ten minutes. After this time the precipitate was collected by filtration through a 2.4cm diameter Millipore filtre (HAWP 02400). The incubation tube is then washed with two 3ml aliquots of 5% TCA which are used to wash the filtre. The filtre was then dried under an infra-red lamp and counted in a Toluene PPO/POPOP scintillation fluid (12.5g PPO + 0.75g POPOP in 2.5 litres of Toluene). The scintillation counter setting used were: 60% Gain; Window-35-0.0.

If samples of the reaction were to be analysed by SDS polyacrylamide gel electrophoresis, 5µl of the reaction was taken into 20µl sample buffer + DTT, boiled for 3 min& run on a 15% acrylamide gel pH 8.8 with a 5% stacking gel pH 6.8. The gel was then stained, destained, dried and exposed.

14. Immunization of Animals and Collection of Antisera

The maximum amount of antigen which can be administered much depends on the purity of the preparation. The minimum amount is

governed by the immunogenicity of the substance. For the RDH-D preparation, about 200μg (0.5 - 1.0mg/ml solution) was found optimal for the initial injections into each young rabbit, (about 2 - 2½Kg body weight). For subsequent, booster, injections, half of this quantity is used each time (immunization with 50μg RDH/ rabbit was tried in 10 rabbits, results were unsuccessful). The antigen is mixed thoroughly with an equal volume of Freund's Complete Adjuvant, plus 1 volume of Freund's Imcomplete Adjuvant (if the volume is too small). For booster injections the antigen is mixed only with Freund's Incomplete Adjuvant. If a number of rabbits are to be injected, it is best to mix the total inoculum by sonication, so that the sample is sterilized while ensuring it is being thoroughly mixed.

Injections were given by sterile syringes and needles, both subcutaneously in multiple sites in the abdomen as well as intramuscularly in the hind legs. Booster injections are given in the same manner, no sooner than 4 weeks intervals. Animals should be bled between the seventh and fourteenth days subsequent to each booster injection. Bleeding is done by cardiac puncture, entering the heart laterally by a sterile syringe needle. Several bleeds may be taken during this time without affecting the amount or the quality of the antibodies.

The sheep are immunized each with 5mg of purified rabbit (anti RDH) Ig as above. Injections are given only intramuscularly into one of the hind legs at each time. The same amount of antigen can be used for each booster injection. Sheep blood is taken from the jugular vein, draining by an infusion 18 gauge needle and tubing set (Gillette Surgical, Middlesex) into a sterile container.

The animal blood is usually collected in a sterile tube or flask, covered by a sheet of clingfilm, and kept at room temperature for 2 - 3 hours till the clotting process is established. Then it

is transferred to $4^{o}C$ and stored overnight. In the morning the blood clot is gently detached from the walls of the container by a glass rod, and after allowing about half an hour for the clot to retract, the antiserum is carefully transferred by a pasteur pipette to a centrifuge tube. The clot is left in the tube in order to retract further and allowing more serum to be collected. The reason for continuing the clotting process in the cold and in sterile containers is only to minimize bacterial contaminations and growth, which could only contribute to unwanted nuclease and protease pools. Usually about 50% (v/v) antiserum is collected from such blood samples. Antisera are centrifuged at low speed for 10 - 15 minutes in order to remove any suspended cells or microorganisms. The clarified antisera are stored at $-20^{o}C$.

15. Covalent Coupling of Ribitol Dehydrogenase to Bromoacetamidoethyl-Sepharose for Purification of Specific Antibodies

Adopted from the procedure of Pedro Cuatrecasas: J. Biol. Chem. 245, 3059 - 3065, 1970.

(a) Preparation of washed, activated Sepharose

In a well ventilated hood, a given volume (say 100ml) of well washed decanted Sepharose-4B is mixed with an equal volume of water, and finely divided cyanogen bromide (250mg per ml of packed gel) is added at once to the stirred suspension. The pH of the suspension is immediately raised to and maintained at 11 by continuous titration with 8M NaOH, (about 50ml). The temperature is maintained at about 20^{o} by adding pieces of ice as needed. The reaction is complete in 8 to 12 minutes, as indicated by the cessation of proton release. A large amount of ice is then rapidly added to the suspension, which is transferred quickly to a Buchner funnel with coarse sintered glass disc, and washed under suction with cold buffer. The buffer should be the same as that which is to be used in the coupling stage (dist.

H2O used in this case), and the volume of wash should be 10 to 15 times that of the packed Sepharose.

(b) Attachment of ethylene diamine to the activated Sepharose

To the moist, washed, activated Sepharose is added an equal volume of cold distilled water containing 2m moles of ethylene diamine for each millilitre of Sepharose (ethylene diamine previously titrated to pH 10 with 6N HCl). The suspension is immediately mixed (in the Buchner funnel) with a glass stirring rod. The entire procedure of washing, adding the ligand solution, and mixing should consume less than 90 seconds. It is important that these procedures be performed rapidly and that the temperature be lowered, since the "activated" Sepharose is unstable. The suspension is transferred from the Buchner funnel to a beaker containing a magnetic mixing bar and is gently stirred at 4^{o}. Although the reaction is essentially complete in 2 to 3 hours, the mixture is allowed to stand at 4^{o} for 16 to 20 hours to ensure complete loss of reactive agarose groups. The substituted Sepharose is then washed with large volumes of distilled water (6 litres here) until it is established with certainty that ligand is no longer being removed. This treatment results in a derivative having about 12μ moles of aminoethyl groups per ml of Sepharose.

(c) Preparation of Bromoacetyl Sepharose Derivatives

Bromoacetamidoethyl-Sepharose can be prepared easily in mild aqueous conditions by treating aminoethyl-Sepharose with O-bromoacetyl-N-hydroxysuccinimide. This derivative of Sepharose can react with primary aliphatic or aromatic amines as well as with imidazole and phenolic compounds. Additionally, proteins readily couple to bromoacetamidoethyl-Sepharose, forming insoluble derivatives in which the protein is located at some distance from the solid support. The following procedure can be used to prepare such derivatives: In 8ml

of dioxane are dissolved 1.0m mole of bromoacetic acid and 1.2m mole of N-hydroxysuccinimide. To this solution, 1.1m mole of dicyclohexylcarbodiimide are added. After 70 minutes dicyclohexylurea is removed by filtration, and the entire filtrate (or crystalline bromoacetyl-N-hydroxysuccinimide ester) is added, without further purification, to a suspension, at 4^{o}, which contains 20ml of packed aminoethyl-Sepharose (2μ moles of amino groups per ml) in a total volume of 50ml at pH 7.5 in 0.1M sodium phosphate buffer. After 30 minutes, the Sepharose is washed with 2 litres of cold 0.1M NaCl. (Quantitative reaction of the amino groups occurs, as shown by the loss of orange colour with the sodium trinitrobenzenesulfonate colour test; I did not perform this test). Reaction of this bromoacetamidoethyl-Sepharose gel with proteins in 0.1M NaHCO3, pH 9.0, for 2 days at room temperature, or for longer periods at lower pH values or lower temperatures, as a 50% (v/v) suspension, followed by reaction for 24 hours at room temperature with 0.2M (0.6ml) 2-aminoethanol to mask unreacted bromoacetyl groups, results in attachment of RDH to bromoacetamidoethyl-Sepharose. In this experiment, about 10mg of pure RDH was used per ml of packed Sepharose, incubated 4 days at 4^{o} with activated Sepharose. Incubation with 2-aminoethanol was 3 days at 4^{o}.

Coupling of the RDH to bromoacetamidoethyl-Sepharose was estimated by measuring A280 of the filtrate; it was better than 65%.

16. Elution of Antibodies From Affinity Column

First, the column is equilibrated with sterile cold PBS (2.73g of KH_2PO4, 6.63g of Na_2H PO4, and 35g of NaCl adjusted to pH 7.2 with NaOH and made up to 5 litres). Then the Ig-fraction of immunized antiserum (see below) is loaded on the column at 10 - 30 ml/hour. The unspecific proteins are washed off with cold PBS, increasing the pump speed gradually up to 50 - 60ml/hour, until the A280nm of the

wash is less than 0.02. Then the column is washed with 0.5M NaCl, buffered as above, to remove loosely attached proteins. The specific antibodies are eluted stepwise by 3M potassium thiocyanate, briefly with PBS, then 1M acetic acid, 0.1M HCl, and 1M HCl. Long contact with HCl must be avoided because it gradually attacks Sepharose. KSCN fractions are immediately dialysed against PBS. Acetic acid fractions are gradually neutralized by 4M Tris, after dialysis both fractions are concentrated by ammonium sulphate precipitation and dialysed again. HCl fractions must be immediately neutralized with NaOH, monitoring with a universal dye indicator. By this procedure one should be able to achieve about 50% recovery of the specific antibodies.

Some antibodies will be precipitated in different fractions. If desired, the precipitated antibodies can be renatured significantly by repeatedly dissolving in 1M acetic acid, neutralizing by Tris, and dialysing. The procedure is economical up to 2-3 cycles. Most of the antibodies recovered this way are quite active. It is important to operate the affinity columns near their maximum capacity to obtain the best yield.

17. Crude Ig-Fraction of Antisera by 50% Ammonium Sulphate Precipitation

An equal volume of saturated ammonium sulphate solution is added slowly, over a period of about 15 - 20 mins, into the stirred antiserum sample. Stirring is continued for about 1 hour, then the precipitated Ig fraction is collected by centrifugation. The precipitate is dissolved in PBS and dialysed overnight with 2 changes of buffer.

18. Ouchterlony Double Immunodiffusion Technique

Pipettes and plates are prewarmed in a 30°C oven. A large, hot water container is needed for immediate rinsing of pipettes. One corner of the glass plate is marked for identification with a diamond pen.

Solutions of 2% agarose (Miles, melted by free steaming) and PBS (twice the normal concentration) containing 20mM EDTA are heated to 56°C.

After mixing equal volumes, 3.5ml of warmed gel solution is added to each 2¼" x 2¼" glass plate, on an absolutely level surface. The solution is poured with a warm pipette from the middle, touching the 4 corners with the pipette tip to ensure even distribution of gel. Plates with gels are kept 20 mins or longer in a "humid box" at 4°C before the wells are made and the antibody/antigen solutions are added. The wells, 3mm in diameter and 5mm separation, are made with a special gel well maker and removed by suction using a disposable pasteur pipette tip; 9µl of sample is placed in each well. Ab is usually placed in the centre well, while the serially diluted Ag samples are placed clockwise starting from the top left well containing the most concentrated Ag sample.

In order to reach optimal Ab/Ag proportions, plates are allowed to remain at 4°C inside a wet box for 3 days.

The procedure for staining and destaining the gels is as follows. First dehydrate by placing a wet 9cm Whatman No. 1 filter paper on top of the gel to avoid sticking. Then several folds of paper-towelling are placed on top of the filter paper, then another gel plate with wet filter is placed on the top and the same repeated. At the end a large glass plate is placed on the top of the tower of gel plates and folded paper towels, a heavy weight is balanced on the top and kept under observation for 2 hours. A longer dehydration period may cause difficulty in separating filter from gel surface and must be avoided. After dehydration, the gels are dried completely by a hot air blower (about 10 mins). The plates are washed in a trough containing PBS

for 2 hours, with occasional shaking allowing the unprecipitated excess proteins to diffuse out. The plates are dried again and then transferred into staining solution (for a necessary period depending on the intensity of the precipitin bands).

The staining solution is: 0.5% CoomassieBrilliant Blue in Methanol/glacial Acetic Acid/H_2O, 9:2:9.

Then the gels are destained in destaining solution (Methanol/ Acetic/H_2O, 50/2/48mls) for the required time. The destained gels are drained vertically onto a paper towelling which is placed under, for several hours till dry.

19. Immunoelectrophoresis

Well buffer (veronal Buffer - Tris Glycine, pH 8.6):

Na-barbitone 32.5g; diethyl barbituric acid 5.18g; glycine 35.13g; Tris 28.25g; EDTA-disodium salt 18.6g; pH adjusted to 8.6, then made up to 5 litres.

Equal volumes of 3% L28 Oxoid Agar and the above Well buffer are mixed and rinsed and poured at 56°C on to prewarmed plates (82 x 82mm). 10ml of agar is added to each plate. Wells are made 2mm in diameter, therefore 5µl capacity, equally spaced between anode and cathode, equidistant from each other, about 1cm apart. Slots, 1mm thick, are equidistant from the wells. 150µl of anti-serum is placed in each slot.

Electrophoresis is carried out at constant current, 15-20mAmps/ plate about 100 volts, till BSA and amido black reach the end. Amido Black, Amido Swartz, Naphthalene Black, they are all synonyms, purchased from Gurr. Electrical contacts between the gel and the electrophoresis buffers are made by wet pieces of wicks, "Johnson's baby" absorbent lint.

Antibody dilutions are usually 5-fold, placed in the troughs from top to bottom of the gel.

20. ^{125}I-Labelling of Antibodies

(Iodination of antibodies is carried out essentially by the procedures of Palacios et al., 1972, and Bouma, III et al., 1975).

Iml of purified anti RDH antibodies (about 5mg/ml) is dialysed against cold PBS, pH 7.8. The antibody solution is transferred to a tapered bottom plastic centrifuge tube with a cap. 0.05ml of (Sigma) lactoperoxidase (0.8mg per ml in PBS pH 7.8) is added, plus 2µl of Na ^{125}I (carrier free, from Radiochemicals, 100mCi per ml, 11 - 17mCi/µg ^{125}I). The reaction is started by the addition of 50µl of H_2O_2, 97µM solution, freshly prepared (1:100,000 dilution of 30% H_2O_2), 25µl of this diluted H_2O_2 solution is added at first, then another 25µl is added after 8 minutes. The reaction is continued for 15 minutes at room temperature (or for a longer period if very hot sample is required) inside a lead pot in a ventilated fume-hood with occasional shaking.

The reaction is stopped by the addition of 1ml of saturated ammonium sulfate solution which precipitates the antibodies. After about ½ hour at 0-2°C, the precipitate is collected by centrifugation at 4000rpm, 15 min,on an MSE bench centrifuge. The antibody pellet is resuspended in PBS and dialysed overnight in the cold. After dialysis, antibodies are passed through an ion exchange column (0.5cm diameter) consisting of 1cm of DEAE-cellulose overlaid with 1cm of CM-cellulose, to be freed from traces of ribonuclease. The A_b peak A280nm is collected. Antibody concentration is determined using the extinction doefficient ($E280^{1\%}$=14.0), and the radioactivity is determined in a LKB 80000 gamma sample counter.

21. DEAE-CM-Cellulose Column Chromatography

A pasteur pipette packed with glass wool at the bottom was HCl washed, rinsed with distilled water, sterilized by autoclaving, together with the tubings and connections. DEAE-52 cellulose was degassed initially by lowering the pH to 2.6 with phosphoric acid and applying

vacuum in a desicator till no more bubbles noticed. Then it was titrated with NaOH to pH 7.2, changed buffer several times to equiliberate with PBS. The degassed DEAE-Cellulose and CM-cellulose were autoclaved for 15 minutes, cooled to 4°C, 1cm of CM-52 cellulose was packed on top of 1cm of DEAE-cellulose in the sterile column with a sterile acid washed pipette. The column was equiliberated with cold sterile PBS. The dialysed clear antibody solution was loaded and then eluted with PBS at 9.6ml/hr.

22. Preparation of Bacterial Polysomes

(a) Preparation of Polysomes for Analytical Use

The procedure of Godson and Sinsheimer (1967) was followed exactly with the only modification that chloramphenicol, 100µg/ml, was added to the culture at 37°C, 5 seconds before it was harvested.

(b) Quantitative Preparation of Polysomes

This is a modification of the above procedure for 1 litre culture of E. coli or K. aerogenes at any cell density.

To 1 litre of a growing bacterial culture at 37°C, chloramphenicol (100µg/ml in 25ml of sterile M9 medium) is added. After 5 seconds the culture is cooled to 0.2°C in 1 min by pouring it into a chilled 3 l conical flask immersed in a methanol-dry ice bath, vigorously swirling the flask to avoid icing at the sides and monitoring the fall in temperature with a thermometer. The cooled culture is immediately transferred into 4 x 250ml sterile, RNase-free and ice-cooled polycarbonate bottles, quickly balanced and centrifuged in a cold Sorvall GSA rotor at 0°C for 2 min at 3000g (4500rpm); total acceleration, spinning and deceleration time = 9 minutes. After discarding the supernatants, the bottles are inverted over clean Kleenex tissues for 1 min to drain in a freezer, and then the walls are wiped with a tissue. It is important to remove as much of the residual growth medium as possible because the cells are to be resuspended and lysed in a very small volume. The cell pellets are quickly and thoroughly

resuspended by repeated blowing through a large mouth plastic pipette in 12ml of 25% w/v "RNAse-free" sucrose made up in 0.01M Tris-HCl, pH 8.1. All operations are carried out on ice and cold containers, solutions and pipettes are used throughout. 3ml of lysozyme-EDTA solutions (equal volumes of cold solutions of 0.85mg of lysozyme per ml made up freshly in 0.25M Tris/HCl pH 8.1, and EDTA 2.7mg/ml mixed shortly before use) is added to the suspension and mixed on ice for 30 seconds, shaking gently from side to side. The suspension is then added to a lytic mixture contained in a plastic centrifuge tube on ice. The lytic mixture, prepared 0.5 - 1 hr before use and kept cold on ice, consists of: 3ml of 5% w/v Brij-58 in 0.1M Tris HCl, pH 7.2; 3ml of 0.1M Mg SO4 solution; 1.5ml of a 1mg/ml DNAse solution (Worthington, "RNAse-free"); 6ml of 1% w/v sodium deoxycholate in 0.1M Tris-HCl, pH 8.1.

The suspension is sucked up and down with a pipette a few times to ensure good mixing and the tube is returned to an ice bath. Within a few minutes the mixture should have visibly cleared and the cells lysed. If the cleaning is slow, the tubes can be removed from the ice bath and stood at room remperature for 2 minutes; in this time the temperature of the mixture will have risen to 10-15^{o}C. After cleaning return the tube to the ice bath. The final lysate should have very little visible optical density.

The lysate is first centrifuged in a Sorvall SS-34 rotor at 6000g for 5 minutes at 0^{o}C to remove the cell debris and membranes. Aliquots of the supernatant containing the polyribosomes (about 15ml) are layered carefully over a cushion (11ml) of 1.5M sucrose in "polysome buffer" (5mM Tris-HCl, pH 7.2, 10mM Mg SO4, 60mM KCl). The tubes are filled to the top with sterile 0.25M sucrose in polysome buffer, balanced and centrifuged in a Beckman L5-50 class H ultra-centrifuge at 2^{o}C and 130,000Xg (26,500rpm) for 12-13hrs. in a Spinco

SW27 rotor, or at 40,000rpm in a Spinco 60Ti rotor for 10 - 11 hours. These tubes can be filled to the top with cold, sterile liquid paraffin. At the end of centrifugation, care is taken to remove the supernatant completely. Then the polysomal pellet is rinsed gently with 0.25M sucrose in "polysome buffer" and is suspended carefully in this medium. After a 5 min centrifugation at 6000g the A260/A280 of the clear polysome supernatant is measured, and it is quick frozen and stored under liquid nitrogen in sterile plastic tubes (Stereline).

A one litre culture is about the maximum one can handle efficiently. If larger quantities of polysomes are required, the lysates from successive 1 litre culture batches can be pooled after storage on ice for up to 30 min. or separately frozen. The ratio A260/A280 of the purified polysome sample is a measure of the purity; ratios of 1.8 - 1.9 are usually obtained. The yield of polysomes very much depends on the cell density of the culture and the efficiency of lysis. Usually about 15mg of purified polysomes and ribosomes is obtained from a 1 litre culture at A650 = 0.6; (13 A260 units is equivalent to 1mg of polysomes, Schechter, 1974).

23. Sucrose Density Gradient Analysis of the Polyribosomes

Polysomes are analysed by velocity sedimentation on linear 0.5 - 1.5M (or sometimes 15 - 30% w/v) sucrose gradients. 0.5- 1.5M gradients can hold a wider range of polyribosomes, therefore they are used throughout this study, unless otherwise stated. Either the 6000Xg supernatant from cell lysate or 10 A260 units of purified polysomes are layered carefully, in a minimum volume, on the cold sucrose gradient (0.5 - 1.5M). Ultracentrifugation is carried out in an SW25.2 swing out rotor of a Spinco L2 ultracentrifuge at $2^{o}C$ for 3 hours at 24000rpm, while applying the brake during decceleration.

Three sucrose gradients are made simultaneously by a Buchler

Gradient Former, using a Pharmacia Pump, and kept on ice covered with Clingfilm. Sucrose solutions are made up in sterile "polysome buffer" using Sigma specially pure "RNAse-free" sucrose and autoclaving the solutions for 5 minutes. The 1.5M sucrose solutions used in the more recent experiments, were supplemented by 10 units/ml of Na-heparin, just before making the gradients (0.8ml of 1000 units/ml heparin solution added to 84ml 1.5M sucrose). Heparin is used as an antiaggregant and as a ribonuclease inhibitor. Nitrocellulose gradient tubes are washed carefully with detergent solution, distilled H_2O,1% SDS solution, then extensively washed with sterile distilled water, finally drained over clean tissues to dry. Gradients are made a few hours before each experiment and stored in ice at 4^oC, covered with a clingfilm. If they are to be stored overnight, a few drops of 5% Brij-58 are carefully layered on top of each gradient.

At the end of centrifugation, tubes are pierced at the bottom, and the gradients are collected through a pump, a spectrophotometer unit (CECIL Instruments Flow Through Spectrophotometer with a W + W Recorder 1100) and an LKB Fraction Collector. Fractions are collected at 2ml/min and each fraction is marked by an automatic event marking pen provided on the recorder instrument. 26 drops (1.5ml) fractions give 36 - 37 fractions. Chart speed of 30cm/hr is appropriate. The fractionation system is filled and equilibrated at first with the same 1.5M sucrose solution used to make the gradients. Since the two starting sucrose solutions will have different absorbance at 260nm, the A260 of both 0.5M and 1.5M solutions are measured by the flow through spectrophotometer, linear increase in absorbance is established, then the difference at every point is subtracted from the A260 tracings obtained by the recorder. Linearity of a gradient can be checked by mixing a dye, 0.1mM

Toluidene Blue (1µl/ml), with the heavy sucrose solution. The change in the A580nm of the gradient is then recorded before or after centrifugation; perfectly linear results are usually obtained. The linearity of the gradient can also be demonstrated by differential absorbance of the starting sucrose solutions, as shown later (see "Diffusion of ^{125}I-antibodies into sucrose gradients after ultracentrifugation"). Radioactivity of each fraction, in the case of ^{125}I, is measured directly in a gamma radiation counter; in the case of ^{3}H, polysomes are first precipitated with trichloroacetic acid, collected on membrane or glass fibre filters, washed and dried, then counted in a suitable scintillation fluid in a β-radiation counter.

24. Quantitative precipitation of RDH polysomes by double antibody technique.

The optimal proportions of the two types of antibodies for maximal precipitation of the primary antibodies is determined on a microscale, using ^{125}I-labelled primary antibodies, incubating samples at +4°C for 12 hours. The primary antibodies are added to the polysomes to an optimal ratio, predetermined in sucrose gradients by Ab/polysome binding, incubated at +4°C for about 3 hours, then the optimal amount of the secondary antibodies are added and incubated further overnight. All antibody/polysome solutions must be supplemented with heparin and Mg^{++} to final concentrations of 10 units/ml and 10mM, respectively, clarified by centrifugation prior to interaction. The double antibody-polysome aggregates are centrifuged at 8000 x g for 15 mins, at 4°C. The precipitate twice is resuspended in solution D (5mM Tris HCl, pH 7.2, 10mM Mg SO4, 60mM KCl, 0.1M sucrose, and 10 units/ml Na-heparin), and centrifuged.

25. Preparation of mRNA from Immune-Precipitated Polysomes.

The RNA from washed immunoprecipitated polysomes is extracted with the aid of solution E (100mM Tris-HCl, pH 9.0; 100mM sucrose; 10mM KCl; 2mM Mg-acetate; and 3% SDS) and phenol. Solution E, 1.5ml, is added at room temperature, and the precipitate is brought to a fine homogeneous suspension by fast repeated blowing from a pasteur pipette. The suspension is mixed with an equal volume of water-saturated phenol, and it is agitated gently at room temperature for 10 mins. After cooling in ice for 5 mins, it is spun in a chilled rotor for 10 mins, at 12,000 x g. The aqueous phase is separated and the phenol phase is re-extracted once at room temperature for 10 mins with 1.5ml of solution F (10mM Tris-HCl, pH 9.0, 0.5% SDS), chilling and phase separations are carried out as above. The aqueous phases are combined and re-extracted twice with phenol at $+4^{o}C$. The RNA is precipitated by adding 0.1 volume each of 2.0M NaCl and 2.0M Na-acetate (pH 5.5), and 2.5 volume of ethanol at $-20^{o}C$, overnight. The RNA precipitate is spun down at $0^{o}C$, at 12,000 x g for 30 mins. The supernatant is carefully pipetted off, the precipitate is resuspended in cold 96% ethanol, re-centrifuged 25 mins as above. The precipitate is dissolved in 1ml of distilled water, precipitated again with NaCl/Na-acetate/ethanol, as above collected by centrifugation, washed twice with cold 96% ethanol, and finally dissolved in distilled water and stored at $-70^{o}C$.

Conversion values (according to Schechter, 1974): 1.4 A280 units for 1mg of Ig; 13 A260 units for 1mg of polysomes; 20 A260 units for 1mg of RNA.

26. SDS-Polyacrylamide Gel Electrophoresis (ICRF)

(a) Sample Buffer

The 5µl protein sample is added to 20µl of the following

solution:

Component	Volume in 3ml	Final Concentration
20% Serva SDS	0.30ml	2%
Glycerol	0.30ml	10%
0.2% Bromophenol Blue	50µl	0.33%
1M DTT	0.30ml	0.1M
1M Tris pH 6.8	0.24ml	0.0625M
Water	1.86ml	
	3.00ml	

DTT is Dithiothreitol, supplied by Sigma. The sample buffer is stored frozen at -20°C.

(b) Running Gel

The receipe shown below is for a 15% polyacrylamide gel pH 8.8:

Component	Volume Used
30% Acrylamide	15.00ml
2.5% Bisacrylamide	1.04ml
Water	2.60ml
1M Tris pH 8.8	11.20ml
20% Serva SDS	0.15ml

This mixture is degassed for 2-3 minutes and then 0.01ml Temed and 0.1ml 10% Ammonium persulphate added.

(c) Stacking Gel

The receipe shown below is for a 5% stacking gel, pH 6.8:

Component	Volume Used
30% Acrylamide	1.67ml
2.5% Bisacrylamide	0.65ml
Water	6.43ml
1M Tris pH 6.8	1.25ml
20% Serva SDS	0.05ml

Temed	5µl
10% Ammonium persulphate	0.05µl

(d) Running Buffer

The electrophoresis apparatus buffer reservoirs are filled with the following buffer:

0.025M Tris base

0.192M Glycine

0.1% Serva SDS

(e) Sample Preparation and Running the Gel

5µl of the E. coli MRE 600 S30 cell free protein synthesising incubation is added to 20µl of sample buffer + DTT and boiled for 3 minutes in a boiling water bath. The sample is then applied to one of the slots in the stacking gel.

The positive lead from the power pack is connected to the lower reservoir and the negative lead to the upper reservoir. The gel is run at 35 volts overnight, with the power pack set at constant voltage, until the bromophenol blue from the sample buffer has just run off the bottom of the gel.

(f) Staining

The stacking gel is cut off and the running gel is placed in the following staining solution for 90 minutes:

25% isopropanol

10% acetic acid

0.05% Coomassie Brilliant Blue R

65% water

(g) Destaining

The stain is poured off and the gel rinsed in water to remove excess surface stain. The gel is then placed in the following destaining solution for 3-4 hours (or longer if required):

7.5% acetic acid

25% methanol

67.5% water

Destaining is made more efficient by floating a piece of foam rubber on the surface. After destaining the gel is washed in running water for 30 minutes to remove as much excess methanol and acetic acid prior to drying the gel. This prevents their vapours from passing through the vacuum pump.

An autoradiographic film such as Kodak XH1 or AP54 or Kodirex (KD 5T) is taped in position over the gel and placed under lead sheets in a dark room cupboard to expose. The films are developed using a Pako automatic processor.

27. Methionine Diagonal Electrophoresis

The protein is first carboxymethylated, under denaturing conditions, to render it more amenable to proteolytic digestion. 30mg of pure, non-radioactive RDH (5mg should be sufficient) was mixed with the in vitro translated, RDH product. This was dialyzed against 5% formic acid at $4^{o}C$ overnight with two changes of formic acid solution. The sample was removed from dialysis and freeze-dried prior to carboxymethylation of the cysteine residues.

5ml of 100mM Tris-acetate buffer pH 8.0 was made up to approximately 10ml with 5g urea, flushed with N2 and added to the dried RDH sample to denature the proteins. Then 3mg Dithiothreitol (DTT) was added to give 10-fold molar excess over RDH thiols (there are 2 Cys/RDH subunit). The mixture was incubated at $37^{o}C$ for 4 hours to reduce all disulfide bonds. Iodoacetic acid was added to give 20 fold molar excess over total thiol, flushed with N2, and pH adjusted to 8.0 with concentrated NaOH. The mixture was

incubated at room temperature for 45 minutes, and 100µl of β-mercaptoethanol was added to quench the reaction.

After dialysis against 5% formic acid, a portion of the sample was freeze-dried, then 5ml of 0.5% ammonium bicarbonate was added. Then, thermolysin, 1:100 (wt/wt) was added and the digestion was carried out at 55°C for about 1¾ hours, when the solution becomes clear. The sample was freeze-dried, the peptides were dissolved in 150µl of pH 6.5 electrophoresis buffer (pyridine - acetic acid - water, 25:1:225 by vol.). The sample was run as a 1cm band on Whatman 3MM paper (or on thinner paper if the radioactivity is not very hot) in high voltage electrophoresis at pH 6.5, for ¾ hours, at 3K volts according to Brown & Hartley (1966). Fluorescent markers DNS-Arg-Arg, DNS-Arg, and dansic acid were applied to aid in calculations of the electrophoretic mobilities of peptides.

A strip containing the peptides was cut from this paper parallel to the direction of electrophoresis and suspended horizontally between two stainless-steel clips. Fluorescent markers were applied. The dry strip was sprayed evenly in a hood with 0.1M iodoacetamide in pyridine-acetate buffer, pH 3.5 (pyridine-acetic-water, 1:10:90), until thoroughly wet but not running. It was then arranged on a glass rack in a desiccator containing a tray of pH 3.5 buffer, and left overnight at room temperature in the desiccator while most of the air initially removed by a vacuum pump. Next day, the strip was removed, dried by hanging in a hood, and rinsed several times with acetone. The dried strip was then stitched across the middle of a full sheet of Whatman 3MM paper and submitted to electrophoresis at pH 6.5 at right angles to the original direction. The fluorescent markers were marked out on the dry paper and the peptides were first revealed by autoradiography, using Kodirex KD5T film, and developed by

Kodak Industrial X-OMAT Processor Model 3, then showed by dipping in cadmium-ninhydrin reagent (Heilmann, Barollier & Watske, 1957).

CHAPTER III

Choice of Bacterial Strains, Kinetics of RDH Enzyme Production and Characterization of Pulse Labelled RNA

A. Introduction

The greatest advantage of studying messenger RNA in the ribitol dehydrogenase system is that a considerable number of mutants are presently available, of both *Klebsiella aerogenes* and of *E. coli*, strains *K-12* and *C* that synthesise large amounts of ribitol dehydrogenase. We thought it was a good idea to begin by studying an *E. coli* K-12 strain which produces the highest level of RDH. The reasons for this choice were that this organism has a well established genetic system which would enable us to carry out other investigations such as those proposed by Rigby *et al.*, (1976): determination of the size of the genetic duplications, the mechanism by which such duplications are produced, and the cause of increased enzyme synthesis in strains which do not carry duplications.

A series of RDH high synthesis mutants had spontaneously evolved in a chemostat from an RDH consitutive *E. coli* K-12 strain, *EA*; they had been characterized by M.J. Gething (unpublished), and appeared suitable for this investigation. Strain *EA* has the genotype, F^+, gal^-, str^r, $rbtC_{101}$, $rbtD^+$, $dalD^+$, *tsx*, Pl^s, λ^s, Tu^s, Tb^r. The enzymic parameters in the cell extracts of this strain and the successive evolvants from it, as determined by M.J. Gething, are shown in Table 3.1.

The parameter used to check for alteration in the catalytic properties of the enzyme was the ratio between the rates of reduction

TABLE 3.1

Spontaneous RDH-High Synthesis Evolvants from E. coli K-12, EA

E. coli K-12 Strain	A.R. (0.5M Xyl./ 50mM Rib.)	RDH activity (Units/ml)	RDH as % total soluble protein*
EA	0.042	2.90	3
EAI	"	5.43	6
EAII	"	14.50	16
EAIII	"	25.10	25
EAIIII	"	29.90	30

*RDH specific activity measurements were carried out at culture A650nm = 1.0. The unit of RDH activity is the reduction of 1 μ mole of NAD^+ to NADH/min under the conditions of enzyme assay, described in "Materials & Methods". The percentage of total protein in the extract represented by RDH, is calculated on the basis of the specific activity of pure RDH; 100 units/mg for wildtype enzyme (J.M. Dothie, Ph.D. dissertation, 1974). All of these bacterial strains were supplied in stab agar (Materials & Methods), by Dr. J.M. Dothie.

of NAD^+ at 0.83mM, with defined levels of xylitol and ribitol (the activity ratio A.R.), which was determined for 0.5M xylitol and 50mM ribitol. The specific activity of RDH in the extracts was also determined as a criterion of whether increased amounts of RDH were being produced by the evolvant strains.

Two fundamental questions are dealt with in this Chapter. First, with the ultimate aim of purifying RDH mRNA, we may ask at which point during culture growth the specific activity of RDH mRNA is maximum? One reasonable answer would be, at the point where the concentration of RDH enzyme is at its peak. Thus, I have determined this point for E. coli strains EAIIII and EA, and for Klebsiella aerogenes AIII (another RDH high synthesis mutant) by measuring variations in RDH activity in extracts throughout culture growth, with respect to total soluble protein, cell mass, and viable cell number.

Secondly, in a high synthesis strain where 30% of the total protein in the cell extract is represented by RDH, one might expect about one third of total pulse-labelled RNA to be RDH mRNA. This is a very large proportion, and in such a case one would expect this mRNA to be represented by some kind of a peak upon fractionation of total cellular pulse-labelled RNA. Therefore we want to know, is it possible to see such a peak? If so, what is the size of this RNA, and could one purify it? I have used the techniques of polyacrylamide gel electrophoresis and Ultrogel AcA-22 chromatography to answer this question, comparing pulse-labelled RNAs extracted from high synthesis and wildtype organisms.

B. Variation of RDH Specific Activity in Growing Cultures of E. coli

It is preferable to grow all bacterial strains, including wild-type, constitutive and high synthesis, in identical media, if their RDH specific activities and their pulse-labelled RNAs are to be

compared with one another. Although RDH genes are constitutively derepressed in high synthesis strain, the synthesis of RDH may be affected if glucose is used as a carbon source, as a result of "catabolite repression". Glucose represses the synthesis of a number of inducible enzymes and transport proteins in E. coli by lowering intracellular level of cyclic AMP (Pastan and Perlman, 1970; Pastan et al., 1974). Other sugars could also bring about catabolite repression, but to a lesser extent. Casamino acids presumably do not catabolite repress, and were therefore used as the sole carbon source.

As shown in Fig. 3.1 , the growth curves of E. coli strains, E A , EAIIII, and CA388, on M9+ 2% casamino acids, are very similar. Culture mass doubling time during exponential growth phase is about 54 minutes in each case. Strain CA388 is the recipient E. coli K-12 strain utilized originally for transfer of RDH genes from Klebsiella aerogenes.

For determinations of RDH specific activities, 200ml M9 + 2% casamino acids media in 500ml bubble culture flasks were inoculated with 1% of fresh cultures grown overnight on the same medium or M9 + 0.2% xylitol for superproducing strains, and incubated at 37°C with sufficient aeration. At various times after inoculation, as indicated in Table 3.2, 11ml samples were removed with a sterile pipette, 1ml was placed in a spectrophotometer cuvette for direct absorbancy measurement at 650nm. The remaining 10ml of the sample was centrifuged for 3 min at 5000 rpm in a chilled Sorvall SS-34 rotor. The cell pellet was resuspended thoroughly in 0.1M Tris-HCl buffer, pH 8.0, containing 5mM EDTA. The volume of the buffer added was approximately proportional to the A650nm of the culture sample (e.g. 2ml to 0.2 A650/ml, and 6ml to 0.6 A650/ml, etc.). The reason

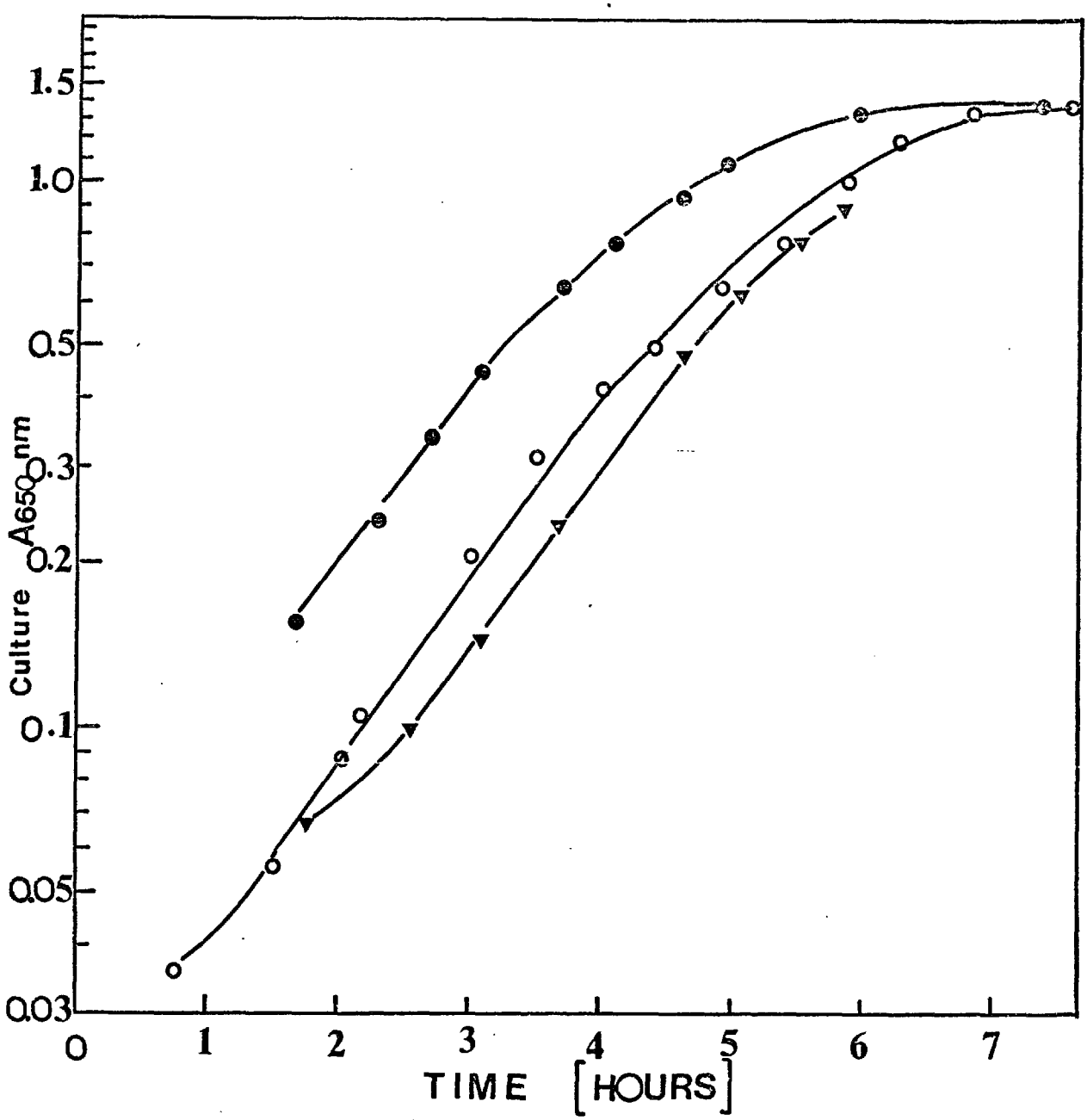


FIG. 3.1. Growth curves of Escherichia coli strains: EA1111(o), EA(▼) and CA388(●), on M9 + 2% casamino acids as the sole carbon source. Absorbancy measurements by Gilford Absorbance Recorder with Unicam SP500 Spectrophotometer(undiluted cultures).

TABLE 3.2

Variations in RDH Specific Activity During Culture Growth in E. coli

Time hr	Time min	A650nm of culture	Viable cell no.	Extract vol(ml)	RDH activity, units in: 1ml of extract	RDH activity, units in: Total extract from 10ml culture	Protein mg/ml of extract	RDH specific activity expressed in: Units/cell mass (U in 10ml ext. / 10 x A650nm)	RDH specific activity expressed in: Units/mg soluble protein	RDH specific activity expressed in: Units/viable cell	Soluble protein (µg) in 10ml ext./ no. of cells in 10ml	
	45	0.036	-	-	-	-	-	-	-	-	-	
1	30	0.056	7.8×10^{8}	-	-	-	-	-	-	-	-	
2	00	.088	9.8×10^{8}	-	-	-	-	-	-	-	-	
2	10	.105	1.05×10^{9}	1	.160	.160	-	0.152	-	1.52×10^{-11}	-	
3	00	.205	1.5×10^{9}	-	-	-	-	-	-	-	-	
3	30	.315	2.0×10^{9}	3	.576	1.728	.205	0.549	2.81	8.64×10^{-11}	3.07×10^{-8}	
4	00	.413	3.1×10^{9}	4	.704	2.816	.198	0.682	3.56	9.08×10^{-11}	2.55×10^{-8}	
4	24	.496	4.5×10^{9}	5	1.080	5.400	.198	1.089	5.45	12.00×10^{-11}	2.20×10^{-8}	EAIIII
4	54	.646	7.2×10^{9}	6	1.776	10.656	.220	1.650	8.07	14.80×10^{-11}	1.83×10^{-8}	
5	22	.776	1.0×10^{10}	8	2.624	20.992	.214	2.705	12.26	21.00×10^{-11}	1.75×10^{-8}	
5	52	1.000	1.7×10^{10}	10	3.920	39.200	.221	3.920	17.74	23.00×10^{-11}	1.43×10^{-8}	
6	15	1.200	2.1×10^{10}	10	4.800	48.000	.240	4.000	20.00	22.90×10^{-11}	1.12×10^{-8}	
6	50	1.320	2.5×10^{10}	10	6.320	63.200	.288	4.788	21.94	25.30×10^{-11}	1.15×10^{-8}	
7	35	1.384	2.8×10^{10}	10	7.200	72.000	.295	5.202	24.41	25.70×10^{-11}	1.05×10^{-8}	
1	45	.067	-	-	-	-	-	-	-	-	-	
2	33	.100	-	-	-	-	-	-	-	-	-	
3	05	.143	1.2×10^{9}	1	.160	0.160		0.112	-	1.28×10^{-11}	-	
3	40	.235	-	-	-	-	-	-	-	-	-	
4	38	.478	$4.2\times10_{9}$	5	.104	0.520	.242	0.109	0.43	1.24×10^{-11}	2.88×10^{-8}	EA
5	02	.622	$6.7\times10_{9}$	6	.160	0.960	.225	0.154	0.71	1.43×10^{-11}	2.01×10^{-8}	
5	30	.780	$9.8\times10_{10}$	8	.144	1.52	.220	0.148	0.65	1.18×10^{-11}	1.80×10^{-8}	
5	50	.900	1.2×10^{10}	9	.160	1.440	.238	0.160	0.67	1.15×10^{-11}	1.71×10^{-8}	

for doing so was to keep the final protein concentration approximately the same and within the permisible range of the protein determination method (see later). The procedure for cell rupture was similar to that described in J.M. Dothie's dissertation (1974). To each 1ml of resuspended cells was added 0.01ml of 10mg/ml lysozyme solution and the suspension was incubated at 37°C for 20 minutes. Then 0.012ml of a 1:1 mixture of 0.1M Mg SO_4 and 0.1M Tris/HCl, pH 8.0 containing 5% Triton was added to each 1ml of the cell suspension and 5 minutes further incubation was carried out. The lysate was then spun at 17,000g for 10 minutes, and the supernatant was the extract which was stored at -20°C.

Protein determination by the Lowry method as modified by Miller (1959), was impossible on these extracts, because of severe interference by Triton with spectrophotometric readings. Other spectrophotometric methods, such as Waddell (1956), and Warburg and Christian (1941), were tried, and they failed for the same reason. It was not a good idea to exclude Triton from these procedures, because uniform and thorough lysis was required throughout this type of experiment.

A recent procedure by Wang and Smith (1975), describing the use of SDS in the Lowry method for determination of protein in presence of Triton X-100, was found extremely useful. All of the subsequent protein determinations in extracts containing Triton were carried out by this method, as is detailed in "Materials & Methods". Enzyme activity in the extracts was determined as described in "Materials & Methods".

The results of this experiment are summarized in Table 3.2. All protein and activity determinations were carried out in duplicate on the same sample. It can be seen from this table that RDH activity in

a given volume of EAIIII culture (10ml) increases by 250 fold from culture A650nm of 0.1 to 1.0. In EA cultures, however, in the same period and in the same volume, RDH activity increases by only 10 fold. The comparisons of the specific activities, however, reveal a very contrasting feature: as illustrated in Fig. 3.2 (a, b and c), whether the specific activity is expressed as units/cell mass, or as units/mg total protein in the extract, or as units/viable cell, the general trend for strain EAIIII is always a continuous rise in RDH specific activity up to the stationary phase. But for strain EA, there is no significant change in RDH specific activity throughout culture growth; in fact, it somewhat decreases after mid-log phase (culture A650nm of 0.6 - 0.8).

A continuous increase in the specific activity of RDH enzyme in EAIIII, indicates continued presence and translation of the corresponding mRNA up to the stationary phase. Also it is probable that the specific activity of RDH mRNA and/or its rate of translation increases continually up to the stationary phase. This was a significant finding, because it meant that one could almost assuredly screen for RDH mRNA in fractionated pulse-labelled RNA prepared from EAIIII cells, grown to near stationary phase, when rarely any other pulse-labelled mRNA is present.

Observation of a somewhat slower rate of increase in RDH activity per cell toward the late log-phase in EAIIII (Fig. 3.2c) is probably the reflection of the variation in cell size with culture age. Reduction in cell size toward the late log-phase gives rise to smaller activity/cell than expected, by contrast with activity/mass, or activity/mg soluble protein. In support of this statement, Herbert (1958) reported that fast-growing K. aerogenes cells are much larger microscopically and have a much greater mean cell mass than slow-growing cells. Also, the results of soluble protein, mg/cell (last

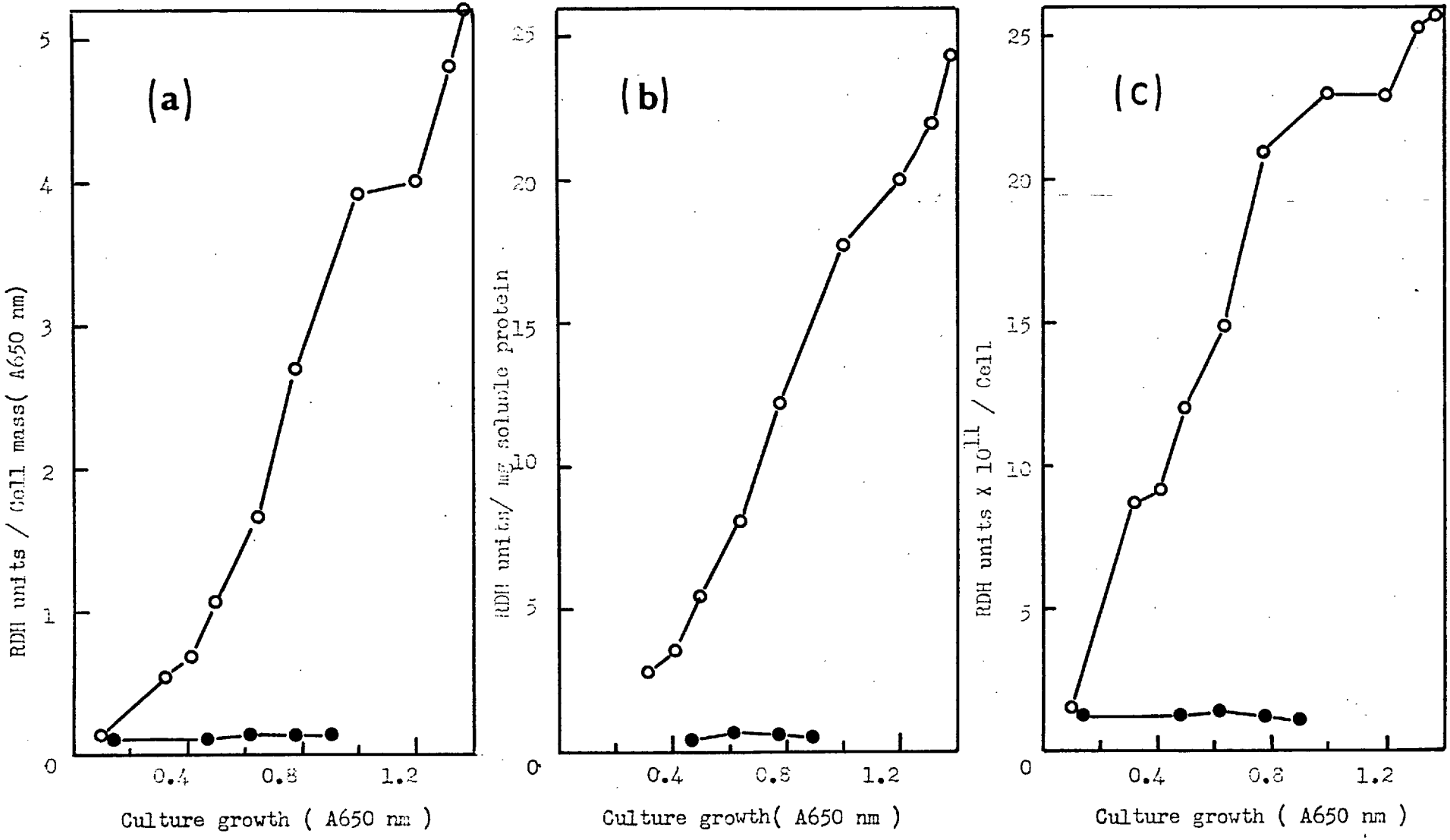


FIG.3.2. Variations in specific activity of RDH enzyme during growth of cultures of E. coli K-12 strains EA1111(○) and EA(●). Specific activities are expressed as: (a) units/ cell mass; (b) units/ mg protein in cell extract; (c) units/ viable cell.

column, Table 3.2) indicate a continuous reduction with culture growth. This is exactly opposite to ones' expectation, that protein concentration/cell should increase with culture age, or at least remain the same. Thus, the explanation lies again in the continual reduction of cell size.

C. Sizing of E. coli K-12 EAIIII Colonies on 0.05% Xylitol/M9 Plates

We can see from Fig. 3.2 (b) that the specific activity of RDH in an extract of E. coli EAIIII grown to A650nm of 1.0 (18 units/mg soluble protein) although quite high and characteristic of a high synthesis strain, is not as high as it ought to be (30 units/mg soluble protein at culture A650nm of 1.0, personal communication from Dr. J.M. Dothie). This suggested that a certain proportion of these cells might have reverted to intermediate and low synthesis types during storage on stab agar. The stabs had probably been stored under adverse conditions during transport from Cambridge to London.

In order to investigate the extent of such reversion, a sample of the EAIIII stab grown overnight on liquid M9 + 0.2% xylitol medium, was serially diluted and spread on M9 plates containing 0.05% xylitol. It had previously been known (Hartley et al., 1972) that one could distinguish faster growing strains simply by colony size on 0.05% xylitol plates, so that one can distinguish high synthesis mutants from wildtype. A homogenous culture of a high synthesis strain should only produce uniform large size colonies after 2-3 days growth on 0.05% xylitol/M9 plates. The number of different size colonies scored on two separate plates were as follows:

	Large	Medium	Small
Plate 1	49	34	40
Plate 2	24	18	20

This result indicated that about half of the bacteria in the stab had segregated to low and intermediate RDH producers.

In order to regenerate uniform very high-synthesis mutants, the EAIIII culture was inoculated into a chemostat, which was operated by Dr. J.M. Dothie. At the same time I began subculturing EAIIII on 0.05% xylitol plates and selecting large colonies, also developing methods for the analysis of pulse-labelled RNA by polyacrylamide gel electrophoresis.

D. Evolution of Mutants with Altered RDH from E. coli EAIIII

A large colony of E. coli EAIIII was purified by five successive subcultures on 0.05% xylitol plates, and then inoculated into a liquid M9/0.2% Xyl medium in a bubble tube and grown overnight. This culture was used as inoculum to start two 200ml cultures simultaneously, one containing M9 + 2% casamino acids, the other M9 + 0.2% xylitol. The object of this experiment was to (i) confirm that the variations in RDH specific activity with culture age on casamino acids/M9 and xylitol/M9 media are similar, (ii) to check whether an improvement in RDH specific activity had occurred after two weeks of selection on 0.05% xylitol plates.

The culture mass doubling time during exponential growth on xylitol/M9 medium was 88 minutes, while on casamino acids/M9 was 55 minutes. The experimental data are presented in Table 3.3, and the plots of specific activities versus the growth points of cultures are shown in Fig. 3.3.

