RESISTANCE TO VERNAMYCIN A IN STREPTOMYCES LOIDENSIS

by

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PREFACE

This dissertation contains the results of work carried out in the Department of Biochemistry at the University of Leicester during the period October 1978 to October 1981.

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This dissertation represents the result of my own unaided work and is not substantially the same as one which has been submitted at any other University.

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CONTENTS

ABBREVIATIONS

Ax	:	absorbance at a wavelength of x nm
ATP	:	adenosine triphosphate
Bis-Tris	:	Bis (2-hydroxyethyl) imino-Tris (hydroxymethyl) methane
butyl-PBD	:	2-(4'-tert-butylphenyl)-5-(4"-tert-biphenylyl) -1, 3,4-oxadioazole
c.p.m.	:	counts per minute
DMSO	:	dimethylsulphoxide
DNase	:	deoxyribonuclease
DTT	:	dithiothreitol
DEPC	:	diethylpyrocarbonate
EDTA	:	diaminoethanetetra-acetic acid
EF-G	:	elongation factor - G
GTP	:	guanosine triphosphate
HEPES	:	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
MBA	:	N,N'-methylene-bis-acrylamide
MES	:	2[N-morpholino] ethane sulphonic acid
NCIB	:	National Collection of Industrial Bacteria
NRRL	:	Northern Regional Research Laboratories
PEG_x	:	polyethylene glycol, average molecular weight x
PEP	:	phosphoenol pyruvate
PTFE	:	polytetrafluoroethylene
poly (U)	:	polyuridylic acid
poly (U,G)	:	polyuridylic, guanylic acid
RNA	:	ribonucleic acid
RNA 30, RNA 50, RNA 70	:	RNA from 30S, 50S ribosomal subunits or 70S ribosomes

mRNA, rRNA, tRNA	:	messenger, ribosomal and transfer RNA
RNase	:	ribonuclease
S	:	Svedberg unit (10^{-13} seconds)
SAM	:	S - adenosyl-methionine
SDS	:	sodium dodecyl sulphate
TCA	:	trichloroacetic acid
Tris	:	tris (hydroxymethyl) aminomethane
TEMED	:	N,N,N',N'-tetramethylethylenediamine

CHAPTER I

GENERAL INTRODUCTION

Chapter 1

1. Biosynthesis of antibiotics and control mechanisms

Antibiotics are often regarded as "secondary" metabolites since, in contrast to "primary" metabolites, their production is not essential for growth of the organism. By far the greatest proportion of known antibiotics are produced by the actinomycetes. Generally, antibiotic production occurs only after cellular growth has terminated and is often the result of a deficiency of one or more nutritional growth-limiting components. In contrast, in defined media, where certain growth factors may be limiting, antibiotic production may occur at the start of cultivation. Accordingly, micro-organisms often produce antibiotics when their growth rate falls below a certain level.

Production of antibiotics can be determined by either chromosomal or extra-chromosomal elements. Often structural genes for antibiotic synthesis can be of chromosomal origin while regulatory genes controlling their expression are located on plasmids (Hopwood, 1978). Hence, extrachromosomal determinants could be responsible where antibiotics of the same (or similar) structure are produced by bacteria of diverse origins. For example, neomycin, produced by Streptomyces fradiae is also produced in species of Micromonospora (Wagman et al., 1973). In contrast, chemically-diverse antibiotics can also be produced by closely-related bacterial strains; accordingly, various strains of Streptomyces griseus can produce streptomycin, cycloheximide, candicidins, cephamycins and several other antibiotics (Stapley et al., 1972).

Evidence for plasmid-determined antibiotic production initially came from "curing" experiments where antibiotic-producing bacteria exposed to UV-light or ethidium bromide subsequently failed to produce antibiotics. While such experiments are not conclusive (Freeman et al., 1977) and often require additional approaches (Hopwood, 1978), the ability to loose two characters

upon curing offers reasonable proof. Accordingly, the ability to loose both methylenomycin production and resistance following loss of the SCP 1 plasmid from Streptomyces coelicolor has been demonstrated by Kirby and Hopwood (1977).

Like other biosynthetic processes, production of antibiotics can be under various control mechanisms. Accordingly, while glucose has been shown to interfere with the biosynthesis of many antibiotics, in only a few cases have specific mechanisms been implicated. For example, glucose has been shown to both repress phosphatase (s) required for neomycin biosynthesis and stimulate inactivation of the antibiotic (Majumdar and Majumdar, 1971; 1972). Similarly, repression of kanamycin biosynthesis by glucose has been reported to result from the effect of the sugar upon N-acetylkanamycin amidohydrolase - the last enzyme in the kanamycin biosynthetic pathway (Satoh et al., 1976).