First, it is clear that variations in RDH specific activity with culture growth on the two media are, as a whole, quite similar. Slightly lower RDH specific activity during early exponential growth on casamino acids/M9 is probably as a result of competition of other operons with RDH gene(s) for transcription and translation; while on

TABLE 3.3

Variations in RDH Specific Activity with Culture Growth After Xylitol Plate Selection for 2 Weeks on 0.2% Xylitol/M9 and Casamino Acids/M9 Media

Time/ hr	min	A650nm of culture	Extract vol(ml)	Increase in A340/min per ml of ext.	Units in 1ml of ext.	Units in total extract from 10ml culture	Protein mg/ml of extract	Specific Activity Units/mg protein in the extract	
4	49	0.090	2	1.74	0.278	0.556	0.080	3.475	0.2% Xylitol/M9
5	40	0.132	2	2.00	0.320	0.640	0.070	4.571	
6	48	0.197	2	4.00	0.640	1.280	0.197	3.249	
8	00	0.290	4	2.90	0.464	1.856	0.103	4.505	
9	23	0.445	4	6.00	0.960	3.840	0.196	4.898	
11	37	0.816	8	6.80	1.088	8.704	0.203	5.360	
23	45	1.600	8x2	14.60	2.336	37.376	0.234	9.983	
4	22	0.260	2	4.40	0.704	1.408	0.314	2.242	Casamino acids/M9
5	35	0.450	4	4.20	0.672	2.688	0.248	2.710	
6	45	0.686	8	5.10	0.816	6.528	0.200	4.080	
7	55	0.906	5x2	7.50	1.200	12.000	0.226	5.310	
9	17	1.131	6x2	11.00	1.760	21.12	0.248	7.097	
10	27	1.306	12	14.40	2.304	27.648	0.266	8.662	
11	33	1.430	8x2	12.60	2.016	32.256	0.234	8.615	
23	45	2.050	10x2	26	4.160	83.200	0.344	12.093	

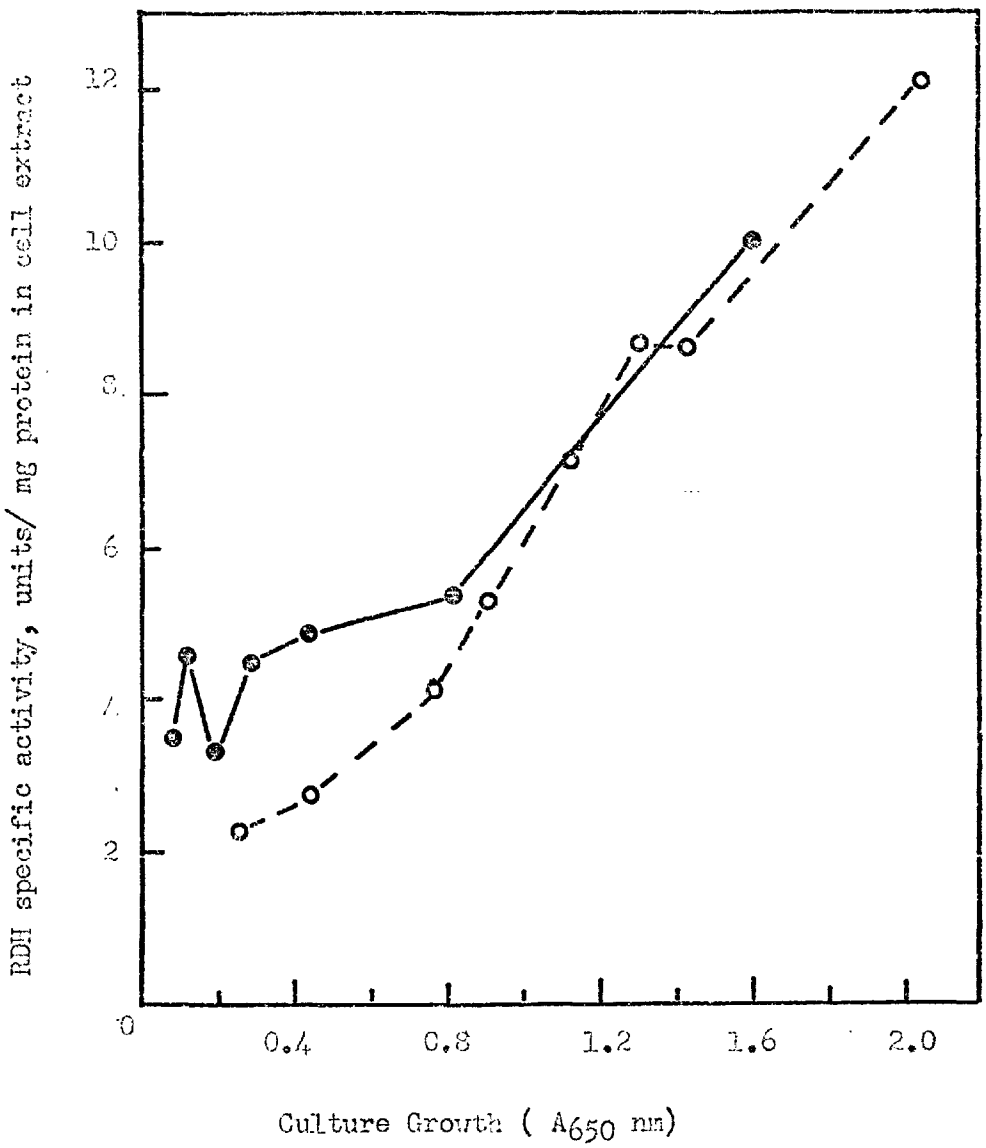


FIG. 3.3. Variation in ribitol dehydrogenase specific activity during E. coli EA1111 culture growth on 0.2% xylitol/M9 (●——●) and 2% casamino acids/M9 (○- -○) media.

xylitol/M9 medium, RDH being the growth rate limiting enzyme, the competition would not be so keen and more RDH enzyme would be made during early exponential growth.

Secondly, the continuous increase in specific activity of RDH up to the stationary phase indicates that the organism is still a high synthesis strain. But, the striking result of this experiment is that the specific activity values are generally much lower than those initially determined, prior to the purification of a large colony from direct subculture of the stab into liquid M9/xylitol medium. For example, at culture A650nm of 1.0, the present specific activity value, Fig. 3.3, is about 6 units/mg protein, while the corresponding value before purification of a colony on xylitol plates, Fig. 3.2 (b) was about 18 units/mg. A decrease in ribitol/dehydrogenase activity in a high synthesis strain with concomitant increase in growth rate of the organism on xylitol/M9 (as demonstrated by larger colonies on 0.05% xylitol/M9 plates) suggested that a structural change in the enzyme from ribitol dehydrogenase to xylitol dehydrogenase (XDH) had taken place (strain EAIIII → EB, Fig. 3.4). Subsequent determinations of the activity ratio, for 0.5M xylitol and 50mM ribitol, after further selection on xylitol plates, proved that the catalytic properties of the enzyme had truly altered. The activity ratio (AR) had increased from 0.042 (the wildtype value) to 0.143 in the new organism (ED), see Fig. 3.4.

In another attempt to regenenate strain EAIIII from the original stab, another large colony was selected from the initial plate (see C above) and used to inoculate a chemostat grown on xylitol. After 2 months (600 generations) a takeover event occurred and several individual colonies of the resulting strain, EE, proved to have increased xylitol/ribitol activity ratios and decreased RDH activity

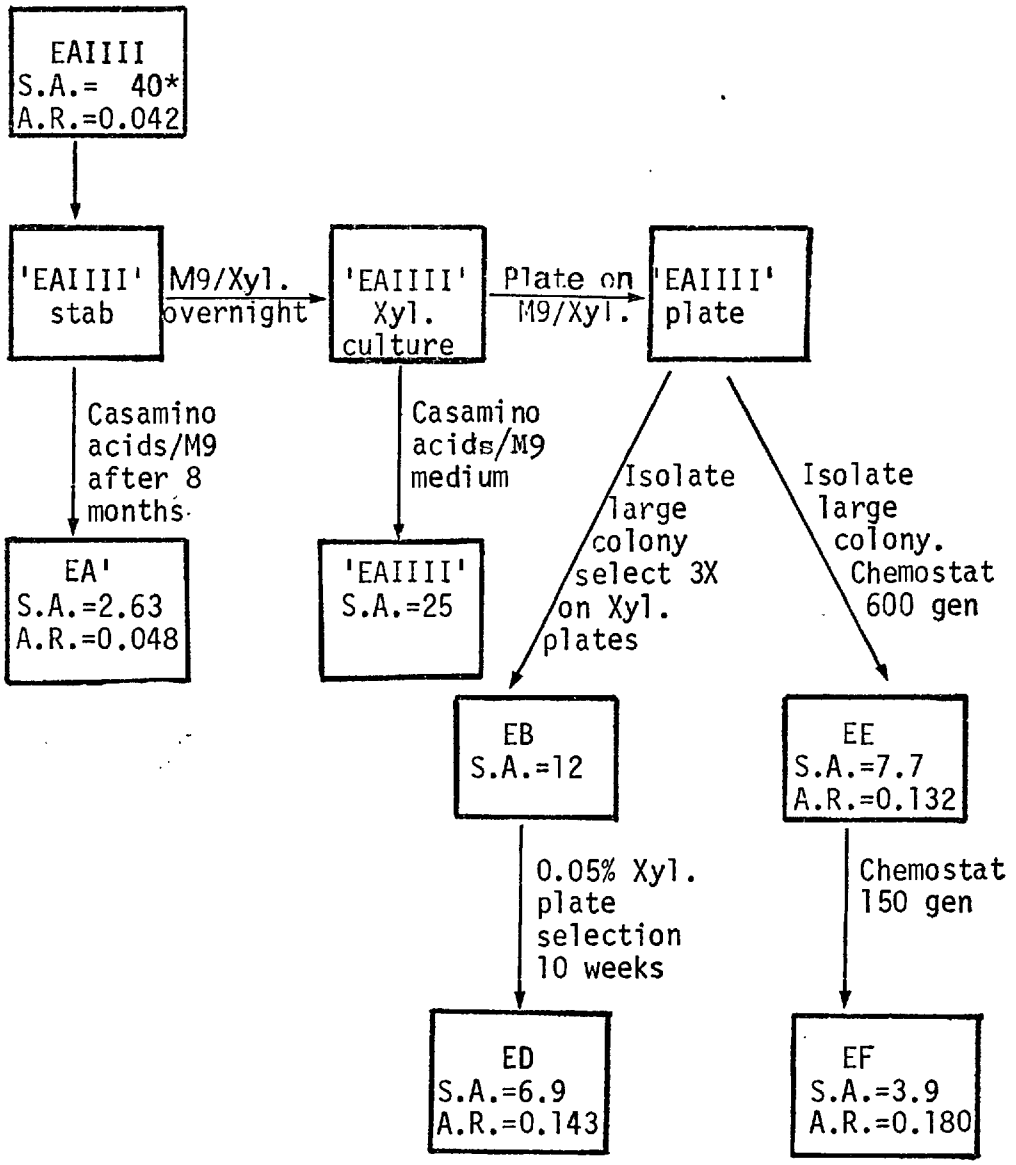


FIG. 3.4. Attempts to isolate E. coli K-12 strain EAIIII from the original stab. S.A. = Specific activity of RDH at the end of growth phase; A.R. = Activity ratio 0.5M Xyl./50mM Rib.; * by personal communication from Dr. J.M. Dothie, also by extrapolation of RDH S.A. at culture A650nm of 1.0 = 30 units/mg; initial letter E, represents an E. coli strain; the second letter refers to the RDH enzyme, A for wildtype enzyme, B, D, E, and F for mutant types with improved XDH activity; the prime above a strain's designation signifies a reversion; sequential additions of single digit numbers after the letters refer to successive mutational steps to RDH high synthesis; no number means not a superproducer.

in extracts (Fig. 3.4). Continuing growth of strain EE in the chemostat gave another takeover after a further 2 weeks (150 generations) resulting in strain EF with a yet greater xylitol/ribitol activity and decreased specific RDH activity (Fig. 3.4).

It appears likely, therefore, that there has been considerable mutagenesis as well as reversion of strain EAIIII on the original stab. The original stock of EAIIII in the stab, after about 8 months had almost completely reverted to low synthesis type, with RDH specific activity of 2.63 and Xyl / Rib AR of 0.048. Attempts to rescue a high synthesis colony from this old stab were unsuccessful. Samples from the original stabs of the intermediate evolvants from E. coli EA (EAI, EAII, and EAIII) were also tested; they all had reverted to low RDH producers.

The unexpected switch in tendency of E. coli strains to alter the catalytic properties of their ribitol dehydrogenase to XDH by spontaneous mutations, in the stab, on xylitol plate and in the chemostat on Xyl/M9, was very surprising. Both Rigby (1971) and Dothie (1974) had reached the conclusion that a series of single point mutations in the RDH structural gene of Klebsiella aerogenes will not lead to a succession of evolutionarily more advanced xylitol dehydrogenases. Their studies showed that while under pressure to grow on xylitol, RDH high synthesis was the predominant evolutionary response of the organism. Only under the conditions of severe mutagenesis and by chemostat selection, could Dothie (1974) select mutants which make better xylitol dehydrogenase. But my results show the contrary to their conclusions: that gradual mutational improvements toward a better xylitol dehydrogenase do take place spontaneously in E. coli. This result is compatible with the results of Clarke and her co-workers for the aliphatic amidase of Pseudomonas aeruginosa (1974): improvements in activity towards several different unnatural

substrates for this enzyme could be isolated by a series of single mutational events.

I noticed that the E. coli high-synthesis strains, EAI, EAII, EAIII, and EAIIII, after a further 8 months storage in the same stabs, all had very slow growth when subcultured into liquid cultures or plates at 37°C, they produced very rough and divided colonies, even on enriched media such as on TYE plates. I could see with naked eye, at least a dozen or so colonies budding or segregating from one another on the same spot. Strain EA, however, gave normal colonies on TYE plates from stabs. It had the expected RDH specific activity (3.44) and xylitol/ribitol AR (0.038).

Such aberrant culture morphology might have been the visible manifestation of some spontaneous high frequency mutations (and repairs) of the RDH superproducer strains when they were maintained for a long period on a rich stab agar, in a struggle to rid themselves of the massive synthesis of a redundant product (RDH). As a result of such hypothetical intense mutagenesis, a few improved XDH mutants could have evolved on the EAIIII stab and subsequently have been selected by subculturing into Xyl/M9 media. However, the strange morphology seems to be a genetic trait of strains EAI, EAII, EAIII, and EAIIII, since repeated single cell cultures retained the new morphology. It is unlikely to be a product of the same mutation that is responsible for high synthesis of RDH in strain EAI, since a parallel RDH-super producer cell line in K. aerogenes, A→AI→AII→AIII, maintained in glycerol stabs in the cold or on Xyl/M9 plates, all produced normal colonies when subcultured into plates at 37° under the same conditions. It may be an adventitious mutation selected during the chemostat growth of EA.

I have also noted that mutational changes in the enzyme to XDH (see Fig. 3.5) always appear to be concomitant with reductions in RDH

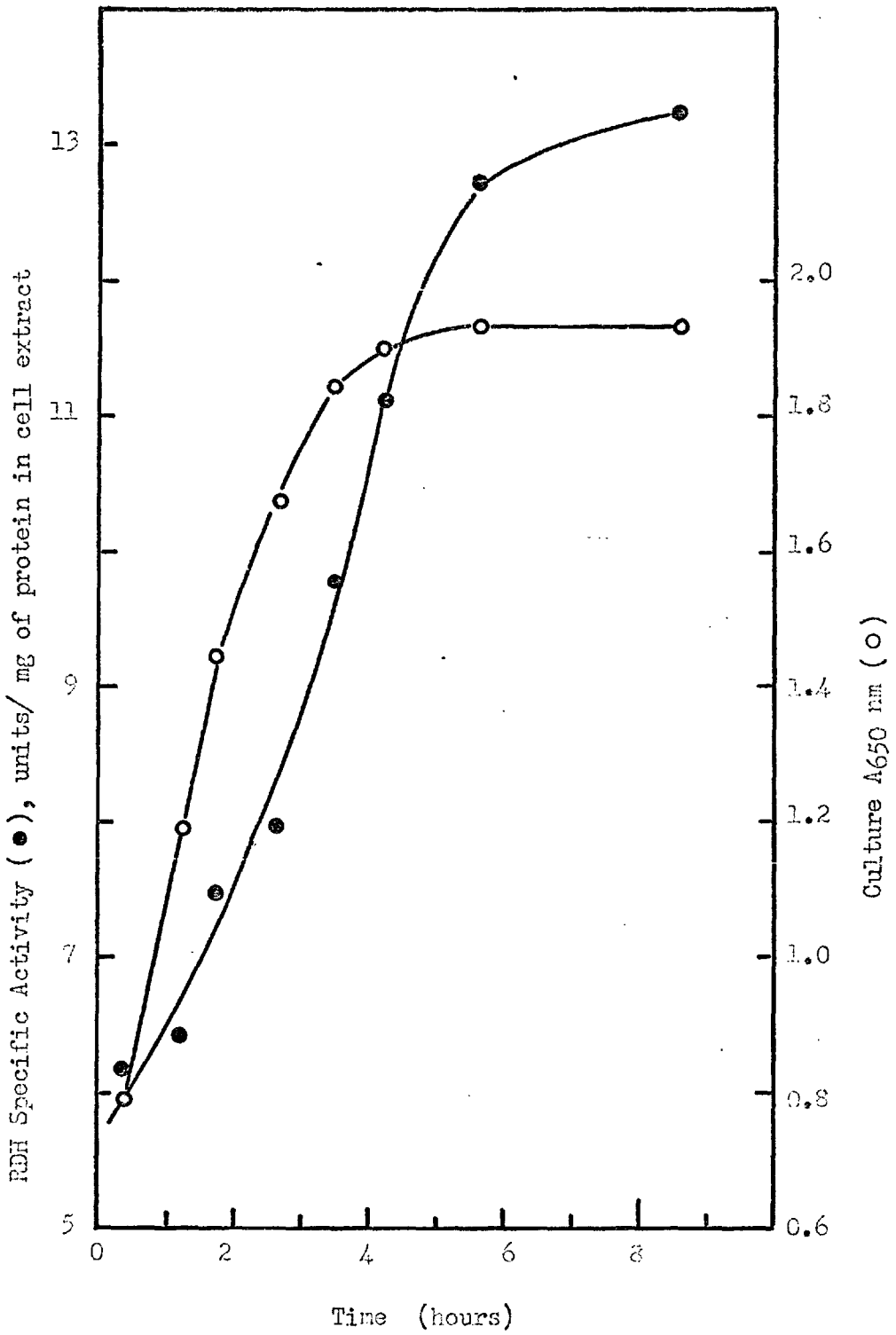


FIG. 3.5. Variations of RDH specific activity and of culture A650 nm of Klebsiella aerogenes A111 with time.

specific activity. In fact the better the XDH produced, the smaller the RDH specific activity appears to be.

E. Variation in RDH Specific Activity During Culture Growth of Klebsiella aerogenes Strain AIII

A 200 ml Xyl 0.2%/M9 culture of high synthesis K. aerogenes AIII was grown at 37°C. Samples were removed for measurements of culture A650nm and extracts were assayed for RDH activity and protein at various times, as described in B above. The results are presented graphically in Fig. 3.5. It is clear that RDH specific activity (units/mg soluble protein) in this strain increases at least up to 3 hours after the culture has entered the stationary phase. Thus, the variation in RDH specific activity with culture growth in this strain, is very similar to that in E. coli strain EAIIII.

The observation of linearity of increase in RDH specific activity with time up to the stationary phase, suggests that RDH mRNA must be present at least to the same proportion at the end of the growth phase as in the mid-log phase. This is a significant observation, because usually little bacterial messenger RNA is synthesized after the mid-log phase. It suggests that RDH mRNA either has an exceptionally extended transcription period in these cultures, or it is unusually stable.

It can be seen from Fig. 3.5 that the maximum specific activity of RDH by the end of K. aerogenes AIII culture growth was about 13 units/mg protein in the extract. It was not possible to increase this specific activity significantly by colony selection on xylitol plates. In the chemostat, however, it was possible to raise the specific activity to 32 units/mg after several weeks, and this value was maintained by continuous subculturing on xylitol plates. The AR (Xyl/Rib) was always close to the wildtype value, 0.042. No spontaneous mutation to a better XDH was ever observed in K. aerogenes

AIII by plate selection or in the extensive chemostat studies described by Rigby et al., (1974).

In order to show that the specific activity of RDH (units/mg protein in the extract) actually increases up to the stationary phase, one must show that this measured increase is not as a result of a continuous fall in the concentration of soluble protein; i.e. the concentration of total protein in the extract should at least remain constant. Fig. 3.6 shows that the total protein in 1ml of extract not only does not decrease, but it actually increases continually up to the stationary phase. But the rate of increase in RDH activity/ml is even greater.

F. Comparative Analysis of Bacterial Pulse-Labelled RNAs from Wildtype and RDH-High Synthesis Strains

Pulse-labelled bacterial RNAs were prepared according to the procedure described in "Materials & Methods". RNA samples (10-30μg) were loaded on 4% polyacrylamide gels inside silicon tubes and run in a buffer containing SDS for 1.5 hours at constant 50 volts. The gels were then scanned inside the tubes for A260nm. For determination of isotope distribution, the gels were removed from the silicon tubes, sliced to 1mm thickness, each slice was digested by hydrogen peroxide inside a capped scintillation vial, and finally counted. These procedures are detailed in "Materials & Methods".

Pulse-labelled RNA samples were compared by this method in pairs, in K. aerogenes AIII and XI Arg^- strains, and in E. coli K-12 strains EA and EAIIII (the latter, before reverting to low synthesis). These comparisons were carried out from culture A650nm of 1.0 to 2.5, corresponding to the periods of highest specific activity for RDH enzyme.

No significant difference could be seen between the radioactivity distribution profiles of each pair, either after a pulse-labelling or

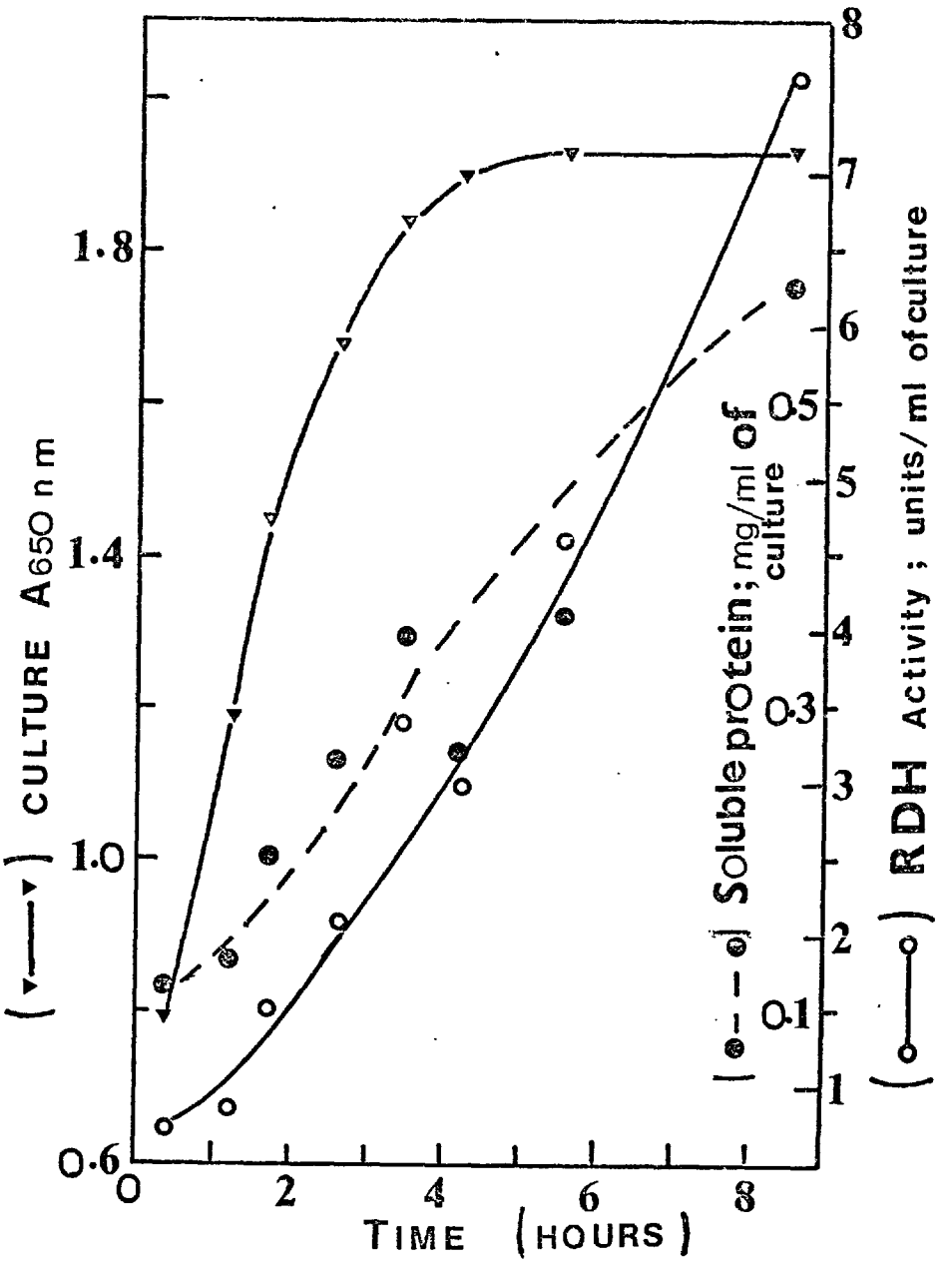


FIG. 3.6. Variations of total protein concentration and of RDH activity in the cell extract with the growth of Klebsiella aerogenes A111 culture.

after a pulse followed by a short chase with excess cold UTP. Fig. 3.7 shows the RNA gel profiles of K. aerogenes strains AIII and XI Arg$^-$; the most contrasting radioactivity profiles ever observed are presented. Apart from the small fluctuating difference from time to time and in different parts of the profile, I could not detect a significant and reproducible difference between the distribution of pulse-labelled RNA extracted from a high synthesis strain and from an appropriate control, either a low synthesis, or a wildtype.

In order to confirm this conclusion, I searched for a different method of RNA fractionation. Gel-filtration using Ultrogel AcA$_{22}$, seemed a suitable method, with fractionation range of 60,000 to 1,000,000 daltons (for globular proteins). A 35cm (length) x 1.3cm (dia) column was packed with this material, equilibrated, and run according to the printed LKB instructions. The elution profile of pulse-labelled K. aerogenes AIII RNA is shown in Fig. 3.8, which shows rather poor resolution for RNA. Most of the RNA was eluted in one broad peak as shown by the radioactivity profile. The radioactivity profiles of XI Arg$^-$ and AIII RNAs were identical.

G. Conclusion

The experiments described show that the specific activity of RDH, either expressed in units/cell mass, or units/mg protein in the cell extract, or units/viable cell, in the high synthesis strains, E. coli K-12 EAIIII and K. aerogenes AIII, increases continuously with culture growth up to a few hours within the stationary phase. By contrast, the low synthesis but constitutive mutants, such as E. coli K-12 EA strain, do not exhibit such increases, in fact the specific activity of RDH decreases to some extent in E. coli strains EA after mid -log phase. Thus, with regard to the

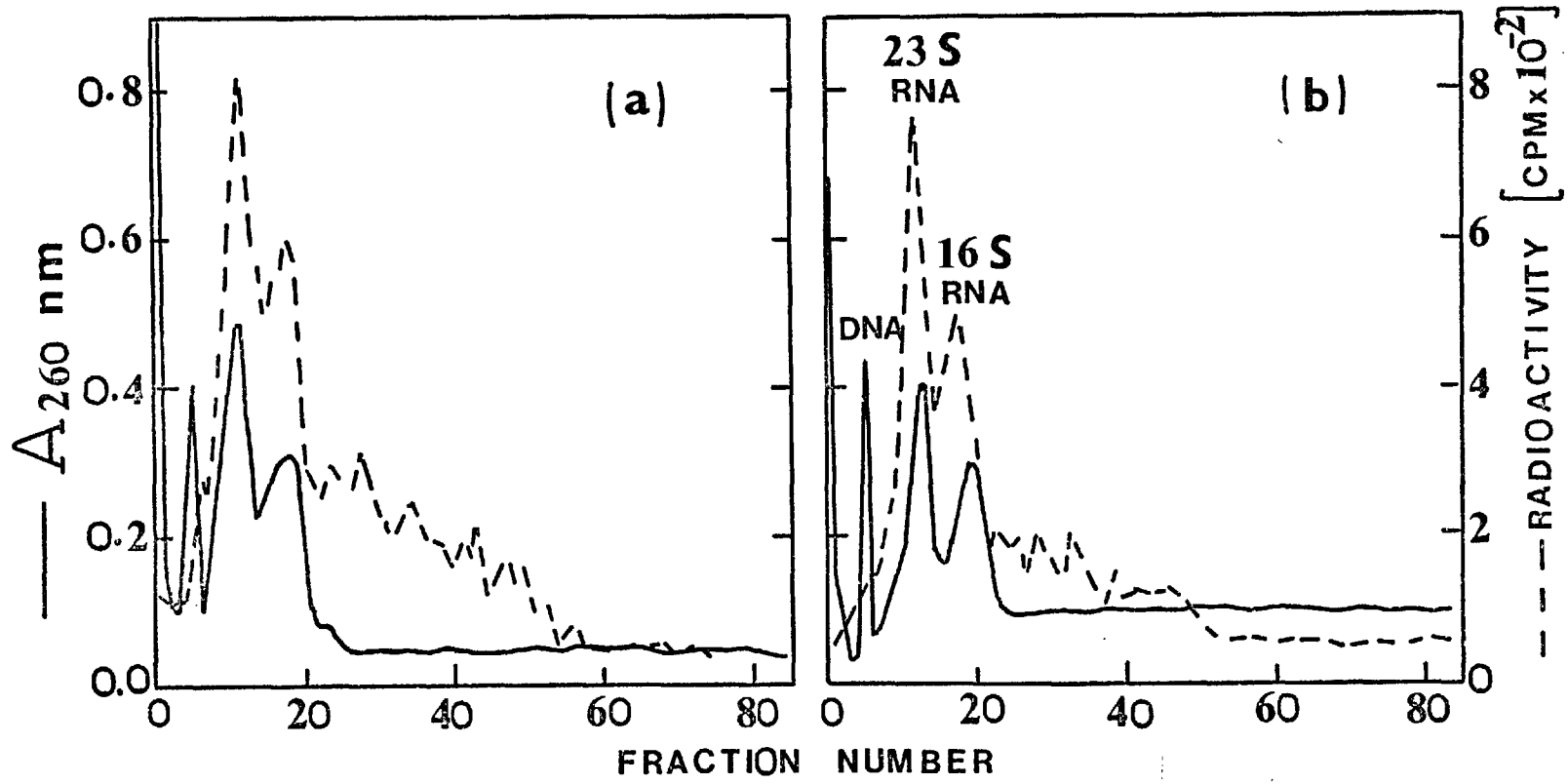


FIG. 3.7. Comparative analysis of pulse-labelled RNAs from K. aerogenes strains A111(a) and X1 Arg⁻(b) on 4% polyacrylamide gels containing SDS. Both cultures were labelled at A650 nm= 2.3 (where the maximum A650 = 2.85), with (5,6-^{3}H)-uridine, 2 µCi/ml for 2 min, then chased with excess uridine triphosphate(10 mM) for 30 sec before RNA extraction. About 10 µg RNA, dissolved in 300 µl TM buffer + 100µl glycerol, were electrophoresed for 1.5 hr at constant 50mV. The gels were pre-run for ½ hr prior to sample loading. Scan rate 1 Cm/min; recorder 60mm/min.

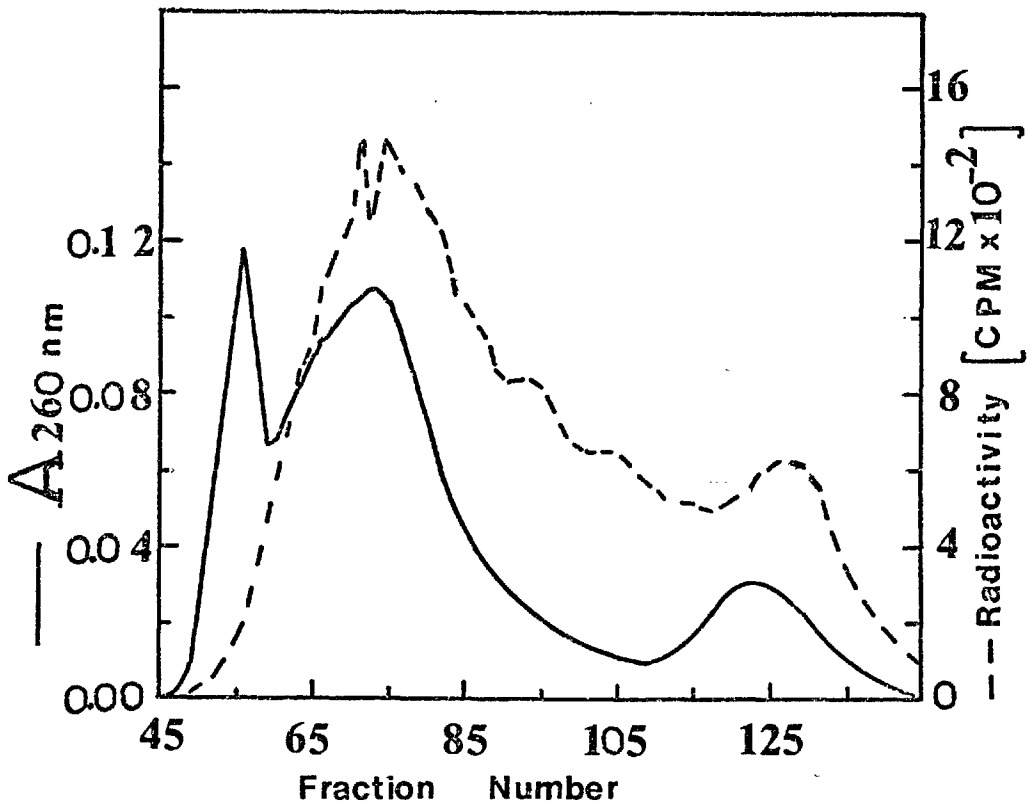


FIG.3.8. Ultrogel AcA22 column chromatography of pulse-labelled RNA from Klebsiella aerogenes A111. 2 min pulse-labelling with ^{3}H-uridine followed by 30 sec chase with cold UTP; elution was with electrophoresis running buffer without SDS; flow rate at 10 ml/hr; fractions, 0.25 ml each were collected for radioactivity measurements.

first fundamental question posed in the beginning of this Chapter, if one assumes a direct correlation between RDH enzyme specific activity and RDH-mRNA specific activity exists, then one must conclude that in high synthesis strains the specific activity of RDH mRNA is maximal at the end of growth period. This is an unprecedented observation, for it is an established fact that the synthesis of bacterial mRNA in general become greatly reduced past the mid logarithmic growth phase. Thus, one is impelled to believe that in the high-synthesis strains either the cell has somehow specifically extended the transcription period of the RDH operon, or the mRNA for this enzyme is unusually stable. In any case, the occurrence of either event should greatly facilitate the purification of this messenger RNA.

Comparative analysis of the size distribution of pulse-labelled RNA from RDH high synthesis and low synthesis strains, by polyacrylamide gel electrophoresis and gel filtration techniques, did not reveal any significant difference between these RNAs. Considering that 30% of the total protein in the cell extracts from high synthesis strains is RDH, one would have expected to see a difference if proportionally more RDH-mRNA existed in these strains.

Therefore, the paradox of ever increasing specific activity of RDH and the considerable proportion of this enzyme in the cell extract on one hand, and the lack of evidence for differential pulse-label corresponding to this mRNA on the other hand, suggests only one thing: that the messenger - RNA for this enzyme could be very stable.

The second objective of the work described in this chapter, to attempt to identify RDH mRNA directly, was based on the assumption that it might be present in huge amounts in pulse-labelled cells of high synthesis strains. There was no evidence of an outstanding difference in mRNA between low synthesis and high synthesis strains,

but this is not surprising if the alternative explanation of a stable RDH mRNA is accepted; a small amount of such mRNA could be capable of making a lot of enzyme if it were translated at least as efficiently as a normal bacterial mRNA.

The unexpected observation of spontaneous mutations to improved xylitol dehydrogenases in the E. coli strain EA prompted further investigation of K. aerogenes by Dr. J.M. Dothie. The extensive chemostat studies with K. aerogenes carried out in Cambridge had failed to yield such spontaneous mutants, but there was a suspicion that the wildtype strain A had mutated during storage: the specific RDH activity in extracts of K. aerogenes strain A from our existing culture collection differed from that observed by Rigby et al., (1974) for the original isolate (strain XI Arg^- of Wu et al., 1968).

A fresh sample of K. aerogenes strain XI Arg^- was obtained from Professor E.C.C. Lin at Harvard, which gave RDH specific activity in extracts similar to that originally observed by Dr. P.W.J. Rigby. However, in several chemostat experiments with this strain, grown on xylitol, spontaneous mutants with improved xylitol dehydrogenases took over the culture. The ability to evolve improved xylitol dehydrogenases spontaneously is not, therefore, an exclusive property of the RDH structural gene when incorporated in the E. coli genome.

The conflict between the experimental results obtained by Rigby et al., (1974) with K. aerogenes in Cambridge and those found here in London by Dothie remains unresolved. It seems likely that a change occurred during storage of K. aerogenes strain A that made it particularly prone to mutation to high synthesis of RDH. Such events would then outnumber the rarer mutations to give improved xylitol dehydrogenases. Whatever the explanation , however, it is clear that one important conclusion of Rigby et al., (1974) must be retracted:

the structural gene for ribitol dehydrogenase can evolve by single point mutations to yield a better xylitol dehydrogenase.

I have noticed that mutations in RDH structural gene to improved xylitol dehydrogenase always appear to be concomitant with reductions in ribitol dehydrogenase specific activity (Fig. 3.5). Indeed the better the XDH produced, the smaller the RDH specific activity appears to be. This may reflect a reduction in the amount of enzyme made in the strains with improved xylitol activity, but it is more likely that it implies a reduction in the activity of the new enzyme towards ribitol. This is in contrast to the observations of Burleigh et al (1974) and Dothie (1974) on strains B and D of K. aerogenes that had enzymes with improved xylitol activity without any appreciable loss of activity towards ribitol or L-arabitol.

CHAPTER IV

A Model In Vitro Translation System

A. Introduction

Purification of a messenger RNA must be monitored at each stage by some specific and reliable method. Translation of mRNA in a cell-free protein synthesizing system and subsequent identification and quantitative determination of the *in vitro* products is probably the most convincing method. Another good, reliable technique is the specific hybridization of mRNA to its complementary DNA. The practicability of the latter technique, however, depends mainly on complete separation of the complementary DNA from total DNA of the organism under investigation. Also, the complementary DNA should be accompanied by as little other (foreign) DNA as possible to avoid excessive 'noise' (background random hybridization, due to unspecific DNA-RNA interaction) which must always be subtracted from specific mRNA-cDNA hybridization.

Thus, during the initial period of this investigation I devoted some time to setting up an *E. coli* cell-free protein synthesizing system. This system was tested successfully to translate synthetic and natural messenger RNAs, as described below.

Contemporary with this work, my colleague, M.A. Neuberger, in this Department, began construction of a λ*rbt* transducing phage to be used in further analysis of the RDH superproducing strains. Such λ*rbt* hybrid DNA would provide a very convenient assay for RDH mRNA.

Protein synthesis was studied essentially by the method of

Nirenberg and Matthaei (1961). An S30 fraction (30,000g supernatant) was prepared from Escherichia coli MRE 600 cells, which are deficient in "magnesium ion-independent"-ribonucleic acid depolymerase (Wade and Robinson 1966).

This preparation contains ribosomes, transfer RNAs, amino acid activating enzymes, as well as initiation, transfer and release factors. Incorporation of two ^{14}C-labelled amino acids, into protein was studied in the presence of synthetic (poly U) and natural messenger (MS2 viral RNA).

The S30 fraction is incubated with amino acid mixture containing one labelled amino acid, in the presence of salts, buffer, GTP, messenger RNA and energy generating system (consisting of ATP, phosphoenol pyruvate and pyruvate kinase). The latter is needed to provide a continuous supply of energy required for peptide bond formation. Controls are run without messenger and without energy generating system. Protein synthesis is measured by determining radioactivity incorporated into the trichloroacetic acid - insoluble fraction which contains mainly proteins. This fraction is washed free of amino acids and nucleic acids (which also may contain some ^{14}C) and radioactivity is measured in a liquid scintillation counter.

B. Cell-Free Protein Synthesis With Frozen Cells From MRE

A frozen cell paste of E.coli MRE 600 was obtained from the Microbial Research Establishment, Porton, Wilts. According to MRE, these cells had been grown aerobically by continuous flow culture in a 16 litre vessel at $37^{o}C$ under glycerol-limitation at a dilution rate of 0.78 hr^{-1} in a medium containing yeast extract, glycerol and salts. Cells were collected in 100 litre lots. Their method is based on one of Elsworth et al., (1968).

TABLE 4.1.

Incorporation of amino acids into protein directed by poly U and MS2 RNA in an E. coli MRE 600 crude extract system, starting with frozen cells from M.R.E., Porton

Additions	Tube Number							
	1,2 µl	3,4 µl	5,6 µl	7,8 µl	9,10 µl	11,12 µl	13,14 µl	15,16 µl
H20	530	530	480	480	440	440	380	380
Salts + GTP	140	140	140	140	140	140	140	140
Amino Acid Mixture with ^{14}C-Phe	130	-	130	-	130	-	130	-
Amino Acid Mixture with ^{14}C-Leu	-	130	-	130	-	130	-	130
S30 (5.15 mg RNA/ml)[a]	200	200	200	200	200	200	200	200
Energy	-	-	50	50	50	50	50	50
Poly U, 2.5 mg/ml	-	-	-	-	40	40	-	-
MS2 RNA, 0.5 mg/ml	-	-	-	-	-	-	100	100
Total	1000	1000	1000	1000	1000	1000	1000	1000
CPM/ml	250	200	400	350	14K	350	2.1K	2.2K
(RNA)					100µg	100µg	50µg	50µg

RNA concentrations are calculated from OD_{260} using a conversion factor, 20 OD_{260} units/ml = 1 mg RNA/ml. In order to obtain the proper concentration of each reagent and to save time while performing a number of assays, various reagents are added in premixed groups (Salts + GTP; Energy source; Amino Acid Mix) as follows:

Salts + GTP	In 1 ml	x 20
1M Tris-HCl, pH 7.8	100 µl	2 ml
2M KCl	20 µl	0.4ml
1.2M Mg-acetate	10 µl	0.2ml
14.3M 2-Mercaptoethanol	0.4µl	8 µl
3mM GTP	10 µl	0.2ml
	140.4µl	2.808ml

Energy Source	In 1 ml	x 15
375mM PEP	20 µl	300µl
50mM ATP	20 µl	300µl
4mg/ml PEP-Kinase	5 µl	75µl
H2O	5 µl	75µl
	50 µl	750µl

Amino Acid Mixture with ^{14}C-Phe	In 1 ml	x 10
Mixture of all amino acids except Leu and Phe, each 2 µmoles/ml	100 µl	1 ml
Leucine (10 µmoles/ml)	20 µl	0.2ml
^{14}C-Phe (25µCi/ml)	10 µl	0.1ml
	130 µl	1.3ml

Amino Acid Mixture with ^{14}C-Leu	In 1 ml	x 10
Mixture of all amino acids except Leu and Phe, each 2 µmoles/ml	100 µl	1 ml
Phenylalanine (10µmoles/ml)	20 µl	0.2ml
^{14}C-Leu (25µCi/ml)	10 µl	0.1ml
	130 µl	1.3ml

The solubility index for all amino acids and the procedure for making up the amino acids mixture are described in "Materials & Methods".

The culture is collected at 4°C and centrifuged in successive 100 litre batches. The cell paste from each 100 litre batch is resuspended in 100 litres of buffer (10mM Tris-HCl; 10mM Mg-acetate; final pH 7.4) at 4°C and again recovered by centrifugation. The cell paste is packed in polythene bags containing 100g frozen rapidly in solid carbon dioxide and stored at -20°C. The procedures for preparation of an S30 fraction from these cells, preincubation to destroy endogenous mRNA, incorporation of labelled amino acids into protein and analysis of the counts incorporated, are described in Chapter 2 "Materials & Methods" (Protein Synthesis in Vitro, Method 1).

The results of one such experiment are shown in Table 4.1, which demonstrates the dependence of *in vitro* polypeptide synthesis on a continuous supply of energy and on added messengers (Tubes 1 - 8). Both poly U and MS2 RNA are capable of directing synthesis of polypeptides under the assay conditions (9, 10, 13 - 16). Poly U is only capable of directing polyphenylalanine synthesis; the control experiment with ^{14}C-labelled Leucine (11, 12) shows no incorporation of this amino acid above the background.

C. Cell Free Protein Synthesis With E. coli MRE 600 Cells Grown By I.C. Pilot Plant To Very Low Cell Density

While studying the literature, I came across some papers (for example, Li and Umbreit, 1966), showing an inverse relationship between the activity of bacterial protein synthesizing systems and the age of culture of the cells. In particular the activity of one of the initiation factors, IF-3, was severely reduced by the age of culture.

In order to obtain a very active S30 fraction, E. coli MRE 600 cells were grown by the Pilot Plant at Imperial College in a fermenter to a low (3.5×10^8) cell density in 45 l of a suitable

medium (described in "Materials & Methods"), at 37°C. Cell density was estimated by monitoring oxygen uptake and by Nephelometer readings (Evans Electroselenium Ltd., Halstead, Essex), using the tabulated and graphical data already obtained by a pilot experiment (E.C. 51, shown in "Materials & Methods").

In the experimental run (E.C. 53), when the oxygen tension was reduced by 0.35% and the Nephelometer read 252 for the undiluted culture (corresponding to a cell density of about 3.5×10^8) the culture was harvested by transferring it quickly to a cooling container, and 15 minutes later the cooled culture was passed at a fairly fast flow rate into a Sharples Continuous Flow Centrifuge system. The total centrifugation time was 20 minutes, and 30g of wet cells were collected. The cells were washed once with 110ml of chilled buffer (0.01M Tris-HCl, pH 7.8; 0.01M Mg acetate; 0.06M KCl; 0.006M 2-mercaptoethanol) and the cell pellet was recovered by centrifugation at 3000 x g, 4°C, 10 minutes. The pellet (23.2g) was quick frozen and S30 fractions subsequently prepared from it according to "Materials & Methods".

Table 4.2 shows the comparative poly U directed protein synthesis, using S30 fraction prepared from I.C. grown young cells, and S30 prepared at the same time from a new batch of MRE frozen cell paste. It is clear from these results that the activity of S30 prepared from I.C. early log. phase cells is at least 6-fold greater than the S30 from MRE cell paste prepared by the procedure described already. Table 4.2 also shows the concentration effect of poly U in the S30 preparation from I.C. cells. 50µg poly U seems to be already approaching saturation in this system (48K CPM for 50µg; 59K CPM for 100µg; and 64K CPM for 200µg). Dependence of each system on energy and external messenger is also indicated.

TABLE 4.2.

Poly U directed protein synthesis; comparison of activities of S30 fractions prepared from E. coli MRE 600 cells grown by MRE (1-4) and cells grown by I.C. pilot plant, harvested at early log. phase (5-16)

Additions	Tube Number							
	1,2 µl	3,4 µl	5,6 µl	7,8 µl	9,10 µl	11,12 µl	13,14 µl	15.16 µl
H20	700	680	650		630	580	560	520
Salts + GTP	140	140	140	140	140	140	140	140
^{14}C-Phe (25µCi/ml)	10	10	10	10	10	10	10	10
S30 (From MRE cell paste), 180 OD_{260}/ml	100	100	-	-	-	-	-	-
S30 (I.C., young cells), 90 OD_{260}/ml	-	-	200	200	200	200	200	200
Energy	50	50	-	50	-	50	50	50
Poly U, 2.5mg/ml	-	20	-	-	20	20	20	20
	1000	1000	1000	1000	1000	1000	1000	1000
CPM/ml	250	7.3K	300	380	350	48K	59K	64K
(RNA)	-	50µg	-	-	50µg	50µg	100µg	200µg

D. Conclusion

A cell-free translation system, based on the procedures of Nirenberg and Matthaei (1961), has been prepared and shown to be active in poly U and MS2 RNA directed incorporation of amino acids into protein. S30 fraction prepared from E. coli MRE 600 bacteria grown by I.C. Biochemistry pilot plant to a low cell density (3.5×10^8 cells/ml culture), cooled and harvested rapidly, is at least 6 times more active in incorporating amino acids into protein than a simultaneously prepared S30 from the same cells prepared by MRE by continuous flow culture in a 16 l vessel, collected in 100 l lots at $4^{\circ}C$ and centrifuged in successive 100 l batches.

CHAPTER V

Purification and Characterization of Monospecific Rabbit-Anti RDH and Sheep Anti-Rabbit Ig Antibodies

A. Introduction

There was a strong indication from the discussion in Chapter III that RDH-mRNA might be quite stable. This, together with the reserved optimism that there might still be a large amount of RDH mRNA present in the superproducer bacteria, encouraged me to investigate the possibility of purification of RDH mRNA by one of the methods currently in use for the purification of eukaryotic messengers; especially since there was no alternative method for pro-karyotic mRNA fractionation available at that time. At this time attempts to insert the RDH gene into a phage genome were in progress, but purification by complementary DNA-mRNA hybridization remained only a prospect and its use was envisaged more as an analytical technique than a preparative one. Some new fractionation methods such as selective trapping of mRNA molecules by their 3'-OH-"poly-A tails" to immobilized oligodeoxythymidene (e.g. Edmonds et al., 1971; Lee et al., 1971; Darnell et al., 1971; Philipson et al., 1971; Gillespie et al, 1972) offer no advantage in bacterial systems, because very few, if any, mRNAs with "poly-A tails" exist.

Purified monospecific antibodies raised against a pure protein in animals, have been known to be capable of interaction with nascent polypeptides of that protein on the polysomes as long ago as 1961 (Cowie et al), and since then it has repeatedly been shown in several systems that antibodies can bind to nascent chains on polysomes (e.g. Warren and Goldthwait, 1962; Williamson and Askonas, 1967; Allen and Terrence, 1968; Schubert and Cohen, 1968; Takagi and Ogata, 1971;

Hamlin and Zabin, 1972).

Palacious et al., (1973) reported on the use of immuno-adsorbents for preparing an RNA fraction that was highly enriched for ovalbumin mRNA. In that study, however, biological purity of the mRNA had not yet been sufficiently established since the procedure employed to isolate the cell-free products programmed by the mRNA preparation was restricted to ovalbumin. Thus possible contamination by non-ovalbumin mRNAs could not be ruled out. Furthermore, fingerprint analysis of enzymic digests of the cell-free products (a crucial test for proving biological purity) was not done.

Schechter (1974) for the first time, described the use of a double antibody technique for the isolation of biologically pure mRNA coding for immunoglobulin light chain from mouse myeloma polysomes. In the first stage antibodies to L chains were allowed to react with polysomes containing nascent L chains to form a soluble antibody-polysome complex. In the second stage another antibody with specificity directed toward the Ig moiety in the complex was added, resulting in the formation of aggregates that precipitated out of solution. Further chemical purification of this mRNA was achieved by using oligo-dT-cellulose. The biological purity of this mRNA (i.e. the capacity to program the synthesis only of L chain in a cell-free system) was found to be 95% (Schechter, 1973). The main criterion employed for determining authenticity and biological purity was the fingerprint analysis of tryptic digest of the total reaction mixture containing cell-free products programmed by the mRNA. Chemical purity (95%) was determined from the amount of rRNA in the mRNA preparation by scanning of appropriate gels.

I therefore decided to investigate the possibility of purification of a bacterial mRNA by a double antibody technique. However, certain

risks pertinent to this particular study seemed obvious from the start. First of all, the idea of purifying and handling bacterial polysomes, itself seemd a formidable task, partly because they are very unstable and partly because they are not so well characterised as eukaryotic polysomes. Secondly, since RDH is a tetramer, there was considerable risk that the majority of antibodies raised against the native RDH protein might not react with the nascent peptides on the polysomes. The extent and the specificity of such interaction would not be known till purified antibodies were prepared and incubated under safe conditions with polysomes.

In this Chapter I shall concentrate on purification and characterisation of monospecific antibodies. The most critical step in purification of antibodies is affinity chromatography. Fortunately certain advances have been made in this field in the recent years which have been of immense help to me in this study.

Much interest has centered on the covalent attachment of biologically active compounds (enzymes, antibodies, and antigens) to insoluble polymers. These derivatives have found special use as immunoadsorbents for the purification of antibodies (Silman and Katchalski, 1966). In principle, the antigen is coupled covalently to a suitable insoluble matrix. Mixtures of antibodies are passed through the column of immunoadsobent. Specific antibodies bind to the matrix-bound antigon, while the other non-specific proteins are washed through. Then the specific antiobodies are eluted under appropriate conditions. Successful application of the method requires that the adsorbent have minimal nonspecific interaction with proteins, that it exhibits good flow properties which are retained after coupling, and that it be mechanically and chemically stable to the conditions of coupling and of elution. Idealy, the gel substance should form a very loose, porous network which permits uniform and unimpaired entry and exit of large macromolecules. The gel particles should preferably be of uniform

size, spherical, and rigid (Cuatrecasas, 1970). A highly porous derivative of agarose, Sepharose, has been described as a suitable matrix for the purification of enzymes by affinity chromatography (Cuatrecasas et al., 1968). Synthetic polyacrylamide gels also appear to be promising as insoluble supports. They are available commercially in beaded spherical form, in various pregraded sizes and porosity (Biogels). A gentle method has been developed for coupling proteins and small molecules to these carbohydrate derivatives by using cyanogen halides (Porath et al., 1967; Axen et al., 1967).

For successful purification by affinity chromatography, the ligand groups critical in the interaction with the macromolecule to be purified must be sufficiently distant from the backbone of the solid matrix to minimize steric interference with the binding process (Cuatrecasas et al., 1968; Cuatrecasas et al., 1970). This can be accomplished by preparing an inhibitor with a long hydrocarbon chain, an "arm", attached to it, which can in turn be attached to the insoluble support. Alternatively, such a hydrocarbon extension arm can first be attached to the solid support. However, extending the position of the ligand further than about 6 carbon or nitrogen atoms did not result in a further increase in binding capacity (Cuatrecasas, 1970).

Bromoacetamidoethyl-Sepharose was prepared in this study according to the procedure of Cuatrecasas (1970), which was used for covalent coupling of both RDH and rabbit-antibodies, in order to purify rabbit-anti-RDH antibodies and sheep anti rabbit Ig antibodies, respectively.

For immunization of animals and timing of injections and bleeding, I have tried to use the recommendations of Hurn and Landon (1970).

The immunogenicity of a peptide is related to its molecular weight; whether or not it normally exists as a polymer; the rigidity of its structure as imposed, for example, by disulphide groups; its

susceptibility to enzymatic degradation; the accessibility of the determinant groups; its content of certain amino acids including phenylalanine, tryptophan, glutamic acid, lysine and especially tyrosine; and its structural relationship to any corresponding peptide in the species under immunization. The antibody response to injected immunogen is increased by the inclusion of adjuvants such as aluminium hydroxide or a mineral oil. The most widely used adjuvant is that introduced by Freund (1951) which consists of a neutral detergent (Arlacel A), paraffin oil and (in the 'complete' form) killed mycobacteria. The detergent, because of its high content of hydrophylic and lipophilic groups, binds both the oil and an aqueous solution of the immunogen, allowing the formation of a stable emulsion. One of the ways in which the adjuvant is believed to work is by releasing the immunogen slowly over the course of weeks, thereby preventing its rapid uptake into the circulation. This avoids rapid degradation of the immunogen by circulating proteolytic enzymes and, in the case of certain hormones such as insulin, minimizes any direct physiological effect of the immunogen on the recipient. The adjuvant also facilitates phagocytosis of the immunogen by macrophages, which is an essential initial step for antibody production. Thirdly, it causes the formation of a local granulomatous lesion which may act as a focus of antibody formation. Finally, the adjuvant causes both local and general stimulation of the reticuloendothelial system with multiplication of macrophages and of immunologically competent lymphoid cells.

Antibody response is virtually independent of immunogen dosage over a wide range, once a certain minimum quantity is exceeded. To this extent, the exact quantity of immunogen administered is of little importance unless scarcity dictates the need for economy. The minimum effective quantity varies from one substance to another. However, the amount required is, in general, substantially less than the dose

levels frequently recommended. With the use of Freund's adjuvent, satisfactory response can often be obtained with primary doses of 100μg per rabbit (even for poor immunogens) and booster doses of half the primary dose. If the immunogen is freely available, and if it is unlikely to have any toxic or undesirable action on the injected animal, doses of 0.5 - 5mg for primary inoculation and 0.25 - 2mg subsequently (depending on purity), are most likely to be effective.

Although certain routes of administration of immunogens are considered more effective than others in producing antibodies, one can still obtain very good response by intramuscular and subcutaneous injections without having to use more drastic conditions such as injecting into the lymph nodes and intra-articular (usually into the knee joints) which could cause a lot of unnecessary pain and discomfort to the animal.

Timing of injections is important in order to obtain the maximum response with minimum effort. Since one of the properties of an oily emulsion is to allow a sustained slow release of antigon over a period of several weeks, the practice of administering injections at weekly intervals is without value where Freund's adjuvant is used (Hurn and Landon, 1970). Antibody levels rise relatively slowly after a primary injection reaching a peak some 6 weeks later. Booster doses should not be given earlier than 4 weeks after the first injection nor more frequently than once each month thereafter. Antibody levels rise to a maximum about 10 days after each booster injection and animals should be bled between the seventh and fourteenth days, several bleeds may be taken during this time without affecting the amount or the quality of the antibody.

B. Immunization of Rabbits with Klebsiella-D RDH, and Collection of Antisera

The pure RDH sample used for all immunizations in this study was the Klebsiella aerogenes-D RDH, one of the purest samples available. It differs only by one amino acid residue from strain A RDH (Hartley et al, 1976). Two ml of RDH solution, approximately 2mg/ml in physiological saline was added to 6 ml of Freund's complete adjuvant and mixed by sonication. Four ml portions of this emulsion were used as the primary injections for two young rabbits (A and B), each weighing 2 - 2½ Kg. Injections were given both subcutaneously in multiple sites in the abdomen as well as intramuscular in the hind legs. Booster injections were given after 5 weeks in the same manner, using half of the initial amounts, 1mg RDH/rabbit, in Freund's incomplete adjuvant.