Nitrogen sources may play a role in the regulation of antibiotic production since production of erythromycin is depressed when nitrogen sources are added to growth media (Smith et al., 1962). However, perhaps the most critical regulator of antibiotic biosynthesis is phosphate. Addition of phosphate to growing cells often results in a delay of onset of antibiotic biosynthesis until the phosphate becomes depleted (Mertz and Doolin, 1973). Addition of phosphate after the onset of antibiotic production can also lead to premature termination of production (Liu et al., 1975; Martin and Demain, 1976). Accordingly, phosphate has been shown to be depleted in the growth medium of Streptomyces griseus prior to the onset of candicidin biosynthesis (Liras et al., 1977). Although phosphate can affect the production of chemically-diverse antibiotics (e.g. peptides, polyene macrolides, tetracyclines) it is not clear at present whether a common regulatory mechanism exists. While phosphate may act directly, it can also act via ATP

(Silaeva *et al.*, 1965; Janglova *et al.*, 1969; Fynn and Davison, 1976) although it is not clear whether the ATP concentration or adenylate energy charge in cells is the true regulatory parameter. Phosphatase may also act as a regulator of antibiotic biosynthesis. A number of biosynthetic pathways e.g. for streptomycin (Miller and Walker, 1970) and neomycin (Majumdar and Majumdar, 1970) contain phosphorylated precursors. Accordingly, phosphatases which cleave antibiotic intermediates (to form active antibiotics) could function *via* feedback inhibition or repression, by inorganic phosphate. Hence *Streptomyces bikiniensis* (the streptomycin-producer) produces a biologically-inactive phosphorylated compound (which may be a streptomycin precursor) in response to high concentrations of phosphate (Miller and Walker, 1969). Furthermore streptomycin phosphatase is inhibited by phosphate (Walker and Walker, 1971).

Finally, production of antibiotics may be under the control of induction processes. Although it is possible to observe the stimulation of biosynthesis of certain antibiotics by exogenously-added compounds, it is not always possible to determine whether these compounds are acting as true inducers or just as antibiotic precursors. Stimulation of streptomycin biosynthesis by 2-S-isocapryloyl-3-R-hydroxymethyl-γ-butyrolactone has been observed by Kleiner *et al.*,(1976). Although the mechanism of stimulation has not been proven, it may involve the production of a compound which decreases the activity of glucose-6-phosphate dehydrogenase. Similarly, the mechanism of stimulation of cephalosporin C production by *Cephalosporium acremonium* by both methionine and its non-sulphur analogue norleucine, remains unclear (Drew and Demain, 1975).

Termination of antibiotic production may occur through depletion of essential antibiotic precursors or by repression of antibiotic-synthesising enzymes (through feedback inhibition by the accumulated antibiotic). In general terms,

the concentration of exogenous antibiotic required to inhibit antibiotic formation is similar to the maximum level of antibiotic produced by the microorganism. Thus, Streptomyces sp. 3022, which can produce approximately 120 μg chloramphenicol per ml, curtails antibiotic production upon addition of similar concentrations of drug to the culture medium. Accordingly, chloramphenicol can repress the activity of arylamine synthetase - an enzyme which is unique to the biosynthesis of chloramphenicol - and which converts chorismic acid into p-aminophenylalanine (Jones and Westlake, 1974). Other antibiotics have also been shown to inhibit their respective synthetase enzymes (Muth and Nash, 1975; Sankaran and Pogell, 1975).

In conclusion, antibiotic production can be encoded by either chromosomal or extrachromosomal elements and be under the control of a variety of mechanisms including carbon catabolite repression, nitrogen metabolite regulation, phosphate regulation and induction.

2. The Role Of Antibiotics

The role of antibiotics in nature is the subject of much controversy. Clearly their biosynthesis imposes a heavy metabolic burden upon the organism and presumably would have been selected against were they not of value. Whilst a number of hypotheses have been proposed in the past, many are no longer seriously considered (Weinberg, 1970) including those implicating antibiotics as either waste products of cellular metabolism or food-storage materials.