The first bleed was done 14 days after the booster injection. At the same time an older rabbit which had not been immunized with RDH was bled. The antiserum from this rabbit (C) was used as a control serum in the following experiments.

C. Development of an RDH-Inactivation Assay-System for Rapid Estimation of Anti-RDH Antibodies

It is possible that antibodies which bind to RDH could inhibit its activity or inactivate it completely. Thus, serially diluted immunized antisera reacted with a known amount of RDH could serve as a quick measure of specific antibody titre in a given sample.

First, the RDH sample should be diluted to an appropriate concentration for enzyme assay (see RDH enzyme assay in Materials & Methods). In the case of RDH-D (approximately 100mg/ml) dilution of about 1:20,000 was found appropriate. Since antibody-antigon reaction has been considered a slow reaction, I thought it was a good idea to carry out such incubations on ice or at $4^{o}C$ to avoid

inactivation of RDH by heat at room temperature, also because eventually antibody-polysome reactions had to be carried out at low temperatures.

However, if one incubates for several hours constant amounts of the diluted RDH solution with constant volumes of serially diluted antisera from immunized rabbits, one would obtain the result shown in Fig. 5.1. First, there is a true inhibition of RDH activity up to a certain dilution of antiserum (1:40, in this case), then there is a rise in RDH activity as a result of insufficient titre of the specific antibodies present. Then, there is a secondary decline in RDH activity, beyond about 1:100 dilution of antiserum. This secondary inactivation is definitely due to insufficient protein concentration, as if serum proteins protect RDH molecules in solution, but below certain concentrations there is no more protection. The situation can be easily rectified by the addition of about 0.5 - 1mg/ml BSA to the buffer used for the dilutions of RDH and antisera, thus no more inactivation due to protein dilution would take place as will be seen in later results.

The next question is, how much time is required to bring about effective inactivation of RDH by the required amount of the immunized antisera? From the result shown in Fig. 5.2 we can see that constant amounts of 1/40 diluted rabbit antiserum added to equal volumes of 1:10,000 diluted RDH-D and incubated on ice for various times, brought about effective inactivation of RDH (>75%) within 10 minutes. Further inactivation took place slowly. No BSA was added to the dilution buffers in this experiment.

Comparative titres of the anti-RDH antibodies in the three rabbit antisera (A, B and C) are indicated in Fig. 5.3. Equal volumes of serially diluted antisera were added to equal volumes of RDH sample and incubated on ice for 50 - 60 minutes (in the case of

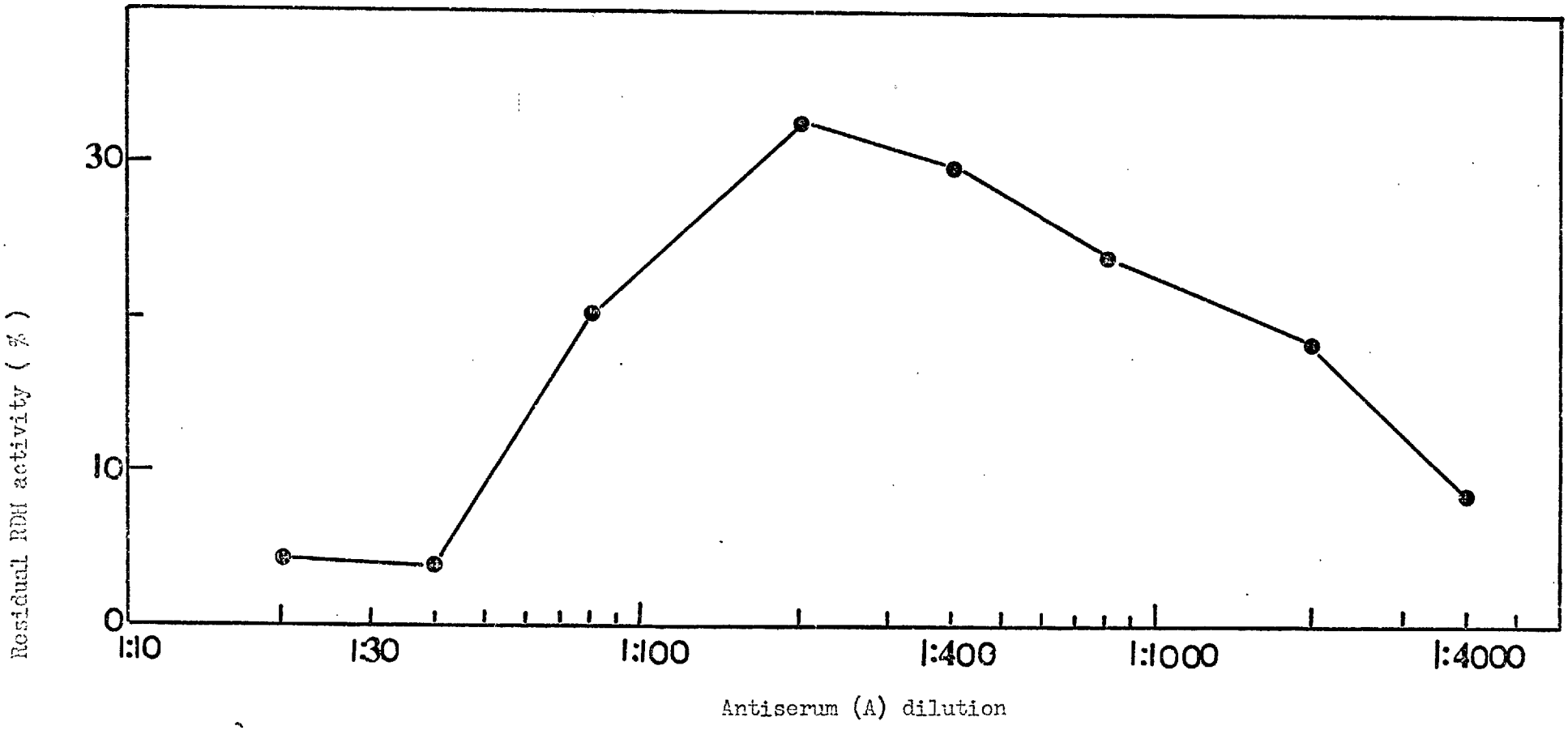


FIG. 5.1. Incubations for 62 hrs at 2°C of 1:10,000 diluted RDH-D samples with equal volumes of serially diluted immunized rabbit-A antiserum.

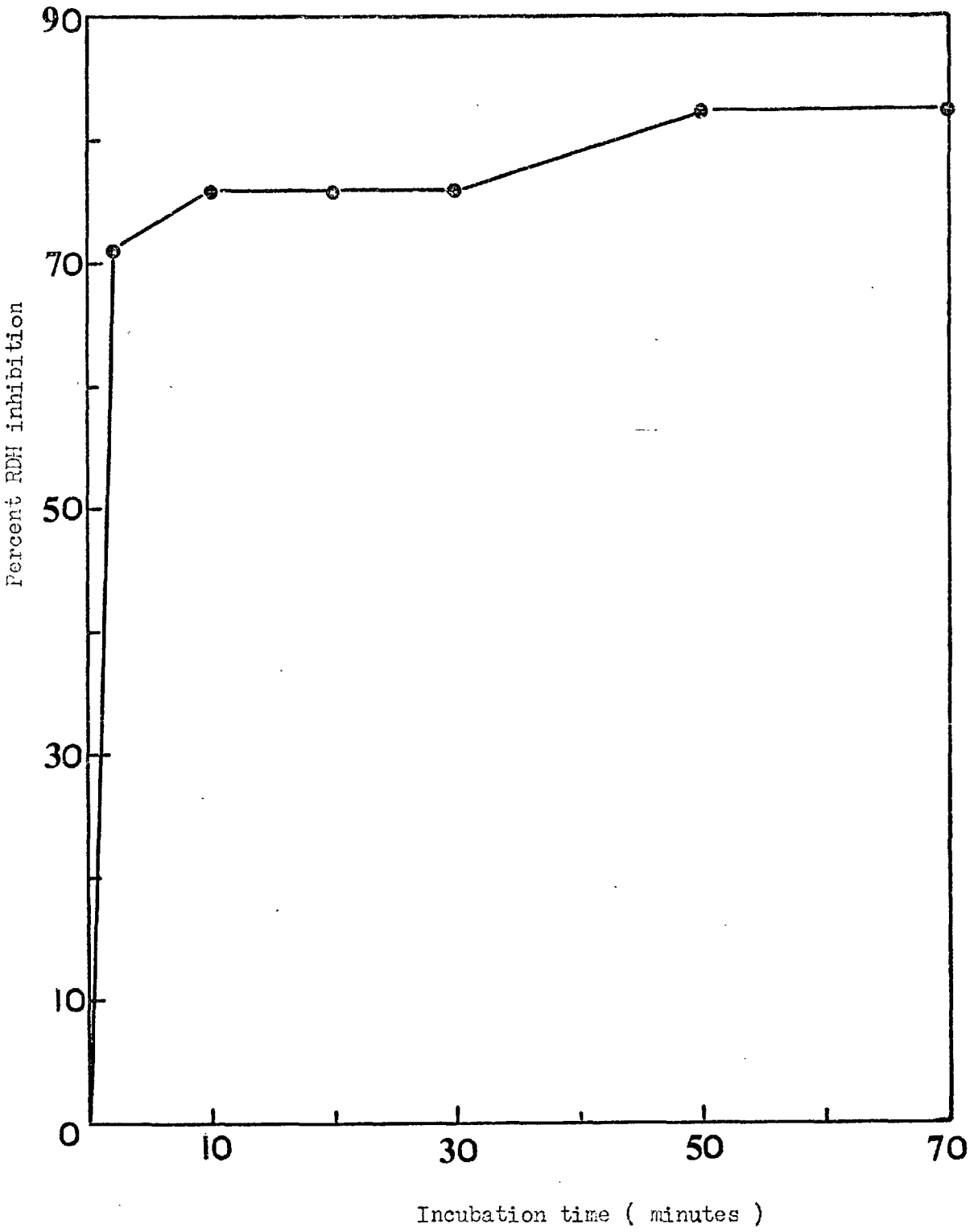


FIG. 5.2. Time course of inactivation of RDH by specific antibodies. 1:40 diluted antiserum-A added to equal volumes of 1:10,000 diluted RDH-D .

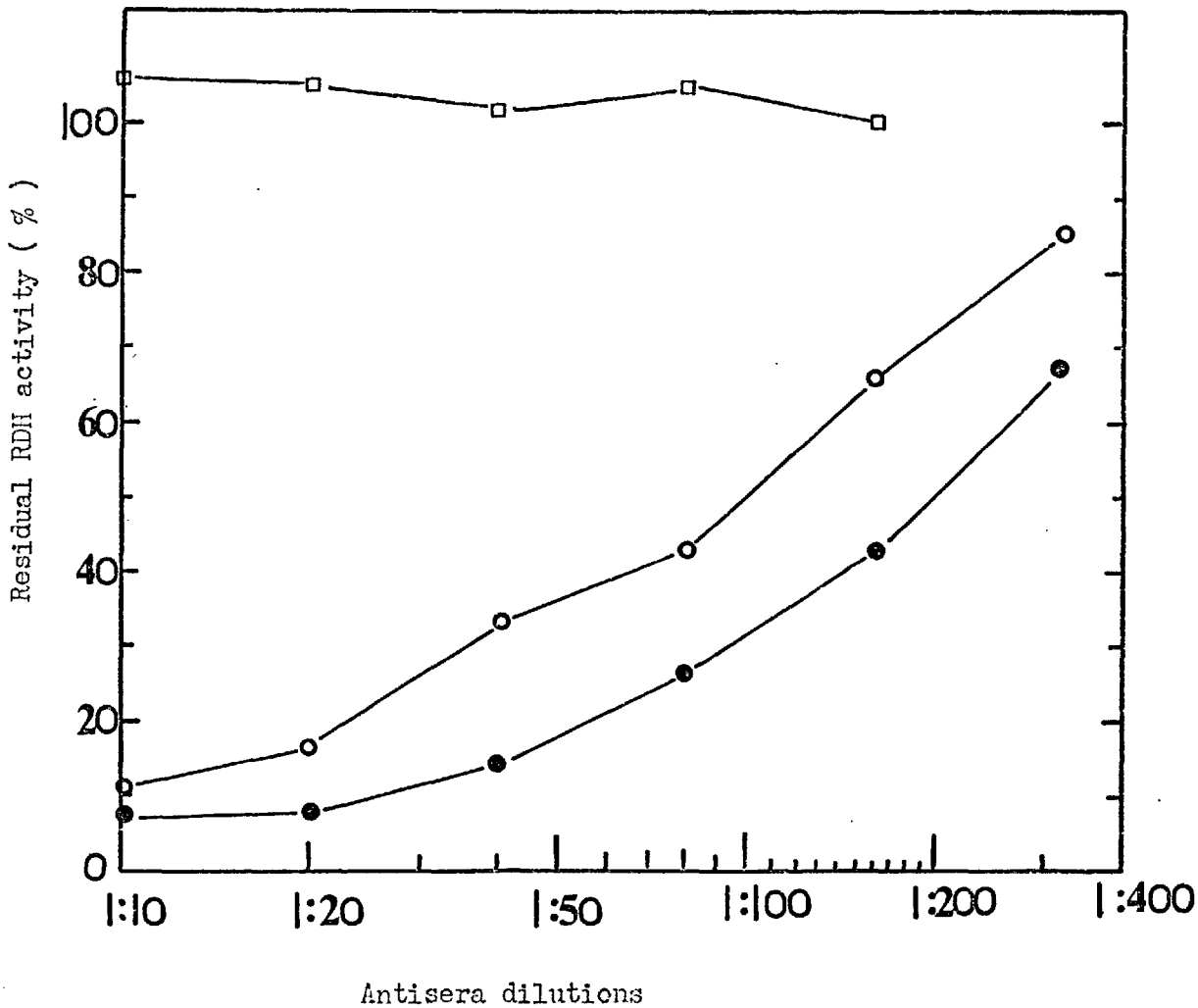


FIG. 5.3. Specificity of RDH inactivation by immune antisera. Klebsiella aerogenes D-RDH (1:10,000 diluted) incubated for 50-60 min at 2°C with serially diluted immune antisera, A (○) and B(●), also with non-immune antiserum C (□) for 100 min.

control serum, C, 100 mins). Percentage of residual RDH activity as a function of antiserum dilution is indicated in each case. First of all there is no inhibition by control rabbit antiserum, C. A and B antisera both inhibit but to different extents. If one draws an arbitary line, say at 50% inhibition across, then one can say twice as much A antiserum is needed as B antiserum to bring about the same amount of RDH inhibition. It is true, of course, that we are talking about a particular class of antibodies which bind and at the same time inactivate RDH. But we do not know at this stage whether all antibodies that bind to RDH inhibit its activity.

For practical purposes, such as calculating the recovery of specific antibodies from columns, we can define a unit of anti RDH antibodies in terms of the ability to inactivate a defined quantity of RDH. Thus, one unit of anti RDH antibody activity is defined as: That amount of antibody which can bring about 50% inactivation of 1μg of RDH. Later we shall compare this inactivation unit with the unit defined by the proportion of antibodies to pure RDH needed for optimal precipitation in double immunodiffusion tests.

D. Immunological Homology of Pure Ribitol Dehydrogenases From Klebsiella aerogenes Strains AN3, D, E and F, also E.coli C

It is important to know to what extent antibodies raised against pure RDH from K. aerogenes D strain will react with the enzyme from other mutant strains of K. aerogenes or from other organisms such as E. coli C-RDH. This is because these antibodies were initially going to be reacted with the polysomes made from the high synthesis strain K. aerogenes AIII, and eventually with polysomes from other strains. (Immunization could not be started with K. aerogenes A-RDH since there was not enough pure A enzyme available at that time). Secondly these results could prove useful in relating the extents of immunological homologies to the enzyme sequences and tertiary structures

which we hoped eventually to determine.

Protein concentrations for the stock RDH samples were all determined simultaneously by the modified Lowry method of Miller (1959), described in "Materials & Methods". Just before incubation with the same antiserum, each RDH sample was diluted to 55µg/ml with 0.1M phosphate buffer pH 7.0. Then 0.5ml samples of each RDH solution were incubated with 0.5ml samples of serially diluted immunized antiserum, incubated on ice for 15 mins. Then each sample was diluted to $^1/5$ by the addition of 4 ml of 0.1M phosphate buffer. 10µl of each sample (5.5µg of enzyme) was added to 1 ml of RDH assay mixture and the increase in A340nm was measured by a spectrophotometer.

Percentage of RDH activity remaining after reaction with antibodies plotted as a function of antiserum titre added is indicated for each RDH sample in Fig. 5.4 The greater the antiserum dilution required to bring about 50% inhibition, the greater the antigenicity of that particular RDH will be. So, if we let the antigenicity of RDH-D = 1.0 (because this was the sample which was immunized), the antigenicity of all other RDH samples would be expressed with respect to this RDH. For example, antigenicity of E. coli C-RDH = $\frac{3.0}{8.0} \times 1.0 = 0.37$, where 3.0 and 8.0 are the antiserum dilutions for 50% inactivation of EC and D respectively determined from Fig. 5.4.

Table 5.1 summarises the degree of antigenic homology between several species of RDH. The difference in apparant antigenicity of the various K. aerogenes mutants is at first sight surprising, since only single amino acid changes are involved: a change of Pro-196 in D for Ala-196 in A for example. However the RDH stock solutions almost certainly contain appreciable amounts of inactivated enzyme, having been stored for long periods. If, as is probable, these inactivated molecules react less well than the native enzyme with that

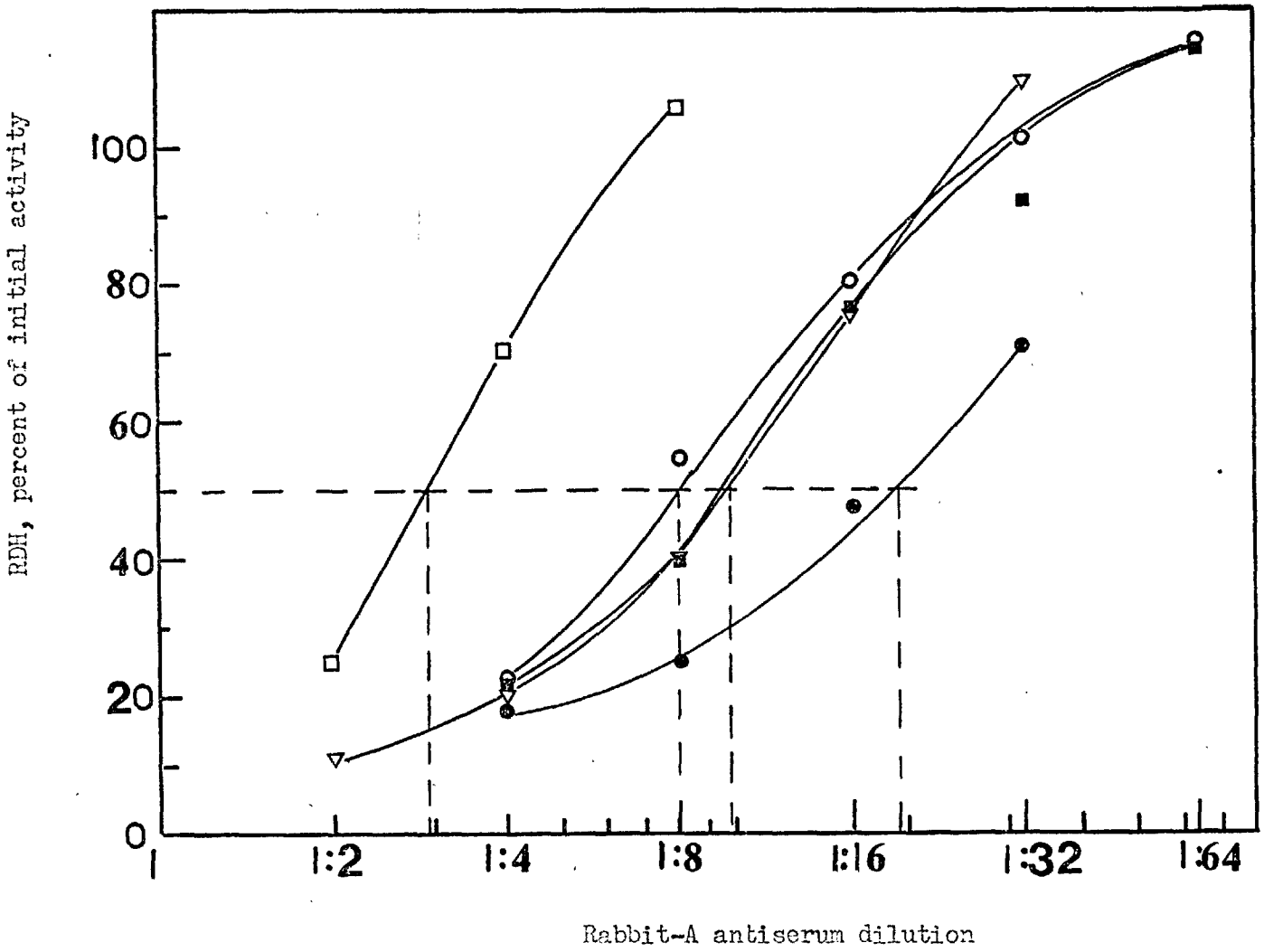


FIG.5.4. Comparative antigenicity of various RDH enzymes to anti-RDH-D immunized antiserum. RDH sources: E. coli C RDH(□); and K.aerogenes enzymes, D (○); E (■); AM3 (▽); and F (●).

TABLE 5.1.

Antigenic homology of various RDH enzymes

Enzyme	Source	Stock solution (mg/ml)	Specific activity (units/mg)	Antiserum dilution for 50% inhibition	Antigenic homology
D	*K. aerogenes* D	110	50.6	8.0	(1.00)
A	*K. aerogenes* AN3	17.6	56.7	9.8	1.23
E	*K. aerogenes* E	13.2	32.0	10.0	1.25
F	*K. aerogenes* F	56.5	26.8	19.0	2.38
EC	*E. coli* C	26	64.0	3.0	0.37

fraction of the antiserum that is inhibitory, one would expect a proportional increase in apparent antigenicity.

The lower antigenicity shown by the E. coli C enzyme is, however, almost certainly a function of the molecular structure. Sequence homologies suggest about 10% difference between the RDH-A and RDH-EC enzymes (Altosaar & Hartley, 1976), but these differences are likely to be located predominantly in the surface of the protein. Hence a much larger proportion of the surface of the enzymes is likely to be different and it is here that structural antigenic determinants will be located.

From our point of view, however, it is clear that anti RDH-D serum cross-reacts effectively with all other species of the enzyme.

E. Purification of Rabbit Anti RDH Antibodies by Affinity Chromatography

(i) Covalent Coupling of RDH to Bromoacetamidoethyl-Sepharose

The procedure for construction of bromoacetamidoethyl-Sepharose-4B was adopted from publication of Cuatrecasas (1970), and is described in "Materials & Methods". In the first step, Sepharose-4B is activated by cyanogen bromide at pH 11.0 and 20°C. The second step consists of attachment of ethylene diamine to the activated Sepharose at 4°C. In the third step, the aminoethyl-Sepharose is reacted at 4°C with freshly prepared O-bromoacetyl-N-hydroxysuccinimide. Fourthly, proteins are readily coupled to the derivative bromoacetamidoethyl-Sephaorse in 0.1M $NaHCO_3$, pH 9.0, for 2 days at room temperature, or for longer periods at lower pH values or lower temperatures, as a 50% (v/v) suspension, forming insoluble derivatives in which the protein is located at some distance from the solid support. Finally, the unreacted bromoacetyl groups are masked with 0.2M 2-aminoethanol at room temperature for 24 hours, or at lower temperatures for longer periods.

Pure Klebsiella aerogenes RDH-D, about 10mg per ml of packed

Sepharose, was incubated for 4 days at $4^{o}C$ with bromoacetamidoethyl-Sepharose-4B, then reacted with 2-aminoethanol at $4^{o}C$ for 3 days. The extent of coupling was estimated as follows: 2ml of RDH solution was added to 25ml of 0.1M $NaHCO_3$, pH 9.0, this solution was added to the Sepharose derivative and the total volume noted = 50ml, i.e. the RDH solution was diluted 25 fold. After 3 days reaction, 0.1ml of the filtrate, was added to 4ml of distilled H_2O (further 1:40 dilution) and the A280/ml of this solution was recorded = 0.026. The A280/ml of an equivalently diluted RDH solution was 0.075, so it is clear that 65% of the protein has been coupled to the Sepharose. We can estimate that about 4.9 A280 units of RDH were coupled per ml of packed Sepharose within the error imposed by the low absorbancy . More accurate estimates could be obtained by including some radioactive protein in the sample and measuring the input and output radioactivities.

(ii) Preliminary Studies on a Test Column

A pilot column was constructed from a pasteur pipette, 0.6cm in diameter. It was packed with glass wool at the bottom, rinsed, and packed at $4^{o}C$ with 1ml of the immunoadsorbent, RDH-acetamidoethyl-Sepharose. The column was washed thoroughly with cold phosphate buffered saline (PBS), pH 7.2 at 10ml/hr. Since RDH is a tetramer, it was suspected that some of the RDH-protein on the immunoadsorbent might be held by subunit interaction. It was necessary to remove such loosely held molecules from the immunoadsorbent so that they do not inactivate the specific antibodies. Also it was important to establish that most of the protein is covalently bound and would not detach from Sepharose during elution. Thus, after the initial PBS wash, the column was washed with 1M acetic acid at 10ml/hour until no further protein was eluted. The total A280 of this eluted RDH was found to be 1.08. Hence of the 4.9 A280 units of RDH per ml of

Sepharose, 1.1 units (22%) were loosely bound and 3.8 units (78%) were covalently bound.

An immunoglobulin fraction of the antiserum was prepared by 50% (v/v) precipitation, with saturated ammonium sulfate (Materials & Methods). The crude immunoglobulin fraction was redissolved in PBS and dialysed in two changes. 19mg of this crude immunoglobulin fraction (in 0.5ml) were loaded on to the column at 7.2ml/hr at 4°C. The column was washed with cold PBS until the A280 was less than 0.01 (about 3 hours at 10ml/hour). The specifically bound antibodies were initially eluted with 1M acetic acid until the A280 reached 0.035. A total of 3.68 A280 units was eluted in this way. Then the elution was continued by 0.1M glycine-HCl buffer, pH 2.6, according to Bouma, III, and Fuller (1975). A further small fraction of antibodies (0.7 A280 units) was eluted, in this way. The antibody fractions were immediately neutralised with 4M Tris during elution from the column. Assuming an extinction coefficient at 280mμ of 1.4 units/mg for immunoglobulin (Schechter, 1974) we can calculate that 3.13mg of the 19mg of crude immunoglobulin fraction (16.5%) were specifically bound to and eluted from the affinity column. It is not possible to work out the capacity of the column from this experiment since it was under-loaded with antibodies.

(iii) Preparative Affinity Column

A 2cm diameter column with a coarse sintered glass disc at the bottom, together with all the tubings and attachments were sterilized by autoclaving. The column was packed with 20ml of the RDH-Sepharose immunoadsorbent; all operations were carried out at 4°C. The column was initially washed with cold sterile PBS; then washed with 1M acetic acid followed by 0.1M Gly-HCl, pH 2.5, to remove loosely bound RDH (Fig. 5.5). Then, after equiliberation with PBS, the column was loaded with 10ml of ammonium sulfate-fractionated antiserum (38mg/ml), at 10ml/hour. Washing was continued with PBS at 36 ml / hour

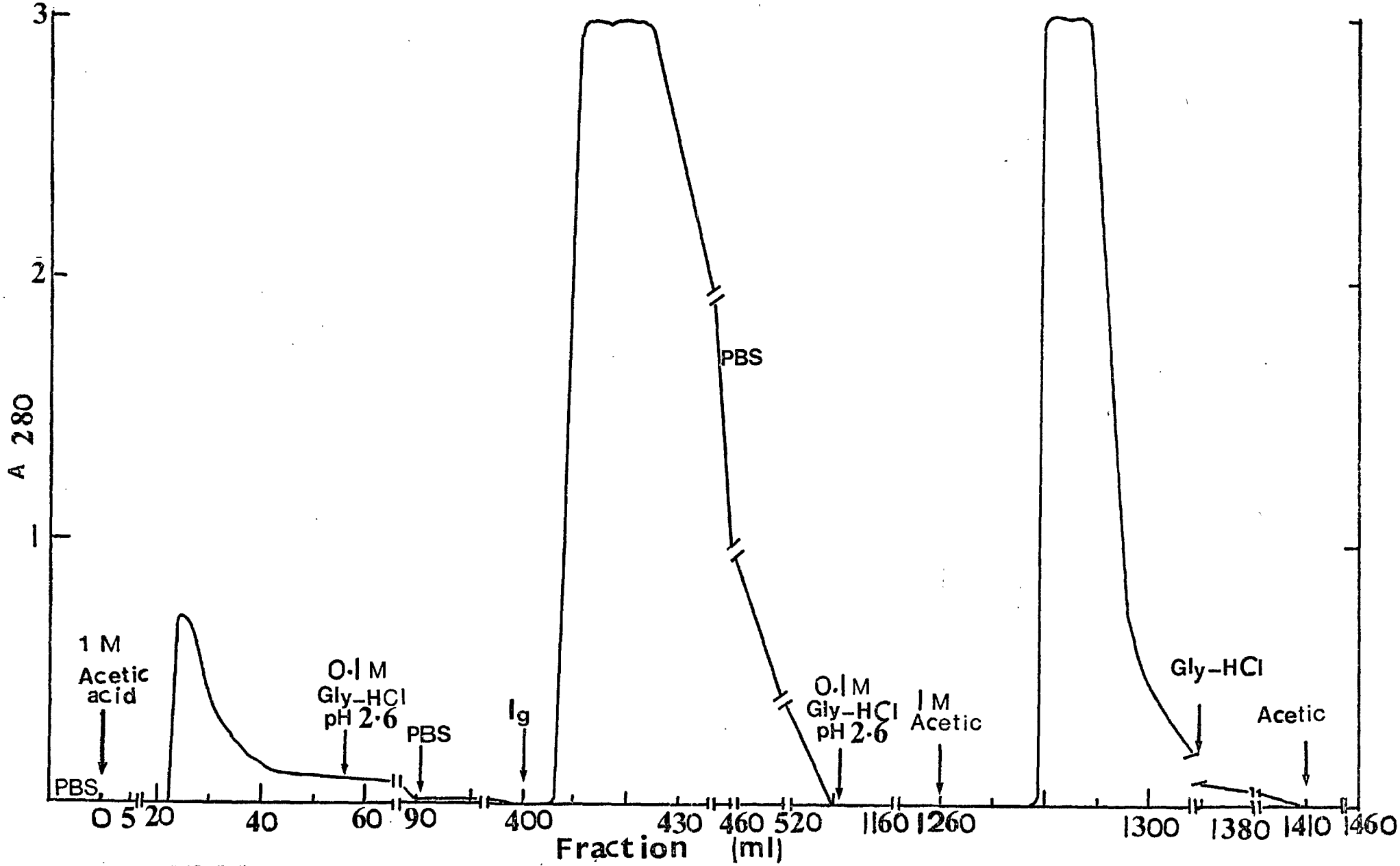


FIG.5.5. Preparative affinity column. Elution with 1M acetic acid and 0.1M Glycine-H Cl, pH 2.6.

till the A280 was about 0.01, approximately 600ml of buffer passed through.

In order to find out whether glycine HCl buffer is a better eluting agent than 1M acetic acid, pH 2.4, the elution of antibodies was first attempted with 0.1M glycine-HCl, pH 2.6. More than 3 bed volumes of this buffer were passed through without any appreciable amount of antibodies eluting off. Then the elution was switched to 1M acetic acid; after 18ml of this acid passing through, a sharp antibody peak appeared as indicated in Fig. 5.5. Elution with 1M acetic was continued till A280nm reached about 0.01; then it was switched to 0.1M glycine-HCl and continued for a long time, but no secondary peak appeared. Hence it appears that 0.1M glycine-HCl, pH 2.6, is not a very good antibody eluting agent.

The amount of the non-adsorbed proteins from this column was estimated spectrophotometrically, as 145mg. The fractions eluted with acetic acid was 64.2mg. The total proteins loaded was 380mg.

Thus, 171mg protein were unaccounted for, which must have stayed on the column. Other methods might be investigated in order to recover this fraction, which may be very strongly bound antibodies.

The non-adsorbed proteins were concentrated by freeze-drying apparatus, dialyzed, then tested for presence of anti-RDH activity in the RDH inhibition assay. No activity was detectable by this assay at all concentrations tested.

The 1M acetic acid fractions, after neutralisation with Tris, were pooled and treated with an equal volume of cold sterile saturated ammonium sulphate. The precipitate failed to dissolve readily in 9mls of PBS, even after dialysis overnight against a large volume of PBS. After centrifugation only 10% of the protein was in the supernatants, and this was found to have some anti-RDH activity.

The precipitate was treated first with glacial acetic acid, then

with urea to about 8M and finally with formic acid in attempts to dissolve it. After dialysis against PBS most of the protein again precipitated, but the supernatant showed further anti-RDH activity. It was combined with the original supernatant to yield a total of 6.4mg of anti-RDH, and stored at -20°C. Attempts to recover further anti-RDH activity from the remaining precipitate by dissolving in glacial acetic acid, dialysis against H_2O and neutralisation with tris failed to yield any more anti-RDH activity.

(iv) Re-use of the Affinity Column

Since I intended to re-use the affinity column for as long as it was profitable, the same column used in (iii) above was equilibrated again with PBS and loaded again with antibodies.

22ml of clear immunized antisera were collected from 45ml of pooled rabbit A and B blood. The A280/ml of this antiserum was 63.8. An immunoglobulin fraction of the antiserum was prepared by 50% ammonium sulfate precipitation. This constituted 35.6% of total serum protein by A280 measurements. 10ml of this crude immunoglobulin fraction (224mg) was loaded on the equilibrated affinity column at 10 - 15 ml/hour. The column was washed with PBS and the antibodies were eluted with 1M acetic acid as described before. The profile of elution (not shown) was similar to the previous experiment. The eluted antibodies were gradually neutralized to pH 7.0 with 4M Tris as they came off the column (approximately 4ml of Tris to 10ml of 1M acetic required). The antibodies were collected in 2 fractions, the main peak, and the prolonged shoulder. They were immediately transferred into sterile dialysis bags and dialyzed in the cold against PBS. The shoulder fraction, prior to dialysis against PBS, was first concentrated by ammonium sulphate precipitation. Following the dialysis, considerable amount of precipitate gathered in both dialysis tubes. The precipitates were removed by centrifugation. The clear supernatants

were combined and saved (Supernatant-1).

Most of the precipitate was soluble in 1M acetic acid, while a small proportion of it, darkish in colour was insoluble and was discarded. The 1M acetic-acid solution was dialyzed against PBS; again some of the proteins precipitated on dialysis and were re-dissolved in 1M acetic acid. The supernatant was saved. This cycle of dissolving the antibodies in 1M acetic acid and dialysis against PBS, was repeated 4 times. Every time a fraction of anti-bodies remained in soltuion. At the end, each supernatant fraction was tested for inhibition of RDH activity. They were all found to contain anti-RDH activities, and the specific activities were no less than the initial supernatant. In fact, supernatant-2 was found to be about 3.5 times as active as supernatant-1 in its ability to inhibit RDH activity. However, since the yield after the second cylce of antibody rescue was rather small, it would not be profitable to carry out this procedure more than twice. The total recovery of units of anti-RDH activity by enzymic inactivation from this column was = 10.3%

Of the 325 A280 units of serum proteins loaded on to the column, 249 were unadsorbed and 38 were eluted with 1M acetic acid. A total of 17 A280 units were recovered in the various supernatants from the latter: 23% of the amount adsorbed. Since only 10.3 units of anti-RDH activity were recovered, about half of the antibodies in the supernatant must have been inactivated.

(v) Determination of the Column Capacity

This was estimated by titrating a sample of the immunoadsorbent with a standardised anti-RDH antiserum and measuring the residual anti-RDH activity in the supernatant.

a) Standardisation of anti-RDH activity of antiserum

First an antiserum had to be standardized with respect to the

number of units of anti-RDH activity it contains. The ammonium sulfate fractionated antiserum from the previous experiment contained 22.4mg protein ml, 5.43mg/ml of it was anti-RDH antibody (by its ability to bind firmly to the immunoadsorbent). 50µl of 1:6000 diluted pure RDH (100mg/ml), i.e., 0.83µg of RDH, was incubated with 50µl of doubling dilutions of this antiserum sample, and the percent RDH inactivation was determined for each sample. Fig. 5.6 shows the plot of this percentage RDH-inhibition as a function of µg of antibodies added to 0.83µg of pure RDH. Corresponding dilutions of the antiserum are also indicated in parallel on the horizontal axis. From this plot it can be seen that 1.58µg of antibodies are needed to inhibit 0.83µg of pure RDH by 50%. That is to say 1.9µg (1 unit) of antibody is needed to inactivate 1µg of pure RDH by 50%. Thus, the total number of units of anti-RDH activity present/ml of rabbit-antiserum = $\frac{5430}{1.9}$ = 2858 u/ml.

b) Titration of immunoadsorbent with antiserum

The RDH-Sepharose in the main affinity column was stirred, and 0.5ml of the packed immunoadsorbent was placed in a centrifuge tube + 2.25ml of PBS. Then 0.25ml successive samples of a standardized immunized antiserum were added to this resin and incubated at 4°C for 1 hour with periodic resuspension. Every time 0.25ml of clear supernatant was removed, and 0.25ml of fresh antiserum was added. The removed supernatant was appropriately diluted and 10µl samples were tested for their ability to inhibit a sample of RDH in the standard assay. This was used as an index of saturation of the immunoadsorbent with specific anti RDH antibodies; thus the capacity of the column.

Table 5.2 shows the percentage inhibition of RDH in its assay brought about by the addition of a 10µl sample of each supernatant following incubations of the immunoadsorbent by the successive additions of the immunized antiserum. It is clear from this result that about

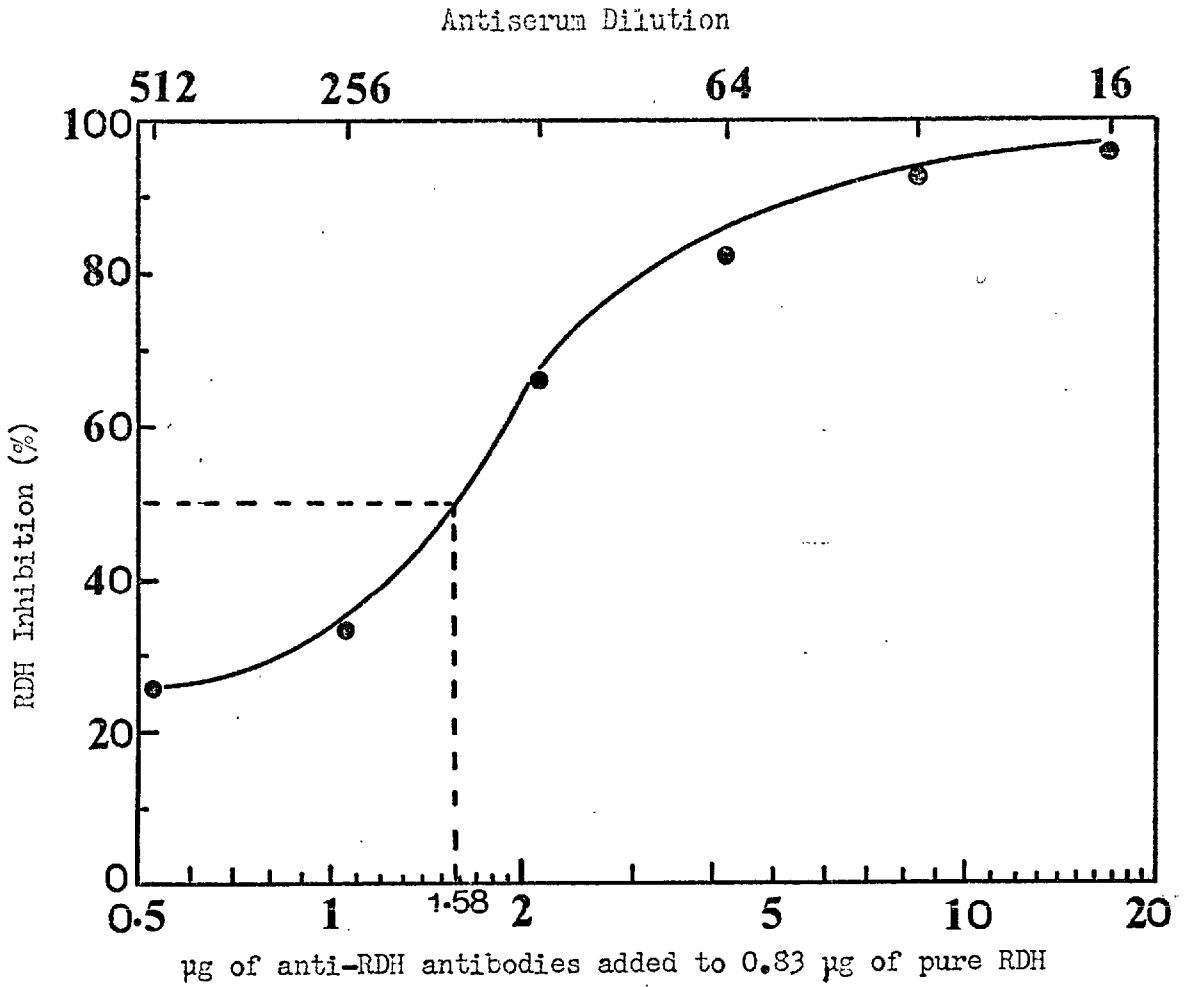


FIG. 5.6. Estimation of nubmer of units of anti-RDH activity present per ml of an immunized rabbit antiserum. Unit of anti-RDH activity is defined as that amount of pure antibody required to inhibit 1 µg of pure RDH activity by 50% in its ribitol assay.

TABLE 5.2

Titration of Anti-RDH antibodies by Sepharose-Bound RDH

Addition No.	Total Units of Antibodies Added per ml of packed RDH-Sepharose	RDH Inhibition (%) by 10µl samples of supernatant
1	1,429	0
2	2,858	0
3	4,287	9
4	5,716	9
5	7,145	17
6	8,574	21
7	10,003	42
8	11,432	48
9	12,861	-
10	14,290	69.7
11	15,719	-
12	17,148	87.5

12000 units of antibody can be added to 1ml of immunoadsorbent before sufficient anti-RDH activity remains in the supernatant to cause 50% RDH inactivation. This represents the capacity of the immunoadsorbent. Considering that it was used twice before, and that a large proportion of the antibodies remained on the resin, I estimate that this immunoadsorbent must have had a capacity of about 14000activity units of anti-RDH antibodies/ml, i.e. 26.6mg of Ig.

I already estimated that 3.8 A280 units (2.7mg) of RDH were covalently coupled to 1ml of Sepharose. Thus, approximately 10 times (w/w) antibody can attach to this coupled RDH. Considering that the average M.W. of an immunoglobulin = 150,000 and that of RDH subunit = 27,000, then one can say 1.77 (approximately 2) molecules of antibody can attach to 1 molecule of coupled-RDH monomer.

(vi) Characterisation of Purified and Crude Anti-RDH Antibodies by Double Immunodiffusion Studies

The details of the double-immunodiffusion technique are described in "Materials & Methods". In these experiments, antibody sample is placed in a centre-well while the serially diluted antigen samples are placed in the peripheral wells which are equidistant from the centre well and from one another. Both the antibodies and the antigen diffuse into the gel. As they encounter one another at some distance within the gel (which depends on the molecular weights and the mobilities of Ab/Ag), they precipitate and form a precipitin band, which would show up better when the soluble antibodies and antigen molecules are washed away and the precipitated Ab/Ag complex is stained. If the antibody titer is much greater than the antigen concentration, then upon prolonged diffusion (usually about 3 days) the antibodies will overcome the antigen, thus the precipitin band will gradually dissolve and push toward the antigen well. The situation will be reversed if the antigen concentration is greater than Ab. Only when

the Ab/Ag concentrations are balanced a sharp stable precipitation band will be formed. Therefore, the technique may be used to quantitate antibodies (as will be shown later) as well as to identify them. If the Ab/Ag samples are left to diffuse for limited time (say overnight) then even unbalanced Ab/Ag precipitation bands can be identified before they have had the chance to redissolve. This is useful for identification of the impurities.

The interaction of crude Ig fraction (by $(NH_4)_2SO_4$ precipitation) of immunized antiserum with doubling dilutions of K. aerogenes AIII-sonicated extract is shown in Fig. 5.7. A diagramatic representation of the same (Fig. 5.8) is also shown. The typical origin and the direction of serially diluted antigen additions are shown in this diagram. One can see that in addition to the major precipitin band, there are at least 3 other precipitin bands due to impurities present. These additional antibodies must have been raised against small amount of impurities present in the purified RDH preparation, which indeed is indicative of the sensitivity of the method.

In order to investigate whether there is significant amount of any of these impurities represented in that RDH preparation, the same antibody sample was reacted with doubling dilutions of 1:100 diluted purified RDH-D preparation. As shown in Fig. 5.9, at least one of these impurities can clearly be recognized by this test. It means that this particular impurity must be present in significant proportion in the purified RDH preparation.

To investigate whether affinity chromatography has helped to remove these contaminating undesirable antibodies, purified antibodies from the last column were reacted with doubling dilutions of K. aerogenes AIII sonicate. This is shown in Fig. 5.10. Very little impurity is noticeable in this case. Thus affinity chromatography has virtually purified anti-RDH antibodies.

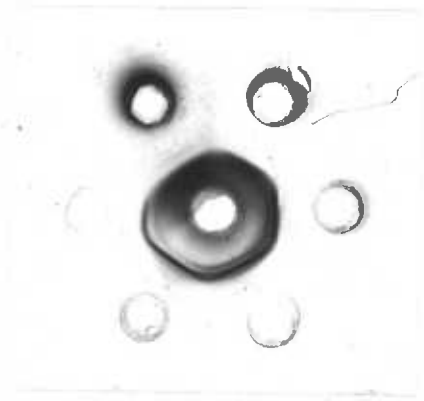


FIG.5.7. Reaction of crude Ig fraction rabbit anti-RDH/ doubling dilutions of K. aerogenes Alll sonicated extract.

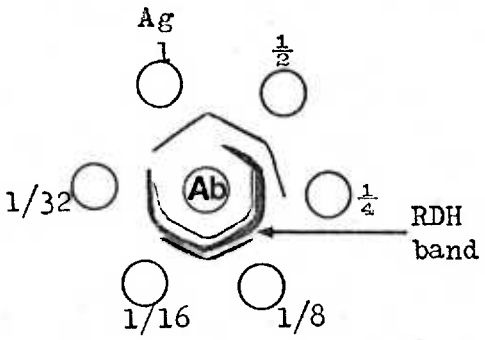


FIG. 5.8. Schematic representation of the precipitin bands present in Fig. 5.7.

FIG.5.9. Reaction of crude Ab/ doubling dilutions of initially 1:100 diluted purified RDH D (100 mg/ml).

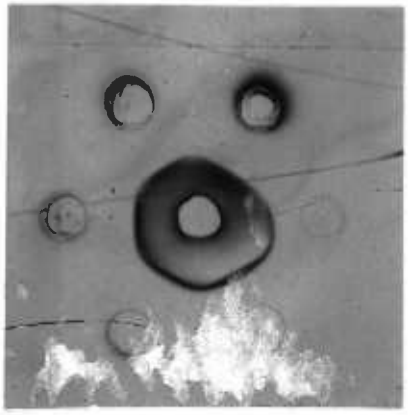


FIG.5.10. Reaction of the affinity chromatography-purified anti-RDH antibodies with serially half-diluted K. aerogenes Alll sonicate.

(vii) Alternative Elution Methods

It is clear from the foregoing results that a large proportion of the specific antibodies remain bound to the affinity column, and the antibodies which have eluted appear to contain a large amount of inactive antibodies. In order to improve on the yield of pure antibodies I searched for alternative elution techniques, e.g. 3M potassium thiocyanate or 0.1M-3M HCl (Cuatrecasas, 1969), and sought to operate the affinity column at its maximum capacity.

The 0.5ml packed, antibody-saturated RDH-Sepharose sample from the studies on the capacity of affinity column (section v, above) was packed into a pasteur pipette column, washed with PBS, then eluted successively with the following reagents: 3M KSCN; PBS; 1M acetic acid; 0.1M HCl; 1M HCl; 3M HCl; PBS.

KSCN fractions were dialysed immediately against PBS, concentrated by ammonium sulphate precipitation, and dialysed again. 1M acetic acid fractions were collected in a tube containing 2 - 3mls of PBS immediately neutralised with 4M Tris, dialysed against PBS, concentrated by $(NH_4)_2SO_4$, and dialysed again. HCl fractions were neutralised immediately with NaOH. The 0.1 - 1M HCl fractions were pooled and dialysed together while the 3M HCl fraction was dialysed separately. All of the precipitates formed were pooled and dissolved in 1M acetic acid, diluted to 0.1M in acetic acid; then the A280nm was measured. The amount of antibodies and the percentage of total antibodies eluted with respect to each reagent added are indicated in Table 5.3. It can be seen that the greatest proportions of antibodies were eluted by KSCN and 1M acetic acid. The total amount of antibodies subsequently eluted by 0.1 - 3M HCl is very small, and perhaps it is not an improvement on the technique, especially since strong acid attacks the Sepharose gradually. From Table 5.3, also we can see that the proportion of insoluble antibodies was greatly reduced, from about 70% previously to about 28.4%.

TABLE 5.3

Anti RDH Antibodies Eluted in Various Fractions from the Second Test Affinity Column

Fraction	Total Ab (mg)	% of All Eluted Frac. by Wt.	Total Activity Units	Sp. Act. U/mg
3M KSCN	2.59	42.2	1100	425
1M Acetic Acid	1.29	19.7	734	569
0.1 - 1M HCl	0.37	6.1	270	730
3M HCl	0.22	3.5	42	191
Precipitated Ab dissolved in 0.1M acetic acid	1.75	28.5	-	-

Table 5.3, also shows the total units of anti RDH activity and specific activity recovered from each fraction. It is interesting to note that the specific activity of the antibodies eluted by 0.1 - 1M HCl is the highest (730), while that of 1M acetic is the second highest (569), 3M KSCN is the third (425), and 3M HCl is the lowest (191).

(viii) Purification of Specific Antibodies by Affinity Chromatography on a Preparative Scale

The reused 20ml RDH-affinity column which had been stored in sterile PBS containing trace amount of Na-Azide, was used again in this experiment. An immunoglobulin fraction of immunized rabbit antiserum (1953 A280 units in 73ml) was clarified by centrifugation and loaded at 48ml/hour on the pre-equiliberated column. The unspecific proteins (total A280 = 1760 units) were washed out rapidly with PBS at 66.4ml/hour. However, after concentration with ammonium sulphate and dialysis against PBS, some anti-RDH activity was detected in this fraction (Table 5.4), due perhaps to the rapid loading and washing.

In order to remove unspecifically or weakly bound proteins from the column, following the PBS wash the column was rinsed with 0.5M NaCl in phosphate buffer. A very small amount of protein with some anti-RDH activity was eluted. Then the specific antibodies were eluted from the column by 3M KSCN and 1M acetic acid successively. Elution by HCl was omitted. The elution profile is shown in Fig. 5.11. The KSCN eluted fractions were immediately dialysed against PBS by collecting them directly into a funnel inserted into a sterile dialysis tubing and held in position by a rubber band. The dialysis tubing was contained in a flask containing PBS and stirred by a magnetic stirrer. 1M acetic acid fractions were continually neutralized by the additions of 4M Tris, concentrated by $(NH_4)_2SO_4$ precipitation and dialysed against PBS.

The RDH-inactivation capacity of various fractions were determined

TABLE 5.4

Estimations of Antibodies Eluted From Preparative Affinity Column

	Fraction	mg	% of Bound Abs. (138)	Activity Units	% of Input Activity	Specific Activity U/mg
1)	Crude Ig Fraction	1395	-	182,500	(100%)	130.8
2)	PBS Wash Proteins	1257	-	8,625	4.73	6.7
3)	0.5M NaCl Wash	4.2	3.0	92	0.05	21.9
4)	Combined KSCN + 1M Acetic	82.3	59.6	88,149	48.30	1071.1
5)	Precipitated Ab Dissolved in 0.1M acetic acid	12.6	9.1	-	-	-
6)	Uneluted Abs.	38.9	28.2	-	-	-

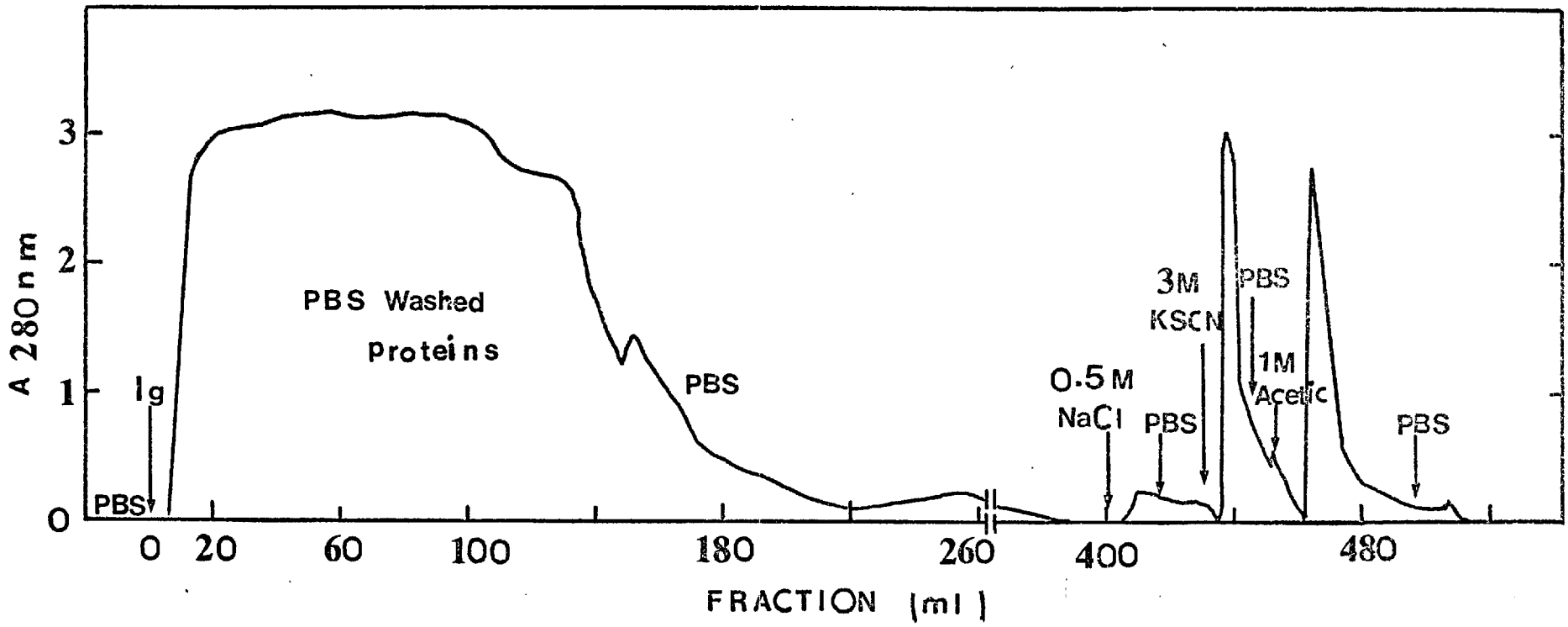


FIG. 5.11. Preparative RDH-Affinity Column. Elution of specific antibodies with 3M KSCN and 1M Acetic acid.

from the appropriate inhibition curves (shown in Fig. 5.12). For example, for the pure Ab fraction (KSCN + Acetic Fractions), 50% inhibition of 50µl of $^{1}/_{6000}$ diluted RDH (0.83µg) was brought about by 50µl of $^{1}/_{295}$ diluted Ab (See.Fig. 5.12). Hence $^{50}/_{295} \times {}^{1.00}/_{0.83}$ is = 0.204µl of Ab i.e. equivalent to 1µg of RDH. The total volume of this Ab fraction was 18000µl. Therefore, the total number of units present = 18000/0.2042 = 88149.

From Table 5.4 we can see that 59.6% by weight of the bound specific antibodies were recovered in the combined 3M KSCN + 1M acetic fractions. This corresponds to 48.3% of total input activity, which is a great improvement on the initial results (10%), and greater than 8-fold purification of the specific antibodies in the Ig fraction has been achieved.

From the data of Table 5.4., one can calculate that about 150mg of specific anti RDH antibodies were present in the total Ig fraction. These antibodies came from 96ml of neat rabbit antiserum. Thus the concentration of specific antibodies in the immunized antisera = 1.56mg/ml.

F. Characterization of Ab Fractions from Preparative RDH-Affinity Column by Ouchterlony Double Immunodiffusion Tests

a. In order to establish the extent of purity of anti-RDH antibodies present in each fraction of the last column, the following tests were carried out:

Fig. 5.13a. Combined pure Ab (3M KSCN + 1M Acetic)/Serial doubling dilutions of K. aerogenes A111 sonicate, up to $^{1}/_{32}$ dilution. Only one band is visible which is rather diffused (unbalanced).

Fig. 5.13b. 0.5M NaCl wash proteins/serial doubling dilutions of K. aerogenes sonicate up to $^{1}/_{32}$. There is not enough specific antibodies present to react with the antigens

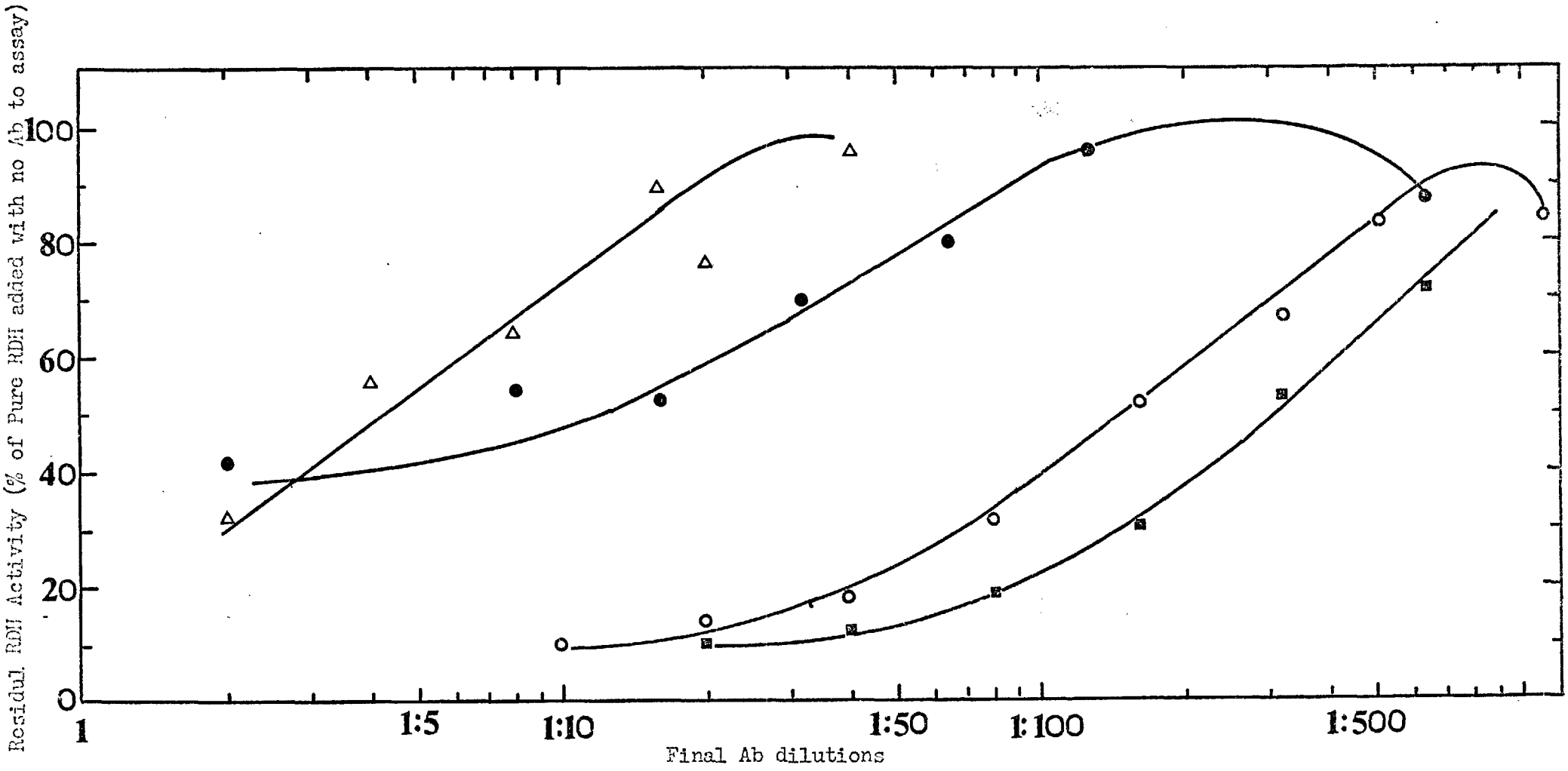


FIG. 5.12. RDH-inhibitory activity of various Ab fractions from a preparative RDH-affinity column.

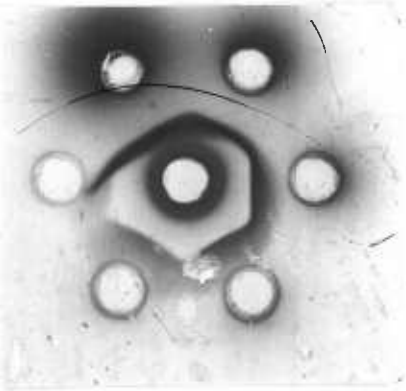


FIG. 5.13a. Pure anti-RDH rabbit Ab/ K. aerogenes Alll sonicated extract, 1-1/32 dilutions.

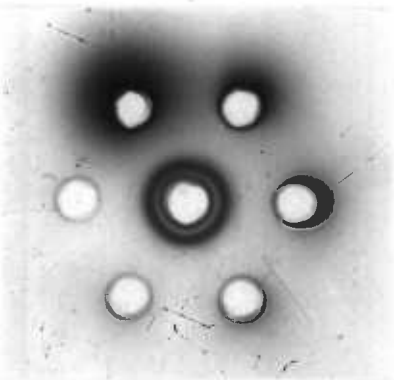


FIG. 5.13b. 0.5M NaCl-wash proteins/ K.aerogenes sonicate 1 - 1/32 dilutions.

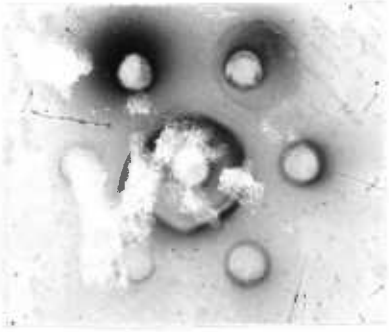


FIG. 5.13c. Crude Ig fraction RDH antiserum/ K. aerogenes Alll sonicate, 1-1/32 dilutions.

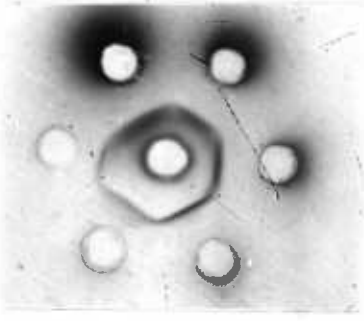


FIG. 5.13d. 3M KSCN Ab fraction/ K. aerogencs Alll sonicate, 1-1/32 dilutions.

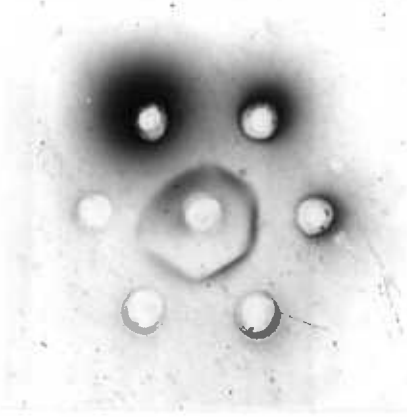


FIG. 5.13e. 1M acetic acid Ab fraction/ K.aerogenes Alll sonicate up to 1/32 dilution.

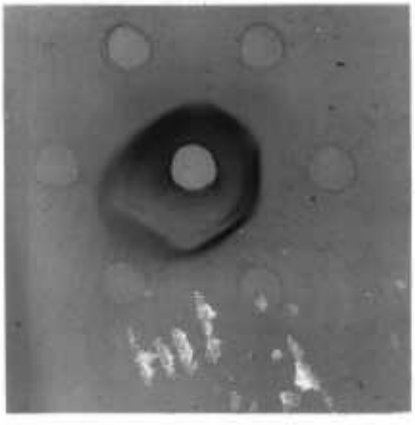


FIG. 5.13f. Crude Ig fraction of anti-RDH immunized rabbit serum / doubling dilutions of initially 1:100 diluted "pure" RDH-D.

in this case.

Fig. 5.13c. Crude Ig fraction/serial dilutions of K. aerogenes sonicate up to 1/32. In addition to the main (RDH) precipitin band, 3 other bands due to impurities are present.

Fig. 5.13d. 3M KSCN fraction/serial dilutions of K. aerogenes sonicate up to 1/32. Presence of only one band supports the specificity of anti RDH antibodies present in this fraction.

Fig. 5.13e. 1M acetic acid fraction/serial dilutions of K. aerogenes sonicate up to 1/32. Again, presence of only one band is indicative of the purity of these antibodies.

b. The following tests were carried out to estimate recovery of the specific antibodies from the affinity column and to show that the antibodies are in fact directed against RDH.

Fig. 5.13f. Crude Ig fraction/doubling dilutions of initially 1:100 diluted "pure" RDH-D. The main precipitin band balancing at about 1/800 dilution is the RDH band. The second precipitin band on the inside, balanced at about 1/400, is due to an impurity in the RDH sample which must be present in a significant proportion.

Fig. 5.13g. Purified Ab/doubling dilutions of initially 1:100 diluted "pure" RDH-D. There is one precipitin band present, and the rough optimal proportion of RDH is at 1:400 dilution.

c. The following tests demonstrate the purity of anti RDH antibodies prepared by affinity chromatography: fine dilution ranges of the Ag against Ab are used. Also they aid in estimating the recovery of specific antibodies from the affinity column.

Fig. 5.13h. Purified Ab/K. aerogenes AIII sonicated extract diluted

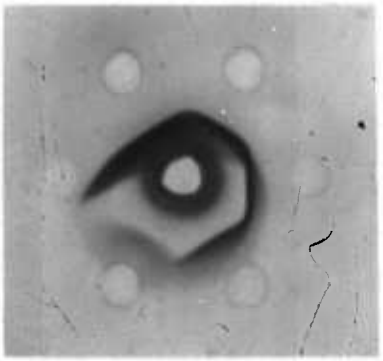


FIG. 5.13g. Purified Ab/ doubling dilutions of initially 1:100 diluted "pure" RDH-D.

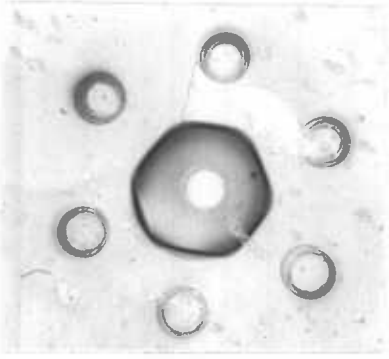


FIG. 5.13h. Pure anti-RDH Ab/ K. aerogenes A111 sonicated extract serially diluted: 10/20; 9/20; 8/20; 7/20; 6/20; and 5/20.

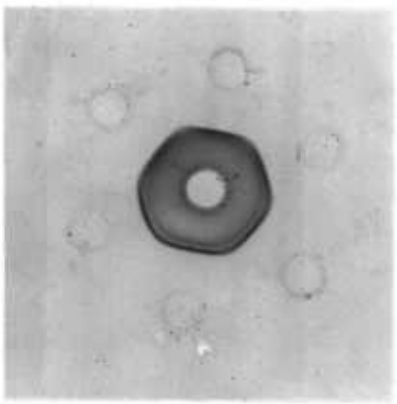


FIG. 5.13i. Crude Ig fraction/ "pure" RDH-D at dilutions of 1/300; 1/400; 1/500; 1/600; 1/700; and 1/800.

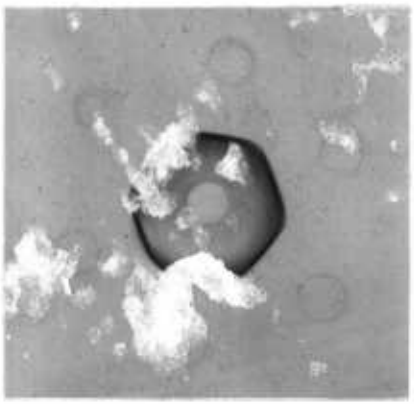


FIG. 5.13j. Pure Ab/ pure RDH diluted: 1/150; 1/200; 1/250; 1/300; 1/350; and 1/400.

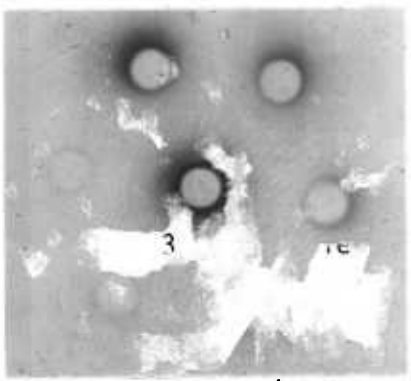


FIG. 5.13k. Pure Ab/ serially half diluted extract of Klebsiella aerogenes strain FG-5.

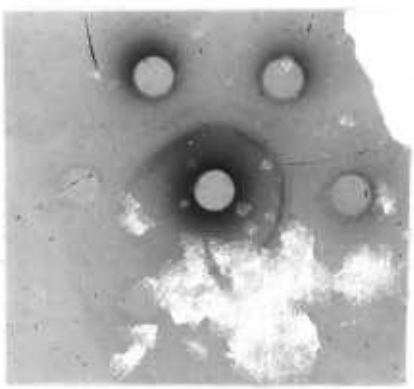


FIG.5.13l. Crude Ig fraction/ serially half diluted K.aerogenes FG-5 strain.

serially $^{10}/_{20}$, $^{9}/_{20}$, $^{8}/_{20}$, $^{7}/_{20}$, $^{6}/_{20}$, $^{5}/_{20}$. Only one clear band is visible. Another doubtful band may be present just inside of the main band. $^{6}/_{20}$ dilution is about the optimal proportion.

Fig. 5.13i. Crude Ig fraction/"Pure RDH" at dilutions of $^{1}/_{300}$, $^{1}/_{400}$, $^{1}/_{500}$, $^{1}/_{600}$, $^{1}/_{700}$ and $^{1}/_{800}$. In addition to the main (RDH) band, a clear second precipitin band due to impurity is shown. $^{1}/_{700}$ RDH dilution is the optimal proportion for this Ab sample with respect to anti-RDH.

Fig. 5.13j. Pure Ab/"Pure" RDH dilutions: $^{1}/_{150}$, $^{1}/_{200}$, $^{1}/_{250}$, $^{1}/_{300}$, $^{1}/_{350}$, and $^{1}/_{400}$. Only one precipitin band is present, $^{1}/_{350}$ dilution of RDH is balanced by pure antibody sample.

Recovery of the specific antibodies from the column can be calculated as follows:

9µl of Crude Ig fraction ≡ $^{1}/_{700}$ diluted RDH

9µl of pure anti RDH Ab ≡ $^{1}/_{350}$ diluted RDH

4.5µl of pure anti RDH Ab ≡ $^{1}/_{700}$ diluted RDH

Thus, 9µl of Crude Ig fraction contains as many specific antibodies as 4.5µl of pure Ab sample; let this be a unit amount of anti RDH Ab.

Total volume of Crude Ig fraction = 73ml

Total volume of Pure Ab fraction = 18ml

Total units: Crude Ig = $\frac{73000}{9}$ = 8111

Pure Ab = $\frac{18000}{4.5}$ = 4000

Therefore, the yield of the specific Ab = $\frac{4000}{8111} \times 100$ = 49.3%

This is in very close agreement to the yield estimated independently by RDH inactivation in its assay (48.3%).

d. The specificity of purified anti RDH antibodies for RDH only and no other protein was tested against a cell extract prepared from a ribitol inducible strain of K. aerogenes, FG5, which was growing in

enriched medium.

Pure anti RDH antibodies reacted with serially diluted FG 5 extract (Fig. 5.13k) show no visiable precipitin band. However, crude Ig fraction reacted with the same extract of FG5 gives 3 precipitin bands corresponding to the 3 impurities, indicating that specific antibodies have been directed against these, while no precipitin band corresponding to RDH is present because no RDH is present in this extract (Fig. 5.13 l).

G. Precipitation of purified antibodies by RDH

The ability of RDH to precipitate the purified antibody was tested at suitable antigen/antibody ratios suggested by the Ouchterlony quantitation.

20 - 160µl samples of RDH (550µg/ml) were added to 100µl of purified antibody and diluted to 1ml with PBS. After 5 hours at $2^{o}C$ the samples were centrifuged and the A280 of the supernatants was measured.

Fig. 5.14 shows that about 50% of the antibody protein was precipitable at optimum Ab/Ag ratio and 50µg of RDH were optimal for precipitation of about 190µg of antibody: a molar ratio of one RDH tetramer to 2.84 molecules of Ig G.

H. Investigation of Purity of Anti RDH Antibodies by Immunoelectrophoresis

The technique of immunoelectrophoresis is described in "Materials & Methods". In principle, as the term indicates, immunoelectrophoresis makes possible differentiation of proteins in solution on the basis of electrophoretic and immunological properties. Electrophoretic migration is generally performed in agar gel. Immunological differentiation of the separated protein fractions is achieved by adding specific immune serum to a groove in the agar block beside the separated protein fractions. Precipitation lines or rings form

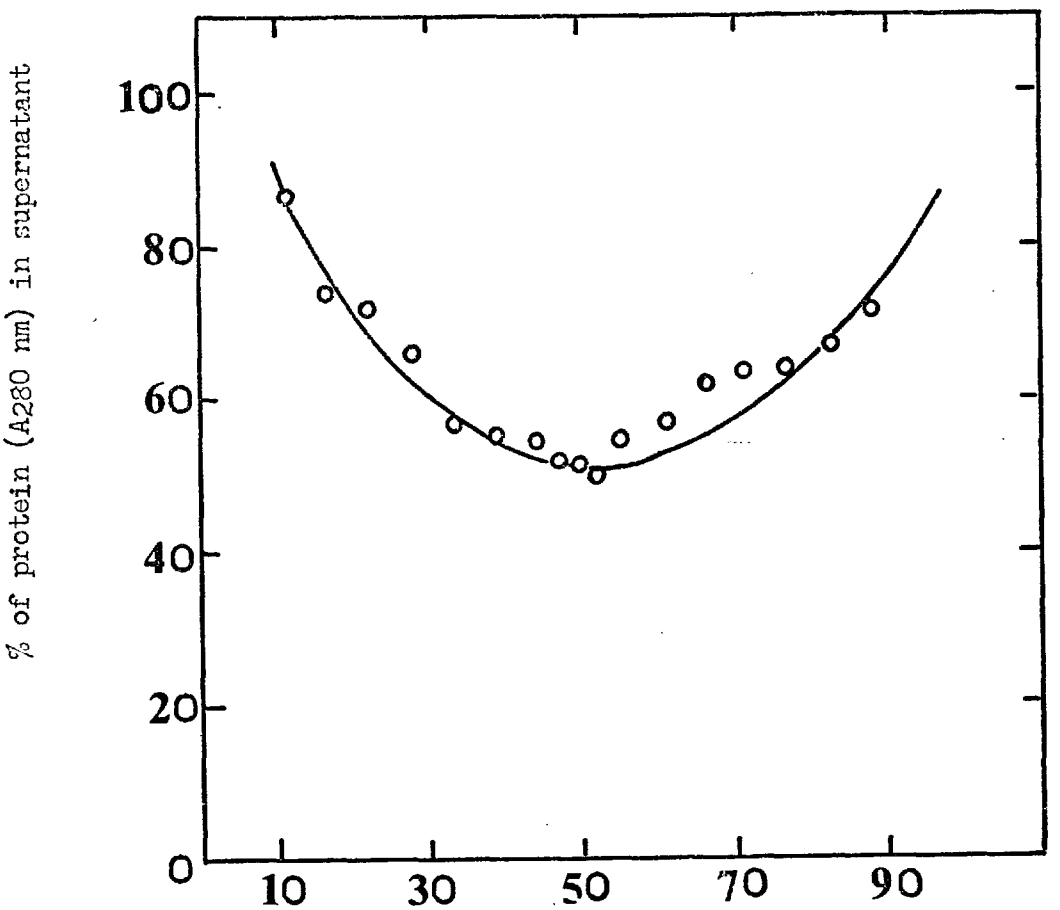


FIG. 5.14. Precipitation of purified anti-RDH antibodies in solution by pure RDH.

within a few hours at the points of contact from diffusion of the electrophoretically separated protein fractionsand of the specific immune serum in the trough. The position of these precipitation lines is determined by the electrophoretic mobility, the rate of diffusion, and the serological specificity of each of the proteins present in the solution under study.

Wells are spaced equally between anode and cathode, and they are equidistant from each other, about 1cm apart. The slot is equidistant from the wells. The result of immunoelectrophoresis is presented in Fig. 5.15. Wells from Nos. 1 - 6 contained 4µl samples of K. aerogenes Alll crude sonicate, or 1mg/ml pure RDH, alternately. After electrophoresis, 5-fold serially diluted purified Ab was added to the slots. Thus from top to bottom, troughs contained respectively 150µl of, 5mg/ml, 1mg/ml, and 0.2mg/ml of pure antibody solutions. Clearly only one band corresponding to RDH is visible in each dilution of Ab, either with pure RDH or with the sonicated extract. Only with regard to well No. 1 (sonicated extract/5mg/ml Ab) a small secondary precipitation line is apparent, but this "spurs" with the original precipitation line, indicating its identity with RDH. Very likely it is due to the presence of significant amount of "nicked" RDH in this preparation. This experiment clearly confirms the monospecificity of the purified antibodies for RDH and no other protein.

I. Re-use of an old affinity column

The original preparative affinity column (Section E (viii) of this chapter) after 13 months storage in sterile PBS containing sodium azide, was re-used with an Ig fraction from a new batch of antiserum.

Table 5.5 shows that this antiserum had lower anti-RDH activity (4.2 units/mg) than the previous sample (130.8 units/mg, Table 5.4), and the antibody fraction eluted with 3M KSCN and 1M acetic acid was also lower in activity (50.8 units/mg compared with 1071 units/mg in

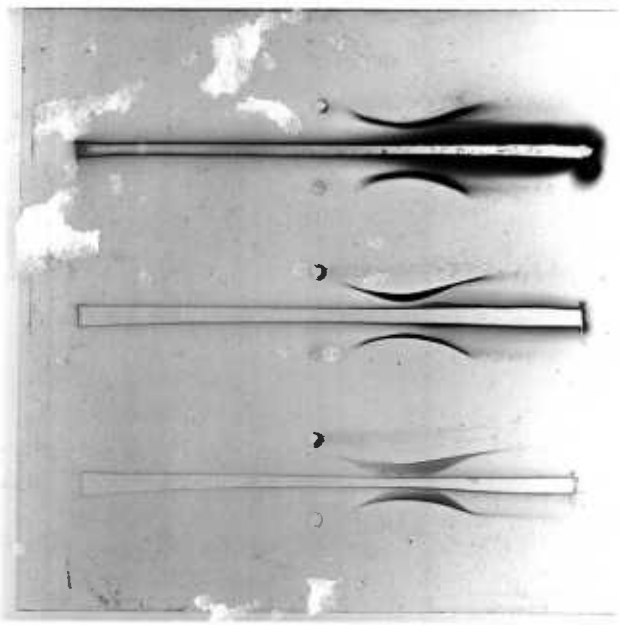


FIG. 5.15. Immunoelectrophoresis of Affinity Chromatography-purified rabbit anti-RDH antibodies. 150 µl of Ab was added to each trough after electrophoresis, from top to bottom, 5mg/ml, 1mg/ml and 0.2mg/ml, respectively. Wells contained from top to bottom alternately 4µl samples of sonicated extract of K. aerogenes A111 and 1mg/ml pure RDH.

TABLE 5.5.

Recovery of Specific Antibodies from an Old Affinity Column

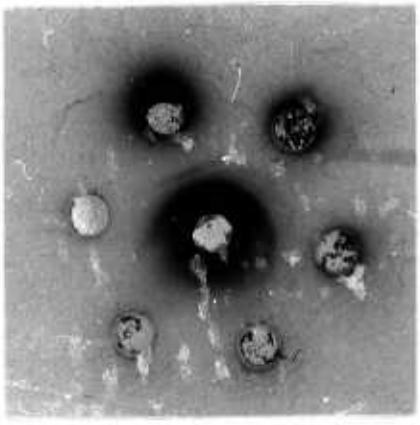
Fraction	mg	Total Units	% of Input Activity Units	Specific Activity (U/mg)
Crude Ig	1918	8000	(100)	4.2
Pure Ab 1st Elution	70	3558	44.5	50.8
Pure Ab 2nd Elution	9.6	<12	<0.15	<1.2

Table 5.4). The unadsorbed proteins eluted with PBS were recycled through this column and a second fraction of bound antibodies was obtained that had negligible anti-RDH activity.

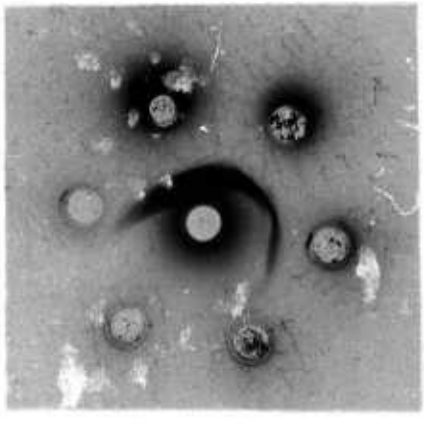
It may be that there is an appreciable fraction of antibodies directed towards 'sequence determinants' in RDH, and that on storage the RDH adsorbed to the column had denatured. Such antibodies would adsorb to denatured RDH but would probably not react with native RDH. They would therefore not inhibit the enzyme activity.

However it is likely that an appreciable proportion of the bound antibodies in this experiment were directed towards impurities in the original antigen. The antiserum used was obtained after a long course of frequent booster injections so that antiboides to impurities would be enriched. Furthermore the column is probably depleted in RDH antigenic determinants since strongly bound antibodies may not have been removed by the washing treatments. The decrease in capacity of the column over the period of use supports this conclusion.

The two purified Ab fractions from the two cycles above were combined, then analysed by Ouchterlony test. Fig. 5.16 shows the reaction of crude Ig fraction against doubling dilutions (up to 1:32) of K. aerogenes Alll sonicated extract. Reaction time was 48 hours. 2 clear precipitin bands are visible. Fig. 5.16b is the reaction of combined purified Ab fractions with the same sonicate and same dilutions. Two clear precipitin bands are again visible, indicating that there is at least one very significant Ab impurity present. The antibody sample will be used for comparison with the very pure and specific antibody fraction prepared previously in the immunoprecipitation of RDH specific polysomes, as discussed in Chapter X.



a. Reaction of crude Ig fraction rabbit anti-RDH antiserum / doubling dilutions (1 to 1:32)of Klebsiella aerogenes strain A111 extract.



b. Combined purified anti-RDH antibodies / doubling dilutions of K. aerogenes A111 extract.

FIG. 5.16. Purification of anti-RDH antibodies on a very old affinity column. Analysis by double - immunodiffusion test.

J. Purification of Sheep Anti-Rabbit-Ig Antibodies on Anti-RDH-Ig-Sepharose Immunoadsorbent

(i) Radioimmunoassay of sheep anti rabbit Ig antibodies

The procedures for immunization of two sheep, collection of antisera and iodination of pure anti RDH antibodies by lacto-peroxidase reaction are described in detail in "Materials & Methods".

2ml of neat rabbit antiserum were mixed thoroughly with 200µl of purified ^{125}I-labelled rabbit anti RDH Ab (593µg/ml). This solution was supplemented with 10 units/ml of heparin and clarified by centrifugation for 10 minutes. 0.1ml aliquots of this clarified labelled rabbit-Ig solution were used to react with serially diluted immunized antisera from two sheep, No. 1 and No. 2. The final volumes of sheep antisera added were, 4, 2, 1, $^1/_2$, $^1/_4$, $^1/_8$, $^1/_{16}$, $^1/_{32}$, $^1/_{64}$ml. All dilutions were adjusted to 4ml with PBS containing 1mg/ml BSA. The reactions were carried out on ice for 2½ hours with occasional shaking. At the end of incubation, the samples were centrifuged for 5 min, supernatants discarded, the precipitated Ab-Ag complexes and the tubes were rinsed with 1ml of PBS, centrifuged again, drained for a few minutes, and finally counted in a gamma scintillation counter. Two sets of controls were included in this experiment. (1) Three samples (1, 2, 3) showing the total radio-activity added initially to each reaction tube containing 0.1ml of rabbit Ab. (2) Three controls (4, 5, 6) containing only iodinated rabbit Ab but no sheep antisera added, all were treated same as the other incubations.

The results of this experiment are presented in Table 5.6. We can see that 0.1ml of rabbit antiserum is precipitated by about ¼ml of sheep No. 1 antiserum, or ½ml of sheep No. 2 antiserum.

Assuming that 15mg IgG/ml of rabbit antiserum (1.5mg IgG/0.1ml) and assuming that at least one sheep Ab binds to one rabbit Ab; then

TABLE 5.6

Titration of Rabbit Ig by Immunized Sheep Antisera

Sheep Antisera Additions (ml)	Precipitated Rabbit Ab (CPM) by Sheep Antiserum No.1	Precipitated Rabbit Ab (CPM) by Sheep Antiserum No.2
4	2680	2705
2	3020	2885
1	2999	2885
1/2	2682	2271
1/4	2413	371
1/8	717	115
1/16	113	87
1/32	99	167
1/64	-	69

Controls	CPM	
1	3873	Total radioactivity in 0.1ml of rabbit antiserum added to each sample, above.
2	3855	
3	3880	
4	51	Same as above, without sheep antisera additions (background).
5	46	
6	44	

there are at least 1.5mg x 4 = 6mg specific anti-rabbit Ig antibodies present/ml of sheep No. 1 antiserum and 1.5 x 2 = 3mg of specific anti rabbit Ig antibodies present per ml of sheep No.2 antiserum. These estimations must be regarded as minimum titres, since it is very likely that more than one sheep Ab can bind to one rabbit IgG.

Sheep No. 1 received both the initial as well as the booster injections into the same leg, but No. 2 received injections in alternate legs. The relative titres of specific antibodies in those two antisera are opposite to the general expectations of immunologists.

(ii) Preparation of Rabbit Ig-Sepharose Immunoadsorbent

Bromoacetamidoethyl-Sepharose-4B was prepared again as described in "Materials & Methods", for coupling to pure rabbit anti-RDH antibodies. Aminoethyl-Sepharose prepared 15 months earlier and stored at $4^{o}C$ in PBS (containing azide) was used in these procedures.

5ml of pure rabbit anti-RDH Ab (8mg/ml) was mixed with 1ml of iodinated rabbit Ig (0.7mg/ml). This solution was added to 24ml of $NaHCO_3$ pH 9.5, and the total volume was adjusted to 30ml. A 5ml sample was removed for radioactivity determination (total c.p.m. = 9870) and then returned into the sample. The 30ml Ab solution (pH 9.5) was added to 20ml packed volume of freshly prepared bromoacetamidoethyl-Sepharose. The suspension was stirred at room temperature for 2 days, followed by reaction for 24 hours at room temperature with 0.6ml of 0.2M 2-aminoethanol to mask unreacted bromoacetyl groups. The radioactivity of a 5ml sample of the supernatant was 630 CPM; thus the total supernatant radioactivity is 6300. Hence 9870 - 6300 c.p.m. are bound to the Sepharose = 36% of the original iodinated rabbit Ig. This corresponds to 14.4mg of Ab coupled to 20ml of Sepharose.

(iii) Affinity Chromatography of Sheep Antisera

105ml of sheep No.1 antiserum was fractionated by 50% ammonium sulfate, the Ig precipitates were dissolved in PBS and dialysed (final volume = 100ml). 14ml of this solution containing at least 14 x 6 = 84mg of specific anti rabbit Ig antibodies were loaded on the pre-equilibrated affinity column. The PBS wash proteins were passed through the column once more. After extensive washing with PBS, the loosely attached antibodies were removed by 0.5M NaCl. Finally the specific antibodies were eluted as before by 3M KSCN and 1M acetic acid. Following dialysis and concentration by ammonium sulphate precipitation, these two eluted fractions were combined and the protein concentration estimated by A280 measurement. 59.0mg of pure soluble antibodies were collected. The PBS precipitated antibodies collected in the dialysis bag were dissolved in 1M acetic acid and after dilution in 0.05M acetic the A280 was measured: the total protein of this fraction was 6.8mg. Therefore, 59 + 6.8 = 65.8mg of sheep Ab was bound to 14.4mg of covalently coupled rabbit Ig-Sepharose i.e. at least 4.6 sheep antibody molecules bound to 1 rabbit Ab molecule.

(iv) Specificity of Sheep anti-Rabbit Ig Purified Antibodies

Reactions of purified sheep anti-rabbit Ig antibodies against pure rabbit anti RDH antiboides (up to 1:32 dilution) is shown in Fig. 5.17. In addition to the major precipitin band (presumably Ig G), 3 other bands of smaller intensities (probably due to other Igs) are present.

Fig. 5.18, shows the reaction of pure sheep anti-rabbits Ig antibodies to K. aerogenes A111 extract (1 - $^{1}/_{32}$ dilutions). Clearly no precipitation band is visible in this case, proving the specificity of these antibodies for rabbit Ig, and no interaction with the bacterial proteins present in the cell extract.

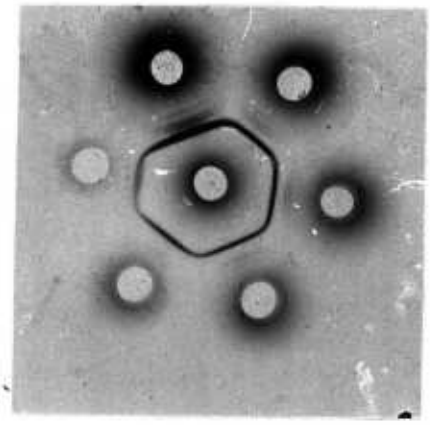


FIG. 5.17. Affinity Chromatography purified sheep anti-rabbit Ig antibodies/ rabbit pure anti-RDH antibodies, up to 1:32 dilutions.

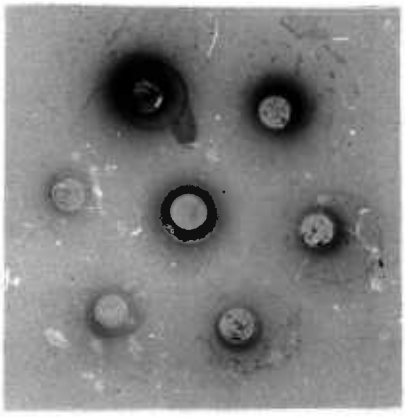


FIG. 5.18 Pure sheep anti-rabbit Ig antibodies/ K. aerogenes Alll sonicated extract, 1 to 1:32 dilutions.

K. Further Purification of Antibodies by DEAE-Carboxymethyl Cellulose Chromatography

Rabbit and sheep antibodies to be used in precipitations of polysomes must be free from traces of ribonuclease. Most of the nucleases are removed by sterile affinity chromatography, however, to be absolutely sure, various researchers (e.g. Palacios et al., 1972; Bouma, III et al., 1975) have recommended the use of an ion-exchange column containing DEAE-cellulose overlaid with CM-cellulose. The procedure for this chromatography is described in "Materials & Methods". Typical elution profiles with PBS for purified rabbit and sheep antibodies are presented in Fig. 5.19 and 5.20 respectively.

L. Relative Activities of Iodinated and Non-Iodinated Antibodies

Since ^{125}I-labelled rabbit anti RDH antibodies were going to be used to identify the polysomes which were engaged in synthesis of RDH polypeptides, it was necessary to determine if any significant activity of specific antibodies was lost through iodination.

Serially diluted iodinated and non-iodinated antibody samples (50µl each) were incubated with 50µl of $^{1}/_{6000}$ diluted pure RDH-D (0.83µg) for 1 hour on ice. 20µl aliquot of each incubation was added to 1ml of RDH assay mixture at 28^{o}C and the increase in A340nm for each sample was measured.

Fig. 5.21 shows the plot of the residual RDH activity (as a percentage of sample with no added antibody) as a function of increasing concentrations of iodinated and non-iodinated antibodies. It is clear from this result that 50% inhibition is brought about by exactly the same amount of each type of antibodies. Therefore, there is no apparent loss of activity through iodination of these antibodies.

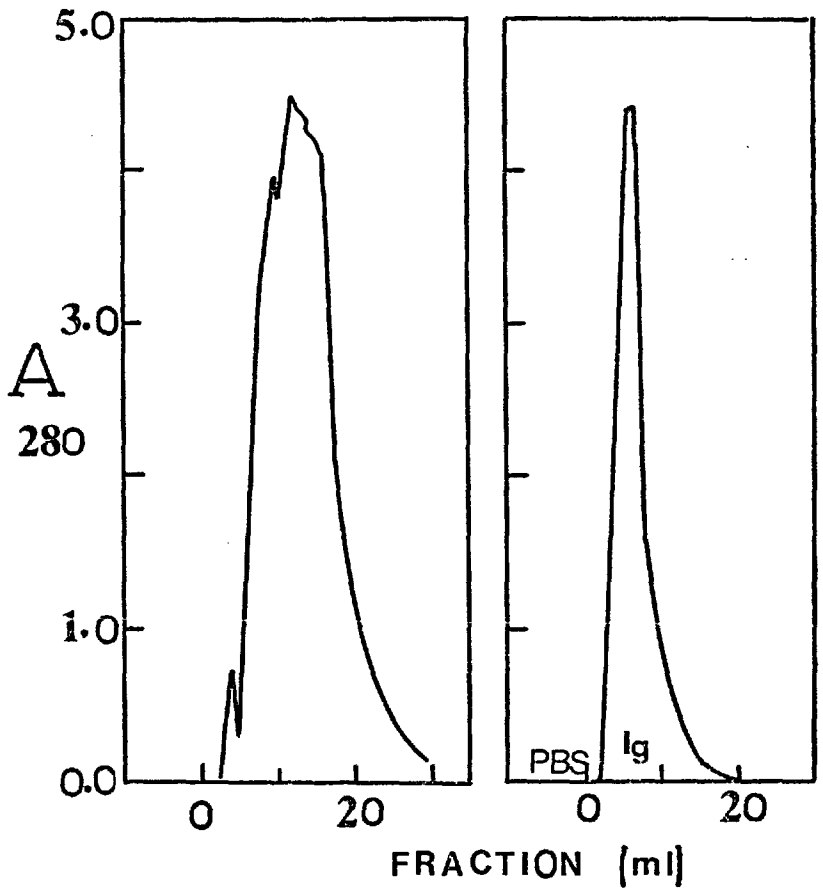


FIG.5.20. Chromatography of sheep-anti rabbit-Ig antibodies on DEAE-CM-Cellulose (2+2 cm,h.) X 1 cm , dia. Elution rate, 24 ml/hr.

FIG.5.19. Chromatography of pure rabbit-anti-RDH antibodies on DEAE-CM-Cellulose (2.5+2.5cm,h.) X 0.5cm, dia. Flow rate, 24 ml/hr.

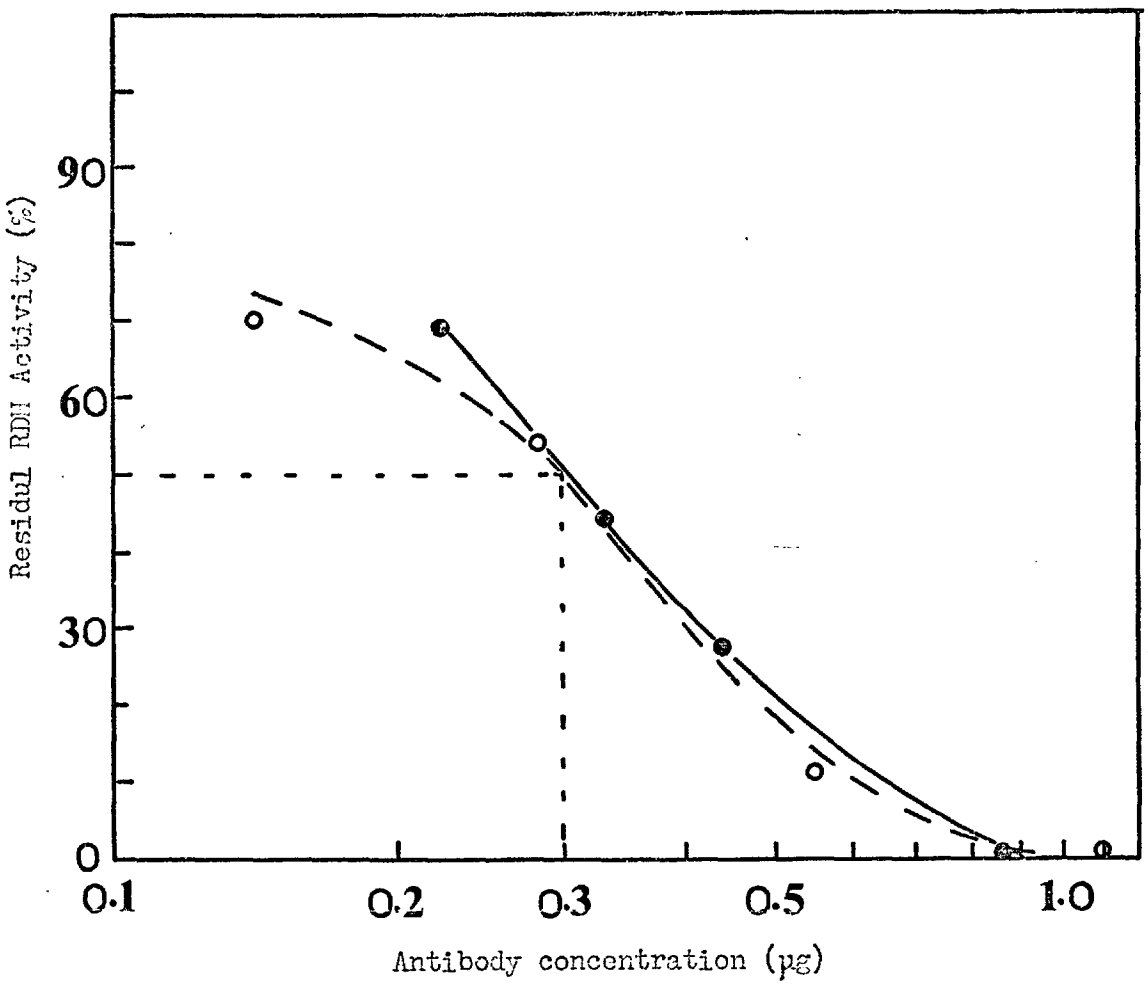


FIG.5.21. Residual RDH activity as a function of increasing concentrations of iodinated (0 - - 0) and non-iodinated(●——●)-rabbit-anti-RDH antibodies present in RDH assay mixture.

M. Homology of Proteins of K. aerogenes, and E. coli, and Characterization of Impurities in Purified RDH sample by Double Immunodiffusion Tests

Fig. 5.22 shows the result of interaction of rabbit anti-RDH immunized serum with a sonicated extract of E. coli K-12 strain CA388. CA388 is the recipiant strain used in transduction studies for transfer of RDH genes from K. aerogenes to E. coli K. Three weak pricipitin bands are present which indicate the following:

(1) All of the three protein impurities present in the purified RDH sample from Klebsiella aerogenes-D have homologous proteins present in E. coli K-12 extract, since they react with the same antibodies, proving that the two organisms must be very closely related.

(2) It had previously been reported (e.g. Rigby 1971) that purification of RDH enzyme from certain high-synthesis strains of K. aerogenes is complicated by the presence of another protein in the cell extract which has similar chromatographic behaviour and electrophoretic mobility to RDH. Moreover this protein (x) seemed to increase in intensity with every step of high synthesis. Therefore it has been suggested that gene amplification in RDH high-synthesis strains may involve a certain segment of the chromosome carrying genes coding for a number of related enzymes, thus (x) could be one of them, possibly ribulokinase, in which case it must be represented by one of these three impurities present in the RDH-D sample. The above result clearly proves that this is not true, at least with regard to RDH-D, since the impurities are also present in E. coli K-12 CA388 which totally lacks the pentitol metabolic pathway and was grown on enriched medium.

Fig. 5.23 shows the result of interaction of anti RDH anti-serum with the extract of E. coli EA1111. This is an identical strain

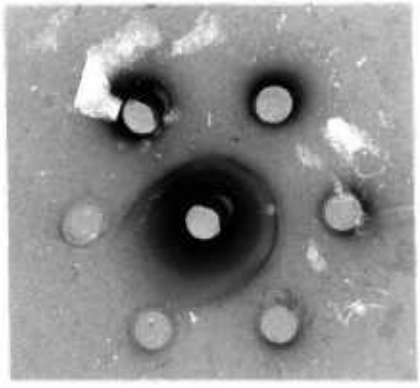


FIG. 5.22. Interaction of crude rabbit-anti RDH antiserum/ extract of E. coli-K-12 strain CA388.

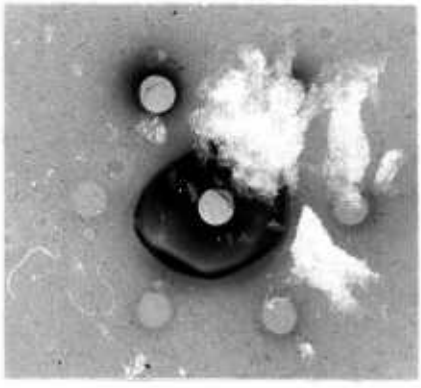


FIG. 5.23. Crude anti-RDH antiserum/ sonicated extract of E. coli K-12 strain EA1111.

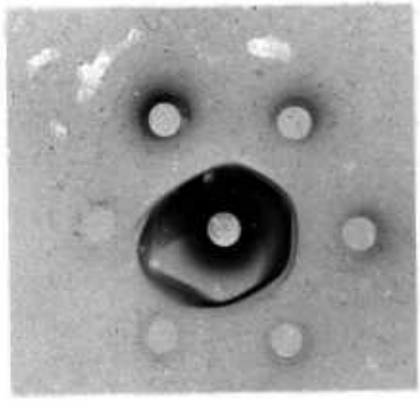


FIG. 5.24. Old affinity column purified rabbit anti-RDH Ab/ E. coli EA1111 extract.

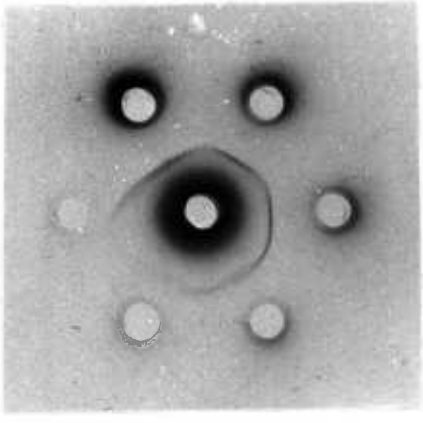


FIG. 5.25. Old affinity column-purified rabbit anti-RDH Ab/ E. coli CA388 extract.

to CA388 except that it has incorporated the RDH genes from Klebsiella aerogenes A strain into its own genome; subsequently it had evolved to RDH high synthesis in a chemostat. The result shows the presence of an extra intense precipitin band due to RDH, in addition to the 3 minor bands present.

Fig. 5.24 shows that the interaction of recently purified rabbit anti RDH antibodies with E. coli EA1111 extract. In addition to the RDH precipitation band an extra band due to one of the impurities is present.

Fig. 5.25 shows the reaction of the same pure antibodies with CA388 extract. The RDH band is absent; only the weaker band due to a single impurity is represented.

N. Conclusion

In this study I have developed methods for making highly purified antibodies directed against RDH by affinity chromatography, starting from an RDH sample which was not very pure itself. The monospecificity of the purified antibodies for RDH have been demonstrated by Ouchterlony double immunodiffusion studies; also by immunoelectrophoresis. Appropriate conditions for elutions of antibodies from affinity column have been worked out. These columns can operate to about 50% efficiency on the basis of recovery by weight and units of activity of specific antibodies. 50% of the purified antibodies are precipitable in vitro by pure RDH. The affinity chromatography conditions have been successfully extended to the purification of sheep anti rabbit Ig antibodies. An assay system based on inhibition of RDH activity was developed for rapid estimation of specific antibodies. Correlations of such estimates with those by optimal proportion by Ouchterlong technique have been worked out.

By RDH inhibition assay, I have shown that antibodies raised

against RDH from Klebsiella aerogenes D strain strongly react with all other Klebsiella aerogenes mutant enzymes tested. Also they react strongly with E. coliC RDH. This immunological homology between E. coli C RDH and Klebsiella RDH is very interesting. Reiner (1975) discovered that E. coli C grows on pentitols and that the genes for ribitol growth map close to position 40 minutes in the E. coli C genome, exactly where the K. aerogenes genes had become incorporated into E. coli K-12 (Rigby et al., 1976). A strain of E. coli C that superproduces RDH has been evolved in a chemostat large amounts of RDH was purified from this mutant and the enzyme has been partially sequenced. Only 4 differences between the K. aerogenes and E. coli C RDH sequences have been found in the 85 residues sequenced so far (Hartley et al., 1976). Hence the enzymes appear to be 95% homologous by sequence. Immunologically, as I described already, E. coli C RDH is 37% homologous to K. aerogenes RDH.

The close homology of the two enzymes points to their relatively recent evolutionary divergence, and suggests either that the two species of microorganisms are close relatives or that they have acquired the ability to grow on pentitols by interspecies transfer of a common metabolic plasmid. More extensive sequence comparisons between other proteins of these organisms would be necessary to distinguish these alternatives. Already I have strong evidence by double immunodiffusion studies in support of the former alternative, that the two organisms are indeed very closely related: Antibodies raised to 3 random proteins present as impurities in Klebsiella aerogenes D RDH preparation, are found to react with exactly 3 proteins in E. coli K, which were shown to have nothing to do with pentitol metabolism.

CHAPTER VI

Purification and Characterization of Bacterial Polysomes

A. Introduction

a) Purification of polysomes

Until 1967 there was no general, satisfactory method to lyse bacterial cells rapidly, in the cold, and with sufficient delicacy to produce undegraded polyribosome preparations. Godson and Sinsheimer described a technique of rapid cell lysis for the preparation of polyribosomes on an analytical scale from several E.coli strains (Godson & Sinsheimer, 1967; Godson, 1967). E.coli cells growing in early logarithmic phase (80-100 ml culture; 1 x 10^8 cells/ml) were chilled in 15-25 seconds to 0-2^{o}C in an alcohol-CO_2 bath, harvested in less than 6 min at 0^{o}C by centrifugation, and lysed by lysozyme-EDTA and a neutral detergent (Brij-58) at 0-2^{o}C over 2-3 minutes. The polysomes released in these cell extracts were believed to be in a state that approximate to their in vivo condition. In fact, no one has yet produced a more satisfactory method or has presented better bacterial polysome profiles by analysis on sucrose gradients than those presented by the above authors.

Melcher (1975) used this method to purify polysomes synthesising β-galactosidase from suitably induced E.coli cells. The culture was initially chilled with an equal volume of ice containing NaN_3 and tetracycline. β-galactosidase specific polysomes in the extract were enriched to 40-50% purity by affinity chromatography on a β-galactosidase substrate analog-Sepharose column. However, the extent of degradation of these polysomes

and the size of the mRNA fragments were not determined.

I have used the method of Godson and Sinsheimer, with a few necessary modifications, to purify undegraded bacterial polysomes from Klebsiella aerogenes and E.coli on a 1 litre scale and at any cell density required (see "Materials & Methods"). Essentially, 1 litre batches of growing bacterial cultures at the required cell density are treated at 37°C for 5 seconds with chloramphenicol, then immediately chilled in less than 1 minute to 0-2°C. Chloramphenicol binds specifically to 50S ribosomal subunit, inhibiting functional attachment of the aminoacyl end of AA-tRNA to the 50S subunit thus preventing transpeptidation, but may also have a direct inhibitory effect on the peptidyltransferase. The antibiotic decreases the rate of polysome breakdown, but ribosomes can slowly progress along mRNA in the absence of peptide bond formation (Pestka,1971).

The cells are harvested in 2 min by centrifugation at 2°C in a chilled GSA Sorvall rotor, quickly resuspended in cold sucrose and lysed by lysozyme-EDTA and neutral detergent. Several such lysates can be accumulated successively on ice for ½ hour, or for longer periods by freezing in a methanol-CO_2 bath, without appreciable degradation of polysomes. After an initial centrifugation at 6000 g to remove cell membranes, the polysomes are pelleted as quickly as possible by ultracentrifugation at 2°C through a cushion of sterile 1.5 M sucrose in polysome buffer. The polysomal pellet is resuspended in buffer, clarified by a centrifugation at 6000 g, and stored under liquid nitrogen. Such purified bacterial polysomes in sterile solutions are stable for several hours on ice. Polysomes stored under liquid nitrogen

after several months, and in spite of periodical thawing and freezing, appear quite stable.

Godson (1967) pointed out that bacterial cells become increasingly difficult to lyse with age. It was recommended that cells be used in early logarithmic phase (less than 2×10^8 cells per millilitre). Also, cells treated with chloramphenicol were said to be slightly more resistant to lysis than normal cells. In my experience, although the older cells may be more resistant to lysis, I have not encountered any great difficulty in lysing chloramphenicol treated cells even at cell densities approaching the stationary phase, under the experimental conditions I have used.

b) The influence of IF3 on polysome profiles

Cell-free extracts from ageing bacterial cultures are generally less active in synthesizing proteins than those derived from logarithmic cultures (Nirenberg, 1963; Li & Umbreit, 1966; Oppenheim et al., 1968; Gonzalez et al., 1968). This loss of activity is noted to be coincident with a decrease in polysome content with concomitant increase in free ribosomes (mostly 70S monosomes), a situation postulated to reflect impaired initiation of the translation process (Gonzalez et al., 1968; Phillips & Franklin, 1969). Stationary phase ribosomes from E.coli are less active even for the translation of artificial templates such as poly U (Li & Umbreit, 1966), but these ribosomes can be reactivated in vitro for poly U translation by a temperature-dependent NH_4Cl washing and by puromycin treatment (Scheps et al., 1971). The latter result suggests that the inability of the ribosomes to respond to poly U is due to their being blocked by unfinished

polypeptide chains. Extracts from stationary phase E.coli, even when functioning with poly U, are inactive with natural mRNA unless crude ribosomal-wash factors are added. This result suggested that these extracts were deficient in some of the initiation factors present in this fraction (Revel et al., 1968; Scheps et al., 1971). Initiation of protein synthesis in prokaryotes has been reviewed by Haselkorn & Rothman-Denes (1973). Further studies by Scheps & Revel (1972) showed that the three initiation factor activities for natural mRNA translation are markedly reduced in extracts from stationary phase E.coli, as compared to growing cells. By using antibodies directed against IF-3,they showed that IF-3 is metabolically unstable, virtually disappearing from E.coli as the cells enter stationary phase. Furthermore, there is evidence for the existence of two distinct IF3's, one recognizing E.coli mRNA, MS-2 RNA, and early T4 mRNA (synthesized in vitro), the other recognizing late T4 mRNA (Revel et al., 1970; Berissi et al., 1971; Lee-Huang & Ochoa, 1971; Lee-Huang & Ochoa, 1973). A mixture of 30S and 50S subunits, IF-1, IF-2, ^{14}C-fmet-tRNA,^{3}H-MS2-RNA and ^{32}P-late T4 mRNA was supplemented with one of the purified IF-3s. IF-3α promoted the formation of 70S initiation complexes (identified by ^{14}C label) containing almost exclusively MS2-RNA, while IF-3β promoted the formation of 70S complexes containing predominatly late T4 mRNA (Lee-Huang & Ochoa, 1973).