Of those currently "in favour" the so-called "competition hypothesis" suggests that production of antibiotics in nature confers a survival advantage to the producing organism. Accordingly, antibiotic production has been demonstrated in natural environments (i.e. soil) and the level of production found to be dependent upon the availability of nutrients (i.e. limiting nutrients favouring antibiotic production) (Brian, 1957; Burkholder, 1959). Antibiotics have also been implicated in the onset of the sporulation process (Katz and Demain, 1977; Mukherjee and Paulus, 1977). Thus, many sporulating bacilli produce antibiotics, and inhibition of spore formation usually coincides with inhibition of antibiotic formation (Sarkar and Paulus, 1972). A similar relationship exists for actinomycetes; while asporogenous mutants of *Streptomyces griseus* and *Streptomyces lavendulae* failed to produce streptomycin and streptothricin respectively, revertants from both organisms were fully capable of antibiotic production (Schatz and Waksman, 1945; Egorov *et al.*, 1971). However, this simple relationship is not always apparent. For example, certain non-sporulating genera (e.g. *Pseudomonas*) are antibiotic-producers. Conversely, it is possible to isolate mutants which can sporulate but not produce antibiotics. Thus, whilst it is tempting to conclude that antibiotic biosynthesis is not the instigator of sporulation,

the possibility still exists that other (perhaps intracellular) antibiotics act as a "trigger" for sporulation (Szabó *et al.*, 1961; Ito and Koyama, 1972).

In addition to possible instigators of sporulation, antibiotics may function in a regulatory capacity. Hence, peptide antibiotics have been implicated as inhibitors of DNA synthesis, cell wall synthesis and modifiers of membrane structure or function - three processes occuring during sporulation (Sadoff, 1972). Alternatively, regulation could occur at the level of RNA synthesis. Accordingly, tyrothricin (a mixture of gramicidin and tyrocidine) has been shown to inhibit RNA polymerase from *B.brevis* and may function by selectively terminating the expression of vegetative genes (Sarkar and Paulus, 1972).

The production of antibiotics has been proposed as a necessary function to maintain cellular biosynthetic machinery where cellular growth is not possible. This implies that primary metabolism can be directed into producing antibiotic precursors for subsequent modification by secondary metabolism. Presumably the antibiotic produced is of little significance; rather, the essence of the hypothesis is the necessity to maintain active primary metabolic processes in the absence of cellular growth (Bu'Lock, 1961). Others (Woodruff, 1966; Weinberg, 1971) hold the view that antibiotic production is an attempt by the producing organism to remove excess primary metabolites, formed as a consequence of unbalanced growth created by a lack of certain growth nutrients.

In conclusion, at present it is not possible to formulate a unifying hypothesis which explains all the available data. Rather, the majority of the evidence points to a role for antibiotics in either conferring a

selective advantage to the producing organism or being involved in cellular differentiation.

3. Resistance To Antibiotics

Microorganisms challenged by antibiotics potentially have a number of mechanisms of resistance at their disposal. Accordingly they can: detoxify or modify the drug (detoxification always leads to inactivation of the antibiotic whereas modification does not); prevent access of antibiotic into the cell; provide an alternative (drug-resistant) metabolic step to that normally inhibited by the drug; modify the target site of the drug such that it no longer binds; reduce the cell's requirement for the inhibited reaction; synthesise a drug antagonist; increase the concentration of the target enzyme such that the drug is "saturated" (Davis and Maas, 1952). At present, no examples of resistance mechanisms involving the last three examples have been found, although this does not imply that they do not exist (Davies and Smith, 1978).

Antibiotic resistance has been observed in producing organisms, bacterial mutants and in clinical isolates of bacteria. Whilst mutants resistant to antibiotics have provided fundamental information regarding many areas of molecular biology (e.g. studies of cell walls, transcription, replication, intermediary metabolism and protein synthesis), clinical isolates of bacteria have provided an insight into the apparent flexibility of micro-organisms when challenged with adverse conditions (i.e. antibiotics).

3.1 Resistance to antimicrobial agents in clinical isolates of bacteria

All the above-mentioned antibiotic-resistance mechanisms (excluding the last three) have been observed in clinical isolates of bacteria. In general the increase in antibiotic-resistant bacteria has resulted from the increased selection pressure imposed by the widespread usage of antibiotics in both clinical and veterinary situations. Such resistance can arise either from

mutation or, more likely, from the inheritance of R-plasmids (or R-factors).

3.1.1. Detoxification R-factors have been implicated in chloramphenicol resistance in enteric bacteria. Resistance has often been shown to result from acetylation of the antibiotic by chloramphenicol acetyltransferase (Okamoto and Suzuki, 1965) and, while the enzyme was found to be expressed constitutively in *E. coli*, in other organisms e.g. *Staphylococcus aureus*, it could be induced by the presence of chloramphenicol or some of its analogues (Benveniste and Davies, 1973). Whilst the presence of R-factor-encoded chloramphenicol acetyltransferases appears widespread in bacterial genera, it is not the only mechanism of inactivation of chloramphenicol; reduction of the p-nitro group of chloramphenicol to give the inactive amino derivative has been reported (O'Brien *et al.*, 1975).