Another interesting property of the IF-3 is its'70S ribosome dissociating activity'. The first step in formation of the initiation complex is the association of IF-3 with the 30S

subunit (Sabol et al., 1970; Sabol & Ochoa, 1971; Sabol et al., 1973; Pon et al., 1972; Dubnoff & Maitra, 1971; Benne et al., 1973). Either IF-3 α or IF-3 β suffices to serve the dual function of preventing the secluding association of a naked 30S subunit with a 50S subunit, while promoting the proper binding of mRNA to the 30S subunit (Lee-Huang & Ochoa, 1971; Lee-Huang & Ochoa, 1973). The binding of IF-3 to the 30S subunit has been sufficiently demonstrated in vitro, using radioactive IF-3; the binding has no co-factor requirements (Sabol et al., 1973; Sabol & Ochoa, 1971; Pon et al., 1972; Benne et al., 1973). The disappearance of IF-3 as the bacterial cells enter stationary phase (Scheps & Revel, 1972) explains the decreased ribosome dissociating activity found in stationary phase cells (Bade et al., 1969), but it is not entirely responsible either for increased ratio of 70S monosome/ribosomal subunits, or for reduction in the average polysome size in the aged cultures, as analysed by sedimentation in sucrose gradients. This situation must not be confused with the profiles of partially degraded polysomes produced as a result of poor isolation techniques or mishandling, which in fact give rise to very similar profiles.

c) Changes in polysome profiles during the growth cycle

Consider an almost inert, non-growing inoculum taken from an old stab or picked from a plate which has been stored in the cold for some time and introduced into a fresh medium under appropriate growth conditions. Common sense tells us that the initial response of the organisms, after receiving the physiological signals from the nutrients, would be (1) to utilize any residual

RNA polymerase to make more polymerase molecules in order to promote general transcription, (2) to make the necessary translation factors to begin protein synthesis on the newly activated, existing ribosomes, (3) to make more ribosomes in order to continue translation, (4) to synthesize metabolic enzymes required to establish the general cellular metabolism. After these initial responses, which could follow each other sequentially, come the DNA replication, membrane synthesis increase in cell mass and eventually cell division. This is why initially there is a lag phase before each culture starts to grow. It is feasible that the translation of mRNAs coding for RNA-polymerase and some earlier synthesized protein synthetic factors may not require certain unstable translation factors such as IF-3, which we know are almost completely depleted from old cultures. Thus, already, in the non-growing (lag) phase we may have continually changing compositions of mRNAs and, therefore, polysomes.

During early and mid logarithmic growth phase, when the cells must increase their mass, replicate their genome and divide as rapidly as possible, certain cistrons will remain permanently derepressed, such as ribosomal genes. Other genes related to various metabolic enzymes and protein factors will be turned on and off in order to maintain the correct proportions of their products. Thus during this period there will be, on the average, a stable proportion of various messages and polysomes present. However, in one respect this may not be quite true; recently there have been reports of a class of relatively stable and abundant mRNAs for outer membrane proteins of E.coli (Hirashima et al., 1974; Levy, 1975). These

messages have a half life of 5.5-11.5 minutes (Hirashima et al., 1973; Lee & Inouye, 1974) to 40-80 minutes (Levy, 1975). These m RNA's accumulate throughout culture growth; thus they will gradually alter the composition of the polysome population.

One can visualize the biological advantage to the organism in accumulating stable m RNA for outer membrane proteins. During rapid growth, massive synthesis of unstable m RNA's will allow the cell to adjust its metabolism rapidly to the process of growth and division. A shortage of outer membrane proteins might then be tolerated and possibly even be an advantage in assisting cell division. As metabolic activity diminishes, new m RNA synthesis will subside and free ribosomes will become available to the stable m RNA and allow completion of the protective layer in the stationary phase. The observation that log phase cells are easier to lyse than ageing cells is in accord with this view. Moreover these relatively stable m RNA's are translated differently from the unstable m RNA's. Later evidence suggests that they are translated on the membrane by ordinary ribosomes but do not seem to require initiation factors such as IF-3 for their translation in vivo. Perhaps they share this property with other membrane bound m RNA's such as those coding for RNA-polymerase and some of the very early synthesized translation factors, or for exported proteins.

In mid log phase the concentration of some essential nutrient falls below the level required for maximum growth. It is here that I have observed a significant change in polysome profiles over a very narrow range of increase of cell density. The unstable m RNA population is depleted within 20 min but

translation continues for a few hours on a class of metastable membrane-bound ribosomes. The majority of these are much smaller in size than the average polysomes of unstable m RNAs, averaging only 3 ribosomes, and they have an average half life of about 75 min.

Some of these small messages probably bind only a single ribosome. This may explain why 70S ribosomes from aged cultures are inactive in translation, even for synthetic m RNAs such as poly U which do not require IF-3 for initiation (Li and Umbreit, 1966). Treatment of such 'ageing ribosomes' with NH_4Cl at higher temperature or with puromycin restores their ability to translate poly U (Scheps et al.,1971), and this is explicable as stripping from a firmly bound small stable m RNA.

The properties of this metastable m RNA are similar to those described for the m RNA of the outer membrane proteins of E.coli (Hirashima et al., 1974; Le & Inouye, 1974; Levy, 1975). It would seem appropriate that in late log phase the cell should begin to concentrate on the synthesis of a tough protective sheath in preparation for the adverse conditions it is likely to encounter in the stationary phase.

B. Comparative Sedimentation Analysis of Bacterial Polysomes present in the 6000 g Supernatant of a Cell Lysate with the Purified Polysomes

In order to show that purified polysomes are not degraded and have similar size distribution to the polysomes present in a freshly prepared cell extract, bacterial polysomes from a 1 litre culture of K.aerogenes A111 were prepared and purified as described in "Materials & Methods" and stored under liquid nitrogen. Then a 6000 x g supernatant from the cell extract of another, 80 ml,

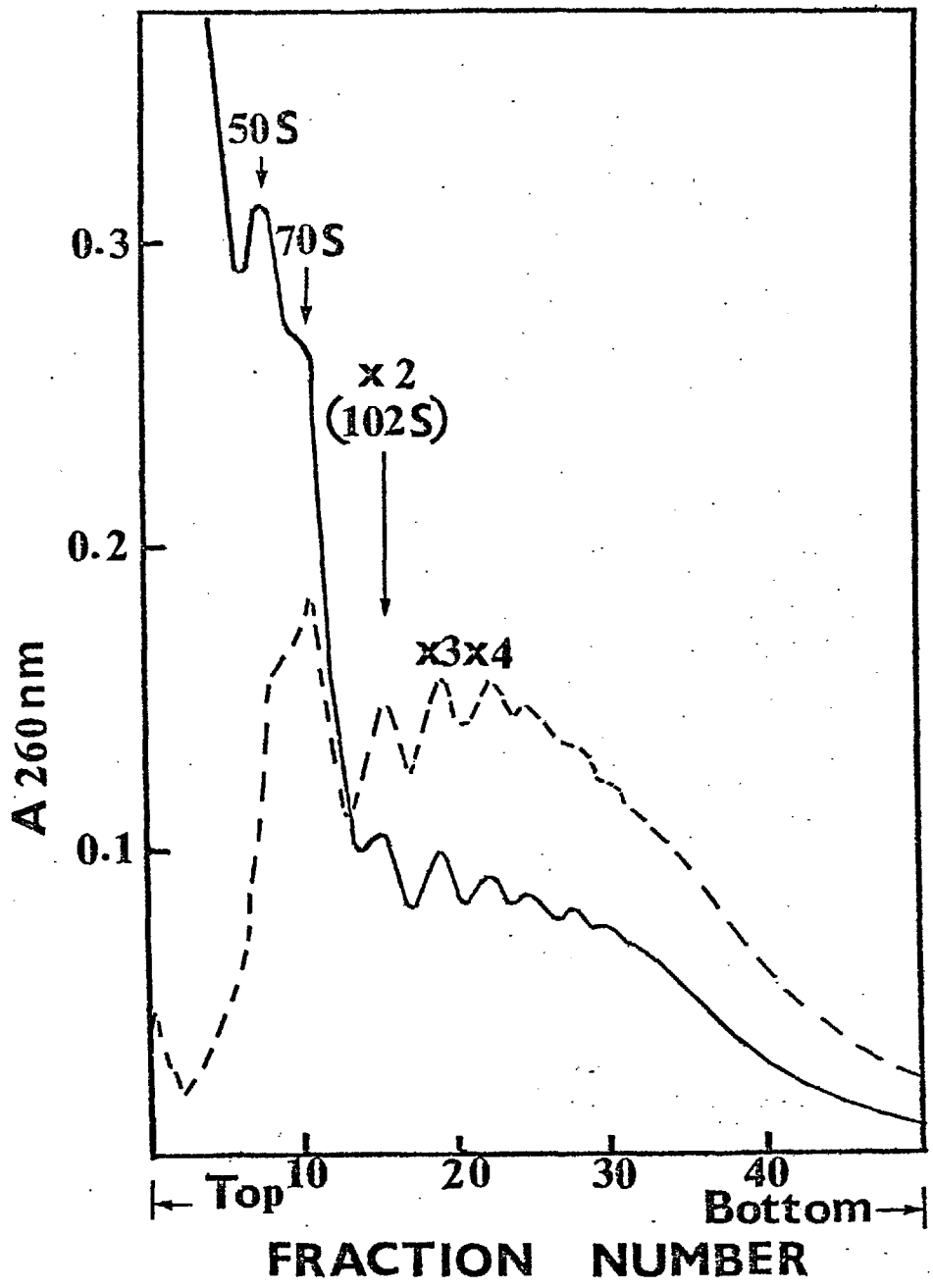


FIG. 6.1. Sucrose gradient (15-30%, w/v) sedimentation analysis of polysomes from Klebsiella aerogenes. (——), A260 of the 6000 X g supernatant of the cell lysate; (– –), A260 of the purified polyribosomes. Ultracentrifugation was conducted in a Beckman L2-65B centrifuge, using an SW 25.2 rotor at 24000 rpm for 150 min at 2°C.

culture of the same cells grown to exactly the same cell density (A650 = 0.55, undiluted) on the same medium (M9 + 0.2% Xylitol) was prepared. The purified polysomes and the lysate samples were loaded on 15-30% sucrose gradients (56 ml each), prepared in parallel, and centrifuged at the same time on an ultracentrifuge. The A260 of various fractions were recorded by a flow-through spectrophotometer and analysed as described in "Materials and Methods".

Fig.6.1 shows the superimposed A260 profiles of the ultracentrifuged lysate and the purified polysome samples. As demonstrated the size distributions of the polysomes in various fractions of the two profiles are very similar. The excess A260 in the 50S and 70S fractions (also to some extent in the di and tri-some fractions) of the cell lysate is due to the contributions of the nucleic acids diffused into the top fractions of the gradient corresponding to the cell lysate, which has a large content of nucleic acids.

C. Dynamics of Bacterial Polysomes Composition

Purified polysome samples prepared from bacterial cultures (K.aerogenes strains as well as E.coli) grown to various cell densities ranging from early logarithmic phase up to near the stationary phase have been studied extensively. The significant results of this study are summarized in Fig. 6.2. Profile 6.2. a) is typical of polysomes purified from early cultures below 5×10^9 viable cells/ml ($A_{650\ nm}$ 0.56) on minimal media such as M9 + 0.2% Xylitol (maximum cell density = 2×10^{11} cells/ml; $A_{650\ nm}$ = 1.9). Notice the typical broad and rounded polysome dome peaking at about pentasomes. Notice also

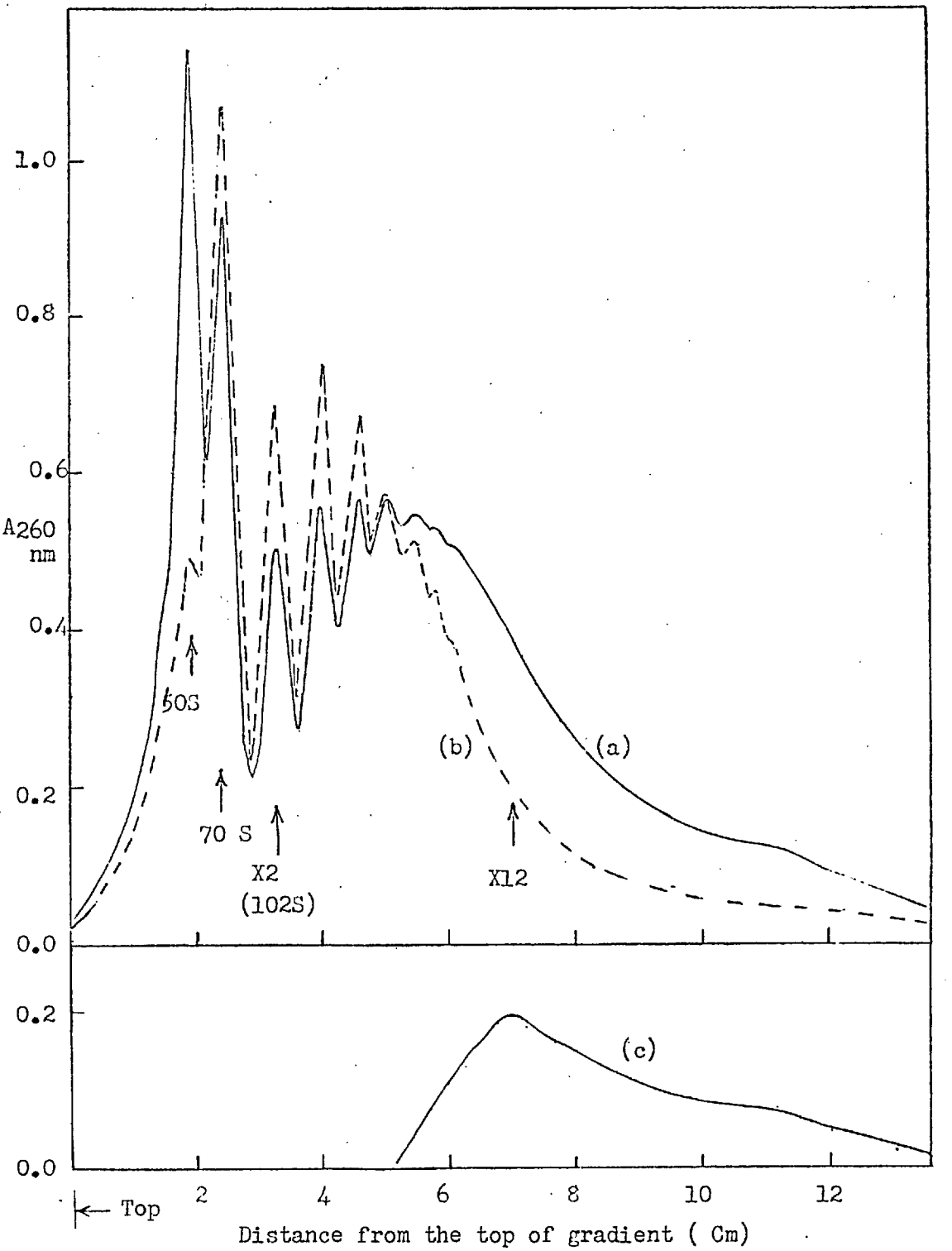


FIG. 6.2. Size distribution of purified bacterial polysomes before and after "the critical culture growth point" . Klebsiella aerogenes Alll cells were grown in LB medium. (a), purified polysomes from the culture harvested at A650nm = 0.3 ; (b), purified polysomes from the culture harvested at A650nm = 0.9 ; (c), the differential absorbances of the two profiles (a) & (b) showing the maximum difference corresponding to about twelve-somes sedimentation. 10 A260 units of polysomes were layered on a 56 ml linear gradient, 0.5 - 1.5 M. Centrifugation was carried out in a swing out SW 25.2 rotor of Beckman L2-65B ultracentrifuge at 24000 rpm, for 3 hr, at 2°C. Gradients were fractionated and the A260 tracings were obtained as described in the "Materials & Methods".

the significant larger polyribosomes shoulder extending to the bottom of the gradient; also the relative proportion of the 70S monosomes to 50S subunits. Different bacterial strains investigated produced similar profiles. Moreover, the type of growth medium, although certainly it must give rise to qualitative differences in the polysomes, does not seem to cause a noticeable difference in the size distribution of the polysomes. In fact, the profile (a) in Fig.6.2 comes from the analysis of K.aerogenes Alll cells grown in a rich medium (LB) to culture A650 nm = 0.30. I must mention that small changes do take place over a period of growth from, say, very early log phase up to mid log phase (culture A650 nm of 0.55). For example, the monosome peak and the oligosomes, X2, X3, X4, may gradually increase slightly, while the proportion of ribosomal subunits may decrease, but the general profile is not very different.

However, the same minimal medium culture, if harvested only about 15-20 minutes after reaching A650 of 0.56, (at A650 $\geq$ 0.6) would yield a significantly different late culture polysome profile, similar to profile 6.2b. Notice that the larger polysomes shoulder is greatly reduced; in fact, in strains other than RDH superproducers and in older cultures it reduces even more. Note the significant increase in the proportion of the oligosomes peaks, X2, X3, X4, and the monosome peak. Note the significant reduction in the ribosomal subunits.

This change in polysome profile also takes place on a rich medium. The transition period takes about the same time (20 min) and at slightly higher culture densities (A650 0.7-0.9 compared with A650 0.6 on minimal) while the maximal growth on a rich

medium such as LB is A650 = 2.8 compared with A650 = 1.9 on minimal. Thus, the signal to stop mRNA synthesis and to alter the composition of polysomes arises on both minimal and rich media at similar times and cell densities and is not critically dependent on the composition of the medium. Fig. 6.2(b) is the polysome profile from K.aerogenes A111 grown on LB broth to A650 nm = 0.9.

As discussed above, I interpret this sudden switch in polysome profile at relatively low cell density as an almost total shutdown in bacterial mRNA synthesis at a critical phase in the culture growth, which could be triggered by exhaustion of some essential metabolite. This effect might be mediated by considerable depletion or modification of RNA polymerase, resulting in general reduction of mRNA transcription. As the mRNA synthesis is discontinued, the unstable polysomes,represented by the heavy shoulder, disappear quickly while the stable polysomes, represented by the oligosomes (1-10 ribosomes), remain longer. The meta-stable mRNAs may even have a greater opportunity to be translated due to lack of competition from unstable mRNAs.

An alternative explanation is a sudden depletion of some of the translation factors such as IF-3. There would then be keen competition among the mRNA molecules for initiation by the remaining active ribosomes, and this would bring about a reduction in the average size of the polysomes.

There is some evidence in support of the first hypothesis against this latter. First, it has been known for a number of years that bacterial mRNA synthesis is greatly diminished after

the mid-logarithmic growth phase; this is why all pulse labelling experiments for bacterial mRNA are routinely carried out at very low cell densities. Secondly, as we shall see later, the average size of the stable RDH polysomes not only does not decrease after the "mRNA synthesis shutdown" but it actually increases. Thirdly, as shown later, the half-life of the oligosomes after mRNA shutdown was found to be 75 min compared with an average half-life of typical unstable bacterial mRNA of 1-2 min. All of these polysomes are extremely sensitive to EDTA and pancreatic ribonuclease, reducing to ribosomal subunits and to 70 S monosomes respectively, while purified DNAse has no effect on them. Also they become pulse-labelled with ^{3}H-uridine as indicated in Fig.6.3.

Two methods could prove my hypothesis that mRNA synthesis does shut down at this critical point. Firstly, if the culture before the "shut down" is pulse-labelled with ^{3}H-uridine for 1-2 minutes, then chased with cold uridine, the rate of decay of label in the heavy shoulder should be greater than the rate in the smaller polysomes fractions. Secondly, addition of specific inhibitors of transcription such as rifampicin to the young bacterial cultures should result in rapid disappearance of the heavy shoulder polysomes, while the decay rate of the oligosomes must be much slower. I have not had time to carry out either of these two experiments.

D. Determination of Size of Polysomes

The number of ribosomes present per polysome at any fraction can be estimated, using a logarithmic plot of the number of ribosomes per polysome versus the log of peaking distance of each visible polysome from the top of the gradient. All the points

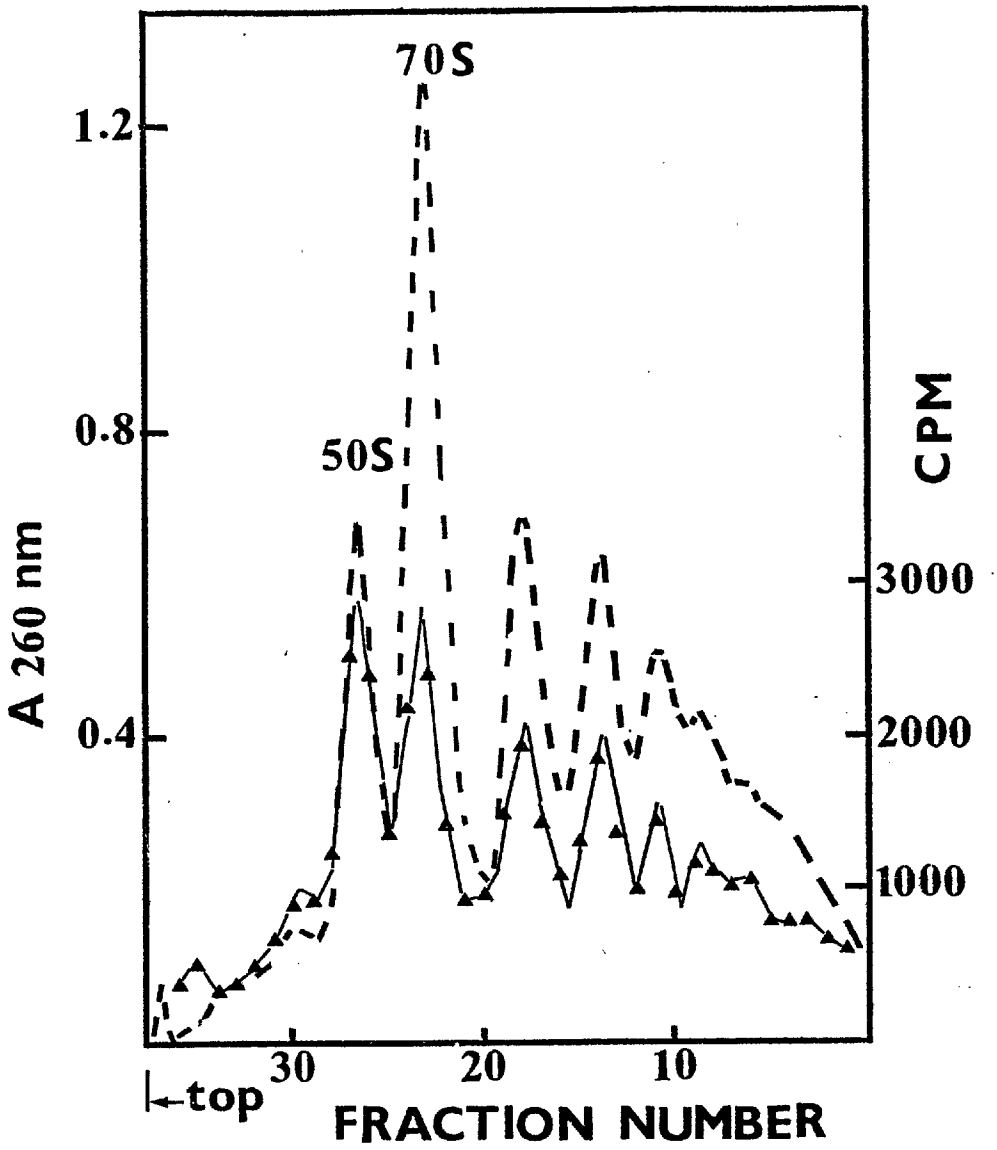


FIG. 6.3. Pulse-labelled bacterial polysomes, just after or about 'mRNA synthesis general shut down'. K. aerogenes A111 cells were grown in 1 litre of M9+0.2% xylitol medium to culture A650nm = 0.6. (5,6)-^{3}H-uridine, aqueous solution (45 Ci/m mole, 0.13 uCi/ml of culture) was added to the culture and incubated for 1.5 min. Then chloramphenicol was added, the cells were harvested, and polysomes were extracted and purified as described in "Materials & Methods". Purified polysomes, 10 A260 units, were layered on 0.5-1.5 M linear sucrose gradients and centrifuged for 5 hr at 25000 rpm at 2°C in a Beckman L2-65B, SW 25.2 rotor. (▲——▲), Radioactivity; (— —), A260 nm.

fall on a straight line as shown, for Fig.6.2 profiles, in Fig. 6.4. Thus, the plot of the differential absorbances of the profiles (a) and (b) in Fig. 6.2 gives rise to a distinct peak. The number of ribosomes per polysome in this particular fraction is estimated to be 12 by the above method.

E. Conclusion

A procedure for quantitative preparation of bacterial polysomes is described which enables one to purify bacterial polysomes at any cell density of a growing culture. Such purified and stored polysomes have been compared by sedimentation analysis with the freshly extracted polysomes on an analytical scale by the method of Godson & Sinsheimer (1967), and shown to be quite comparable.

The observation is made that at a particular point in late log phase of a bacterial culture, a distinct change in the profile of polysomes takes place. The "usual", widely distributed polysome profile with the characteristic broad, heavy shoulder extending to greater than 50-somes virtually disappears and is replaced by a much shorter polysomes (oligosomes) population. This observation is interpreted as two populations of polysomes coexisting in the young cultures. The first is related to the majority of unstable bacterial mRNAs with an average half-life of 1-2 min, corresponding to enzymes and metabolic proteins. The second population is related to a class of metastable mRNAs coding mainly for the structural and outer membrane proteins which cannot compete too well with the unstable mRNAs for initiation of translation. But after the "general mRNA shutdown", the second stabler population will be expressed fully up to the

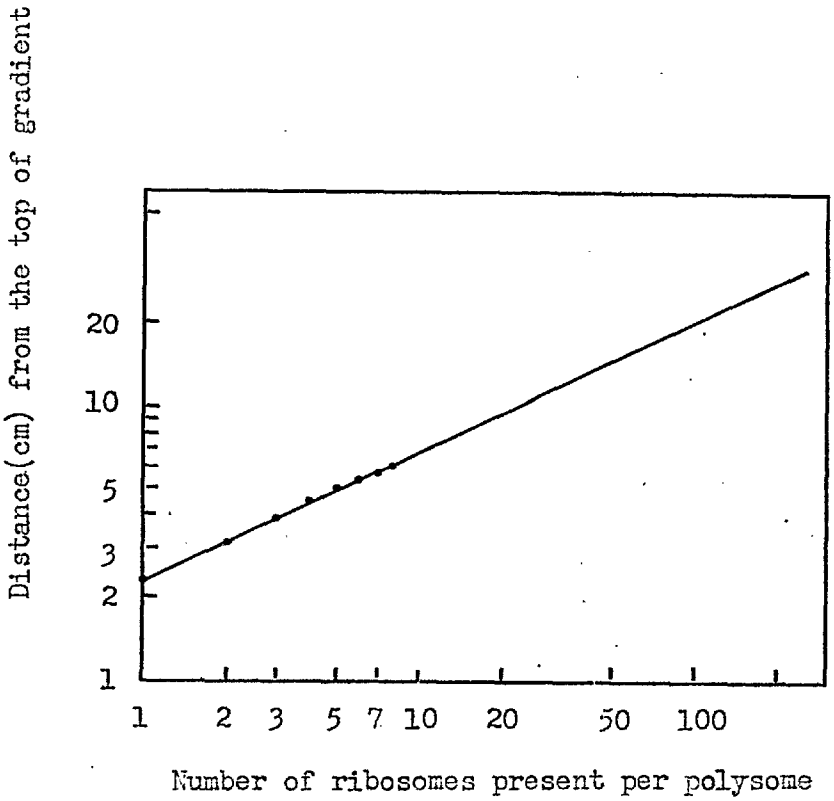


FIG. 6.4. Velocity sedimentation distances of the polysomes in Fig. 6.2 as a function of the number of ribosomes present per polysome in each fraction. A double logarithmic plot.

end of culture growth. Some evidence in support of this interpretation is mentioned. I shall return to this subject later when this view is strengthened by further experiments.

A simple method is described for estimating the size of polysomes at any particular fraction in the sucrose gradients, by extrapolation of a plot of the log of peaking distance of the visible oligosomes from the top of the gradient as a function of the log of number of ribosomes present per polysome, where all the points lie on a straight line.

CHAPTER VII

Radioimmunological Identification of Polysomes Synthesizing Ribitol Dehydrogenase Polypeptide Chains

A. Introduction

We have seen in Chapter V that the purified anti RDH antibodies are 50% by weight precipitable by pure RDH. This indicates that at least 50% of these antibodies can bind to native RDH. What we need to know is to what extent these active antibodies will react with the nascent RDH polypeptides attached to polysomes under the experimental conditions used. It is quite possible that RDH, being a tetramer, the antibodies may not react at all with the nascent chains.

A further hazard arises from the unavoidable conditions used for Ab/polysome incubation, i.e. low temperature (0-2^{o}C), limited incubation time (usually less than 1 hour), and rather diluted incubation mixtures. It would be an advantage to use high specific activity ^{125}I-antibodies, provided the antibody activity is not greatly diminished and the polysomes are not affected in any way. ^{125}I-labelled purified antibodies with initial specific activities of about 30,000 CPM/µg, prepared by lactoperoxidase reaction (Materials and Methods) and used throughout this study, showed no reduction in their capacity to inactivate RDH in its assay (Chapter V).

B. Binding of Different Concentrations of ^{125}I-Anti-RDH to purified K.aerogenes A111 Polysomes

Purified bacterial polysome samples were incubated on ice with

various amounts of pure ribonuclease-free ^{125}I-anti-RDH-D antibodies. They were then analysed by velocity sedimentation on linear sucrose gradients. As long as reasonable salt and buffer concentrations were maintained, the polysomes were very stable at all concentrations of ^{125}I-antibodies added (up to 500 µg/10 A260 of polysomes tested) and for different incubation periods (up to 1 hour investigated), as judged from the comparative sucrose gradient sedimentation profiles with polysomes which were not incubated with the antibodies (profile not shown). The radioactivity associated with the polysome fractions as a result of the incubation of 10 A260 units of K.aerogenes A111 polysomes with purified ^{125}I-anti-RDH antibodies increases up to about 30 µg of antibodies added, whereafter the antigenic determinants appear to be saturated with the antibodies.

Fig. 7.1 illustrates that the ^{125}I-radioactivity bound to 10 A260 units of A111 polysomes did not increase significantly when the incubated ^{125}I-antibodies were increased from 30 to 150 µg. Some of the radioactivity in the ribosomal and the smaller oligosome fractions is due to some diffusion of the large amounts of unreacted radioactive antibodies layered over the gradients.

C. Diffusion of ^{125}I-Antibodies into Sucrose Gradients

In order to show that the radioactivity in polysome profiles such as Fig. 7.1 represents bound antibody rather than artifacts arising simply from diffusion of antibodies, comparative experiments were carried out with and without polysomes, using equal amounts of the same ^{125}I-antibody solution on identical gradients and by simultaneous centrifugation. Fig 7.2a shows the diffusion of antibodies

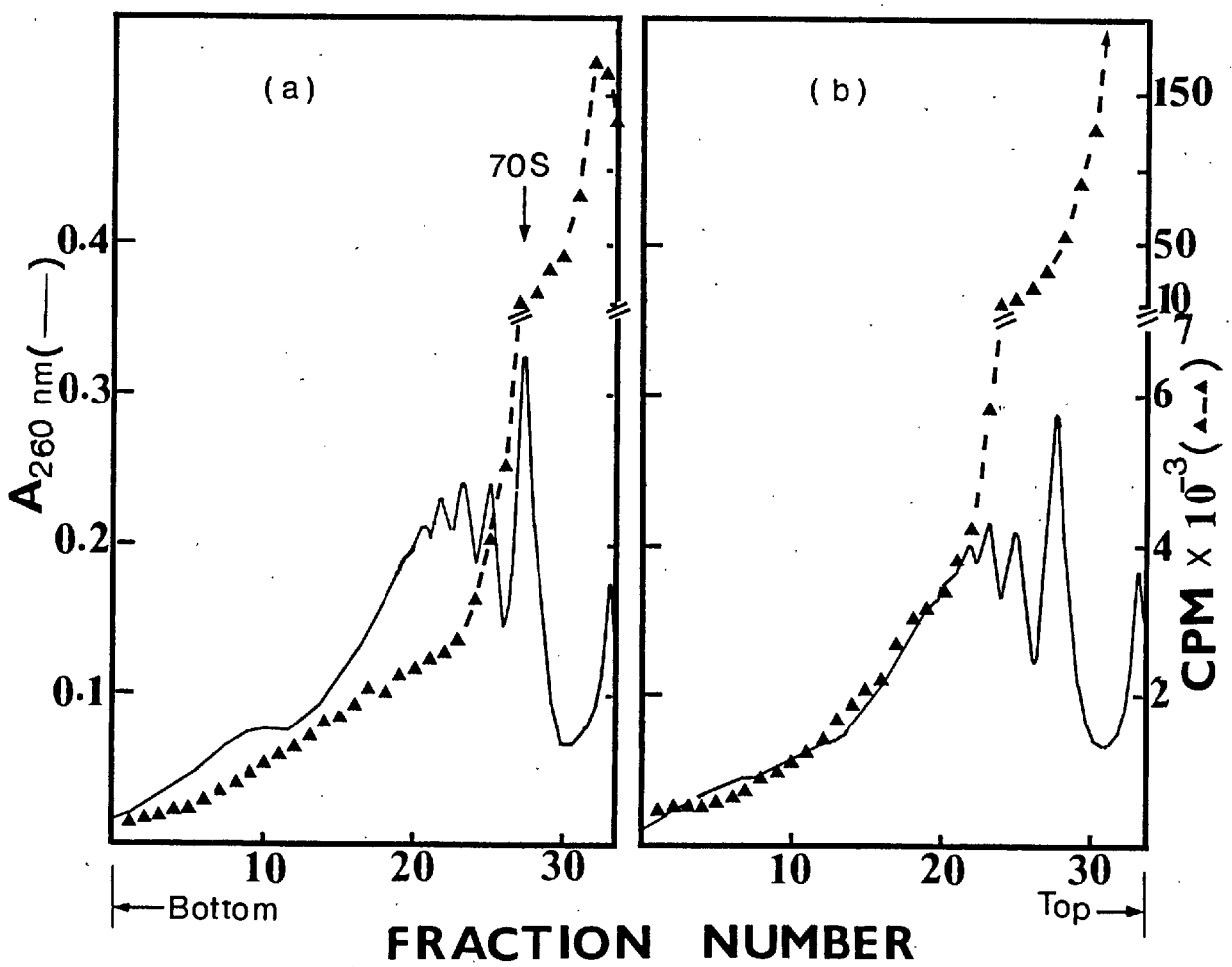


FIG. 7.1. Binding of different concentrations of ^{125}I-anti-RDH antibodies to Klebsiella aerogenes Alll polysomes. Purified polysomes (10 A260 units in 1.5 ml), prepared from K. aerogenes Alll cells grown on M9/xylitol medium to culture A650nm = 0.6, were incubated at 4°C for 1 hour with (a) 30µg and (b) 150µg of ^{125}I-anti RDH antibodies (specific activity, 17000 CPM/µg). After the incubation, the polysomes were layered over a 56 ml continuous sucrose gradient (0.5-1.5M) and centrifuged in a Beckman SW 25.2 rotor at 2°C, 24000 rpm, for 2 and 3/4 hours. Fractions (1.7 ml) were collected from the bottom to measure the radioactivity.

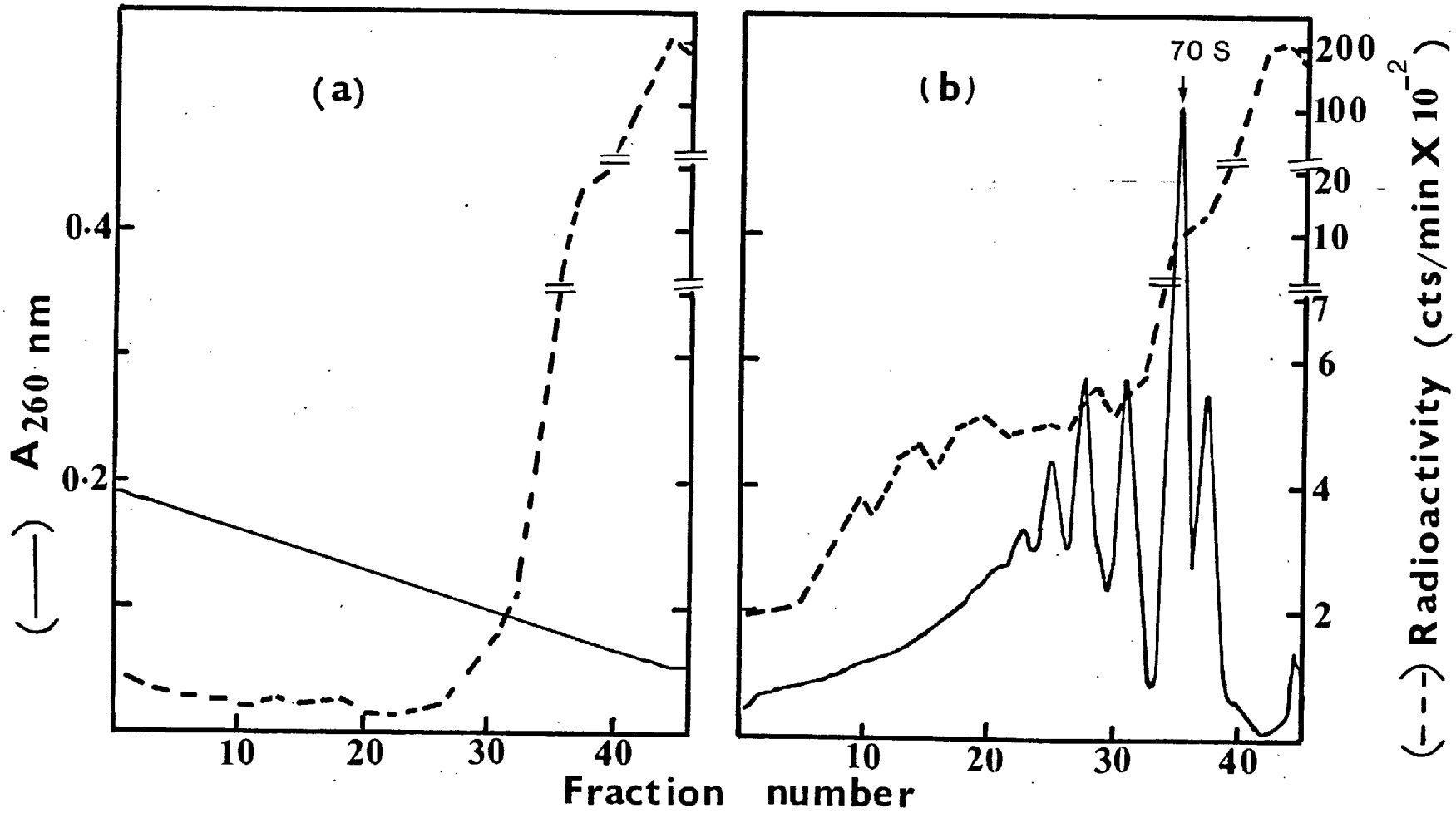


FIG.7.2. Diffusion of ^{125}I-antibodies in sucrose gradients. (a), 5µg of purified ^{125}I-anti-RDH antibodies (specific activity = 28000 CPM/µg) in 0.5ml were layered over a 57ml linear sucrose gradient(0.5-1.5M); (b), 5µg of the antibodies as in (a) were incubated on ice with 10 A260 units of K. aerogenes AlIl polysomes in 0.5 ml for 40 min, then the mixture was layered on a gradient as in (a). Both samples were centrifuged in a Beckman SW 25.2 rotor at 2°C for 3 and ½ hours at 24000 rpm . A260 in (a) is due to 0.5-1.5M sucrose.

after centrifugation in the absence of added polysomes, while in Fig. 7.2b an identical anti-RDH sample was pre-incubated with 10 A260 units of K.aerogenes Alll polysomes, then the mixture was layered on another similar gradient and centrifuged on the same rotor. From the comparison of these profiles, it is clear that significant amounts of radioactivity have diffused into the top 1/3 of the gradients. The difference between (a) and (b) radioactivity profiles represents the radioactivity bound to the polysomes. This diffusion can be minimized to some extent, as we shall see in the later profiles, by reducing the volume of sample layered; also by more careful application of the sample.

Fig.7.2a also shows the linearity of the sucrose gradient as an outcome of differential absorbances of the starting sucrose solutions (0.5-1.5 M). This is the actual A260 tracing obtained by a continuous flow spectrophotometer and an automatic recording unit. Such differential absorptions are determined for every set of experiments and the necessary adjustments are made with respect to each polysome profile absorbance.

D. The Specificity of Interaction of Pure ^{125}I-anti-RDH Antibodies with RDH-Synthesizing Polysomes

In an attempt to determine the degree of specificity of interaction of ^{125}I-anti-RDH antibodies with polysomes specifically engaged in synthesis of RDH polypeptides, purified K.aerogenes polysomes prepared from a high synthesis strain (Alll) and an inducible strain (FG-5) were compared. Alll cells were grown on M9 + 0.2% xylitol medium, while FG-5 cells were grown in an enriched broth (LB) medium. Both cultures were grown to A650 = 0.65.

Equal amounts of the two purified polysome samples were incubated with equal quantities of ^{125}I-anti-RDH antibodies; then they were analysed by co-sedimentation on identical sucrose gradients as shown in Fig.7.3. It can be seen that the general pattern of association of ^{125}I-antibodies with FG-5 polysomes (Fig.7.3a) is very similar to that of Alll polysomes Fig.7.3b. However, the specific activity of interaction of the iodinated antibodies with polysomes (CPM/A260) is twice greater for Alll polysomes than for FG-5 polysomes. This suggests that although there is some specific interaction between ^{125}I-pure anti-RDH antibodies and RDH synthesizing polysome, there is also an equal amount of non-specific interaction with other bacterial polysomes in the preparation, since the FG-5 preparation did not contain any RDH specific polysomes.

E. Attempts to Minimize Non-Specific Antibody-Polysome Interactions by Neutral Detergents

The mono-specificity of the purified antibodies for RDH was clearly demonstrated in Chapter V. Therefore, it was highly unlikely that these antibodies could bind strongly to anything else in the bacterial extracts. In an attempt to eliminate the non-specific interactions such as those shown in Fig.7.3a, which most probably are due to protein-protein interaction, neutral detergents (Brij-58 and deoxycholate) were added to the antibody-polysome incubation mixture. It can be seen from the comparative profiles in Fig.7.4 (a,b and c) that though additions of the neutral detergents have slightly decreased the non-specific interactions, they certainly did not provide a solution to the problem.

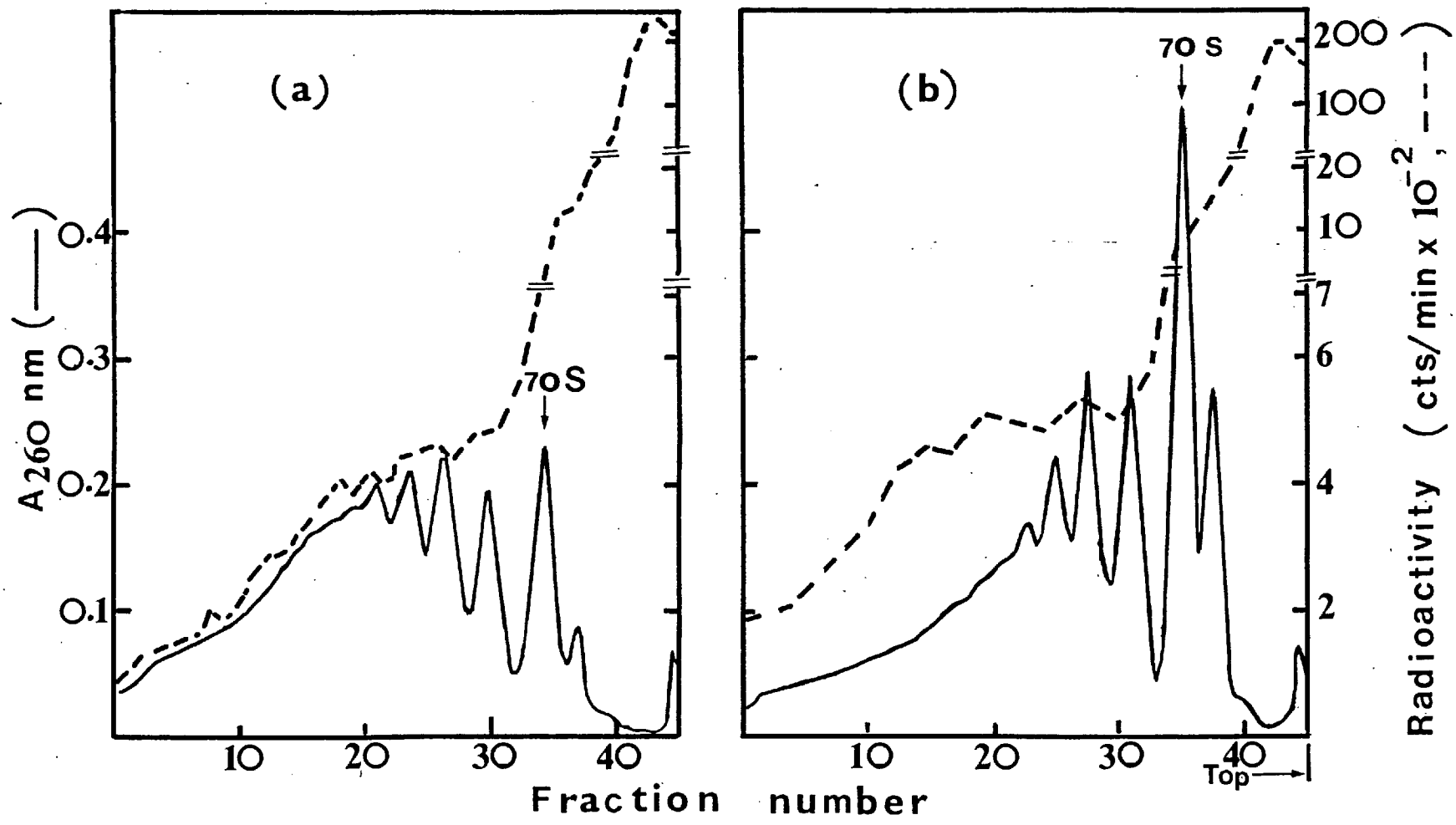


FIG. 7.3. Interaction of purified ^{125}I-labelled anti RDH antibodies with purified polysomes from Klebsiella aerogenes strains FG-5 (a) and A111 (b). 5µg of the iodinated antibodies were incubated with 10 A260 units of polysomes in 0.5ml, on ice, for 40 min. Ultracentrifugation was in a Beckman SW 25.2 rotor in 0.5 - 1.5M continuous sucrose gradients at 24000 rpm, for 3 and ½ hours, at 2°C.

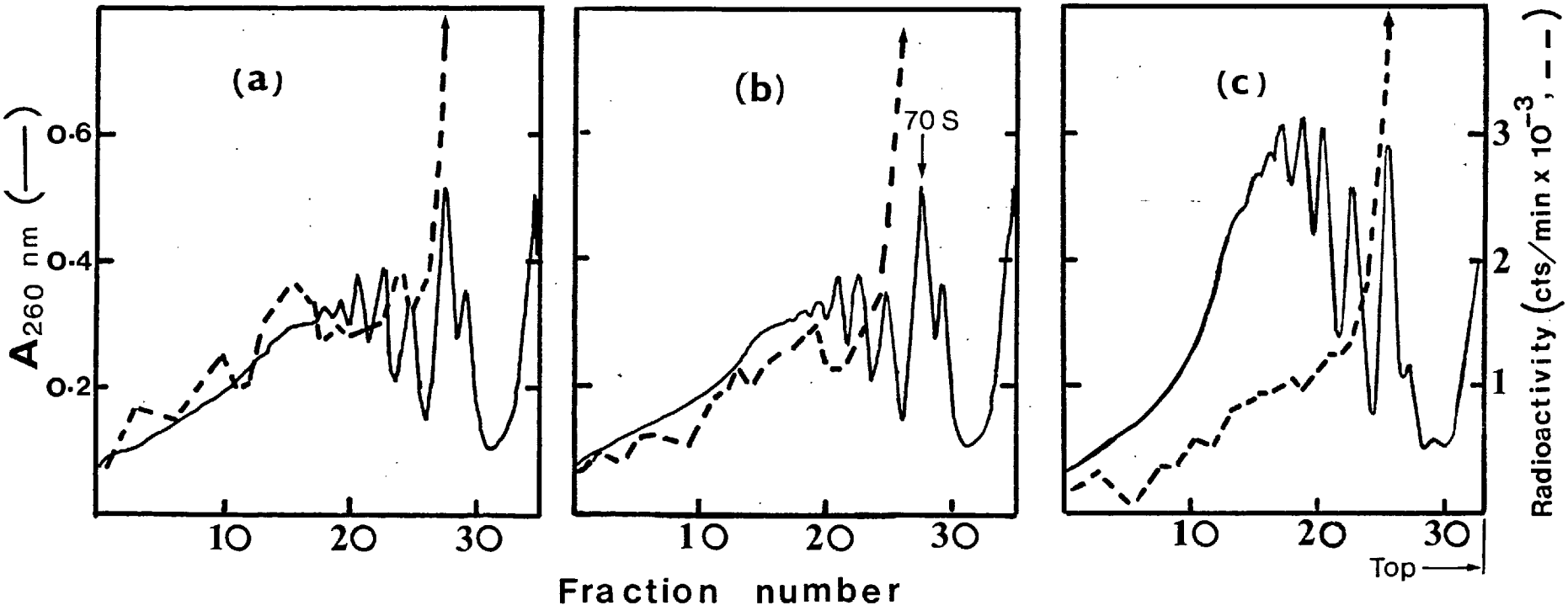


FIG. 7.4. Interaction of anti RDH antibodies and bacterial polysomes in presence of neutral detergents. Polysomes (10 A260 units) in 1.8ml of 0.25M sucrose in "polysome buffer" were incubated with 10ug of ^{125}I- anti RDH antibodies on ice for 45 min, then they were layered on a 56 ml continuous (0.5 - 1.5M) sucrose gradient and centrifuged in a Beckman SW 25.2 rotor at 24000 rpm for 3 hrs at 2°C. Profiles (a) & (b) are from K. aerogenes A111 polysomes; (c) is from K. aerogenes FG-5 polysomes. Incubation mixtures (b) & (c) also contained Brij-58, 0.8% (w/v), and deoxycholate, 0.2% (w/v).

F. Demonstration of Specific Interaction of Anti-RDH Antibodies with Polysomes containing Nascent RDH Chains in the Presence of Heparin

Since the neutral detergents and the high concentrations of sucrose (which is generally known to be a good anti-aggregant) could not jointly eliminate the non-specific binding of antibody to polysomes, then the most likely cause of non-specific interactions might be the formation of salt bridges between antibody molecules and polysomes, greatly facilitated by the presence of high concentrations (10 mM) of divalent cations, magnesium in particular, which are strictly required to maintain stable polysome structures. Inclusion of a suitably chosen competitor for non-specific salt bridge formation in the antibody/polysome incubation mixtures could provide a solution to this dilemma. The competitor must be a macromolecule, and contain a large number of monovalent acidic groups. Heparin seemed to fit these requirements very well, thus it was investigated in this study. It is an acid mucopolysaccharide of molecular weight about 17,000 in which the repeating unit (Fig.7.5) consists of D-glucuronic acid with an O-sulfate group probably at C-2, and D-gulcosamine N-sulfate with an additional O-sulfate group at C-6. Both the linkages of the polymer are alternating 1,4. It is not certain whether all or only a portion of the glucuronic acid residues in heparin carry an O-sulfate group. Both linkages are α-D.

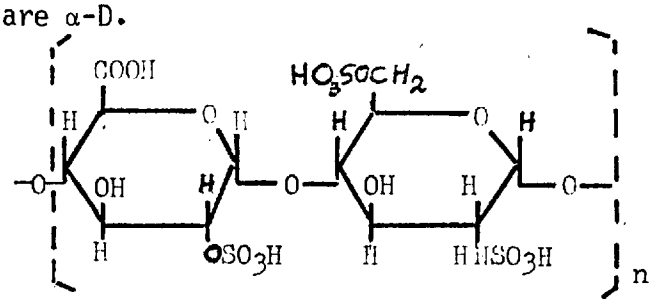


Fig 7.5. Postulated repeating unit of heparin (White,Handler and Smith, (1964).

The influence of heparin in eliminating the non-specific interactions is shown in Fig.7.6. The presence of only 10 units/mg of heparin in the antibody/polysome incubation mixtures completely prevented any attachment of ^{125}I-pure anti-RDH antibodies to the polysomes from K.aerogenes FG-5, (inducible) strain (Fig.7.6a). However, under the same conditions the specific binding of the pure anti-RDH antibodies to the polysomes carrying nascent RDH polypeptides, present in the K.aerogenes A111 polysome preparation, became apparent by clear contrast, as shown in Fig.7.6b.

G. Effect of RNAse and EDTA on Immune-Identified RDH Polysomes

Since RDH comprises a large proportion of the proteins of K.aerogenes A111 cell extract, it may be argued that some of this native RDH could have contaminated the polysome preparation, thus the observed radioactivity associated with these sucrose gradient fractionated polysomes may not be the result of interaction of the specific antibodies with the nascent RDH present on the polysomes, but may be due to sedimentation of the antibody/native RDH complexes. To prove that this is not the case, crucial tests with RNAse and EDTA were conducted. By these treatments, before or after incubation of the appropriate polysomes with ^{125}I-antibodies, the radioactivity in the sucrose gradients must co-migrate with the A260 profile of the 70S monosomes, the degraded polysomes, and the ribosomal subunits.

The results presented in Fig.7.7 clearly show that the radio-immune-identified material in the sucrose gradients is both sensitive to RNAse and to EDTA. As a result of limited digestion with pancreatic ribonuclease the sedimentation of the radioactivity followed exactly the sedimentation profile of the degraded polysomes, mainly into

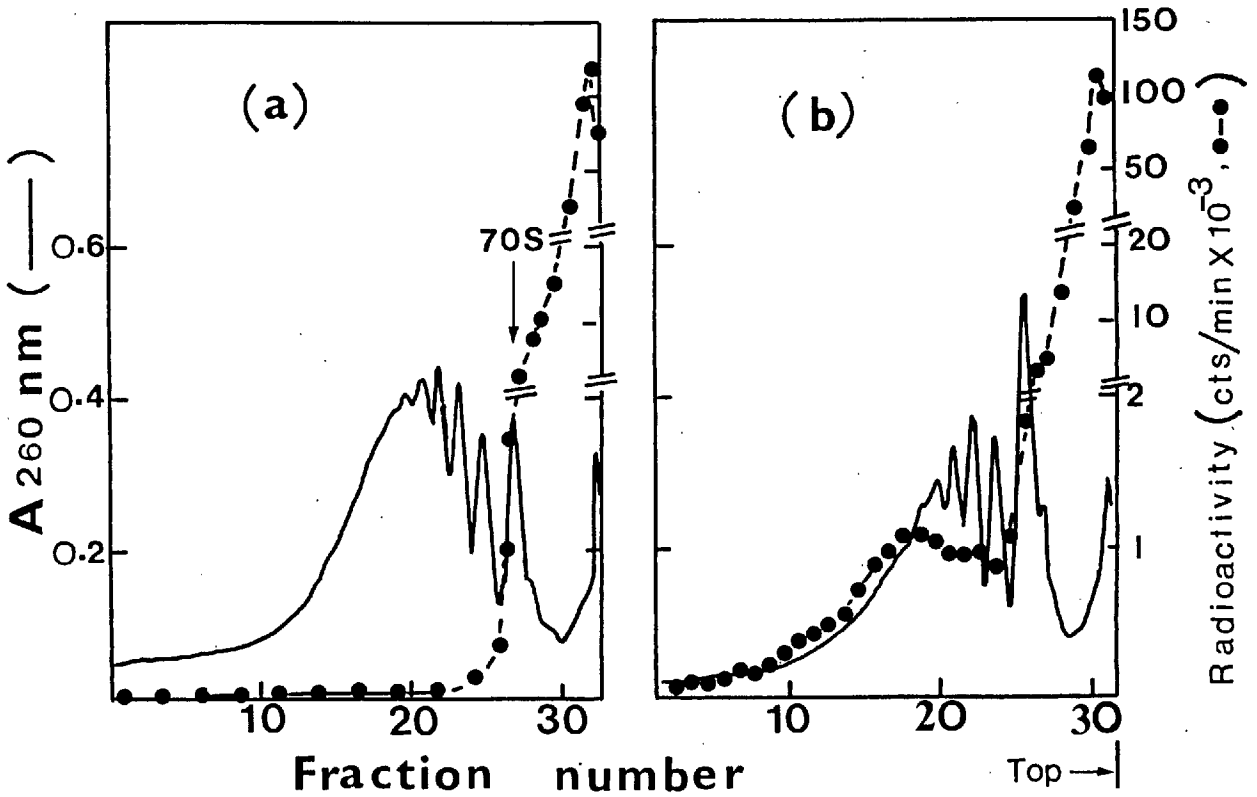


FIG. 7.6. Specific interactions of polysomes involved in RDH synthesis with ^{125}I-pure anti RDH antibodies in presence of heparin. Polysomes (10 A_{260} units) in 1.8 ml of "polysome buffer" containing 10 units/ml of Na-heparin and 0.1M sucrose were incubated for 45 min on ice with 30µg of ^{125}I-anti RDH (specific activity = 7000 cts/min per µg). (a), purified polysomes from K. aerogenes FG-5 strain grown on LB medium up to culture A650 = 0.6; (b), purified polysomes from K.aerogenes Alll strain grown on LB medium up to culture A650=0.75. Both samples were centrifuged in 56 ml continuous (0.5-1.5M) sucrose gradients in a Beckman SW 25.2 rotor at 24000 rpm for 3 hr at 2°C

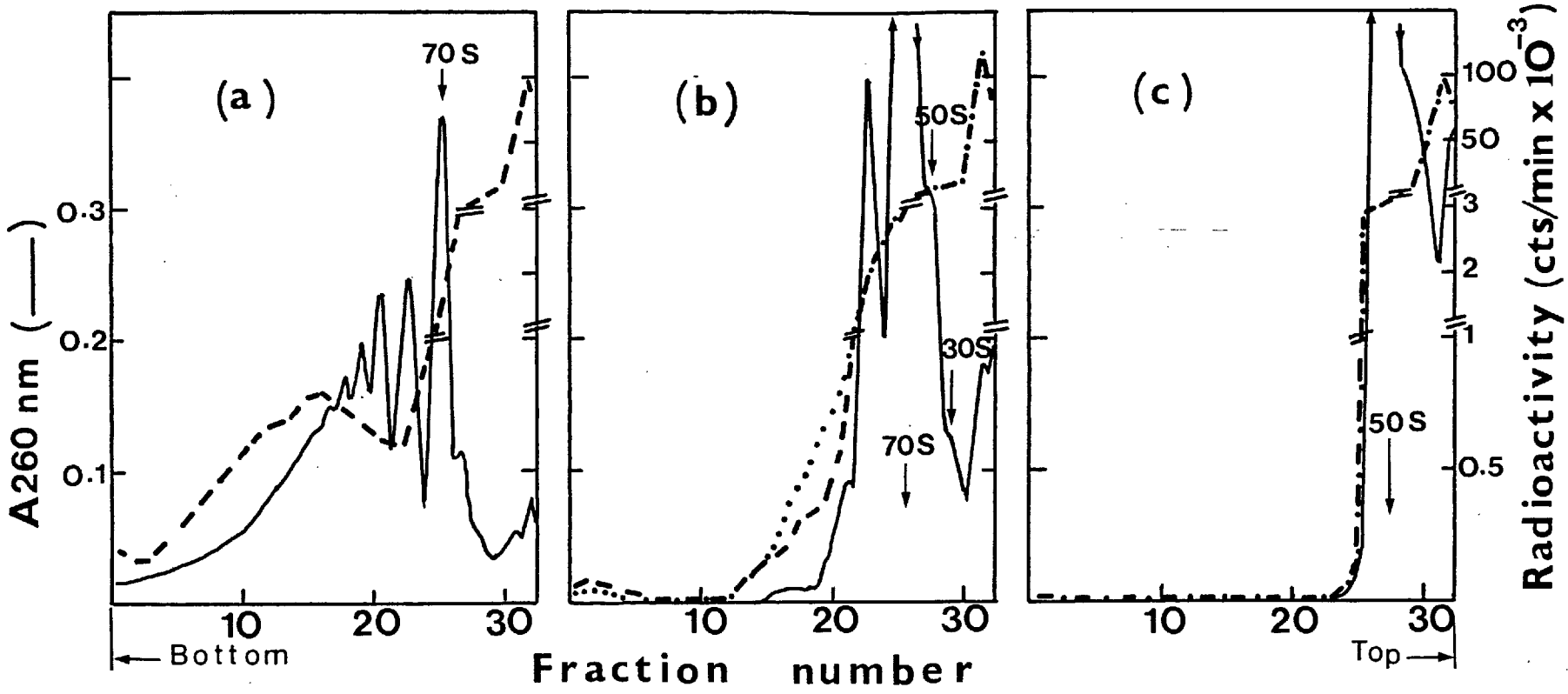


FIG. 7.7. Effects of polysome breakdown by RNAse and EDTA on the binding of anti RDH antibodies. Klebsiella aerogenes Alll polysomes (10 A260 units in 0.8ml) were incubated with 30µg of ^{125}I-labelled anti RDH antibodies and treated as follows: (a), no disruption; (b), enzymic digestion with 10µg/ml of pancreatic ribonuclease-A for 15 min at 2°C either before (- - -) or after (• • •) antibody incubation; and (c), chemical dissociation with 30 mM EDTA for 20 min either before (- - -) or after (• • •) antibody incubation. Centrifugation was at 24000 rpm for 3 hrs at 2°C in 0.5 - 1.5M continuous sucrose gradients in a Beckman SW 25.2 rotor.

the monosome fraction (Fig.7.7b). Treatment of the polysomes with EDTA resulted in restriction of the radioactivity to the upper sucrose gradient fractions, corresponding to the characteristic pattern of antibody diffusion. The polysomes were degraded mainly to the 50S and 30S ribosomal subunits (Fig.7.7c).

H. Conclusion

Polysomes involved in the synthesis of "wild type" RDH from Klebsiella aerogenes high synthesis strain AIII were identified by the binding of monospecific ^{125}I-labelled antibodies directed against the mutant RDH enzyme (D) from the same organism. These interactions were highly specific when heparin was included in the antibody/polysome incubations. They were only 50% specific in the absence of heparin. Neutral detergents, Brij-58 and deoxycholate, were ineffective in reducing non-specific interactions. The specificity of the purified antibodies for RDH synthesizing polysomes was clearly indicated by the total absence of any interaction between the same antibodies and the polysomes prepared from an isogenic K.aerogenes strain which is inducible with respect to the RDH synthesis (FG-5) which was grown on the same broth medium.

The radio-immune-identified RDH polysomes are very sensitive to both pancreatic ribonuclease and to EDTA, co-sedimenting exactly with the degraded polysomes-mainly the monosome fraction - when treated for a limited time with RNAse, and with the ribosomal subunits fractions after EDTA treatment. Such complete sensitivity of the immuno-identified complexes to the reagents which specifically degrade polysomes (particularly ribonuclease) has not been previously demonstrated. Collagen-synthesizing polysomes identified by anti-

collagen antibodies were shown to be only partially sensitive to pancreatic ribonuclease, while the total polysome population was completely degraded in the same experiment (Pawlowski et al., 1975). The radio-immune complexes formed by the incubation of the anti-fibrinogen and rat hepatocytes polysomes were shown to be almost completely insensitive to pancreatic ribonuclease digestion, while they were partially sensitive to EDTA (Bouma III et al., 1975).

The difference between my results and those of these other workers probably lies in the fact that the RDH polysomes were treated with excess anti-RDH antibodies. Under such conditions, only one of the valencies of each divalent antibody will bind to antigen. When ribonuclease cuts between the ribosomes, all the polysomes including the ^{125}I-antibody bound polysomes would be reduced to 70S monosomes. In the previous studies referred to, the antibodies were not added in complete excess (for anti-fibrinogen, only 5 µg of antibodies were added / 10 A260 units of polysomes). Under such conditions, the second valency of an antibody molecule will also bind to an antigen, most probably to a nascent chain attached to a neighbouring ribosome on the polysome string. (This is by the way why antibody/polysome complexes do not effectively precipitate). Treatment with pancreatic ribonuclease degrades the unprotected m RNA between the ribosomes, but the ribosomes will still be held together by the antibody-nascent chains. Similarly, after EDTA treatment the antibody would cross-link the ribosomal subunits, so that rapidly sedimenting material would remain.

Bouma et al. (1975) explained the lack of sensitivity to ribonuclease of fibrinogen antibody-polysome complexes by postulating

some type of peptide assembly of nascent chains still attached to the polyribosome with a small cytoplasmic pool of completed chains. They suggested that some completed subunits exist in cytoplasmic pools and that these unbound subunits could begin disulfide bridging (assembly) with NH_2-terminal alignment to a nascent polypeptide chain prior to its release from the polysome. This complex would be identified by the anti-rat fibrinogen antibody and could be sterically protected from RNAse digestion (Bouma III et al., 1975). However, one can argue that similar results should have been found in our experiments since RDH is a tetramer and there is certain to be a pool of completed subunits which could bind to nascent chains and protect against ribonuclease digestion , as postulated by Bouma et al. (1975). Since we find no such protection, we suggest that the experiments with fibrinogen antibody and polysomes may have been artifactual and should be repeated with fully saturating antibody.

CHAPTER VIII

Characterization of Polysomes Involved in the Synthesis of Ribitol Dehydrogenase and of Naturally Stable Bacterial Polysomes from Klebsiella aerogenes AIII

A. Sedimentation Profile of RDH-Polysomes Before and After "General mRNA Transcription Shut Down"

It was indicated in Chapter VI that in logarithmically growing bacterial cultures two distinct populations of polysomes appear to be present. First, there is a relatively stable population consisting mainly of oligosomes (1-10 ribosomes), peaking at trisomes. Overlapping with these oligosomes there is present a second population of unstable polysomes, very heterogeneous in size, stretching up to at least 50-somes and with a broadly sedimenting peak at about 12-15-somes. While the initial population is believed to be engaged in the synthesis of the smaller structural proteins, such as the outer membrane proteins, the labile, larger polysomes must be engaged in the synthesis of various enzymes and metabolic proteins. In the logarithmically growing cells there is a definite balance between the relative proportions of these two populations. However, at a particular cell density of the culture in the late log phase (corresponding to culture A650 = 0.56 in minimal salt medium) a sudden shift in the total polysome profile takes place (presumably as a result of termination of mRNA transcription); the unstable polysomes dissappear almost entirely while the stabler oligosomes persist for a few hours until the culture eventually reaches the stationary phase.

RDH-synthesizing polysomes have been identified using ^{125}I-monospecific antibodies (Chapter VII). Examination of the size distribution profile, by sedimentation in continuous sucrose gradients, of the ^{125}I-immunoidentified RDH synthesizing polysomes which were prepared before the "mRNA transcription shut down" shows that the number of RDH polysomes present in each fraction decreases as a linear function of the increase in size (number of ribosomes/polysome), giving rise to a "triangular" sedimentation profile with the base of the triangle at the monosomes peak and the apex of the triangle at about 36-somes, as shown in Fig. 8.1a. The major reason for this size distribution is that the RDH-mRNA molecules are in competition with hundreds of other labile bacterial messages for translation. As a result of a limited number of translation factors being present per cell, the sedimentation distribution of RDH polysomes becomes a function of the statistical loading of ribosomes on RDH-mRNA molecules, thus producing the observed heterogeneity. Other speculative but probably less significant reasons for this "triangular"sedimentation distribution of RDH polysomes are: very intense initiation of transcription with respect to RDH operon, and some heterogeneity occurring in the length of a portion of RDH mRNA molecules while they are in coupled transcription-translation.

When the sedimentation distribution of RDH polysomes after the presumed "transcription shut down" is examined (Fig. 8.1b) a distinct change is noticed. The "triangular" size distribution profile of RDH polysomes is converted to a dome-shaped profile. The latter distribution pattern is characteristic of the stable polysomes which have been prepared from various tissues of the higher organisms, specifically immunoidentified and analysed by

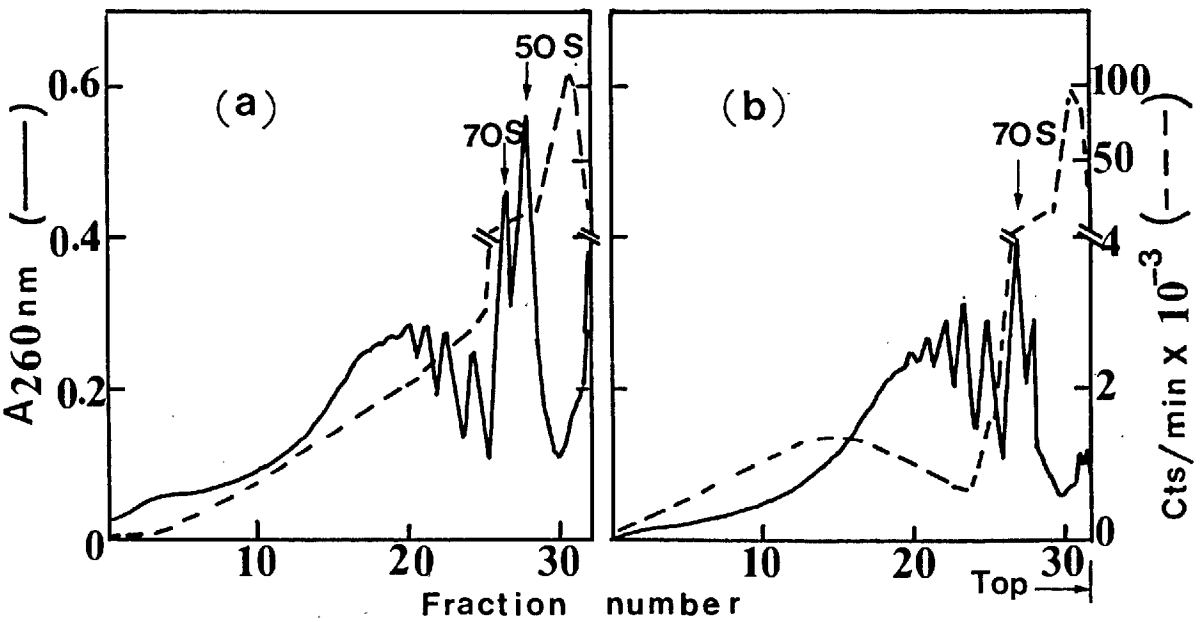


FIG. 8.1. Size distribution of RDH-synthesizing polysomes before and after "the general transcription shut-down". A 1 litre M9/0.2% xylitol culture of Klebsiella aerogenes Alll was grown at 37°C . One half of this culture (a) was harvested at A650nm= 0.30, while the cells from the other half (b) were collected at culture A650nm= 0.56. The cells were lysed and the polysomes purified according to the procedures described in "Materials & Methods". About 10 A260 units of each polysome sample was incubated with excess (30µg) of ^{125}I-labelled anti-RDH (specific activity about 8000 cts/min per µg) in 1.85 ml for 45 min on ice, then the sample was layered carefully on 56ml of a continuous 0.5-1.5M sucrose gradient, and the polysomes sedimented by a 3hr centrifugation at 24000 rpm at 2°C in a Beckman SW 25.2 rotor. A260 and the radioactivity profiles were obtained as described in "Materials & Methods".

sucrose gradient sedimentation (e.g., Palacios et al., 1972; Bouma III et al., 1975; and Pawlowski et al., 1975).

The reason for this sudden change in the sedimentation profile of RDH polysomes before and after "transcription shut down" is probably as follows. As a result of rapid depletion of the labile mRNA population after the "shut down" the entire cellular translation system will become available to the naturally stable mRNA population and to the selectively stabilized RDH mRNA. However, certain rate limiting translation factor(s), perhaps IF-3 for example, will become entirely devoted to the translation of stable RDH-mRNA molecules (which share their origin and other properties than stability with the labile mRNA fraction), since the same factor(s) do not seem to be required by the naturally stable mRNAs. This explanation is supported by the observation that after "transcription shut down" the number of ribosomes loaded per RDH mRNA increases considerably in spite of the vast excess of the naturally stable mRNA over RDH mRNA. The ^{125}I-radioactivity associated with the RDH polysomes, peaks at about 15-somes and stretches smoothly to about 50-somes. It can be seen from Fig. 8.1(a, b) that the proportion of the smaller RDH-synthesizing polysomes (oligosomes) has greatly decreased.

The above observation is very significant, because not only does it provide strong evidence for the stability of RDH-mRNA (the accumulation of larger RDH polysomes together with the disappearance of smaller RDH polysomes), but it also suggests that the naturally stable bacterial mRNAs do not have exactly the same translation factor requirements as the labile bacterial messages (no increase in size of the A260 polysome profile). Perhaps they do not require such factor(s) as IF-3, which is normally very rapidly

depleted in the aging bacterial cultures. This conclusion is independent of the fact that all of the bacterial mRNA may be translated by the same pool of ribosomes.

B. Effect of the Rich Medium on the Frequency of Translation of Stable RDH mRNA by Ribosomes

Purified polysomes prepared from K. aerogenes AIII cells which have been grown on a rich broth medium (such as LB) up to just after "transcription shut down" (culture A650 = 0.9), interacted with ^{125}I-specific anti RDH, and sedimented on a sucrose gradient (0.5 - 1.5M), give rise to the characteristic dome-shaped antibody/RDH polysome profiles. However, these RDH polysomes are much slower sedimenting than when the culture was grown similarly on a minimal medium containing 0.2% xylitol as the sole carbon source. As shown in Fig. 8.2, the sedimentation of RDH-polysomes just after "the transcription shut down" on rich medium extends up to 25-somes and the radioactivity peaks at about 7-somes, while by comparison on M9/Xylitol medium (see Fig. 8.1b) RDH-polysomes extend up to about 50-somes and the corresponding radioactivity peaks at about 15-somes. This observation indicates that in some way, the cells are able to suppress the translation of stable RDH mRNA molecules when the products become redundant or unnecessary.

C. In Vivo Stabilities of RDH-mRNA and the Naturally Stable Bacterial mRNA

The extents of in vivo stabilities of the 'naturally stable' bacterial mRNA and of RDH mRNA from K. aerogenes AIII were investigated by measuring the decay rates of the corresponding polysomes after the specific blocking of transcription process by the simultaneous additions of two antibiotics with different modes

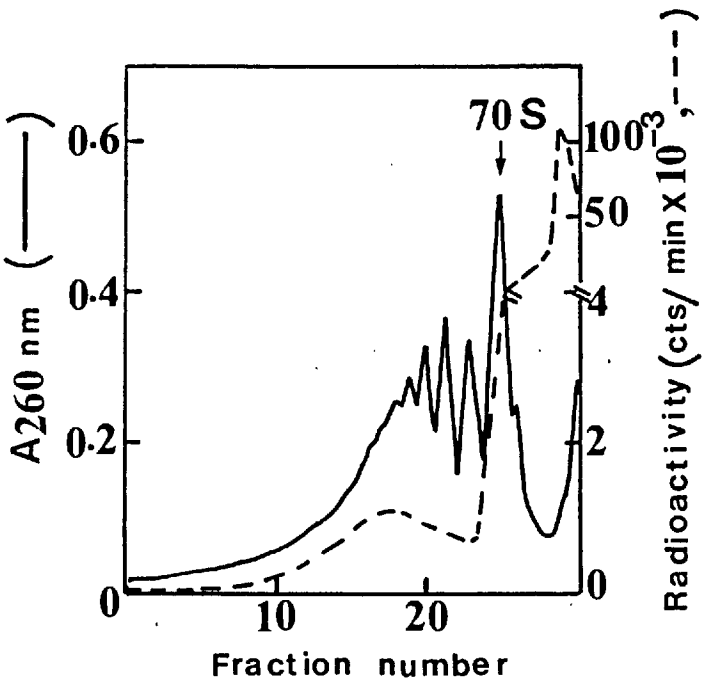


FIG. 8.2. Effect of the rich medium on the frequency of the in vivo translation of stable RDH mRNA. A 500 ml LB medium was inoculated by 20ml of an M9/xylitol culture of K. aerogenes A111 growing at late log phase. When the culture A650nm reached 0.9 (i.e., just after "transcription shut down"), the cells were harvested & lysed, and the polysomes were purified as described in "Materials & Methods". The polysomes and the ^{125}I-labelled pure anti-RDH were incubated together and sedimented on a sucrose gradient as described in Fig. 8.1.

of action, rifampicin and iodouracil, to the culture. Rifampicin is a potent inhibitor of DNA-dependent-RNA polymerase of bacterial (Wehrli et al., 1968) and chloroplast (Surzycki, 1969) origin; it binds specifically to the β-subunit of RNA polymerase (Rabussay D. & Zillig W., 1969; Heil, A., & Zillig, W., 1970). A concentration of 5μg/ml rifampicin strongly inhibits the incorporation of (^{14}C)-uracil into TCA-insoluble material of bacterial origin (Calvori et al., 1965; Hartman et al., 1967).

A 2 litre culture of K. aerogenes AIII was grown at 37°C on M9 + 0.2% xylitol up to culture A650 = 0.6; then the antibiotics were added. 5-Iodouracil was dissolved ½ hours before addition in 25% ammonia, the pH was adjusted to 9.0 with 1N HCl, and 15ml was added to the culture to give a final concentration of 0.1g/l, approx. 0.2mM and final pH of 7.0. Rifampicin (10μg/ml of culture) was weighed shortly before addition, and was added as dry powder at the same time as iodouracil. At 0, 8, 25 and 65 minutes after addition of antibodies, the cells from 500ml portions of the culture were harvested and lysed as in "Materials & Methods". After an initial 6000 x g centrifugation for 5 minutes to remove the cell debris and membranes, the entire population of polysomes plus ribosomes and 30S and 50S ribosomal subunits was collected by a centrifugation for 15 hours at 40,000rpm in a Beckman 60Ti rotor at 2°C, filtering through a cushion of 1.5M sucrose. The supernatant was carefully removed without disturbing the pellet, and the pellet was not rinsed.

Polysomes (approximately 20 A650 units) in 1.5ml were incubated with excess (55μg) of pure ^{125}I-anti RDH antibodies (specific activity = 4200 CPM/μg) for 60 min on ice, then layered on 57ml of

0.5-1.5M linear sucrose gradients and centrifuged for 3 hours at 24000rpm in the Beckman SW25.2 rotor at 2°C. The A260nm and the radioactivity profiles of each sample after sedimentation were obtained as described in the "Materials & Methods", and they are shown in Fig. 8.3(a-f). It is clear from the comparisons of these profiles that the break down of the polysomes (the "stable" polysomes) begins shortly after the addition of antibiotics to the culture, the A260nm of the polysome fractions has reduced and it has given rise to an equivalent increase in the 70 S monosomes and the ribosomal subunits fractions, this change is noticeable even 8 minutes after addition of the antibiotics. However, there is not much visible change in the specific activity of RDH synthesizing polysomes (the radioactive 'hump'/total A260nm).

The precentage of the "stable" polysomes and the RDH-synthesizing polysomes remaining at various times after additions of rifampicin and 5-iodouracil to the K. aerogenes AIII culture, calculated from the profiles a-f in Fig, 8.3., are shown in Table 8.1. When these percentages are plotted as a function of time after transcription block, as shown in Fig. 8.4., it can be seen that the "stable" polysomes have decayed linearly with a half-life of 75-80 minutes, while the RDH-polysomes appear perfectly stable with no measurable decay. The solid line shown passing through each set of points is the statistical line of best fit (regression line of x on y) which was computed by the method of least squares.

A superstable RDH mRNA with no decay may seem unreal, but it will become even more convincing if we refer to Fig. 3.6. and see that RDH enzyme activity increases linearly for at least 8 hours after the transcription shut down (culture A650nm = 0.6 on M9/

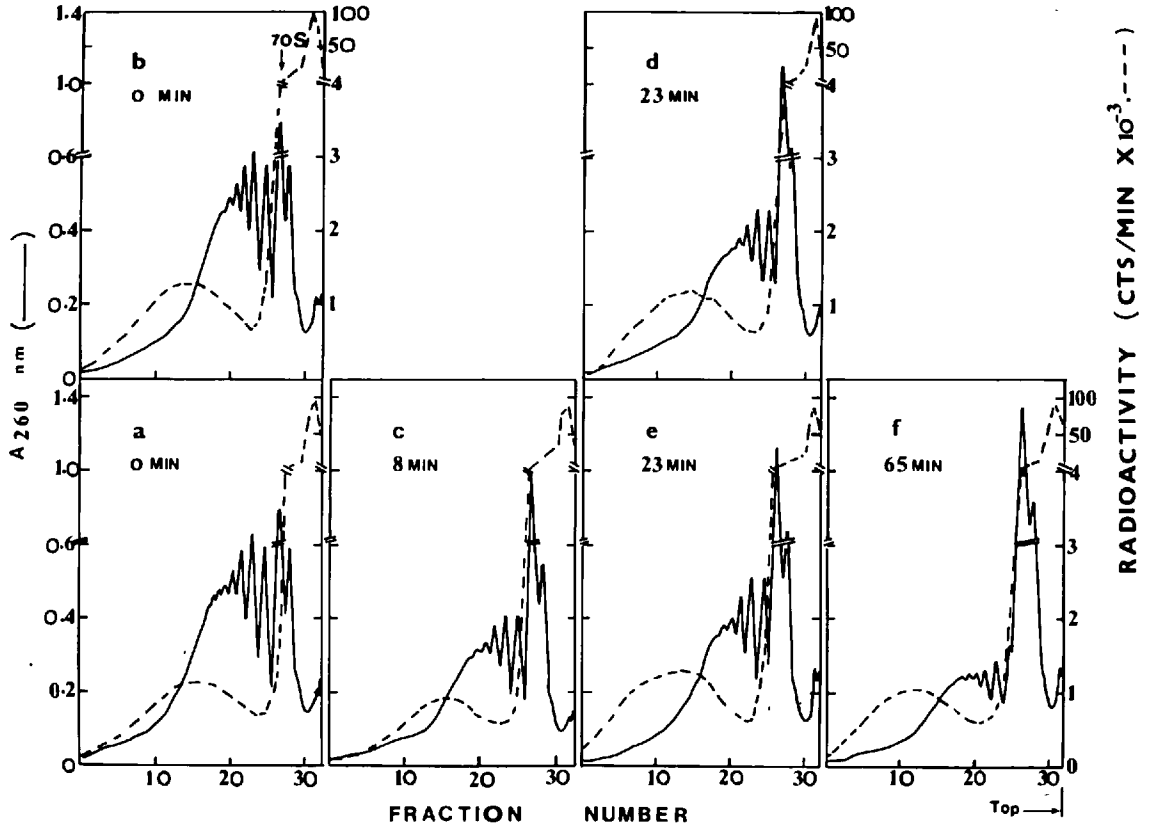


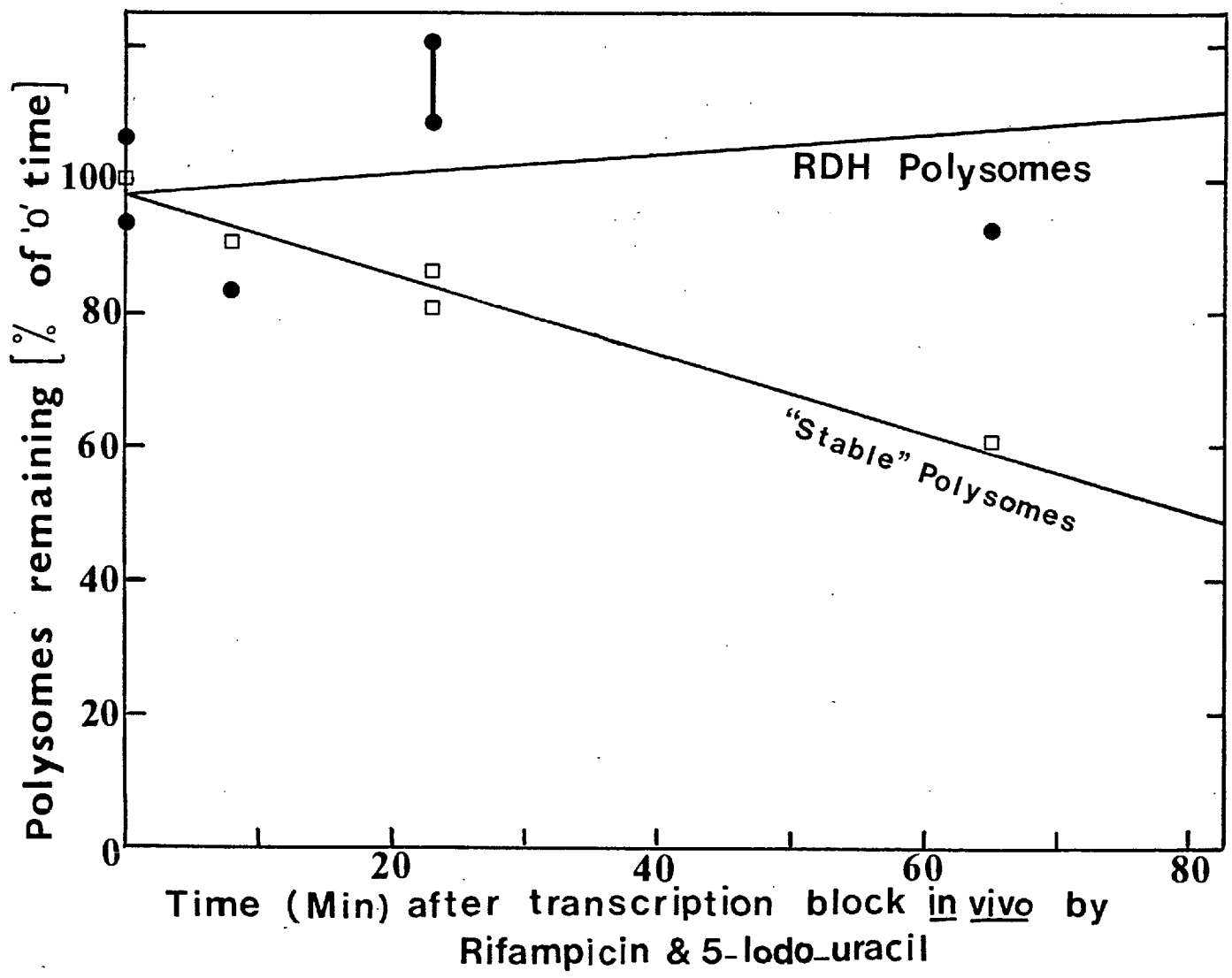
FIG. 8.3. In vivo stabilities of the RDH-synthesizing and the naturally stable polysomes from K. aerogenes A111 after additions of rifampicin (10μg/ml) and 5-iodouracil (0.2 mM) to the M9/xylitol culture at A650 nm = 0.55. The cell harvest times after additions of the antibiotics to the culture are indicated on sedimentation profiles a-f. The centrifugation conditions are as in Fig. 8.1.

TABLE 8.1

The Percentages of the "Stable" and the RDH Synthesizing Polysomes Remained at Different Times After Additions of Rifampicin and 5-Iodouracil to the Culture

Profile (Fig.8.3)	Minutes after transcription block	Total A260 units of polysomes + ribosomes + ribosomal subunits	A260 units associated with "stable" polysomes (fractions 14-25 inclusive)			RDH-synthesizing polysomes (total CPM present in fractions 1-21 inclusive)		
			Obs.	Adjusted with respect to total A260	% of zero time	Obs.	Adjusted with respect to total A260	% of average value of the two '0' times
(a)	0	20	-	-	-	18793	18793 (AV=20116)	93.4 (100)
(b)	0	(20)	11.8	11.8	(100)	21439	21439	106.6
(c)	8	15.8	8.4	10.7	90.7	13223	16744	83.2
(d)	23	18.4	8.8	9.5	80.9	20074	21829	108.5
(e)	23	19.3	9.8	10.2	86.5	23449	24341	121.0
(f)	65	18.3	6.6	7.2	60.9	16948	18559	92.3

FIG. 3.4. The percentages of the naturally stable polysomes and the ribitol-dehydrogenase super stable polysomes of K. aerogenes Alll survived as a function of time after the specific in vivo transcription block by the additions of the antibiotics, rifampicin and 5-iodo-uracil to the culture.



0.2% xylitol medium), i.e., about 3 hours after the culture had reached the stationery phase (this was the final determination).

D. Estimation of the Length of RDH mRNA from the Maximum Number of Ribosomes Attached per mRNA Molecule

Since we have established the superstability of RDH mRNA, then the large degree of heterogeneity in sedimentation of radioimmune-identified RDH polysomes observed in sucrose gradient can only be attributed to variability in the number of ribosomes engaged in translation of each mRNA molecule. The use of excess antibodies/polysomes in these experiments ensures that there will be no significant dimerization or polymerization of the polysomes, as judged by the smoothness of the heavy shoulder of radioactivity in each profile. Dimerization, trimerization, etc., should produce a number of periodic peaks.

In Chapter VI it was shown that the number of ribosomes present per polysome at any fraction in a sucrose gradient sedimentation can be estimated, from a double logarithmic plot of the peaking distance of each visible oligosome from the top of the gradient as a function of the number of ribosomes present per polysome. By this method, the range of ribosomes attached to each RDH mRNA was estimated both before and after "The general mRNA shut down" for K. aerogenes AIII, (Table 8.2). From this Table it can be seen that before "shut down" about 66% of RDH mRNAs are translated by only 1-4 ribosomes, while after the "shut down", 65% of the RDH mRNAs are translated by greater than 10 ribosomes.

The maximum number of ribosomes attached per RDH mRNA is about 55. Assuming that this is the largest number of ribosomes

TABLE 8.2

Proportional Representation of Number of Ribosomes Attached Per RDH-mRNA for K.aerogenes AIII Cells Growing on M9 + 0.2% Xylitol Before and After "General mRNA Shutdown", at Culture A650nm = 0.6

No. of Ribosomes	Before Transcription Shutdown			After Transcription Shutdown		
	CPM	Proportion	%	CPM	Proportion	%
1	2300	2300	31.6	0	0	0
2	2300	1150	15.8	100	50	1.8
3	2350	783	10.8	200	67	2.5
4	2200	550	7.6	550	137	5.1
5	2000	400	5.5	700	140	5.2
6	1900	316	4.3	800	134	5.0
7	1800	257	3.5	1000	143	5.3
8	1750	219	3.0	1050	131	4.8
9	1600	178	2.4	1200	134	5.0
10	1450	145	2.0	1250	125	4.6
11-15	1300	100 x 5	6.9	1250	96 x 5	17.8
16-25	700	35 x 10	4.6	1200	60 x 10	22.2
26-35	240	8 x 10	1.1	1000	33 x 10	12.2
36-45	0	0	0	700	17 x 10	6.3
46-55	0	0	0	300	6 x 10	2.2

that an RDH mRNA can accommodate (i.e. the ribosomes almost touching one another), also assuming the length of messenger occupied by each ribosome is 20 nucleotides (Morse, Mosteller and Yanofsky, 1969), then the RDH mRNA would be about 1100 nucleotides in length. This is long enough to contain information coding for one RDH polypeptide of about 250 residues long. However, if the ribosomes on a maximally loaded RDH mRNA (55-somes) on the average are 10 nucleotides apart from each other, then the 1650 nucleotides long mRNA could possibly bear coded information for 2 polypeptides.

E. Termination, or Modification of Transcription at *mid* Log-Phase?

Disappearance of the heavy shoulder of bacterial polysomes with concomitant changes in the size distribution of RDH superstable polysomes at *mid* log phase (culture A650=0.55) indicate that transcription with respect to labile messages (including the selectively stabilized RDH superstable mRNA) is terminated or greatly decreased at that point in culture growth, but it does not indicate that the same applies to the naturally stable bacterial mRNA. It is quite possible that the transcription of the stable mRNA corresponding to the structural proteins will go on (and even be enhanced as a result of certain modification of transcription process at late log) while the transcription with respect to the labile mRNA (which correspond to various enzymes and metabolic proteins) is specifically terminated. In order to distinguish between these possibilities, we must determine whether or not the ratio of "stable-polysomes" to "RDH-polysomes" increases when measured at some time after this "shut down" point. Coupled to this question, we are asking another practical question: Can we enrich the superstable RDH polysomes by simply allowing the bulk of the

bacterial polysomes ("stable polysomes") to decay naturally in an aging culture after the "general transcription shut down"?

The answer is yes: The "shut down" occurs generally, at least with respect to mRNA, and the RDH polysomes can be enriched by allowing the natural decay of the "stable" polysomes to proceed after the general transcription shut down. Thus, a 2 litre culture of K. aerogenes AIII strain was grown on M9+ 0.2% xylitol at 37°C up to culture A650 = 0.50, then it was subcultured into equal halves. One culture was harvested at A650 = 0.55, the other at A650 = 1.02. The cells were lysed and the polysomes were purified as in section D, above. Approximately equal amounts of the two polysome samples were incubated on ice for 1 hour with saturating quantities of ^{125}I-anti RDH (55µg), layered on to 0.5-1.5M continuous sucrose gradients, centrifuged and analyzed as before.

Fig. 8.5 shows the comparative radioactivity and A260 sedimentation profiles of the two polysome samples harvested at (a) the beginning of "trancription shut down" (culture A650 = 0.55), (b) approximately 1 hour after "shut down" (culture A650 = 1.02). It is quite clear by looking at these profiles that while the relative proportion of the RDH-synthesizing polysomes is unchanged, there is a significant reduction in the "stable polysomes" (A250 units associated with di-deca-somes fractions) and an equivalent increase in the A260 of the ribosomal fractions.

The relative proportions of each type of polysome are computed from the appropriate areas under the profiles in Fig. 8.5 (a and b) and are shown in Table 8.3. If we look at the lines (1) and (2) we can see that the proportion of the total polysomes/(polysomes + ribosomes + subunits) has decreased form about $^{2}/_{3}$ ($^{8.3}/_{12.7}$) to about $^{1}/_{2}$ ($^{7.3}/_{15.5}$), only one hour after the "shut down". If the

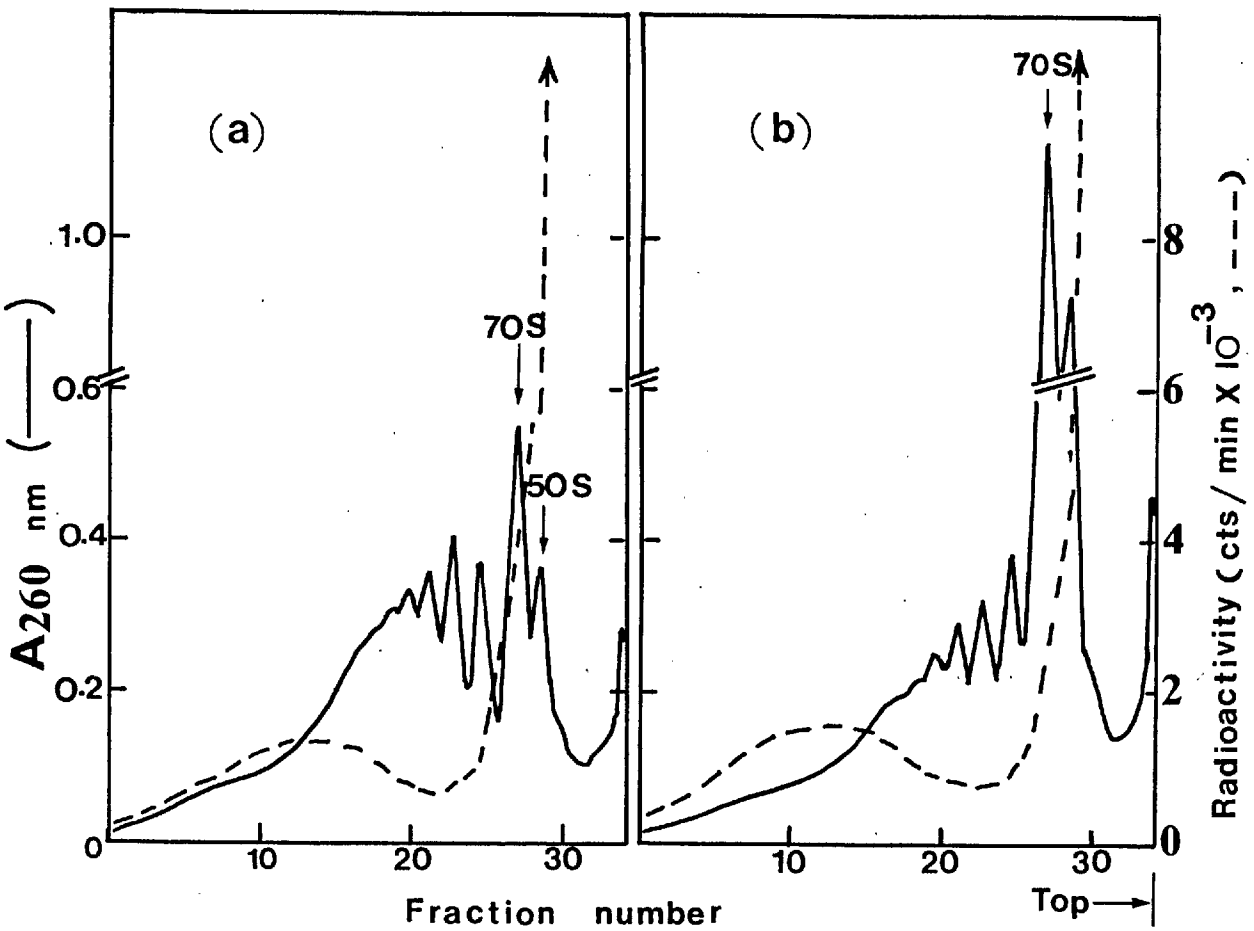


FIG. 8.5. Natural decay of the 'stable' bacterial polysomes and the stability of the RDH-super stable polysomes from Klebsiella aerogenes strain Alll cells, grown on M9+0.2% xylitol medium, after the "general transcription shut down". The polysome sedimentation profiles correspond to: (a), the cells harvested at culture A650nm= 0.55; and (b), at A650= 1.02.

TABLE 8.3

Calculation of the relative amounts of RDH- and "Stable" - polysomes

		At the Beginning of "Transcription Shut Down" (Culture A650=0.55)	Approx. 1 hour after "Transcription Shut Down" (Culture A650=1.02)	
(1)	Total A260 Units (Polysomes + Ribosomes + Ribosomal Subunits)	12.7	15.5	
(2)	A260 Units of total Polysomes (from 2 x 70S to the largest)	8.3	7.3	
(3)	A260 units of the "Stable Polysomes" (di-deca-somes)	6.3	5.1	
(4)	Total radioactivity Associated with RDH-Polysomes (Fractions 1-22)	2.04×10^4	2.50×10^4	
(5)	Ratio: "Stable - Polysomes"/RDH Polysomes; (3)/(4)	3.1×10^{-4}	2.2×10^{-4}	
(6)	Specific Activity of RDH-Polysomes; (4)/(1)	1.6×10^3	1.6×10^3	
(7)	Specific Activity of "Stable Polysomes"; (3)/(1)	0.50	0.33	(66%)

transcription were to become modified in favour of "stable mRNA", then the ratio "stable polysomes"/RDH-polysomes would have increased, but in line (5) we observe that the opposite result is obtained, in fact the ratio has decreased from 3.1×10^{-4} to 2.2×10^{-4}, proving that the "transcription shut down" occurs generally, applying to both stable and labile mRNA. Lines (6) and (7), respectively, show that the specific activity of RDH-polysomes with respect to the entire population of polysomes + ribosomes and subunits is unchanged, while the specific activity of the "stable polysomes" has decreased to 65% of its value at the beginning of "transcription shut down" (culture A650 ='0.55). RDH-polysomes have been considerably enriched in the polysomal fractions, and for practical purposes this is quite useful, since by appropriate timing of centrifugation one can pellet only the polysomes while leaving the monosomes and smaller particles unprecipitated in the sucrose layer.

F. Cellular Location of the "Stable"-Polysomes and the RDH-Super Stable Polysomes

The locations of the "stable" polysomes and the super stable RDH polysomes in K. aerogenes AIII cells were investigated for two separate reasons. The observed characteristics of the 'naturally stable' polysomes are: (1) size - about 200 nucleotides long, (2) half life - about 75 minutes, (3) relative abundance - greater than 70% of the total polysome population at culture A650 = 0.6, (4) exclusive translation at the late log phase for the normal cells. From this one would expect that these polysomes may be engaged in the synthesis of outer membrane proteins, the polypeptide products of which would be exported through the inner into the outer membrane. Therefore, it would be highly probable that the majority of these "stable" polysomes should be firmly attached to the membranes

via their nascent polypeptides which are in the process of being transported into the outer membrane as they are being synthesized. In contrast, since the products of RDH polysomes are released into the cytoplasm, one would not expect their polysomes to be membrane bound unless the selection for stability has somehow involved such attachment.

Godson (1967) reported that if E. coli cells harvested in the early log phase were shocked by lysozyme-EDTA and subsequently treated with Brij 58 or Lubrol W only, 70% of the RNA was released, i.e., most of the polyribosomes. The polyribosomes that remained attached to the low speed sediment could be released with deoxycholate and DNAse, and when these were examined on a sucrose gradient, they did not appear to differ from the released polyribosomes. If deoxycholate and DNAse were added to the lytic mixture, all but a few per cent of the RNA was released immediately from the slow speed sediment, although in that case the DNA was sompletely degraded.

Hence, the selective use of these detergents, Brij 58 and deoxycholate seemed like a good technique for separating membrane bound and free polyribosomes from one another. I used this method to investigate the locations of the "stable" and RDH-superstable polysomes in the cell and to see whether this would yield a purification of RDH-polysomes.

Thus, an M9 + 0.2% xylitol culture of K. aerogenes AIII was grown to culture A650 = 0.55; then it was split into two equal halves. The cells harvested from one half were lysed in the usual way, by lysozyme-EDTA/Brij 58/deoxycholate/and DNAse, the cells from the second half were lysed only by lysozyme-EDTA/Brij 58. The polysomes from each sample were purified by the standard method.

The yield of the total polysomes + ribosomes from the second preparation was only 25% of the first standard method, indicating that 75% of the polysomes + ribosomes in that sample had remained bound to the membranes which were removed by slow speed centrifugation.

About 13 A260 units of each polysome sample was incubated with 55µg of ^{125}I-labelled anti RDH; layered on a continuous 0.5 - 1.5M sucrose gradient, sediemented and fractionated under standard conditions. One glance at the comparative sedimentation profiles of these polysomes samples, Fig. 8.6 (a and b) clearly shows that in the case where deoxycholate and DNAse were left out, the A260 corresponding to the "stable" polysome fractions has greatly diminished, while the A260 corresponding to the larger polysome fractions has significantly increased, also the ribosomal subunits and the 70S monosomes constitute a large proportion of the total A260 units in profile (b), Fig 8.6. ^{125}I-anti RDH-bound RDH-polysomes show a very significant enrichment when deoxycholate and DNAse were left out.

The comparative data calculated from these profiles are shown in Table 8.4. The total amount of each polysomes + ribosomes preparation (A260 units) reacted with ^{125}I-anti RDH and analyzed on a sucrose gradient is shown in the first line. The A260 units, corresponding to the polysomal fractions (2 x 70S and larger) of each sample are indicated in the second line. Total ^{125}I-radioactivity in the total polysome fractions, representing RDH-polysomes in each sample, is shown in the third line. The fourth line shows the specific activity of RDH-polysomes in the total polysomes in each case (CPM/A260 units). The specific activity of RDH-polysomes in (b) has increased >3-fold (from 2.6×10^3 to 8.2×10^3). The specific activity of RDH-polysomes in the entire polysomes + ribosomes +

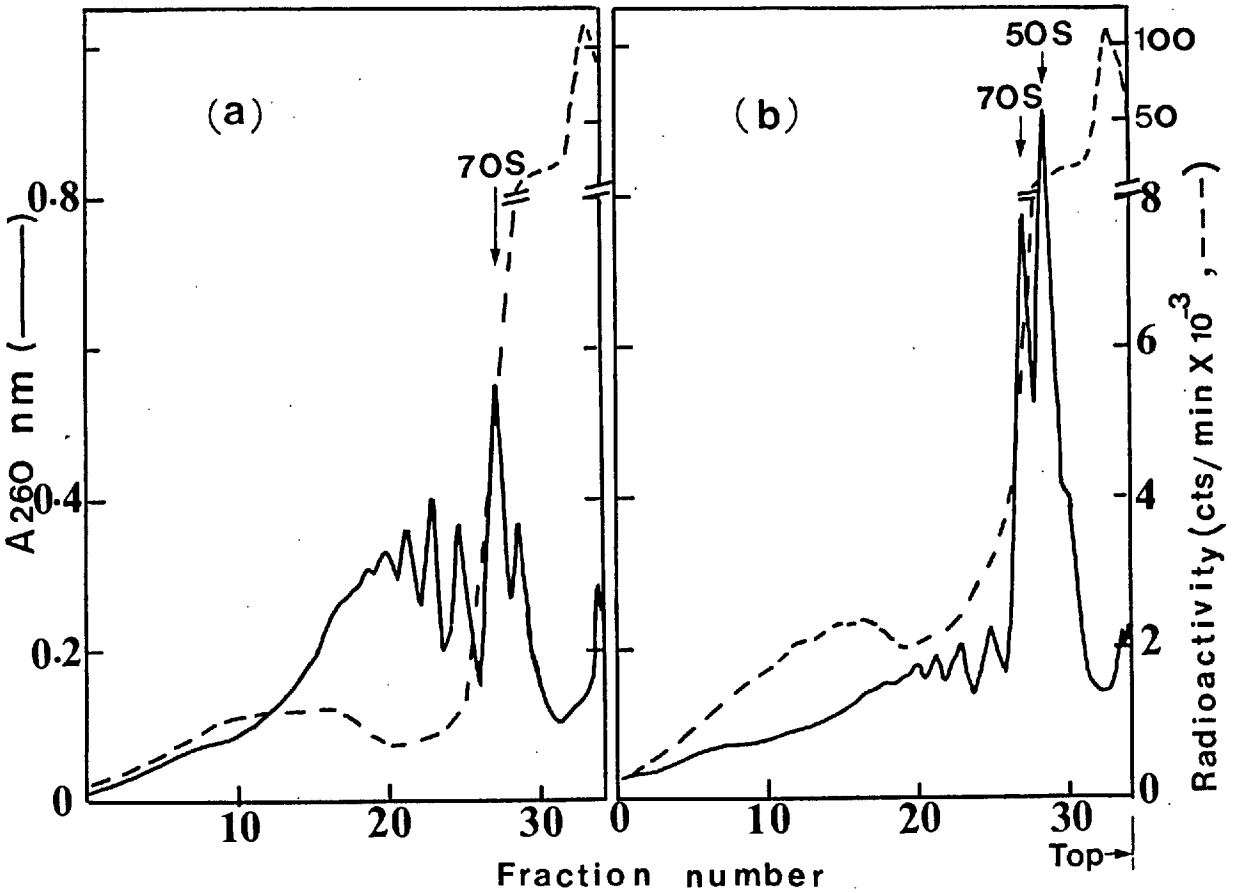


FIG. 8.6. Cellular locations of the "stable" polysomes and the RDH super stable polysomes in Klebsiella aerogenes Alll. (a), cell rupture was by the standard technique, lysozyme-EDTA/ Brij 58/ deoxycholate/ DNAse; (b) lysis was by lysozyme-EDTA/ Brij 58 only.

TABLE 8.4

Enrichment of RDH-Polysomes Extracted from K. aerogenes AIII by Selective Use of Detergents During Cell Lysis

		Lysis by Lysozyme/ EDTA + Brij 58 + Deoxycholate + DNAse	Lysis by Lysozyme/ EDTA + Brij 58 - Deoxycholate - DNAse
(1)	Total A260 units (Polysomes + Ribosomal subunits)	12.5	13.9
(2)	A260 units of Polysomes only (2 x 70S and Larger Polysomes)	8.3	5.3
(3)	Radioactivity Associated with RDH-Polysomes (Fractions 1-25)	21400	43600
(4)	Specific Activity of RDH-Polysomes in Total Polysomes Fractions: (3)/(2)	2.6×10^3	8.2×10^3
(5)	Specific Activity of RDH-Polysomes in the Entire Polysomes + Ribosomes + Ribosomal Subunits Population: (3)/(1)	1.7×10^3	3.1×10^3

ribosomal subunits population is shown in the fifth line, about 2-fold enrichment is achieved in this case (from 1.7×10^3 to 3.1×10^3).

From these data we can conclude (i) that the naturally "stable" polysomes are membrane bound, this conclusion strengthens the idea that they are engaged in the synthesis of outer membrane proteins, (ii) RDH-super-stable polysomes occur free of membranes in the cytoplasm, (iii) RDH-polysomes can be enriched at least 3-fold by this method, simply by avoiding the use of deoxycholate and DNAse during the cell lysis and selectively sedimenting polysomes while leaving ribosomes in solution

G. Conclusion

Studies on the size distribution profiles of the total bacterial polysomes and the ^{125}I-anti RDH bound-polysomes at early and late logarithmic growth of culture and observations of the decay rates of bacterial polysomes with or without additions of the specific inhibitores of transcription to the culture, strongly suggest that two distinct classes of mRNA of different stabilities exist in normal bacterial cells. The first is a very labile population, corresponding presumably to the synthesis of most bacterial enzymes and metabolic proteins. These mRNAs are very efficiently translated by active ribosomes and they are very heterogeneous in size; some of them could have greater than 50 ribosomes attached per molecule of mRNA.

The second class of bacterial mRNA is a comparatively more homogeneous population, rather short in length (about 200 nucleotides long, since each mRNA appears to be attached to 1-10 ribosomes, under the most favourable conditions at late log phase). They are

believed to be engaged in the synthesis of small structural proteins such as the outer membrane proteins. Their average half-life estimated by polysome decay, equals 75-80 minutes.

In the RDH-high-synthesis strain, K. aerogenes AIII, the RDH mRNA falls into a special class of its own. Though it appears to have many of the characteristic features of the labile mRNA, it displays an unusual stability with no measureable decay. Both the present studies on RDH polysomes as well as the previous studies on the kinetics of RDH-enzyme synthesis (Chapter III) strongly support this conclusion.

At late log phase there is a sudden depletion of the labile polysomes population and a concomitant increase in the average number of ribosomes attached to superstable RDH-mRNA with virtual disappearance of the smaller RDH polysomes. This I interpret as termination of transcription process at the late log phase which could occur either generally or only with respect to the labile mRNA. However, joint considerations of the decrease in specific activity of the "naturally stable" polysomes, constancy of the specific activity of RDH superstable polysomes, and the increase in the ratio of RDH polysomes/"stable" polysomes at the late log, suggest that the shut down of mRNA synthesis applies generally.

Fractionation of bacterial polysomes by using appropriate detergents demonstrated clearly that the super stable RDH-polysomes are free in the cytoplasm, while the majority of the "naturally stable" mRNAs are attached to the cell membranes. The latter observation lends further support to the notion that the polypeptide products of these membrane bound, stable polysomes are continuously transported into the outer membrane while they are being made.