Clinical resistance to β-lactam antibiotics (e.g. penicillins and cephalosporins) can also occur by detoxification through the widespread occurrence of β-lactamases in both Gram-positive and Gram-negative organisms. Whilst β-lactamases can be either chromosomal or plasmid-determined, there are greater numbers of chromosomal-encoded variants of the enzyme (Davies and Smith, 1978). However, whilst 80% of the plasmid-encoded enzymes constitute only two variants of the enzyme, their broad distribution throughout the bacterial genera is probably a reflection of the common presence of the relevant genes on transposons (which transpose at high frequency) (Hedges *et al.*, 1974). Surprisingly, not all β-lactam antibiotics are substrates for β-lactamases. Methicillin, for example, is completely refractory to the enzyme. Whilst methicillin-resistant strains of *Staphylococcus aureus* have been isolated, resistance appears to be the result of morphological changes in the bacterial cell envelope (Sabath, 1977).

Detoxification of heavy metals (i.e. mercury) has been observed in both

Gram-positive and Gram-negative bacteria. In E. coli the mechanism appears to involve reduction of the metal salt to metallic mercury, thereby increasing its vapour pressure with subsequent evaporation from the culture medium (Komura and Izaki, 1971; Komura et al., 1971).

3.1.2. Modification Whilst detoxification or modification of antimicrobial agents might ultimately appear to give the same result, there are subtle differences. Whereas detoxification of antibiotics usually occurs in the culture medium, antibiotic modification appears to occur intracellularly (antibiotic in the culture medium remaining unchanged). Accordingly, resistance to aminoglycoside antibiotics is usually the result of a plasmid-encoded antibiotic modifying enzyme. Much evidence implicates the periplasmic space in Gram-negative organisms as the location of aminoglycoside-modifying enzymes although, since the enzymes are not released from spheroplasts they may be associated with the inner cell membrane (Davies and Benveniste, 1974). In general, aminoglycoside-modifying enzymes can modify antibiotics by N-acetylation, O-phosphorylation or O-nucleotidylation. Whilst little is known regarding the uptake of aminoglycosides by sensitive organisms it seems likely that existing membrane transport systems could be utilised (i.e. antibiotics being transported on systems designed for structurally-analogous fuel or carbon sources) (Alper and Ames, 1978; Bryan and Van Den Elzen, 1977; Höltje, 1978). Since, in general, antibiotic in the culture medium does not get inactivated, it must be assumed that modification results in a blockage in the transport system. Alternatively, the rate of modification could equal the rate of influx of antibiotic. No attempt will be made to catalogue all the classes of aminoglycoside-modifying enzymes since their range is extensive. Rather, examples from each group will be given. Accordingly, kanamycin A can be

inactivated by an acetyltransferase (Okamoto and Suzuki, 1965) found in an R-factor-carrying strain of E. coli. Umezawa et al., (1968) demonstrated the inactivation of streptomycin by an R-factor encoded adenylylating enzyme. Similarly, streptomycin can be inactivated by phosphorylation (Ozanne et al., 1969).

3.1.3. Interference with drug transport Since antibiotics may well enter sensitive cells via established transport mechanisms, it is not difficult to imagine modifications to those transport systems resulting in prevention of drug access. Probably the most thoroughly investigated system is that of tetracycline resistance. Resistance to this drug may result from inhibition of normal transport systems (Franklin, 1967). Furthermore resistance may coincide with production of a membrane protein (the so-called "Tet" protein) which was found in plasmid-containing minicells exposed to inducible concentrations of tetracycline. Whilst synthesis of the protein has been observed in cell-free extracts of E. coli to which plasmid DNA had been added (Yang et al., 1976) extracts of non-induced cells were unable to synthesise Tet protein in the absence of inducer (i.e. tetracycline). However, as yet, a causal relationship between production of the Tet protein and tetracycline resistance has not been established. Furthermore, since the uptake of tetracycline by both tetracycline-resistant and sensitive cells is similar, resistance may involve an active pump mechanism to remove drug from the vicinity of the ribosomes (Davies and Smith, 1978).

3.1.4. By-pass mechanisms Only two instances of by-pass mechanisms (i.e. a drug-sensitive enzyme being replaced by a drug-resistant counterpart) have been observed. Resistance to sulphonamides in E. coli, Citrobacter spp. and Klebsiella pneumoniae resulted from the target enzyme, dihydropteroate

synthetase, being replaced by a plasmid-encoded, drug-resistant alternative (Wise and Abou-Donia, 1975). Similarly, resistance to trimethoprim (an antibiotic which normally inhibits dihydrofolate reductase in sensitive bacteria) was found to be the result of a new drug-resistant target enzyme (Amyes and Smith, 1974), produced together with the drug-sensitive counterpart.

3.1.5. Target site modification Although target site modifications by mutation are quite prevalent, only one well-established case of plasmid-mediated target site modification exists, notably the so-called MLS-resistance phenotype in both staphylococci and streptococci, where resistance to macrolides, lincosamides and streptogramin B antibiotics is the result of 23S ribosomal RNA becoming dimethylated by a plasmid-encoded enzyme (Lai and Weisblum, 1971; Weisblum *et al.*, 1971; Allen, 1977; Yagi *et al.*, 1975).

3.2 Resistance to antibacterial agents in bacterial mutants

Studies of antibiotic-resistant bacterial mutants have aided greatly the present understanding of the mechanism of protein synthesis. The apparent specificity of antibiotic action coupled with the assumption that antibiotics act where they bind has enabled a detailed picture of the protein synthetic machinery to emerge.

Mutants resistant to streptomycin have been isolated from *E. coli* and found to contain an altered ribosomal protein S12 (Ozaki *et al.*, 1969) whilst spectinomycin-resistant mutants contain an altered S5 protein (Bollen *et al.*, 1969). Resistance to erythromycin can arise by mutations in either ribosomal protein L4 or L22 (Wittman *et al.*, 1973); the mutation in ribosomal protein L4 may also confer resistance to other macrolide antibiotics (Tanaka *et al.*,

1971a). Alternatively, resistance to erythromycin can be conferred by alterations of ribosomal RNA (Lai and Weisblum, 1971). Gentamycin-resistant mutants appear to contain an altered ribosomal protein L6 (Kühberger et al., 1979). Kasugamycin-resistance in E. coli can arise either from failure to methylate two adjacent adenine residues close to the 3′-end of 16S ribosomal RNA (Helser et al., 1971; 1972) or through an alteration in ribosomal protein S2 (Okuyama et al., 1974). Curiously, resistance to viomycin in a mutant of Mycobacterium smegmatis has been shown to be located in the RNA of either the large or small ribosomal subunit. (Yamada et al., 1978).

In contrast, mutations affecting the "soluble" factors of protein synthesis have been found. Accordingly, resistance to fusidic acid (a steroidal antibiotic which inhibits functions associated with elongation factor-G) has been shown to result from an alteration in the G-factor (Bernardi and Leder, 1970).

Finally, two instances of the loss of a ribosomal protein conferring antibiotic resistance have been observed. Accordingly, mutants of Bacillus megaterium and Bacillus subtilis lacking a ribosomal protein serologically-related to E. coli ribosomal protein L11, have been shown to be resistant to thiostrepton (Cundliffe et al., 1979; Wienen et al., 1979).

3.3 Resistance to antibacterial agents in producing organisms

It might be expected that antibiotic-producing organisms do not need to protect themselves against their toxic products since, in general, antibiotics are not produced until cell growth has terminated. However, as already discussed, under certain conditions of growth (e.g. nutrient deficiency) antibiotic production can occur at the start of cultivation. Under these conditions, the producing organism is inevitably exposed to its toxic product.

3.3.1. Resistance due to permeability loss. *Streptomyces sp.* 3022 a (a chloramphenicol-producer) can be rendered resistant to the drug by growth in sub-inhibitory concentrations of chloramphenicol. While ribosomes from the induced strain were found to be sensitive to chloramphenicol *in vitro*, the presence of a chloramphenicol hydrolase could not account for the levels of resistance observed *in vivo* (Malik and Vining 1971; 1972). Accordingly, resistance has been attributed to a change in permeability to the drug. Presumably chloramphenicol hydrolase could function to scavenge endogenous chloramphenicol. While resistance to chloramphenicol in clinical isolates of bacteria is often the result of a chloramphenicol-acetyltransferase, no such enzyme was found in another producer of chloramphenicol, namely *Streptomyces venezuelae* (Shaw and Hopwood, 1976). Presumably therefore, the organism must adopt an alternative mechanism of chloramphenicol resistance.

Streptomyces griseus, a streptomycin producer, appears to be sensitive to streptomycin during exponential growth phase but resistant upon production of the antibiotic (Cella and Vining, 1975). While ribosomes from the producer were found to be sensitive to streptomycin *in vitro*, resistance *in vivo* coincided with a reduced uptake of radiolabelled drug by the mycelia. Accordingly, it was concluded that resistance was due (at least in part) to changes in permeability to the antibiotic. However, intracellular streptomycin was found to be partially inactivated by phosphorylation, a situation observed by others (Miller and Walker, 1970). Since streptomycin phosphate is not only biologically inactive, but is also the biosynthetic precursor of streptomycin it is tempting to speculate that the resistance mechanism could involve both changes in permeability plus phosphorylation of intracellular streptomycin, followed by export *via* the conventional pathway for streptomycin.