It appears that the stable, membrane bound mRNAs may have different translation factor requirement from the labile mRNAs (including the superstable RDH mRNA which essentially belong to the latter class). The frequency of attachment of ribosomes to RDH superstable mRNA increases significantly after the transcription shut down, in spite of the vast excess (70-90%) of the "stable" mRNA present in the cells, both before and after the general transcription shut down. One can see a possible link between this observation and the unusual puromycin resistance of the biosynthesis of the outermembrane proteins (lipoproteins), reported by Hirashima et al., (1973). On that basis they had also suggested that the envelope proteins of E. coli appear to be biosynthesized in a somewhat different manner from that of the cytoplasmic proteins. Another interesting observation is that when the K. aerogenes AIII cells were grown on a rich broth medium when the accumulation of RDH enzyme is no longer needed by the cells, the superstable RDH mRNA molecules appear to be significantly less loaded with ribosomes, as if the cells somehow manage to suppress the translation of a temporarily unwanted but constitutively made product.

The length of the RDH-superstable mRNA can be estimated from the combination of the maximal load of ribosomes and the number of nucleotides covered by a single ribosome (Morse et al., 1969). It is at least 1000 nucleotides long, but considering the heterogeneity of ribosome load per mRNA molecule as observed with RDH mRNA it could be up to 1700 nucleotides long, i.e. it could code for one or two polypeptide(s).

Because the RDH polysomes are found to occur free in the cytoplasm, it proves that the stability of RDH mRNA is not caused

by some form of association with the membranes, but this explanation may still hold true with respect to the metastable membrane bound mRNAs.

The finding that one can considerably purify RDH super-stable polysomes (a) by aging the culture, (b) by avoiding the use of deoxycholate and DNAse, which then would leave the "stable" polysomes membrane bound, and (c) by appropriate sedimentation timing, is of great technical value. Also it cuts on the costs of such expensive products as electrophoretically purified "RNAse-free" deoxyribonuclease, when quantitative yields of polysomes are required.

CHAPTER IX

Hybridisation of RDH-mRNA to Complementary DNA (λ^{rbt^+}-DNA)

A. Introduction

The unusual stability of RDH mRNA of the high synthesis strain K. aerogenes AIII has been demonstrated in the last chapter. However, the validity of the experiments involving the specific interaction of the polysomes with ^{125}I-labelled anti RDH might be questioned. For example, since RDH enzyme constitutes 30% of the total soluble protein of K. aerogenes AIII it may be said that some of the RDH protein becomes non-specifically attached to ordinary polysomes and subsequently attracts the ^{125}I-labelled anti RDH antibodies.

But such a hypothesis does not explain the distinct change in the sedimentation pattern of RDH polysomes as a result of the general transcription shut down, nor does it explain specific enrichment of RDH polysomes (a) by aging the culture after the transcription termination and (b) by selective isolation of the cytoplasmic polysomes using Brij-58 and lysozyme-EDTA only. Secondly the stability of this mRNA is independently supported by the characteristics of RDH enzyme production described in Chapter III. Thirdly the polysomes reacted with the specific anti RDH were initially purified by passage under ultra-centrifugation through an 11ml layer of 1.5M sucrose, which is impermeable to soluble proteins and small molecules. Fourthly, in the rare event of any contamination of polysomes by RDH, presence of heparin in the polysome/antibody incubation mixture also in the sucrose gradients, as it was shown in Chapter VII,

eliminates any non-specific binding of proteins to polysomes.

Nevertheless, the ideal way to show that the immunoidentified polysomes actually contain RDH mRNA is to extract that mRNA, place it in an in vitro protein synthesizing system and demonstrate that it can programme the synthesis of RDH polypeptide for some length of time. The second best method is the specific hybridization of the polysomal RNA (containing RDH mRNA) to the complementary DNA. The latter seemed the easier of the two methods since the RDH gene by this time had been integrated into the genome of λ-bacteriophage by one of my colleagues in this Department (M. Neuberger, Ph.D. Thesis 1977). Therefore, the hybridization method was initially tried to provide further support for the following points:

(a) The fractionated polysomes actually contain RDH-mRNA.

(b) Considerable enrichment of RDH polysomes (therefore of RDH-mRNA) can be achieved by (i) selective extraction of the polysomes from aged cultures using Brij-58, (ii) sucrose gradient fractionation.

(c) RDH mRNA is very stable in K. aerogenes AIII strain.

B. Preparation of Polysomal RNA for Hybridization to λ^{rbt^+}-DNA

Two 200ml cultures, (i) K. aerogenes FG-5 (RDH low synthesis, inducible strain), growing on M9 + 2% casamino acid + 0.2mM guanine, (ii) K. aerogenes AIII, growing on the same medium + 0.2% xylitol, were supplemented at early logarithmic growth with 5,6-^{3}H-Uridine (1μCi/ml) and long labelled for about 4 hours until the culture A650nm reached 0.8. The cells in each case were harvested and lysed in the standard way (including deoxycholate and DNAse) and the polysomes were purified by passage through a cushion of 1.5M sucrose and pelleting. The RNA from ¼ of each polysome sample was purified by phenol extraction ("Materials & Methods"). The two RNA samples were designated RNA-1 and RNA-4, corresponding

to FG-5 and AIII polysomes respectively. Comparative hybridisation of RNA-1 and RNA-4 to $\lambda\frac{rbt^+}{}$-DNA should indicate if AIII polysomes contained RDH mRNA.

The remaining ¾ portion of each of the above polysome samples was layered on a continuous 0.5 - 1.5M sucrose gradient and centrifuged in a Beckman SW25.2 rotor, at 24,000rpm for 3 hours. Fractions 1-20 and 21-36 in each case were pooled separately. Fractions 21-36 should be considerably richer in RDH-polysome content in the case of AIII polysomes. Polysomes in each lot of pooled fractions were pelleted by centrifugation in a Ti-60 rotor at 40,000rpm overnight. The RNA from each pelleted polysome sample was purified by phenol extraction and designated, RNA-2 and RNA-3 (corresponding to fractions 1-20 and 21-36 respectively of FG-5polysomes) and RNA-5 and RNA-6 (corresponding to fractions 1-20and 21-36 respectively of AIII polysomes). Comparative hybridization of these RNA samples should indicate whether RDH-mRNA is predominantly associated with the rapidly sedimenting polysomes of AIII strain only.

Another AIII culture (500ml) was grown in the same medium as above with 1μCi/ml of 5,6-^{3}H-Uridine up to culture A650 = 0.8. After the addition of chloramphenicol, the culture was split into 100ml and 400ml portions. The cells harvested from 100ml portions were lysed by the standard technique (lysozyme-EDTA/Brij-58/ deoxycholate/and DNAse). The cells from the 400ml portions were lysed without deoxycholate and DNAse. Polysomes from each lot were purified as before. Polysomes from the 100ml culture were directly phenol extracted, and the RNA prepared from them was called RNA-7. Polysomes from the 400ml culture were divided into ¼ and ¾ portions. The ¼ portion was phenol extracted, and the RNA

from this was designated RNA-8. The ¾ portion of the latter polysome sample was layered on 0.5-1.5M sucrose gradient and centrifuged as above. Fractions 1-22 and 23-36 were separately pooled, the RNAs extracted from the corresponding pelleted polysomes were designated respectively, RNA-9 and RNA-10. Comparative hybridization results with respect to RNAs 7 and 8 should demonstrate the enrichment of RDH mRNA by leaving the "stable" bacterial messenger bound to the membranes. RNAs 9 and 10 will show further enrichment of RDH mRNA by sedimentation in sucrose gradient.

If these experiments are successful, then they automatically provide further proof of stability of RDH mRNA in AIII, because the ^{3}H-uridine introduced into the culture will be completely taken up by the stable cellular RNA within 20 minutes and the presence of ^{3}H-RDH mRNA 4 hours after addition of the labelled uridine to the medium could only mean that this mRNA is very stable.

Another direct way of determination of the stability of RDH mRNA in AIII cells would be to grow a saml culture (20-30ml) for a certain length of time in the presence of ^{3}H-uridine, adding rifampicin to the culture, removing 2ml aliquots of the culture at zero time and at various intervals after the addition of rifampicin, then (a) measuring incorporation of label into acid insoluble material, (b) extracting the RNA and hybridizing to increasing amounts of the complementary DNA up to saturation, thus measuring the decay (or stability) from various saturation plateaus.

C. DNA-RNA-Hybridization

All of the procedures used for ($\lambda \frac{rbt^{+}}{\quad}$) phage preparation and purification, separation of the phage DNA strands, and separate DNA-strand / RNA hybridization, are described in detail in Miller's

"Experiments in Molecular Genetics" (1972).

These procedures entail first of all the induction of the phage multiplication in the logarithmically growing lysogenic bacteria which carry the appropriate gene, harvesting the cells at a suitable time, then lysing the washed cells in presence of 1-2% chloroform in a phage buffer by freeze - thawing. After removal of the cell debris by two successive centrifugations at 15,000 x g for 10 mins, the phage particles are purified by two successive CsCl ultracentrifugations, (an initial "block gradient" centrifugation followed by an equilibrium centrifugation); the appropriate phage band is separated each time. After dialysis, the phage DNA is extracted by precipitation of capsid proteins with SDS and KCl. After purification of phage DNA, hybridization may be followed in two ways, either using denatured DNA-bound to nitrocellulosefilters, or using the strand of DNA complementary to RDH mRNA. This is done by denaturing the DNA, annealing it to poly UG and separating the two stands by an equilibrium CsCl gradient centrifugation. Since the filter method is very inefficient and requires large amounts of DNA for sensible results, the separated strand method was used as a preferred choice in this study. After hybridization, the unhybridized RNA is digested with pancreatic ribonuclease and the protected mRNA-DNA hybrids are trapped by means of the single strand "DNA tails" to the nitrocellulose filters, washed, dried and radioactivity counted.

In an initial hybridization attempt, an L-strand $\lambda\frac{rbt^+}{}$-DNA sample, donated by M. Neuberger, which had been stored at 4^oC for a few months, was used. Not surprisingly, it did not hybridize at all, either to the polysomal RNAs (section B, above) or the λ-mRNA with much higher radioactivity (prepared by M. Neuberger). It was concluded that this DNA sample was completely degraded.

In the next two attempts $\lambda \underline{rbt^+}$ induced frozen cell pastes, prepared by I.C. Pilot Plant under the supervision of M. Neuberger, were used. After following the whole procedure of phage purification to the end twice, no phage could be detected either by block gradient or by equilibrium centrifugation. The phage titre was determined afterwards; it was much too low to be useful for any DNA preparation.

Next, an undenatured DNA sample (400µg), which had been prepared from an earlier made batch of cell paste and was donated by M. Neuberger, was used. This DNA sample was denatured, annealed to poly U,G, and fractionated by equilibrium CsCl gradient centrifugation, as described in Miller (1972). Thus, in an acid washed tube, 0.25ml of 1% sodium-N-lauroylsarcosinate (made up fresh from 35% stock solution, Koch-Light Lab. Ltd.) was added to the 1.6ml (400µg) DNA+ 400µg of solid poly (U,G). The volume was brought up to 2.5ml by addition of 0.65ml of 10^{-3}M EDTA and the pH adjusted to 8.5 with 10µl of 1N NaOH, using narrow range pH indicator paper. The mixture was heated to 92°C for 3.5 minutes. The temperature of the mixture was measured by placing a thermometer in a parallel tube filled with the same amount of liquid. The time was marked from the point at which the temperature reached 90°C. After 3.5 mins, the tube was chilled in an ice water mixture and 1.7ml of ice cold 10^{-3}M EDTA immediately squirted into the tube. 5.62g of CsCl was added to the mixture, and the refractive index was adjusted to 1.4035 $\pm$ 0.0001 (10^{0}, 26' exactly) with solid CsCl and 10^{-3}M EDTA. The sample was placed in a polyallomer tube, overlayered with mineral oil, capped, and spun at 37,000rpm,20°C,for 40 hours in a Spinco angle 40 rotor. At the first attempt to collect fractions by a pump/flow through spectrophotometer/and fraction collector set up, the UV cell got blocked and about 20%

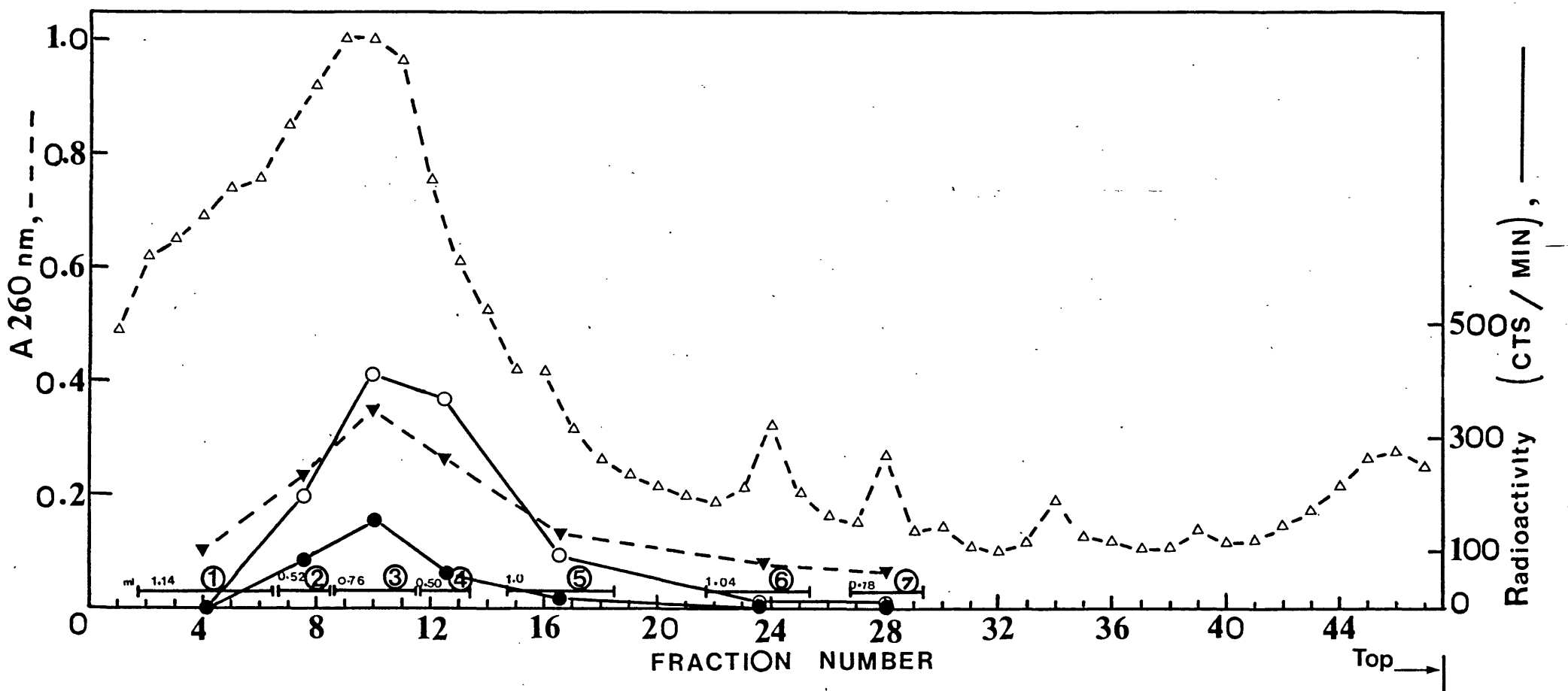


FIG. 9.1. Separation of the poly(U,G)-annealed-denatured $\lambda \frac{nh\dot{b}^{+}}{}$ - DNA strands by CsCl equilibrium sedimentation, and hybridization of the single stranded complementary DNA to pulse-labelled RNA from the RDH-high-synthesis strain, K. aerogenes A114. △ - - - △, A260 nm before hydrolysis with NaOH; ▼- - - ▼, A260 nm after hydrolysis of poly(U,G) with 0.25M NaOH and subsequent dialysis; O——O, hybridized radioactivity, using the sample oxidizer for analysis; ●——●, hybridized radioactivity, by liquid scintillation counting; ① -⑦, the pooled fractions; ml, vol of each pooled sample.

of the solution from the bottom of the gradient was lost as a result. The rest of the solution was transferred to another tube, its density was readjusted and re-spun for 40 hours. The second time, the fractions were collected manually, 0.2ml of 2 x SSC (1 x SSC = 0.015M trisodium citrate + 0.15M NaCl) was added to each fraction and the A260nm was measured. The U.V. absorption profile is shown in Fig.9.1. Since most of the U.V. absorbing material appeared in one major peak near the bottom of the tube, there was some uncertainty about the separation of the two strands and about the actual yield. Therefore certain fractions, shown in solid straight lines in Fig. 9.1, were pooled separately (total of 7 pooled groups), 1M NAOH was added to each sample to the final concentration of 0.25M to hydrolyze poly (U, G), then dialysed. The A260nm (due to DNA) was measured again after dialysis which is also indicated in Fig. 9.1. Each sample was then heated for 4 hours at 67^{o} to anneal any cross-contaminating DNA strands and stored at -20^{o} overnight. The total volume of each pooled sample after dialysis is indicated over each representative horizontal bar in Fig. 9.1.

A constant amount of pulse labelled RNA (RDH-high-synthesis strain, K. aerogenes AII4, labelled for 2 mins with 200μCi 5,6 - ^{3}H-Uridine at early log phase, donated by M. Neuberger) was hybridized to equal volumes of each pooled, dialyzed DNA sample (1-7). Controls contained the same amount of RNA and no DNA. The hybridization procedure was exactly according to Miller (1972). The results are shown in Table 9.1. The hybridization was carried out twice with different amounts of DNA/RNA, the radioactivity of one series was measured by liquid scintillation technique, the other, by sample oxidizer, which is a more sensitive method. Both

TABLE 9.1.

Hybridization of λ^{rbt^+} - L-Strand DNA to Pulse-Labelled RDH mRNA

Pooled-Frac. No.	"DNA" (µl)	2XSSC (µl)	RNA (µl)	Obs. CPM	Net CPM (Less control)	
1	25	170	5	79	(0)	Liquid Scintillation
2	25	170	5	182	90	
3	25	170	5	248	156	
4	25	170	5	151	59	
5	25	170	5	111	19	
6	25	170	5	85	(0)	
7	25	170	5	91	(0)	
Control	-	195	5	92	(0)	
1	47	145	8	65	(0)	Sample Oxidizer
2	47	145	8	271	200	
3	47	145	8	483	412	
4	47	145	8	441	370	
5	47	145	8	167	96	
6	47	145	8	82	11	
7	47	145	8	83	12	
Control	-	192	8	71	(0)	
R	-	-	-	39	-	

R, Rinsing from sample oxidizer

results are shown in Table 9.1. They are also shown parallel to the A260 profiles in Fig. 9.1. It is clear that most of the hybridizable counts correspond to the main DNA (A260) peak, i.e. the peak A260 contains L-strand (complementary) DNA. The heavier strand (R-strand) must have already separated in the first run and have been lost with the bottom 20% of the gradient when the spectrophotometer cell got blocked. Judging by the hybridizable radioactivity as well as the A260 profile after 0.25M NAOH hydrolysis of poly (U,G) and dialysis, we can tell that very little DNA was present. Most of the U.V. 260 absorbing material at the bottom of the gradient before hydrolysis must have been due to poly (U,G). This DNA certainly was insufficient for the saturation-hybridization experiments intended to be done with the polysomal RNA samples 1-10 previously prepared (section B, above) which are at least 10-fold less radioactive than the above pulse labelled RNA sample. Much greater quantities of L-strand $\lambda\frac{rbt^+}{\quad}$-DNA would be required to proceed with those experiments. To be sure about this, the main DNA containing fractions were combined (Fig. 9.1 - pools 2, 3 and 4), then samples of this DNA solution were hybridized against polysomal RNA-4. As expected, there was little difference between the radioactivity of the hybridized samples and the background.

D. Conclusion

Hybridization of the long labelled polysomal RNA from the RDH-high-synthesis strain K. aerogenes AIII with the complementary DNA (λ^{rbt^+}-L-strand DNA) was attempted. Although the hybridization technique appeared to work, it is impossible to carry out the desired experiments with the very small amount of DNA available. It would be necessary to spend a few more weeks to purify sufficient L-strand DNA, and to repeat the RNA preparation with much higher amounts of

radioactive uridine fed to the culture. At this time purified sheep anti-rabbit IgG antibodies were made available and conditions were all set for double immunoprecipitation of RDH-polysomes. Since there was a good chance that the *in vitro* translation of the purified RNA might succeed and provide even more convincing and interesting results, the hybridization studies were inconclusively abandoned at this point, and purification of RDH-mRNA and its *in vitro* translation begun instead. The polysomal RNA samples 1-10 prepared already would not be wasted as they could always be used later either for hybridization, or for *in vitro* translation.

CHAPTER X

Purification and In Vitro Translation of Ribitol Dehydrogenase Messenger RNA from Klebsiella aerogenes A111

A. Introduction

Monospecific antibodies (anti-RDH rabbit antibodies, and anti rabbit-Ig sheep antibodies) were purified by affinity chromatography and were made free from traces of ribonuclease by sterile DEAE- and CM-cellulose chromatography (Chapter V). Purified bacterial polysomes (Chapter VI) were shown to be very stable when incubated with such purified antibodies for more than one hour (Chapter VII). We have seen that iodinated rabbit anti-RDH antibodies specifically bind to polysomes which carry nascent RDH chains, while they do not bind at all to ordinary bacterial polysomes (Chapter VII). Also, we have learnt that RDH mRNA *in vivo* is extremely stable (Chapter VIII). Thus, there were sufficient reasons to believe that one might be able to produce biologically pure RDH mRNA using the specific immune-precipitation of RDH polysomes, which would be translatable in an appropriate cell-free system.

Initially I shall describe the double-antibody precipitation technique used to isolate polysomes carrying nascent ribitol dehydrogenase chains. Then the results of *in vitro* translation of RDH mRNA, prepared by phenol extraction of immune-precipitated polysomes, and subsequent analysis of the product of this translation will be presented.

B. Conditions for Immune-Precipitation of RDH Polysomes

It is almost impossible to precipitate polysomes by using

only mono-specific antibodies raised against a particular protein, such as RDH, because the divalent antibodies tend to cross-link adjacent nascent polypeptides on the same polysomes rather than the nascent chains on separate polysomes. A second antibody directed against the first type of antibodies (or some other method of immobilization of the initial Ab/polysome complex, such as formalin-fixed Staphylococcus aureus cells - Mueller-Lantzsch and Fan, 1976) is necessary to bring about effective precipitation of the appropriate polysomes. Optimal conditions must be worked out for each system because of variability in the quality of antibodies raised and purified, and the number of available antigenic sites per nascent polypeptide.

Perhaps the ideal way to determine the optimal proportions of each type of antibodies and polysomes for quantitative immunoprecipitation of polysomes by this double antibody method is that used by Schechter (1974). Constant amounts of the total polysome population (for example 20 A260 units) are incubated with replica series of increasing amounts of the first Ab; then increasing amounts of the second Ab (anti Ab 1 antibodies) are added. A number of precipitation curves of polysomes as a function of antibodies concentrations are constructed, as shown schematically (Fig.10.1). From the plateaus of these curves one can decide what amounts of the two types of antibodies are required in order to obtain maximal immune-precipitation of the polysomes.

I have used a slightly less rigorous method to explore appropriate ratios of polysomes and antibodies 1 and 2, since the saturation level for binding Ab 1 to polysomes had already

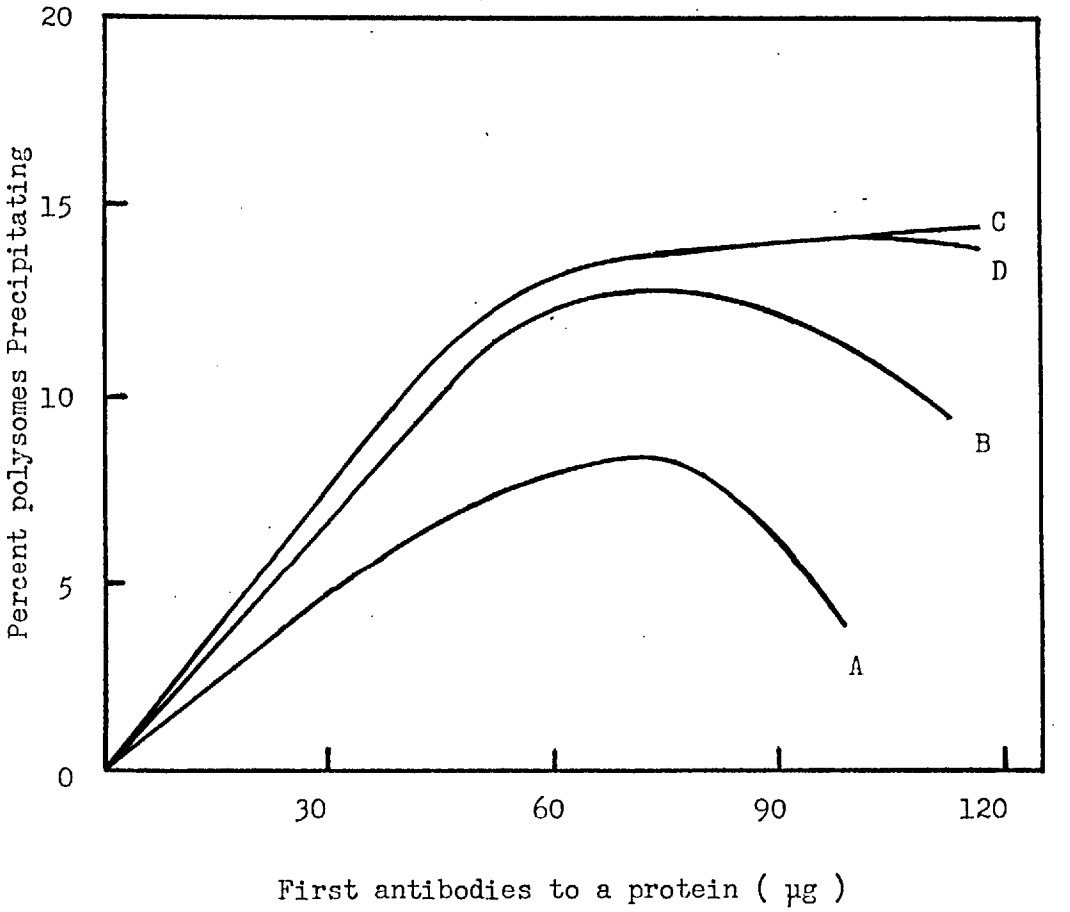


FIG. 10.1. Schematic of precipitation curves of polysomes as a function of two types of antibodies concentrations, according to the method of Schechter (1974). Amounts of the second antibodies (directed against the first antibodies) added to the Ab-1/polysome-complex are, for example: A = 600 µg; B = 900 µg; C = 1200 µg; and D = 1500 µg.

been determined (Chapter VII). Ratios of Ab 2 required to precipitate Ab 1 were determined on a microscale. Then Ab 2 was added in this ratio to a mixture of Ab 1 and polysomes at the previously determined saturation level (30 µg of antibody/ 10 A260 units of K.aerogenes A111 polysomes isolated at culture A650 = 0.6 - 0.7. A typical experiment was as follows:

A 1 ml sample of the purified rabbit anti-RDH (5.034 mg/ml) was mixed with 0.25 ml of ^{125}I rabbit anti-RDH (0.593 mg/ml; 300,000 CPM/ml) and made up to 10 ml with PBS. Aliquots of this labelled antibody (100 µl) containing 52 µg Ab and ca. 600 CPM were reacted with 200 µl of various concentrations of sheep anti-rabbit Ig antibodies (5.517 mg/ml serially half-diluted with 0.1 M phosphate buffer pH 7.0 containing 1 mg/ml of bovine serum albumin). After 1 hour's incubation on ice, the precipitates were collected be centrifugation at 3000 Xg for 10 minutes, washed once with 2 ml of cold buffer and spun again. After decanting the supernatants the tubes were inverted to drain for a few minutes and the precipitates were counted directly in a LKB 80000 Gamma Counter.

Fig.10.2 shows the percentage of input radioactivity recovered in the precipitate as a function of multiplicity of sheep antibodies/rabbit anti-RDH antibodies. It can be seen that about 15-20 fold sheep anti-rabbit Ig antibodies in excess of rabbit antibodies are needed to bring about near maximal (about 85%) precipitation of the rabbit antibodies under the experimental conditions. It should be pointed out that this result is considerably influenced by the temperature of the incubation mixture, the total reaction time, and the volume of the reaction mixture. For quantitative immunoprecipitation of

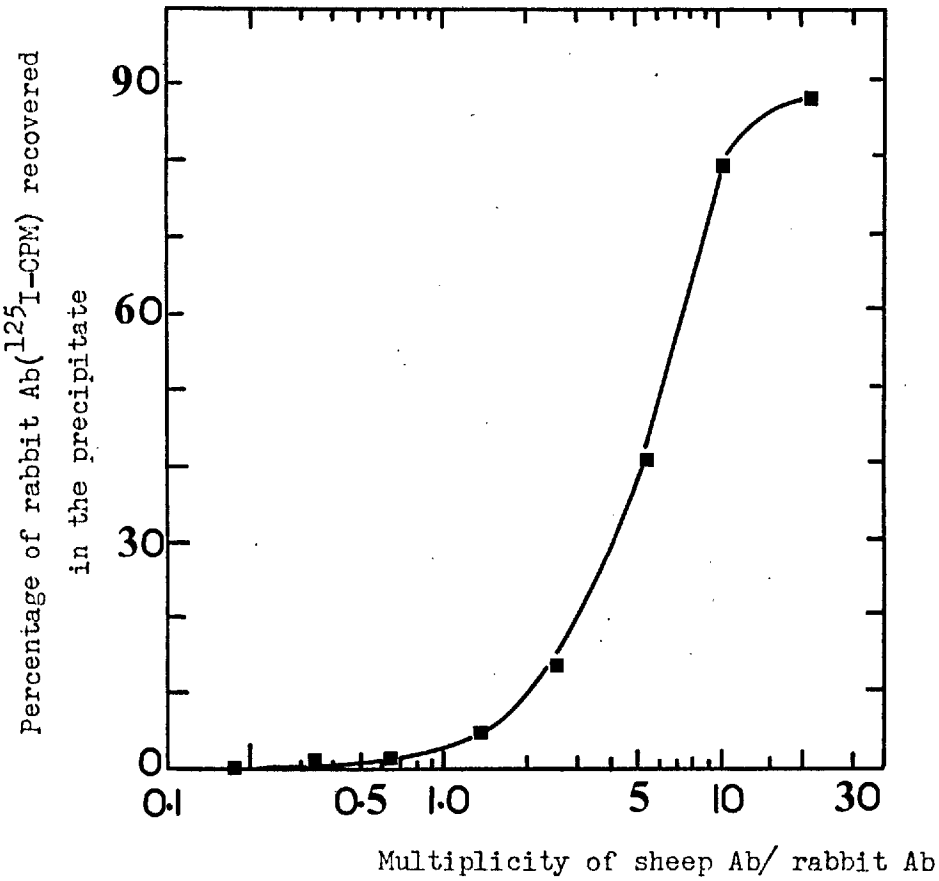


FIG. 10.2. The percentage of rabbit anti-RDH antibodies recovered in the precipitate as a function of multiplicity of sheep anti-rabbit-Ig antibodies/rabbit anti-RDH antibodies. Incubation, 1 hr on ice.

RDH polysomes (see below), 30 μg of rabbit anti RDH Ab: 10 A260 units of total A111 polysomes: 20 x 30 μg sheep anti-rabbit Ig Ab were used. These proportions are rather comparable with the proportions used by Schechter (1974), 10 A260 units of polysomes: 58 μg of goat anti L-chain antibodies: 12.4 x 58 μg rabbit anti-goat Ig antibodies, for the precipitation of the immunoglobulin light chain polysomes.

C. Double-Immune-Precipitation of RDH Polysomes from Klebsiella aerogenes High Synthesis Strain A111

(a) Antibody preparations

The purified rabbit anti-RDH antibodies were as follows:

Rabbit Ab-1 was a ribonuclease-free preparation (1.545 mg/ml) purified by affinity chromatography and DEAE-CM-cellulose chromatography (Chapter V), - the purest sample I ever prepared. These antibodies were shown to be monospecific for RDH by the criteria of double immunodiffusion and immuno-electrophoresis.

Rabbit Ab-2 was another purified rabbit-anti RDH Ab preparation (8 mg/ml) which had been purified by affinity chromatography several months later (see Chapter V). The double-immunodiffusion test had shown the presence of antibodies to a second protein (impurity) since a second precipition band could be seen upon reaction with K.aerogenes sonicated extract. This was also used to see whether any polysomes carrying nascent peptides complementary to these contaminating antibodies exist in the mid-log-phase bacterial polysome population and also to find out whether DEAE-CM-cellulose chromatography is absolutely required to safeguard against any trace of ribonuclease in the Ab preparations. Both of these Ab preparations (rabbit Ab-1,

and rabbit Ab-2) were supplemented with 0.1 M Mg SO_4 solution, to make the Ab solutions about 10 mM in Mg^{++}, and with sodium-heparin (1000 units/ml) to a final concentration of 10 units/ml. All of the Ab solutions were clarified by centrifugation and kept on ice.

Sheep anti rabbit-Ig antibodies, purified by affinity chromatography and DEAE-CM-cellulose, (2.772 mg/ml) were also made 10 mM in Mg^{++} and 10 units/ml in heparin, clarified and kept cool.

(b) Polysomes

Several left-over, purified polysome samples which had been stored in liquid nitrogen for 6-12 months, were pooled and clarified by a centrifugation at 6000 Xg for 10 minutes. All of these samples were originally isolated from K.aerogenes Alll cells grown on xylilol/M9 medium to culture A650 nm of 0.6 - 0.7 (i.e. after the 'general mRNA synthesis shut down'). The A260 nm of the pooled, clarified polysomes was =70.7/ml; the ratio A260/A280 of the polysomes in distilled water at 1 : 50 dilution was 1.97 and at 1 : 100 dilution was 1.92. These clarified polysomes (6 ml) were supplemented with 60 µl of Na-heparin (1000 units/ml), divided into 4 and 2 ml portions, and stored on ice.

(c) Precipitation

To the 4 ml Alll polysome sample in the tube No.1 (280 A260 units) were added 560 µl of rabbit Ab-1 (+ 56 µl of 0.1 M Mg SO_4 + 6 µl heparin). To the 2nd polysome sample in the tube No.2 (140 A260 units) were added 56 µl of rabbit Ab-2. Both tubes were incubated on ice for 35 minutes. Then 6 ml of sheep anti-rabbit-Ig Ab (+ 60 µl heparin + 600 µl of 0.1 M Mg SO_4)

were added to tube No.1, and 3 ml of the same Ab (+ 30 µl of heparin + 300 µl of Mg SO_4) were added to tube No.2. Both tubes were incubated for a further 45 minutes on ice. The double Ab-polysome precipitated complexes were collected by centrifugation at 8000 Xg for 15 minutes, at 4°C and the supernatants were combined and stored overnight at 4°C.

Each one of the precipitates was resuspended in 1 ml of solution D (0.1 M sucrose, 10 units heparin/ml, 5 mM Tris-HCl, pH 7.2, 10 mM Mg SO_4, and 60 mM KCl) and centrifuged again (tube No.1 accidentally fell to the floor and most of its contents was spilt). The clear washing solutions were carefully removed leaving precipitates yielding RNA-1 and RNA-2.

The combined supernatants from both immunoprecipitations, after storage overnight at 4°C, gave rise to more precipitate which was centrifuged and washed as before; the RNA extracted from this precipitate was called RNA-3. It was interesting to find out whether this RNA-3 contained pure RDH mRNA, since the double Ab/polysome incubation time could then be extended in order to obtain better yields.

^{3}H-Polysomes (10 A260 units), long labelled, from K.aerogenes strain FG-5 (an RDH inducible strain) grown on casamino acids/M9 medium were incubated as above with rabbit and sheep antibodies. The precipitate collected after 2 hours did not contain any significant radioactivity, indicating the absence of polysomes which react with anti-RDH antibodies in this population.

D. Preparation of mRNA from Immune-Precipitated Polysomes

The RNA from each of the above three washed precipitates was suspended in 1.5 ml of solution E (100 mM Tris-HCl, pH 9.0;

100 mM sucrose; 10 mM KCl; 2 mM Mg OAc; and 3% SDS) at room temperature by fast repeated blowing from a pasteur pipette. The suspension was mixed with an equal volume of water-saturated phenol, agitated gently at room temperature for 10 minutes, cooled on ice for 5 minutes, and spun in a chilled Sorvall SS-34 rotor for 10 minutes at 12,000 Xg (10,000 rpm). The aqueous phase was separated and the phenol phase re-extracted once at room temperature for 10 minutes with 1.5 ml of solution F (10 mM Tris-HCl, pH 9.0, 0.5% SDS); chilling and phase separation were done as above. The aqueous phases were combined and re-extracted twice with phenol at +4^{o}C. The RNA was precipitated by adding 0.1 volume each of 2.0 M NaCl and 2.0 M NaOAc (pH 5.5), and 2.5 volume of ethanol at -20^{o}C, overnight. The RNA precipitate was spun down at 0^{o}C, 12,000 Xg for 30 minutes. The supernatant was carefully pipetted off, the precipitate was resuspended in cold 96% ethanol and re-centrifuged 25 minutes as above. The precipitate was then dissolved in 1 ml of distilled water, precipitated again with NaCl/NaOAc/ethanol, as above, collected by centrifugation, washed twice with cold 96% ethanol, and finally dissolved in 0.1 ml of distilled water and stored at -70^{o}C. 10 µl of the sample was mixed in 1 ml of distilled H_2O for estimation of RNA concentration and yield as shown in Table 10.1.

The proportion of the immune-precipitated RDH-polysomes in the total polysome population can be estimated as follows: The weight of polysome RNA is approximately 0.82 mg on the assumptions indicated in Table 10.1. Assuming 50% loss of RNA during phenol extraction (already estimated from RNA

TABLE 10.1

Estimation of RNA from immune-precipitated polysomes

	Total Vol.ml	A260nm $x10^{-2}$	A280 $x10^{-2}$	$\frac{A260}{A280}$	$\frac{A260}{ml}$	$\frac{mg}{ml}$	Yield* (mg)
RNA-1	0.1	0.185	0.099	1.87	18.5	0.93	0.093**
RNA-2	0.1	0.385	0.223	1.73	38.5	1.93	0.193
RNA-3	0.2	0.233	0.121	1.89	23.3	1.17	0.241

* Assuming 20 A260 units = 1 mg of RNA (Schechter, 1974).

** Most of this RNA was spilt before extraction (see text). Assuming yields as for RNA-2 a recovery of 0.39 mg. might have been expected.

extraction of the ^{3}H-uridine labelled polysomes), and allowing about 2-fold increase for the weight of the proteins originally present in the polysomes, thus the estimated weight of the immune-precipitated polysomes would be:

$$0.82 \times 2 \times 2 = 3.28 \text{ mg.}$$

The total A260 nm of the polysomes used was 420 which = 32.3 mg (using the conversion factor by Schechter, 1974, 13 A260 units for 1 mg of polysomes). Therefore, the proportion of the immune-precipitated (RDH) polysomes in the total polysome population under the culture conditions described was about 10%.

E. Translation of RDH mRNA in a Crude E.coli Cell-Free System

Translation of ribitol dehydrogenase messenger RNA in a crude E.coli MRE600 cell-free system was carried out according to 'Protein Synthesis in Vitro, Method II' (described in Materials and Methods). This translation and the running of SDS gel were conducted at Imperial Cancer Research Fund by Dr. Robert Harvey, rather than with the crude E.coli system described in Chapter IV, since the relevant solutions had been stored for over 2 years. Dr. Alan Smith's laboratory at I.C.R.F. had several in vitro protein synthesis systems in current use, and it seemed to us likely that the naked RDH-mRNA might not survive in the crude E.coli system. I therefore took advantage of Dr. Smith's kind offer to perform the experiments in his laboratory.

The three RNA samples purified by specific immunoprecipitation of RDH polysomes and subsequent phenol extraction (in D, above), were tested in the E.coli MRE600 crude in vitro system for their ability to stimulate incorporation of amino acids into protein, using ^{35}S-Met. Pure Qβ viral mRNA was used in parallel as a control. Table 10.2, also Fig.10.3, show the results of these in vitro translations under physiological conditions: 9 mM Mg^{++}

TABLE 10.2

Protein synthesis directed by RDH mRNA samples 1, 2 and 3 in E. coli S30 fraction at 9mM Mg^{++}

		TUBE NUMBER										
		1	2	3	4	5	6	7	8	9	10	11
Premix*	µl	20	20	20	20	20	20	20	20	20	20	20
Water,	µl	5	4	4	2	-	4	2	-	4	2	-
5µg/µl QβRNA,	µl	-	1	-	-	-	-	-	-	-	-	-
0.93µg/µl RNA-1,	µl	-	-	1	3	5	-	-	-	-	-	-
1.93µg/µl RNA-2,	µl	-	-	-	-	-	1	3	5	-	-	-
1.17µg/µl RNA-3,	µl	-	-	-	-	-	-	-	-	1	3	5
		25	25	25	25	25	25	25	25	25	25	25
CPM X 10^{-2}/ 2µl		67	833	88	145	221	160	364	590	107	167	278
(RNA)	µg	0	5	0.93	2.79	4.65	1.93	5.79	9.65	1.17	3.51	5.85

Incubations for 30 min at 37°C. 2µl was taken for counting and the remainder of each sample saved at -70°C.

* E. coli Premix	In 25 µl	X 12
	µl	µl
1M Hepes pH 7.0	1.25	15
2M NH_4Cl	1.25	15
0.1M Mg-Acetate	1.50	18
1M 2-Mercaptoethanol	0.25	3
0.05M ATP	1.00	12
0.07M PEP	1.50	15
0.01M GTP	0.75	9
AA mixture - Met	1.00	12
^{35}S-Met	3.00	36
E. coli S30	7.50	90
Water	1.00	12
	20.00	240

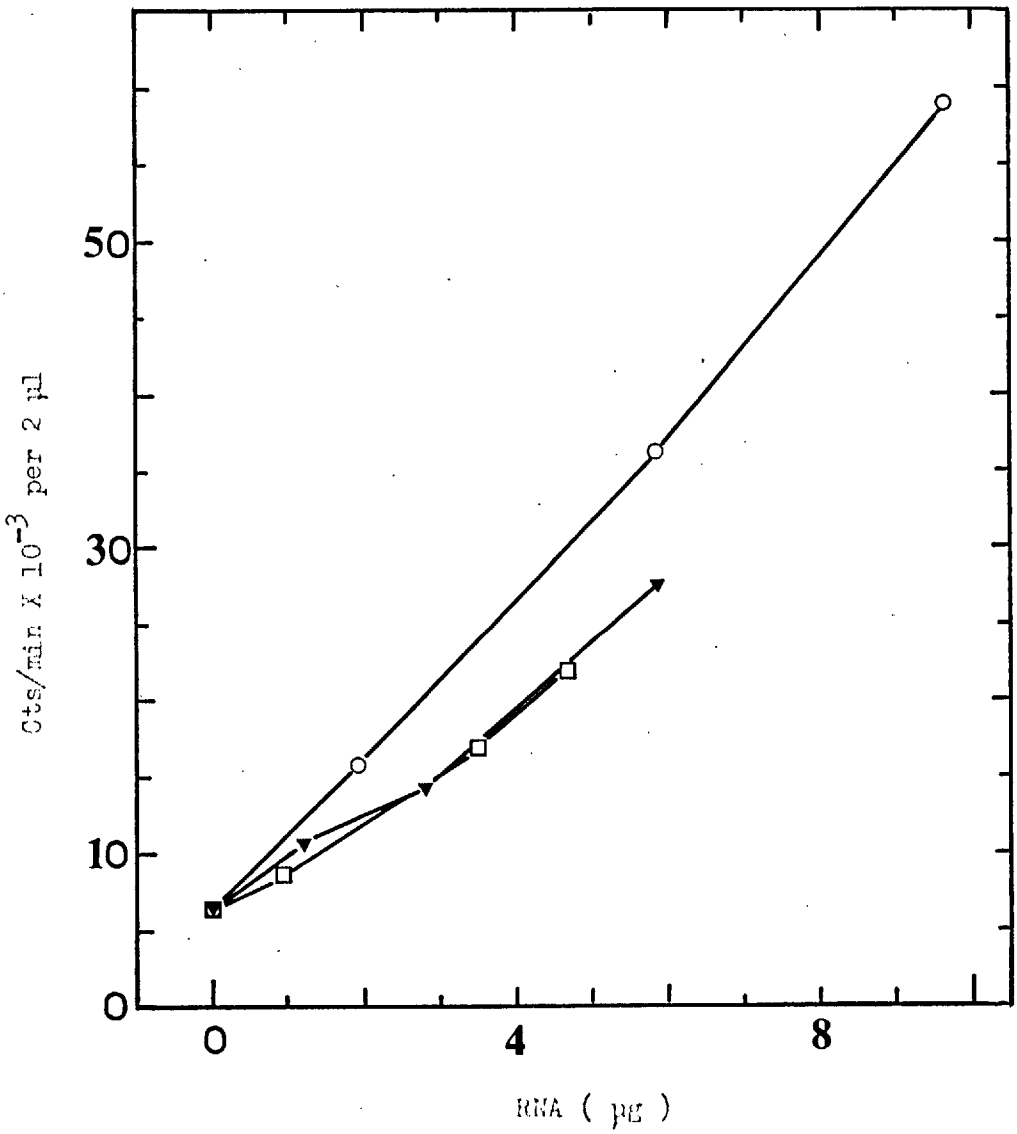


FIG. 10.3. Incorporation of ^{35}S-Met into protein directed by purified RDH-mRNA samples 1-3 from K. aerogenes strain A111 at 3 different concentrations of each mRNA sample. All incorporations were carried out for 30 min at 37°C. (□), RNA-1; (○), RNA-2; and (▼), RNA-3.

and 37°C, for 30 minutes. Increasing concentrations of each RNA sample were used. All three samples are shown to be very active in stimulation of amino acids incorporation into protein, and the incorporations are linearly proportional to the amount of RNA added. Tube No.1 is the complete E.coli system without added mRNA; thus the counts incorporated in this sample are due to endogenous mRNA present in the system. Tube No.2 contains Qβ RNA. Tubes Nos.3-11 all contain different amounts of RDH mRNA as indicated.

F. Analysis of the Product of In Vitro Translation by SDS Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel for the analysis of the E.coli cell-free translation product (Section E, above) was prepared and run as described in "Materials and Methods", Method II. 5 µl of the reaction mixture was taken into 20 µl of sample buffer + DTT, boiled for 3 minutes and run on a 15% acrylamide gel, pH 8.8 with a 5% stacking gel, pH 6.8. The gel was then stained, destained, dried, exposed to an autoradiographic film, and the film was developed. The autoradiograph and the dried gel could be directly superimposed by using radioactive dye markers on the four corners of the gel paper; thus the position of the pure RDH protein, run in a parallel well as a control, could be marked accurately beside the autoradiograph.

Fig.10.4 shows the photograph of the Coomassie Blue stained dry SDS-gel, showing RDH-A11 in well No.14, RDH-D in well No.13, and E.coli S30 proteins in wells No.1 and 3-12. Wild type RDH (A) and the mutant RDH (D) migrate to the same extent, as previously shown by Dothie (1974).

Figs.10.5, 10.5a and 10.5b, show the autoradiographs of this gel after 3 days, 20 days and 20 days (negative print)

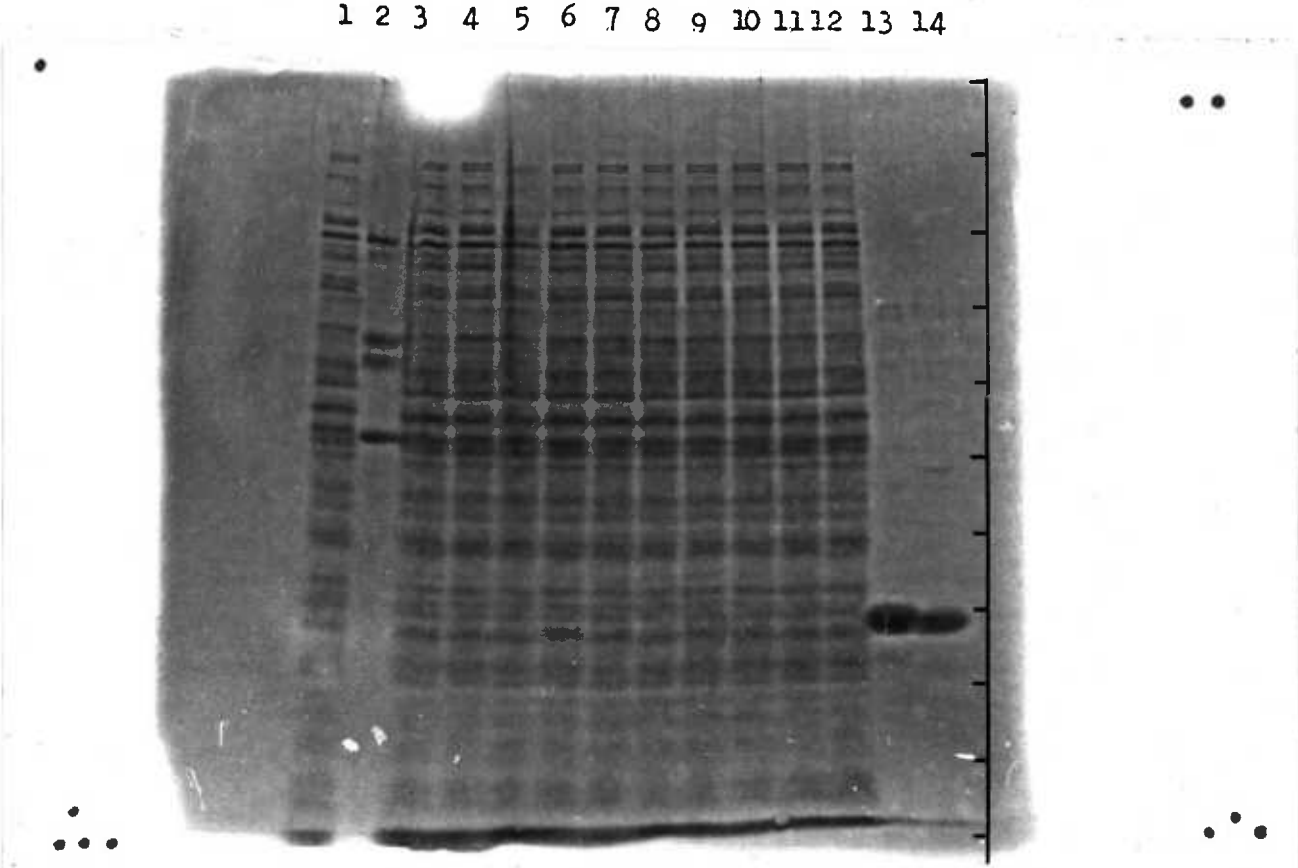


FIG. 10.4. Coomassie Blue stained and dried SDS-polyacrylamide gel electrophoresis, showing RDH (A11) in well #14, RDH (D) in well #13, and E. coli S30 fraction proteins in wells #1 and 3-12.

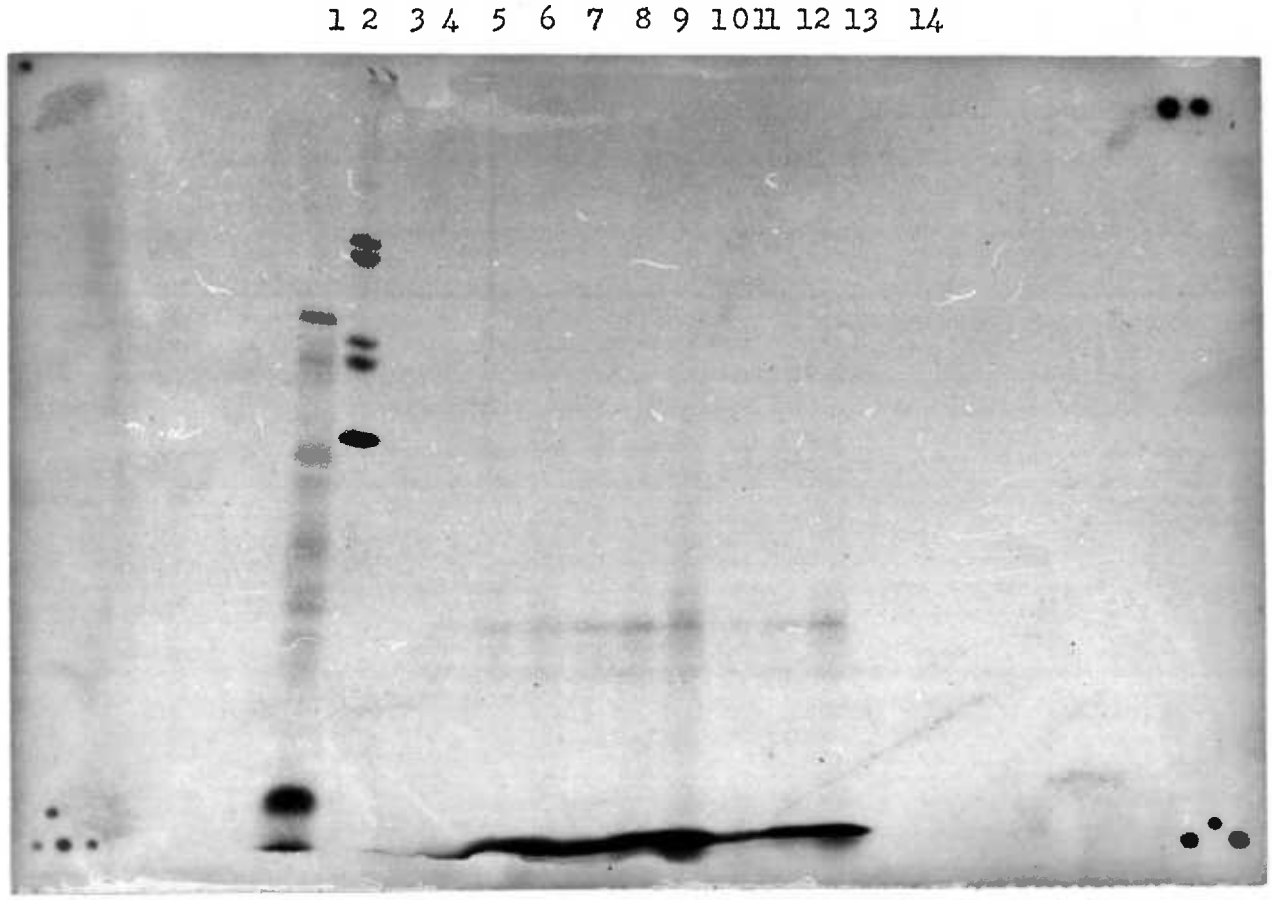


FIG. 10.5. Autoradiograph of the SDS-polyacrylamide gel shown in Fig.10.4, 2 days exposure to the gel.