3.3.2. Resistance due to antibiotic modification Curiously, resistance to streptomycin in the producing organism Streptomyces griseus may well be different from that in another streptomycin-producing organism, Streptomyces bikiniensis. As already discussed above, resistance to streptomycin in the former organism appears to involve both changes in permeability and antibiotic modification. In contrast, resistance to streptomycin in Streptomyces bikiniensis may be due solely to antibiotic modification (Piwowarski and Shaw, 1979). Whilst the onset of antibiotic resistance in both organisms was essentially similar, decreased uptake of radiolabelled drug in resistant cells from Streptomyces bikiniensis was not observed. Similarly, ribosomes from both organisms were comparable regarding their response to drug in vitro. In support of previous work (Miller and Walker, 1969) a streptomycin-phosphorylating enzyme was observed in extracts of Streptomyces bikiniensis and it was suggested that this enzyme may be sufficient to render the producing organism resistant to streptomycin.

Antibiotic modification has also been implicated in resistance to viomycin and capreomycin by the producing organisms Streptomyces vinaceous and Streptomyces capreolus respectively. Both antibiotics are potent inhibitors of protein synthesis and while both producing organisms are resistant to their toxic products in vivo the ribosomes from each are sensitive to their respective antibiotics in vitro (Skinner and Cundliffe, 1980). Accordingly, resistance to the antibiotics is conferred (at least in part) by novel antibiotic-modifying enzymes. While viomycin and capreomycins 1A and 11A could be inactivated by phosphotransferases isolated from supernatant fractions of either producing organism, all the capreomycins (i.e. 1A, 1B, 11A and 11B) but not viomycin could be inactivated by an acetyltransferase isolated from Streptomyces capreolus. Although these enzymes may be sufficient to render the producing organisms resistant to their respective

drugs it does not negate the possibility that alternative resistance mechanisms could also be employed (for example, the cell membranes could be impermeable to the drugs).

3.3.3. Resistance due to target site modification The best documented case of antibiotic resistance in a producing organism being conferred by target site modification is that of thiostrepton resistance in *Streptomyces azureus*. Thiostrepton inhibits protein synthesis in sensitive bacteria by binding to 50S ribosomal subunits. In contrast, 50S ribosomal subunits from *Streptomyces azureus* were found to be refractory to the drug *in vitro* (Cundliffe, 1978). Following construction of both native and hybrid 50S particles using ribosomal RNA and ribosomal proteins from both the producing organism and a thiostrepton-sensitive organism (i.e. *Bacillus stearothermophilus*) it was possible to implicate *Streptomyces azureus* ribosomal RNA as the determinant of thiostrepton resistance. Furthermore a SAM-dependent methylase, obtained by a salt wash of *Streptomyces azureus* ribosomes, was capable of rendering ribosomal particles from *Streptomyces coelicolor* resistant to thiostrepton. Subsequent analysis of the particles revealed the presence of a methylated residue in 23S RNA; a similar methylated residue was found in *Streptomyces azureus* 23S RNA. More recently, methylation of *Streptomyces coelicolor* 23S RNA has been shown to coincide with the appearance of 2'-O-methyl-adenosine (Cundliffe and Thompson, 1979).

An examination of four other microorganisms producing thiostrepton-like antibiotics has revealed that they too possess 23S RNA which confers resistance to their respective (and related) drugs (Thompson and Cundliffe, 1980). Similarly, resistance to nosiheptide (also a thiostrepton-like antibiotic) has been shown to involve specific pentose-methylation of 23S ribosomal RNA (Cundliffe and Thompson, 1981).

In view of the apparent potency of thiostrepton (and thiostrepton-like antibiotics) it is perhaps not surprising that their respective producing organisms should engage a suitable constitutive resistance mechanism that renders their ribosomes totally refractory to indigenous drug. Accordingly, it is tempting to speculate that organisms producing other, very potent, antibiotics should also adopt a constitutive antibiotic resistance mechanism possibly involving modifications to ribosomal RNA. Indeed, Streptomyces erythreus, the erythromycin producer appears to fall into this category (see below). However modification of ribosomal RNA is not the only means of rendering ribosomes resistant to thiostrepton. As discussed later, ribosomes from a mutant of Bacillus megaterium lacking ribosomal protein BM-L11 are significantly more resistant to thiostrepton in vitro than corresponding wild-type ribosomes (Stark and Cundliffe, 1979 a). However, whilst it is possible for thiostrepton to inhibit partially polyphenylalanine incorporation by Bacillus megaterium mutant ribosomes, the ribosomes from Streptomyces azureus are totally resistant to drug when assayed under similar conditions. Accordingly, while the mutant can tolerate thiostrepton, the producing organism appears to adopt a much more effective resistance mechanism.

Streptomyces erythreus (the erythromycin producer) contains ribosomes refractory to erythromycin (Teraoka and Tanaka, 1974). Conceptually-similar experiments to those outlined above have been employed to establish, in detail, the mechanism of resistance to the drug. In essence, resistance to erythromycin is the result of di-methylation of two adenine residues in 23S ribosomal RNA (R.H. Skinner, personal communication). Furthermore, the ribosomes are rendered cross-resistant to both lincomycin and streptogramin B; accordingly, the producing organism appears to adopt a similar, if not identical, mechanism of resistance to erythromycin as

certain clinical isolates of bacteria exhibiting the so-called MLS-phenotype.

The only other documented case of a ribosome-based antibiotic-resistance mechanism is that of resistance to the aminoglycoside antibiotic, istamycin, in the producing organism Streptomyces tenjimariensis (Yamamoto et al., 1981). Not only is the organism resistant in vivo but its ribosomes are refractory to the drug in vitro. Furthermore the ribosomes are cross-resistant to kanamycin A, neamine, ribostamycin and butirosin A. However the resistance determinant has yet to be isolated to either ribosomal subunit and, ultimately, to ribosomal RNA or ribosomal proteins.

Other target site modifications resulting in antibiotic resistance but not involving the protein-synthetic machinery have been documented. Accordingly, Pseudomonas fluorescens, the producing organism of pseudomonic acid (a competitive inhibitor of isoleucyl-tRNA synthetase) contains a pseudomonic acid-resistant isoleucyl-tRNA synthetase (Hughes et al., 1980). However, resistance to actinomycin in the producing organism Streptomyces antibioticus is more difficult to categorise. Accordingly, whilst Marshall et al., (1968) found a reduced uptake of radiolabelled actinomycin in stationary phase cultures of the producing organism (compared with organisms in exponential growth phase), Jones (1976) found the presence of actinomycin-binding proteins in the stationary phase cells. In addition, the transcription machinery (normally the target of the drug) appeared to be unique since the combination of both DNA template and homologous RNA polymerase was refractory to actinomycin, whereas hybrid transcription systems were not (Jones, 1976). Hence it is difficult to establish which resistance mechanism contributes most to the normal functioning of the organism. Conversely, all three could act in a cooperative fashion.

Whilst resistance to actinomycin in Streptomyces antibioticus may involve

a modified RNA polymerase, this certainly appears to be the case with the rifamycin-producing organism, *Streptomyces mediterranei*. Accordingly, the producing organism can grow in the presence of high concentrations of rifampicin (a semi-synthetic derivative of rifamycin). Furthermore, DNA-dependent RNA polymerase from the producing organism is resistant to rifampicin *in vitro*, by comparison with extracts from either *Streptomyces griseus* or *E. coli* (Watanabe and Tanaka, 1976).

4. Reconstitution Studies, Antibiotics And Ribosomes

Ribosome reconstitution has enabled functional roles to be ascribed to certain ribosomal proteins. As will be discussed elsewhere, ribosomal subunits can be dissociated either to a limited extent and subsequently reassociated (i.e. partial reconstitution) or dissociated completely into constituent RNA (s) and ribosomal proteins for subsequent reconstitution (i.e. total reconstitution). Although partial reconstitution techniques were developed first (as a result of their relative simplicity) they have been overshadowed by the development of total reconstitution techniques, which have ultimately allowed detailed assembly maps of ribosomal proteins on to ribosomal RNA for both 30S and 50S ribosomal subunits to be constructed (Held *et al.*, 1974; Roth and Nierhaus, 1980).

4.1 30S ribosomal subunit Whilst partial reconstitution techniques for 30S ribosomal subunits demonstrated, for the first time, the requirements for individual ribosomal proteins for the functional activity of ribosomal particles, significant advances were not made until the development of the total reconstitution procedure. Subsequently, "single component omission" experiments could be performed to establish the roles of individual ribosomal proteins in the various partial reactions of protein synthesis. Such an approach does suffer from the drawback that where a number of ribosome functions are impaired, unequivocal interpretation of the results is difficult. This is due to the possibility that the absence of the ribosomal protein could result in faulty assembly of the particle, which could ultimately be reflected in a general loss of ribosome activity. However, certain unequivocal interpretations were possible. Accordingly, *E. coli* ribosomal protein S12 was shown to be important for initiation of protein synthesis (Ozaki *et al.*, 1969) while particles deficient in ribosomal protein

S1 had a pronounced increase in translational error frequency (Nomura et al., 1969). Also, ribosomal proteins S3 or S5 were shown to be required for the binding of dihydrostreptomycin (Schreiner and Nierhaus, 1973).