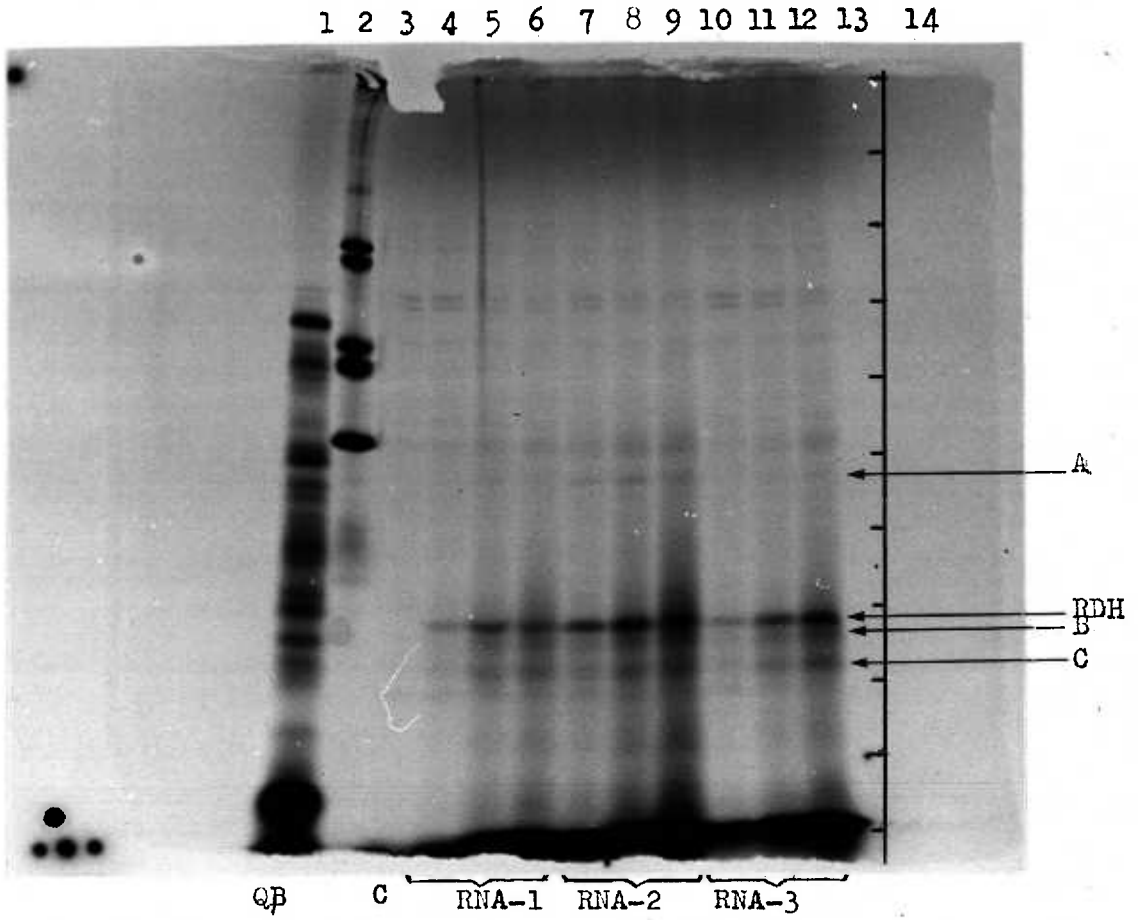


FIG. 10.5a. Autoradiograph of the SDS-polyacrylamide gel shown in Fig. 10.4, 20 days exposure to the gel. C, is the control sample, complete <u>in vitro</u> system without added RNA. Qβ, is the incubation with Qβ mRNA added. 2, contains radioactive calibration proteins.

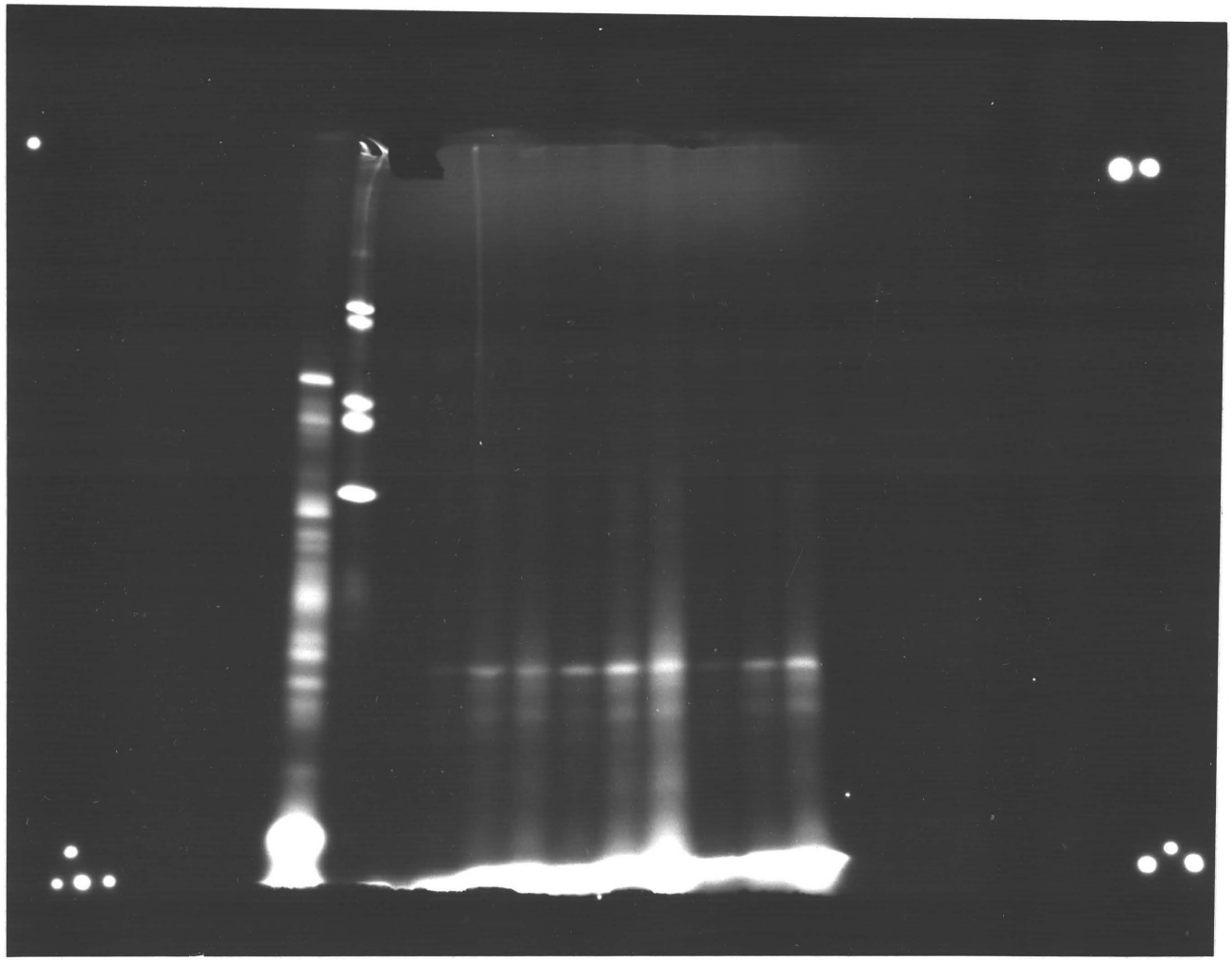


FIG. 10.5b. Photonegative of Fig. 10.5a.

exposure, respectively. It is quite clear from these results that in the reaction tubes which were supplemented either by RNA-1, 2 or 3, (slots 4-12 on the gel), by far the major product of in vitro translation is a polypeptide which migrates electrophoretically exactly to the same extent (72 mm) as wildtype RDH on the SDS-polyacrylamide gel, as indicated. The fact that the product of in vitro translation appeared as a clear sharp band, representing a homogeneous polypeptide, is a good indication of the stability of this messenger RNA. The mobility of the radioactive band is exactly that of whole RDH monomer. Had the mRNA been unstable one would have expected to see at best only fragments of the protein.

Comparisons of the RDH-mRNA-containing samples on the SDS gel (slots Nos.4-12) with the control, containing the complete protein synthesis complement without any added mRNA (slot No.3), Fig.10.5a, indicate the presence of a few minor radioactive bands such as A, B and C, indicated in Fig.10.5a. They could have originated from accidental trapping of other polysomes in the double antibody-polysome complex precipitate, since the precipitate was only washed once.

It is interesting to note that all three RDH mRNA samples, RNA-1, RNA-2 and RNA-3, produced almost identical results. Thus, the antibodies in rabbit-Ab-2 directed against a protein impurity present in the original RDH sample apparently could not find any exclusive class of polysomes to react with in that polysome preparation. This is encouraging because it implies that one need not be over-cautious about removing every last trace of antibody impurity in order to obtain very clean RDH-messenger-RNA; many such antibody impurities are probably directed against proteins which have unstable mRNAs.

The finding that RNA-3, prepared from the material aggregated overnight in the supernatant, is as pure and as active as RNA-1 and RNA-2, is another useful observation. It suggests that the antibody/polysome incubations could be extended for longer periods in order to obtain greater yield of mRNA. In that case less of each type of pure antibodies could be used which, in turn, would result in greater specificity of Ab/polysome precipitation. Sterile DEAE-CM-cellulose column chromatography of the antibodies appears not essential provided that the affinity chromatography has been carried out under sterile and ribonuclease-free conditions and washed extensively with sterile buffer, prior to elution of the specific antibodies.

G. Characterization of the Product of In Vitro Translation by Met-Diagonal Electrophoresis

Conventional tryptic and chymotryptic fingerprints of RDH are not very useful since many of the peptides are insoluble. Thermolysin digests the protein readily but produces a large number of small peptides. Hence to test whether the products of in vitro translation, described above, contain methionine sequences characteristic of RDH, a thermolysin digest of the radioactive protein was examined by the methionine diagonal electrophoretic technique of Tang and Hartley (1967).

In this method an enzymic digest is submitted to paper electrophoresis at pH 6.5 and then treated with iodoacetamide at low pH. This converts methionine to its positively charged sulphonium salt. When electrophoresis is carried out at right angles to the original direction most peptides will be on a diagonal, but those containing methionine lie off this diagonal because of their altered mobility.

The in vitro translation mixture from Tube 4 (Table 10.2) contains 7.2×10^3 CPM/μl in precipitable protein of which

about half is probably in endogeneous E.coli proteins (see control tube 1 - Table 10.2). About 15 µl of this mixture were added to 30 mg of pure RDH(D) as carrier. This mixture was acid denatured, carboxymethylated and digested with thermolysin as described in Chapter II (Section 23). About 20% of the product was submitted to methionine diagonal electrophoresis as described in the Methods chapter above.

Figure 10.6 shows the spots revealed by the cadmium-ninhydrin stain. There are two acidic methionine peptides and a closter that were originally neutral, but an expected basic peptide (CAM-Met-Lys-His-Ser) has been run off the paper.

Analysis of the radioactive peptides was attempted, before ninhydrin staining, by autoradiography using Kodirex Film (KDST), but no radioactivity could be detected even after 5 days exposure. There were clearly insufficient counts for this technique.

A more sensitive detection method is the Spark Chamber (Birchover Instruments Ltd., Hitchin, Herts., England) which consists of a number of wire spirals with central anode wires placed in a suitable gas mixture (such as 90% Argon and 10% Methane) and between which a high voltage is applied. A charged particle such as a β particle passing through the detector will produce ionization of the gas which creates an electrical breakdown leading to a visible spark at that point. The Spark Chamber will thus produce sparking rates in proportion to the activity of spots on a chromatography medium. By photographing the sparks with an extended exposure a result similar to an autoradiograph is obtained but with an exposure time approximately 1000 or more times shorter than would have been necessary with

FIG.10.6. Met diagonal electrophoresis (pH 6.5/pH 6.5) of RDH thermolytic digest; analysis by Cadmium Ninhydrin.

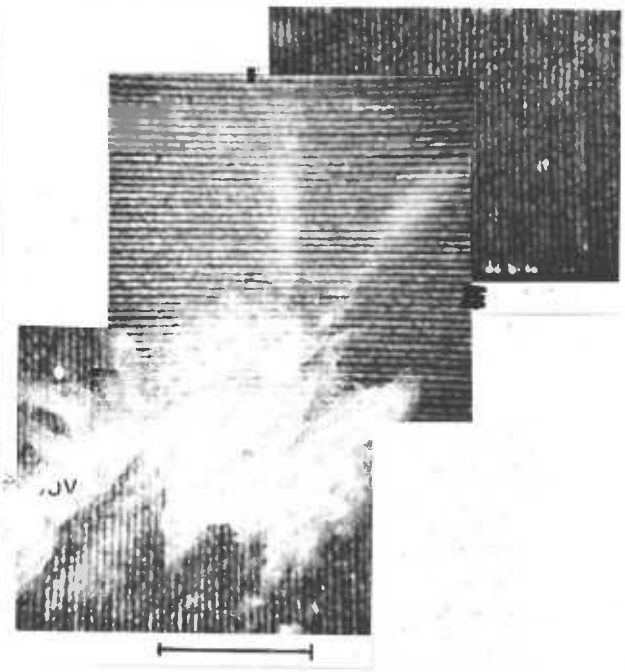


FIG.10.7. Met diagonal electrophoresis (pH 6.5/pH 6.5) of RDH thermolytic digest; analysis by Spark Chamber.

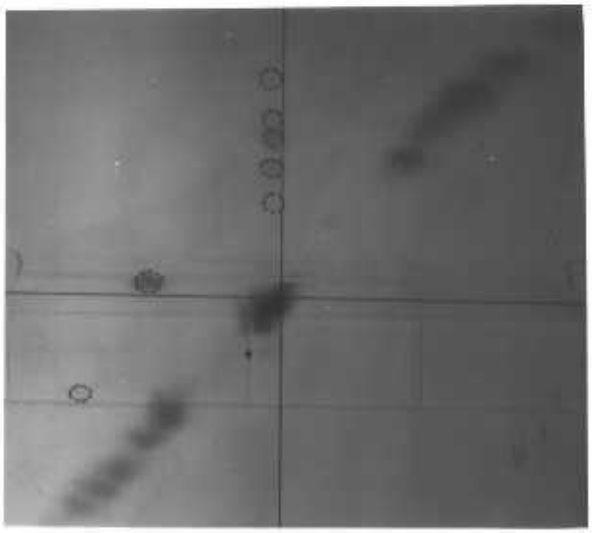
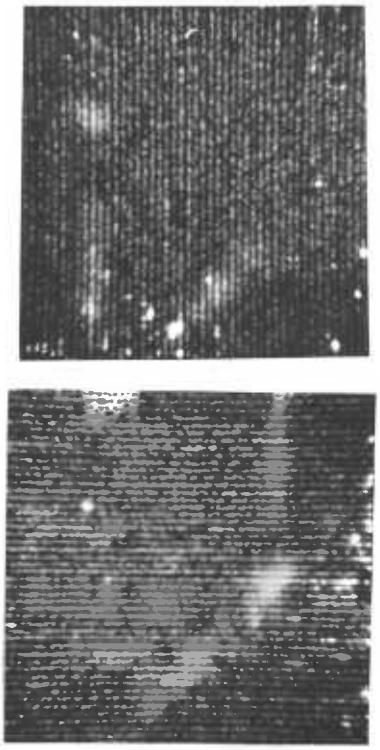


FIG. 10.6. Encircled acidic and neutral peptides.



A: Top part of Fig.10.7, showing the CM-Met radioactive spot. B: Middle portion of Fig. 10.7, showing parts of acidic and neutral regions.

conventional autoradiography using photographic film. The detector is capable of imaging Alpha, Beta and Gamma emitting isotopes.

The ninhydrin-positive (RDH) spots on the 3 mm paper (photograph shown in Fig.10.6), any other radioactive area which could be detected by the Spark Chamber (Fig.10.7), and several other control areas were cut out from the 3 mm paper, and each sample was counted inside a glass vial containing a suitable scintillation fluid (toluene containing 0.1 g/l of dimethyl POPOP and 6 g/l of PPO) for 10 minutes. The result of 'diagonal electrophoresis' of the thermolytic digest of the mixture of pure, non-radioactive RDH and ^{35}S-Met-labelled in vitro translation product is shown diagramatically in Figure 10.8. The ninhydrin positive spots are drawn in solid lines, while the other areas that were cut out for radioactivity determinations are shown by broken lines. Mobilities in first and second dimensions with respect to aspartic acid are indicated on this diagram. The radioactivity (CPM) of each area or spot after background deduction is indicated beside it on the diagram. The background was 53 CPM and the control areas from the 3 mm paper which were not radioactive all had almost the same CPM with maximum deviation of $\pm$5 CPM. Therefore, any radioactivity in excess of 5 CPM on the diagram is significant. The authenticity of these counts was checked by the close agreement with the photographic result obtained by the Spark Chamber (Fig.10.7). The distribution of radioactivity by the two methods were superimposable, using a magnification device provided on the Spark Chamber.

The significant result of this experiment is that every ninhydrin positive Met-containing RDH peptide which lies off the

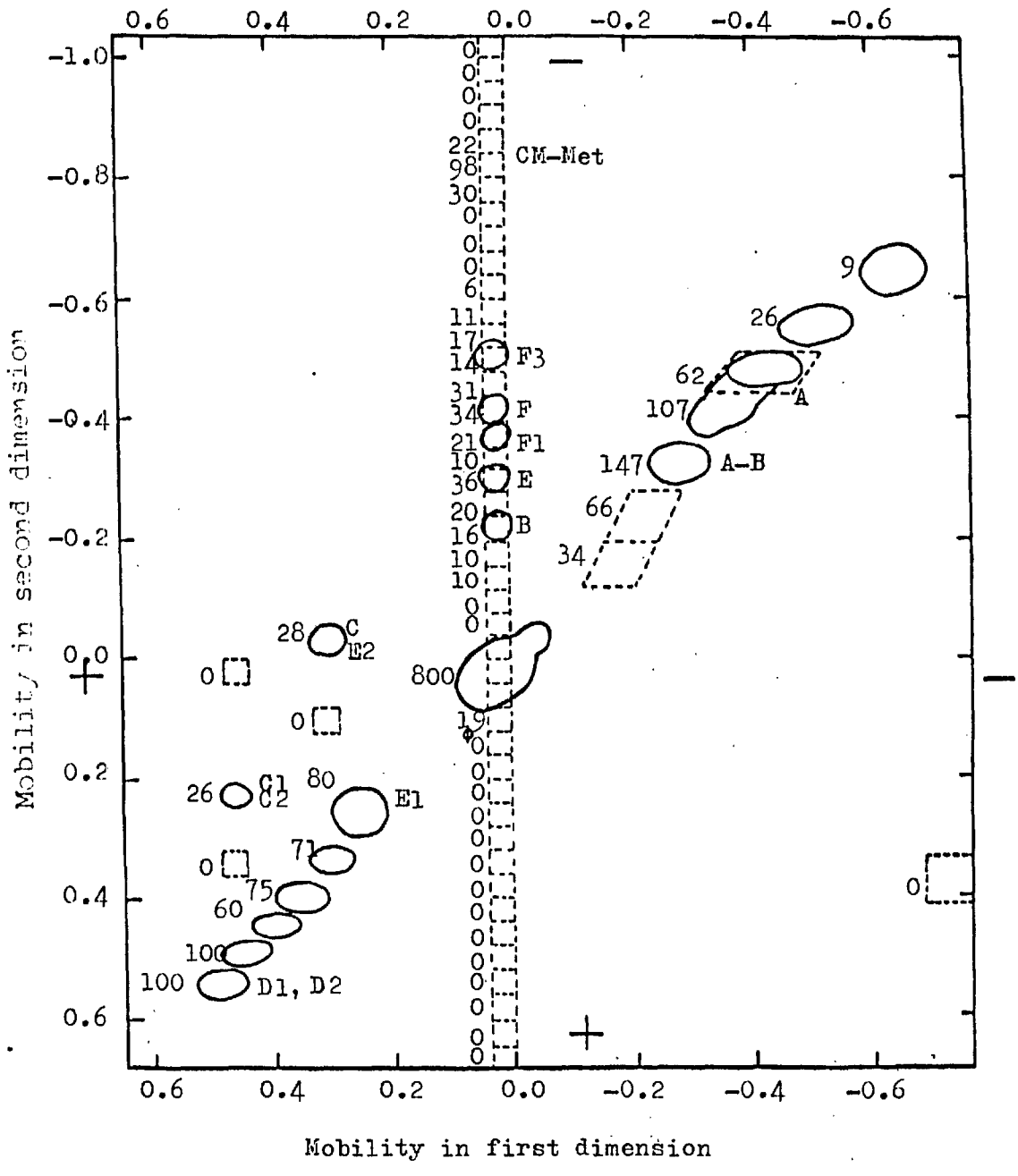


FIG. 10.8 .Methionine diagonal (pH 6.5/pH 6.5) of a thermolytic digest of carboxymethyl-ribitol dehydrogenase and the <u>in vitro</u> translation product. The peptides were applied originally at the spot marked ϕ in the horizontal strip, not shown. After electrophoresis at pH 6.5 and treatment with iodoacetamide, the strip was stitched to a full sheet of paper and submitted to electrophoresis at pH 6.5 in the vertical direction. The electrophoretic mobility of peptides relative to aspartic acid is indicated. The number beside each peptide or area is its corresponding CPM by liquid scintillation counting after background subtraction. CM-Met, is S-carbamoylmethylmethionine. The expected sequences of the Met-containing RDH peptides indicated alphabetically are shown in Table 10.3.

diagonal contains radioactivity. This proves that the in vitro translation product must have been RDH. Regretably, as a result of incomplete iodoacetamide reaction some of the Met-containing RDH peptides did not differentiate from the diagonal. Even the peptides which have emerged from the diagonal, one can see to have only partially reacted. The basic N-terminal peptide, Met-Lys-His-Ser, could have run off the paper if one assumes the charge on His in this particular case to be close to +1 rather than +0.3. The most convincing peptides are the two acidic spots which are well clear of the diagonal and are well resolved. Each of them, however, could be a mixture of two peptides (C, E_2; and C1, C2, refer to Table 10.3) since other partially digested peptides are represented in the neutral region. It was possible to assign Met-containing thermolytic sequences from RDH (Fig.10.9) to various peptides obtained after 'diagonal electrophoresis', by pairing the observed and predicted mobilities of various peptides, as shown in Table 10.3.

A radioactive and not ninhydrin stained spot is present which is neutral in the first dimension and basic in the second, it is identified by its relative mobilities (0, -0.86) to be (^{35}S-labelled)-CM-Met, which had probably originated from either insufficient initial dialysis or cleavage of ^{35}S-methionyl-tRNA bonds.

H. Conclusion

Conditions have been described for immune-precipitation of ribitol dehydrogenase polysomes by a double antibody technique, using purified rabbit anti-RDH antibodies and sheep anti-rabbit Ig antibodies. RNA isolated by phenol extraction from these immune-precipitated polysomes is capable of directing protein

TABLE 10.3

Electrophoretic mobilities of Met-containing RDH thermolytic peptides at pH 6.5 before and after alkylation by iodoacetamide

Peptide	Sequence	1st Dimension		2nd Dimension		Predicted Mobility		Observed Mobility	
		M.W.	Charge (+2)	M.W.	Charge (+3)	1st (-0.71)	2nd (-0.94)	1st	2nd
A	Met-Lys-His-Ser	592	+1.3	652	+2.3	-0.47	-0.72	-0.45	U
B	Val-Ser-Ser-Met-Asn-Thr-Ser	742	0	802	+1	0	-0.29	0	-0.25
A-B	Met-Lys-His-Ser-Val-Ser-Ser-Met-Asn-Thr-Ser	1334	+1.3	1454	+3.3	-0.28	-0.61	-0.30	U
B1	Val-Ser-Ser-Met-Asn-Thr-Ser-Leu-Ser-Gly-Lys	1164	+1	1646	+2	-0.24	-0.36	—	—
C	Leu-Met- Gln-Ala-Asp-Gln	704	-1	764	0	+0.33	0	+0.35	-0.06
C1	Val-Asp-Leu-Met-Gln-Ala-Asp-Gln	918	-2	978	-1	+0.52	+0.26	+0.48	+0.24
C2	Leu-Met-Gln-Ala-Asp-Gln-Val-Asp-Asn	1050	-2	1110	-1	+0.48	+0.24	+0.48	+0.24
D	Leu-Asp-Asp-Trp-Pro-Lys-Ala-Lys-Met-Asp-Glu-Ala	1255	-3	1315	-2	+0.61	+0.41	Not analysed	
D1	Leu-Leu-Asp-Asp-Trp-Pro-Lys-Ala-Lys-Met-Asp-Glu-Ala	1368	-3	1428	-2	+0.58	+0.39	+0.54	U
D2	Leu-Asp-Asp-Trp-Pro-Lys-Ala-Lys-Met-Asp-Glu-Ala-Leu-Ala-Asp-Gly-Ser	1514	-3	1574	-2	+0.54	+0.37	+0.54	U
E	Leu-Met-Gln-Pro	487	0	547	+1	0	-0.33	0	-0.32
E1	Leu-Ala-Asp-Gly-Ser-Leu-Met-Gln-Pro	930	-1	990	0	+0.26	0	+0.26	U
E2	Leu-Met-Gln-Pro-Ile-Glu	729	-1	789	0	+0.32	0	+0.35	-0.06
F	Leu-Phe-Met	409	0	469	+1	0	-0.43	0	-0.43
F1	Val-Leu-Phe-Met	508	0	568	+1	0	-0.37	0	-0.38
F2	Leu-Phe-Met-Val-Thr-Arg-Ser-Lys-Asn	1150	+1	1210	+2	-0.23	-0.44	—	—
F3	Phe-Met	296	0	356	+1	0	-0.52	0	-0.52

Mobilities are expressed relative to the mobility of aspartic acid, where the mobility of aspartic acid from DNS-Arg marker relative to DNS-OH is 0.67. The primary peptides are represented by single letters, A to F. U, is undifferentiated peptide in second dimension due to inadequate iodoacetamide application.

FIG. 10.9 . Complete sequence of ribitol dehydrogenase (Morris et al., 1974). Met-containing thermolytic sequences are indicated. Solid lines represent primary peptides, broken lines represent partial or secondary peptides.

synthesis in a crude E.coli cell-free system. Analysis of the ^{35}S-labelled in vitro translation product by SDS-polycrylamide gel electrophoresis and by Met-diagonal electrophoresis of the thermolytic digest, positively identify this major product to be ribitol dehydrogenase.

The proportion of the double-antibody-precipitated RDH polysomes in the total polysome population of Klebsiella aerogenes strain A111 at culture A650 nm of 0.6 - 0.7, was estimated to be about 10%.

Characterization of ^{125}I-Ab bound RDH polysomes by velocity sedimentation on sucrose gradients (Chapter VIII) showed that an RDH mRNA molecule is associated on the average with about 17 ribosomes (this can be calculated from the data in Table 8.2). Thus on the weight basis RDH mRNA should represent only about 1% of the total RNA extracted from immune precipitated polysome. From Table 10.2 one can calculate that about 3.2 μg of pure Qβ mRNA is equivalent to 9 μg of the purified RDH mRNA in the ability to direct incorporation of ^{35}S-Met into protein. That is to say, pure RDH mRNA must be at least $3.2 \times \frac{100}{9} = 35.5$ times more active than Qβ RNA in this respect. Considering that the addition of rRNA to the protein synthetic system (as in the case of purified RDH mRNA) is inhibitory to the translation process, it is quite remarkable that RDH mRNA can still incorporate amino acids 35 times better than a pure and stable viral (Qβ)mRNA. This suggests that the mutant RDH mRNA could contain more efficient translation iniation (and possibly termination) features.

CHAPTER XI

DISCUSSION

Monospecific and ribonuclease free antibodies have been shown in this study to be very useful for quantitative preparation of a biologically pure mRNA from bacterial cells that contain a multitude of different mRNA species. In addition they have enabled me to observe and to investigate some novel aspects of bacterial messenger RNA. For instance the discovery that the bacterial cell is capable of increasing the synthesis of a particular protein which is greatly in demand, by making the mRNA which codes for that protein singularly stable, without apparently affecting the stabilities (or instabilities) of other mRNA species, is very interesting. It enables us to make realistic speculations about the mechanism of in vivo degradation of bacterial mRNA, and what could make a mRNA stable or unstable.

Equally interesting is the observation of the sudden "general transcription shutdown" at a particular cell density of culture, at the *mid* log phase, while cell division continues thereafter for at least 6-7 doublings. Investigation of the precise mechanism that brings about this transcription switch-off may prove quite fruitful. As a result of this transcription shutdown a distinct and rapid shift takes place in the sucrose gradient-sedimentation distribution of RDH-polysomes, specifically identified by ^{125}I-anti-RDH. Indeed, this particular observation provided the major clue to the discovery of unusual stability of this mRNA from the RDH high-synthesis strain K.aerogenes A111.

By using the techniques of double immuno-precipitation and phenol extraction, I have purified intact RDH mRNA from polysomes

extracted from K.aerogenes Alll cells which had grown up to late log phase, just past the "transcription switch-off point", where the RDH mRNA was expected to be enriched while the risk of contamination by all the different labile mRNAs was expected to be minimal. Indeed, the comparitive in vitro translation of RDH mRNAs, prepared by using either highly purified or partially purified anti RDH antibodies, have shown that those expectations are correct; there were no significant amounts of other polysomes present to react with these contaminant antibodies, as revealed by the SDS-gel electrophoresis analysis of the in vitro translation products. Therefore, absolutely pure antibodies are not required under these conditions to yield highly purified stable RDH mRNA. Biological purity of this messenger RNA (i.e. its capacity to programme the synthesis of only RDH polypeptide in a cell-free system) is extremely high; at least 50% of the ^{35}S-labelled protein seems to migrate in a single band with pure RDH monomer on SDS gels. Further chemical purification of this mRNA should be relatively easy, by applying various techniques such as separation of mRNA from ribosomal subunits in sucrose gradients either under low Mg^{++} concentration or after puromycin treatment, RNA gel electrophoresis and/or hybridization to L-strand $\lambda \frac{rbt^+}{}$ (complementary)-DNA techniques may also be used if necessary.

Studies of the kinetics of RDH enzyme production, the binding of ^{125}I-labelled specific anti-RDH antibodies to polysomes carrying nascent RDH polypeptides, and the cell-free synthesis of RDH in crude E.coli S30 extract programmed by the purified Klebsiella aerogenes Alll RDH-mRNA indicate that this RNA is extremely stable, both in vivo and in vitro. ^{125}I-specifically-labelled RDH polysomes do not show any decay up to 65 min measured

after addition of Rifampicin and 5-iodouracil to the culture (Chapter VIII). Also, by far the major product of *in vitro* translation of purified RDH-mRNA, as analyzed by SDS-Polyacrylamide gel, is the complete RDH monomer (Chapter X).

Such a highly stable bacterial mRNA must have been selected in the chemostat experiments under continuous pressure to grow these cells on a poor substrate as xylitol. At present the only evidence suggesting that wild-type RDH-mRNA is not stable comes from studying a parallel cell line (EA) in *E.coli* K-12 which has incorporated the wild type *K.aerogenes* RDH gene into its own genome. On the basis of enzyme production the constitutive (but not high-synthesis) strain EA does not seem to have stable RDH-mRNA, while the evolvant high-synthesis strain EA IIII shows the same kinetics of enzyme synthesis as *K.aerogenes* AIII; it must have stable RDH-mRNA (see Chapter III). The mechanism by which such stability has been conferred on RDH-mRNA is unknown at present. I have ruled out the possibility that this mRNA is protected from nuclease digestion as a result of some kind of association with cell membranes since polysomes of this stable RDH-mRNA are found free in the cytoplasm. However this explanation is not ruled out for a number of naturally semi-stable (non-RDH) bacterial messages, which I have found their polysomes to be firmly bound to cell membranes, and I suspect that these polysomes are engaged in the synthesis of exported and outer membrane proteins.

It is unlikely that 'blocked' or 'capped'-5'-ends, such as occur in most eukaryotic mRNAs (Furuichi et al., 1977; Shatkin, 1976; Both et al., 1975; Kozak and Shatkin, 1976), could be the principle reason for stability of RDH mRNA, since such modifications obviously require specific enzymes. 3'-end modifications such

as 'poly A tails' (Edmonds and Camela, 1969; Gillespie et al., 1972; Darnell et al., 1972) also are unlikely to be the major reason for this RDH-mRNA stability, because it is now established that mRNA degradation in bacteria occur from the 5'- to 3'-end (Morikawa, Imamoto, 1969; Morse et al., 1969; Schlessinger et al., 1977). Thus, the most probable mechanism for evolution of this stable mRNA would be point mutation(s) in the 5'-end non-translated region of mRNA leading to destruction of a specific ribonuclease binding site (or, less likely, creation of certain secondary structures in the same region which would be resistant to the 5'-end nuclease cleavage). Determination of the 5'-end structure of this stable RDH-mRNA, together with that of corresponding region of the unstable (wildtype) RDH mRNA (as could be deduced from sequencing the appropriate DNA fragment from restriction enzyme digests of $\lambda \frac{rbt}{}$) could elucidate the precise mechanism of the inherent bacterial mRNA instability as well as its mutation to a stable form.

It is important to point out that an increasing number of point mutations in the critical 5'-end region of the mRNA could yield a series of stepwise mRNA stability mutants, producing different levels of enzyme high synthesis in each strain. Clearly, it is important to establish whether or not the succeeding RDH-high synthesis mutants in a particular cell line evolved in a chemostat have stabler RDH-mRNA at every step of evolution. The stabilities of RDH mRNA from different mutants could be measured, following addition of rifampicin to the culture, either directly, by hybridization of RDH-mRNA to $\lambda \frac{rbt}{}$DNA, or indirectly from kinetics of enzyme synthesis or binding of ^{125}I-anti RDH to polysomes.

The enzymic mechanism of bacterial mRNA degradation from the 5'-end has been a controversial subject for a number of years (see Chapter I, "Introduction"). At least one serious hypothesis (Altman and Robertson, 1973), that the degradation from 5'-end is brought about as a result of combined action of various endo-and-3'-exo-nucleolytic ribonucleases, now appears to be wrong - because RDH mRNA from the K.aerogenes A111 strain which produces wildtype enzyme is extremely stable both in vivo and when it is presented naked to the crude E.coli cell-free system. Ribonuclease V (Kuwano et al., 1969) is the only known 5'-ribonuclease at present which seems to satisfy the requirements for bacterial mRNA degradation in vivo.

The trp operon mRNA transcribed from the PL promoter of the λ N gene with a 5'-terminal λN message (Yamamoto and Imamoto, 1975) has a similar functional stability (i.e. capacity to serve as template for in vivo enzyme production) as the trp mRNA from uninfected cells (half-life of 1-2 minutes), and PL trp mRNA seems to be translated as efficiently as the normal trp mRNA. In contrast to this functional stability, PL trp mRNA shows a significantly greater chemical stability than the normal trp mRNA. Also this stabilization of trp mRNA produced from the PL promoter increases with time after phage infection (chemical half-life increases up to greater than 23 min in the later times after phage infection). It was inferred that (1) the rate limiting step to initiate bulk mRNA degradation is determined by a sequence located at or near the 5' end of the messenger RNA; (2) functional inactivation of each mRNA is regulated independently of bulk chemical degradation of the message; and (3) the chemical stabilization requires a modification of the decay trigger, possibly by a phage-specified protein such as the N and/or tof

gene product(s) that might bind to the mRNA.

I find these inferrences are, first of all, somewhat contradictory. Functional inactivation (for example, by endonuclease cleavage at specific sites) in both PL trp mRNA and in normal trp mRNA occurring at the same rate, implies that endonuclease cleavage occurs at least as frequent as 5'-exonuclease initiated at a specific site. Thus with the efficient 5'-terminal endonuclease cleavage of normal trp mRNA, one would expect the chemical stability of the rest of the message to be the same as the PL-trp mRNA, but apparently it is not so.

Secondly, RDH mRNA neither appears to be cleaved in vitro nor does it degrade in vivo when transcription is terminated by the addition of antibiotics rifampicin and 5-iodouracil. Furthermore, chemical stabilization as in PL trp mRNA, postulated to require modification of the decay trigger by a phage coded-protein does not apply to RDH mRNA, since this apparently transcribed from its own promoter in the uninfected cells.

The mechanism of enzyme evolution has been a topic of considerable interest among scientists. One of the most interesting theories holds gene duplication as the initial step in the evolution of new enzymes (e.g. Hartley, 1966; Rigby et al., 1974). Evidence for gene duplications based on comparisons of tertiary structures of different related proteins is apparently well documented and has been extensively reviewed by Dothie (1974), also by Hartley (1974) and Rigby et al. (1974). Experimental enzyme evolution with the RDH system has yielded several RDH high-synthesis strains, some of which have been particularly attributed to gene duplication and triplication. Evidence for these duplications is gathered on three types of statistics:

(1) A gene duplicated strain after overnight growth on broth reverts to a single gene strain with a high frequency of the order of 10^{-3}. Reversion of a point mutation would occur at a frequency of about 10^{-6}.

(2) Revertants of a gene duplicated strain (A11) all give rise to small colonies on 0.05% xylitol plates and all of these produce wild type enzyme levels. Revertants of a gene triplicated strain (such as A111 or A211), some have enzyme levels like A11 and produce intermediate size colonies on 0.05% xylitol plates, while others are wild type in behaviour and produce small colonies on 0.05% xylitol.

(3) Frequency of mutation to RDH^- - A single gene copy strain, A or A1, produces RDH^- mutants with certain frequency. A presumed gene doubled strain gives rise to RDH^- phenotype with square of the probability of A, this is a very infrequent event. A gene triplicate produces RDH^- with cube of the probability of A, this in fact is never observed because of very low probability.

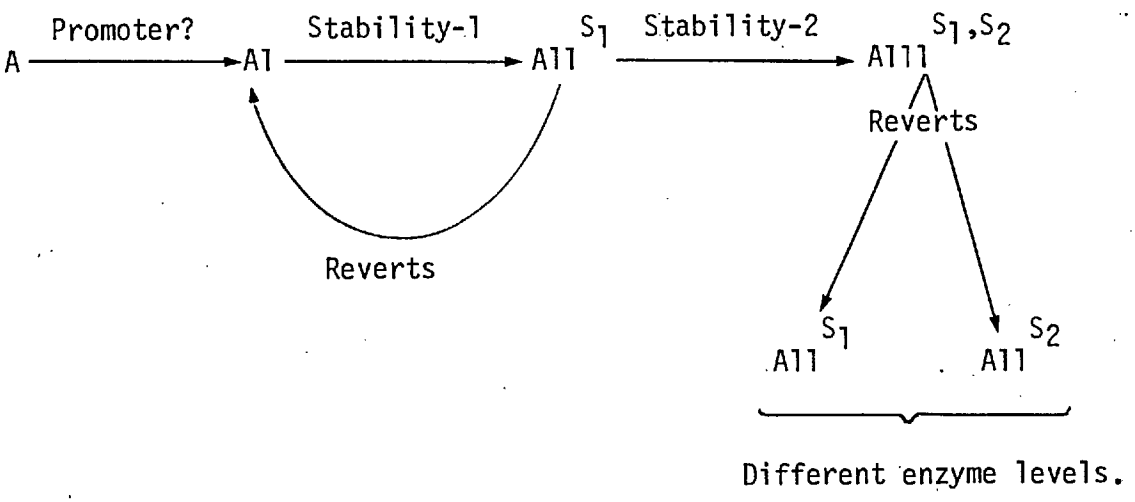
One may ask the question: could these statistical phenomena be explained by a mutation to mRNA stability (or a few successive mutational events leading to stabler mRNA after each event), as an alternative model to the theory of gene duplication? The answer is that it is not entirely improbable:

(1) The segregation frequencies observed by Rigby et al. (1974) may be artefactual. A strain producing large amounts of RDH may have a lower growth rate on rich medium than the wild type. Hence overnight growth on rich medium, as used in the above experiment, would enrich the culture in the revertant relative to the superproducer. Hence the observed frequencies of around 10^{-3} for strain A11 could actually have been as low as 10^{-6},

which is the accepted range for reversion of a single point mutation. Higher apparent reversion frequencies for hyperproducers would reflect greater growth disadvantage on the rich broth medium prior to screening.

(2) Segregation patterns of the apparent gene-duplicated and triplicated strains can be explained as follows:

If the step AI ⟶ AII is a mutation to mRNA stability of large magnitude and the step AII ⟶ AIII is an additional mutation to further stability of the mRNA, then the revertants of AII could only produce small colonies on 0.05% xylitol and have wild type enzyme level, while the revertants of AIII would be two types, AII^{S_1} and AII^{S_2}. If these two stability mutations confer different mRNA stabilities, therefore, different enzyme levels in the corresponding mutants, then we expect to observe say intermediate size colonies with AII-type enzyme production for AII^{S_1}, and smaller colonies with near-wild type enzyme levels for AII^{S_2}. These can be shown diagrammatically as follows:



(3) The low frequency of mutations to RDH^- by AII and AIII can be explained by postulating that mutational events to RDH^-

in the high-synthesis may still result in production of vast amounts of a mutant protein which might partially function as a poor enzyme to metabolize xylitol/ribitol and make the RDH$^-$ mutants still phenotypically RDH$^+$, thus producing small colonies but very few RDH$^-$ colonies. On the other hand, the same mutation in a low synthesis strain may not have enough of this mutant protein to metabolize sufficient quantities of the substrate, so more frequently it would give rise to apparent RDH$^-$ mutant colonies.

Fortunately, we are now in an extremely strong position to test these hypothesis and assign definite gene duplication steps to each mutant in question. Purified ^{32}P-labelled stable RDH-mRNA can easily be extracted in quantitative amounts from bacterial polysomes. This may be used to titrate the DNA prepared from various high synthesis and low synthesis mutants by DNA-RNA hybridization techniques, in order to establish which strains contain more than one copy of the RDH gene. Furthermore, the length of the DNA-RNA hybrid measured accurately by heteroduplex electron microscopy should tell us whether such gene duplications are in tandem or are randomly scattered in various sites of the bacterial chromosome.

I have not yet determined the length of RDH mRNA, but this should be easy by sedimentation analysis on SDS-sucrose gradients, and by gel electrophoresis. The maximal number of ribosomes loaded on this mRNA, together with the figure of 20 nucleotides covered by each ribosome and no space between the ribosomes during derepression of trp operon (Morse et al., 1969), tell us that RDH mRNA should be at least 1000 nucleotides long. But the great heterogeneity of the number of ribosomes loaded per RDH mRNA observed both during early and late log phase would tell

us that this estimation may not be very accurate and RDH mRNA could in fact be a great deal longer. If this is proven to be true, then it raises the question, how is it that other putative polypeptide(s) coded by this possibly polycistronic mRNA are not synthesized as frequently as RDH? The idea of 'translational promoters' in this regard is very intriguing, some examples of the existence of such 'translation promoters' with differential efficiencies in RNA bacteriophages and in bacteria were mentioned in Chapter 1 (Introduction). Preliminary results in this study show that RDH mRNA is translated at least 35 times more frequently than Qβ RNA. Therefore, different translation initiation promoters must have different binding affinities for ribosomes.

Whether the stable RDH mRNA from K.aerogenes Alll has also an evolved and more efficient translation promoter than the other labile bacterial messages we do not know. But the chances are that it has not, since from close examination of polysome profiles (Chapter VIII) RDH-mRNA does not seem to compete for in vivo translation any stronger than other (labile) bacterial mRNAs during early logarithmic growth phase, this could explain the 'triangular' pattern of sedimentation of RDH-specific polysomes on sucrose gradients during early log phase.

Direct Watson-Crick base pairing between the 3' terminus of 16S RNA and a specific sequence of nucleotides near the AUG codon in the 5'-end of messenger RNA seems to contribute to the recognition and binding of protein synthesis initiator regions by bacterial ribosomes (Shine and Dalgarno, 1974; Steitz and Jakes, 1975). Shine and Dalgarno noted that certain nucleotide sequences are common to the ribosome-binding sites of most of the coliphage cistrons sequenced and, in particular, that all coliphage RNA ribosome-binding sites sequenced to date contain

all or part of the purine-rich sequence 5'-AGGAGGU-3' in a similar relative position on the 5' side of the initiator codon AUG. This sequence is complementary to a region at the 3' end of the E.coli 16S ribosomal RNA, OH-AUUCCUCCACUAG (5'), (Shine and Dalgarno, 1974). Although such complementary sequences in certain cases may be sufficient for formation of stable initiation complexes, in a lot of cases this may not be strictly true. Some very efficiently translated mRNAs have only 3 or 4 bases complementary to the sequence at the 3'-end of 16S ribosomal RNA. Additionally, these sequences are not all the same distance from the initiating AUG codon. Most of these sequences are 6 - 10 nucleotides apart from the 5' side of the initiator codon, but some are much farther apart (e.g. in lac-mRNA the only complementary region, GGA is 25 bases away from the initiator AUG codon; gal-mRNA UAAG complementary sequence is 16 bases apart from AUG). Furthermore, it has been shown that an intact 3'-end of 16S rRNA is not required to form stable initiation complexes in vitro (Ravetch and Jakes, 1976).

I have observed that almost every E.coli or coliphage mRNA sequenced up to now can form some kind of a hairpin-like secondary structure adjacent to the 5' side of the initiating codon, as shown in Fig.11.1. These structures could play an important part in recognition and stable attachment of the mRNA-ribosome. There may be a special groove provided in the 30S ribosomal subunit to accommodate such hairpin structures, furthermore, the invariable sequence complementarity between the 3'-end of 16S RNA and the 5'-side of the hairpin would considerably strengthen this interaction. There is certain amount of variation in the shape and the size of this hairpin loop which could be an important factor in the observed

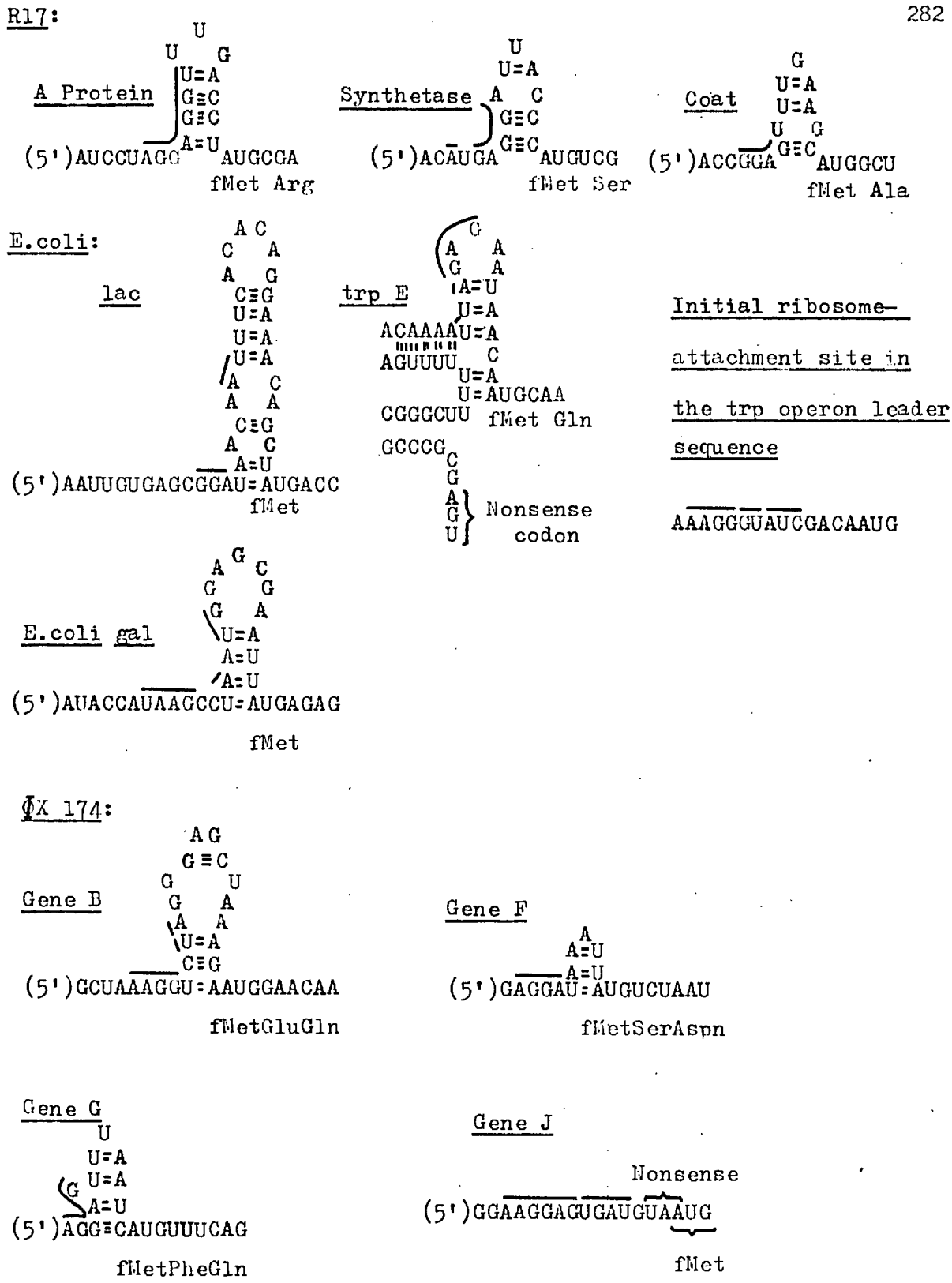


FIG. 11.1. Structures in the non-translated 5'-initiation sites of various mRNAs contributing to E.coli ribosome binding. Solid lines above represent the nucleotide regions which can form Watson-Crick base pairing with 3'-end of 16 S ribosomal-RNA.

heterogeneity in the rate of initiation of translation of various messages. In fact the trp mRNA initiator site contains three adjacent hairpins as shown in (Fig.11.1). Some loops like the lac initiator are very long. Both of these factors could contribute to the stability of the initiation complex and the rate of translation. It would be interesting to see what the stable RDH-mRNA initiator region looks like, and whether it is different from the wildtype RDH mRNA initiator. I have screened several RNAs, including the entire 5' sequences of the above mRNAs and I could not see a secondary structure adjacent or near the 5' side of the non-initiating AUG codons. The initiator sequence corresponding to the J-gene mRNA of ϕX 174 (Fig.11.1) cannot form a hairpin secondary structure, but this site can form a possible ten base-paired complex (if a single base is looped out of the 3'-end of 16S rRNA). Thus, both the complementary initiating sequence to the 3'-end of 16S rRNA as well as the hairpin secondary structure adjacent to the AUG codon seem to be important in the binding of ribosomes to mRNA.

The information gained from this study raises several interesting questions. It is well known that the translation initiation factors, in particular IF-3, become greatly depleted from the aged bacterial cultures. But stable RDH mRNA is very efficiently translated up to hours in the stationary phase. Does this indicate that the presence of a population of normally labile, cytoplasmic mRNAs is required for maintenance of activity of these otherwise labile initiation factors, and the reason that they normally become depleted in the aged cultures is because of general transcription shut down?

A class of metastable bacterial polysomes which are membrane bound have been recognized in this work which may be

related to the structural outer membrane and excretory proteins. Comparative studies of polysomes in this work, as well as previous studies of Hirashima et al. (1973) suggested that these membrane-bound bacterial mRNAs are translated in a different way from the cytoplasmic mRNA. These polysomes can now be fractionated easily from the cell extracts by the selective detergent extraction method used in this study, and the mRNA can be purified by the techniques described by Hirashima et al., 1974. Then one could study the translation of these purified messages *in vitro* in presence or absence of membranes, and investigate the mechanism of export of their products.

E.coli K-12 is unable to utilize pentitols; it is unlikely that it could carry the permease gene(s) for the transport of pentitols. Then how does *E.coli* K-12 which has incorporated the RDH gene from *K.aerogenes* into its own genome transport ribitol or xylitol? Has *E.coli* K-12 acquired the appropriate permease gene as well? $\lambda \frac{rbt^+}{}$ lysogen in *E.coli K-12* allows the organism to grow on xylitol immediately (M. Neuberger, 1977). It implies that this prophage probably carries the necessary permease gene. In that case one should be able to select temperature-sensitive (permease) mutants of $\lambda \frac{rbt^+}{}$ which could support the host cell growth on xylitol at the permissive temperature, and not at unpermissive temperatures, while synthesizing RDH constitutively during growth in broth at both temperatures.

A high synthesis mutant which produces a very stable RDH mRNA such as *K.aerogenes* A111 theoretically can go on synthesizing more and more RDH under continuous culture till the cells become packed with RDH and burst. But, of course, this never happens and the cells must reach a stable level of RDH eventually. Then how is this stable level of RDH maintained?

One can speculate that certain proteases indiscriminately remove the extra amount of proteins present in the total cellular protein pool. RDH being removed by this process is not replaced as quickly, since its synthesis is in competition with hundreds of other proteins.

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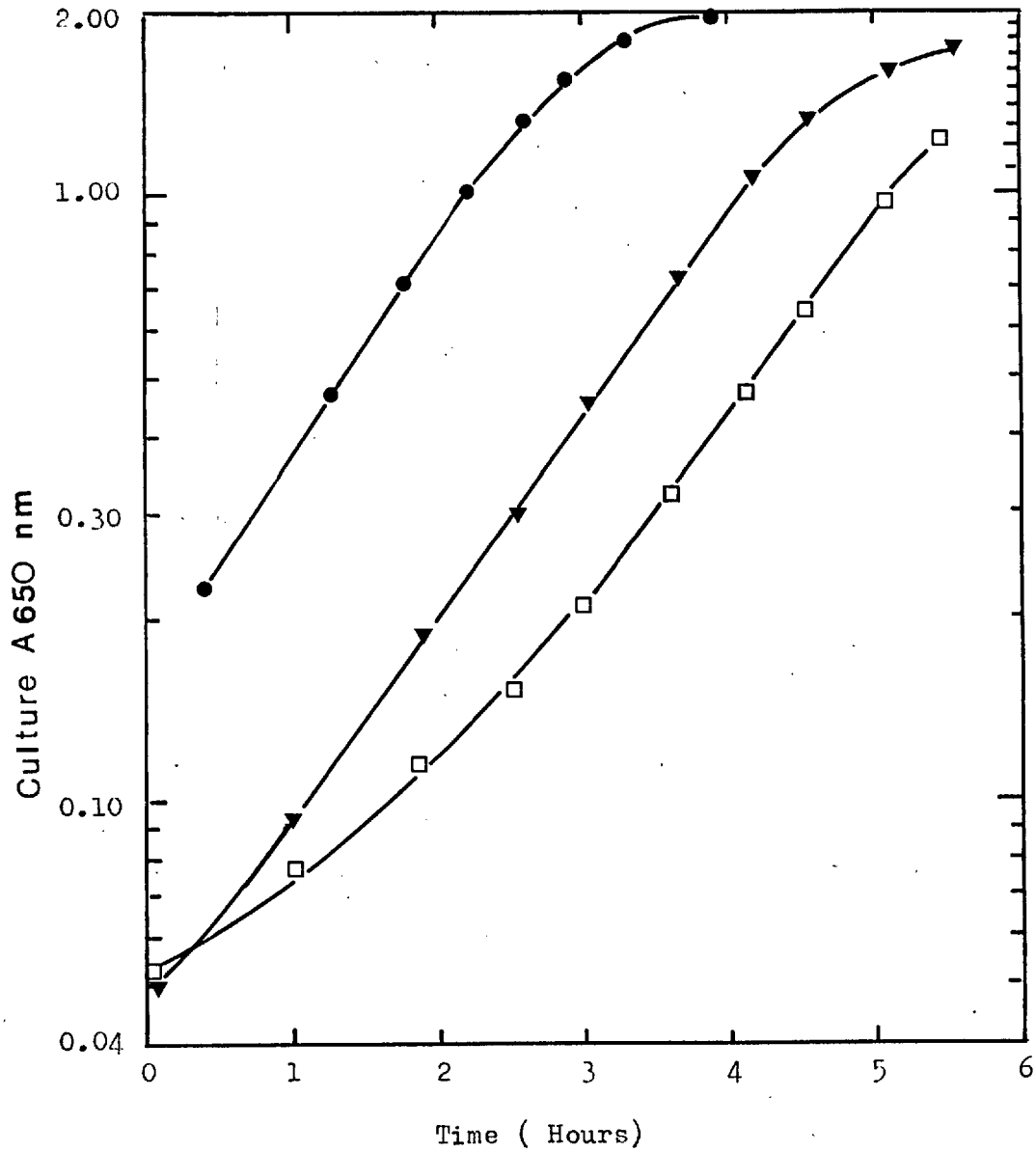
Appendix

Growth curves of Klebsiella aerogenes A111



(●): An overnight M9/ Xyl culture of K. aerogenes strain A111 was inoculated by a single 5-weeks old colony, which was stored on a M9/ Xyl plate at 4°C, and grown at 37°.A sample from this fresh overnight culture was streaked on to a M9/ Xyl plate, which was grown for two days at 37°. A sample from this fresh plate was inoculated into a 30 ml M9/Xyl medium, the increase in A 650 of this culture (undiluted) is shown.

(□): A colony from the above 5-weeks old plate was directly inoculated into 30 ml of M9/ Xyl , the growth of which is shown.

(▼): A 3-days old colony was directly inoculated into a 30 ml M9/ Xyl medium, the growth is indicated.

The lag periods of the three cultures are not shown.