4.2. 50S ribosomal subunit Partial reconstitution methodology for 50S ribosomal subunits allowed significant advances in the understanding of ribosome structure and function to be made. At the simplest level, when ribosomal protein L7/L12 was removed from the 50S ribosomal subunit by washing in ethanol and ammonium chloride, a marked loss of both EF-Tu and EF-G-dependent GTPase activity resulted. Subsequent reconstitution of the protein onto the ribosomal subunit resulted in a return of the various activities (Hamel et al., 1972). Early studies using the partial reconstitution technique had implicated ribosomal proteins L6, L11 and L16 in the reconstitution of the peptidyl transferase centre (Nierhaus and Montejo, 1973; Dietrich et al., 1974; Moore et al., 1975). However, with the advent of the total reconstitution technique, it was possible to demonstrate that when ribosomal proteins L6, L10, L11 and L16 were added individually to a corresponding core particle, no peptidyltransferase activity was observed. However, the combination of all four proteins resulted in high levels of activity. By omitting each of the ribosomal proteins in turn, it was possible to establish that ribosomal protein L16 was responsible for activity, whilst any two of the remaining three proteins were required for the assembly of ribosomal protein L16 into the mature particle (Nierhaus, 1980).

Partial reconstitution techniques have also been employed to show the requirement of ribosomal protein L16 for the binding of chloramphenicol (Nierhaus and Nierhaus, 1973), virginiamycin S (de Béthune and Nierhaus, 1978) and, together with ribosomal protein L15, erythromycin (Teraoka and Nierhaus, 1978).

Preliminary reconstitution studies with the thiostrepton-producing organism Streptomyces azureus have ultimately resulted in a detailed understanding, at the molecular level, of certain RNA/protein interactions within the ribosome. Thiostrepton inhibits initiation, elongation and termination phases of protein synthesis by preventing the interactions of various supernatant protein factors with the ribosome (Cundliffe, 1981). Streptomyces azureus is protected from thiostrepton due to the possession of a modified drug target site. Reconstitution studies enabled the site of thiostrepton resistance to be localised to 23S RNA (Cundliffe, 1978). Subsequently, it has been shown that 50S ribosomal subunits of the producing organism contain 23S RNA modified by a single pentose methylation (resulting in formation of 2'-O-methyladenosine) (Cundliffe and Thompson, 1979). While intact 50S ribosomal subunits from thiostrepton-sensitive organisms are not substrates for the "thiostrepton-resistance" methylase, both core particles (i.e. 50S particles lacking certain ribosomal proteins) and naked 23S RNA can be methylated with concomittant resistance to thiostrepton (following reconstitution with ribosomal proteins). Core particles prepared from E. coli 50S ribosomal subunits were found not to bind radio-labelled thiostrepton when assayed by gel filtration (Highland et al., 1975). However, upon reconstitution of the core particles with ribosomal protein L11, thiostrepton was shown to bind the particle with high affinity. More recently, it has been shown that a complex of 23S RNA and ribosomal protein L11 is sufficient to form a binding site for thiostrepton (Thompson et al., 1979). Enzymic digestion (with T1 ribonuclease) to remove unprotected RNA has revealed that 61 nucleotide bases (i.e. residues 1052 - 1112 inclusive - see Brosius et al., 1980) of the 23S RNA are protected by L11 (E. Cundliffe - personal communication). Furthermore, the site of ribose-methylation (conferring resistance to thiostrepton in the producing organism) is located in the L11-protected fragment. The thiostrepton-binding site

has also been localised with respect to the three-dimensional structure of the 50S ribosomal subunit. Accordingly, 50S ribosomal subunits containing bound thiostrepton were reacted with anti-thiostrepton serum and examined by immune-electron microscopy. A single antibody binding site was found in the centre of the "seat" region. Furthermore the region is in close proximity to one of the antigenic sites (i.e. site A) of ribosomal protein L11 (Stöffler et al., 1980).

Thiostrepton has also been shown to inhibit the so-called "stringent response". Accordingly, when bacteria are starved of amino acids, the regulatory nucleotides guanosine tetra- and penta-phosphates are synthesised in response to stringent factor (a supernatant protein). The regulatory nucleotides affect the synthesis of certain species of RNA. Thiostrepton appears to prevent either the binding of stringent factor to the ribosome, or pyrophosphate transfer (from ATP onto GTP or GDP, to yield the regulatory nucleotides) (Cundliffe, 1981). A thiostrepton-resistant mutant of Bacillus megaterium has been shown to lack a ribosomal protein (BM-L11) immunologically-related to E. coli ribosomal protein L11 (Cundliffe et al., 1979). Furthermore, the mutant is unable to synthesise tetra- and penta-phosphates in vivo (thereby exhibiting the "relaxed" phenotype). While ribosomes from the mutant could not support synthesis of the regulatory nucleotides in vitro, reconstitution of purified protein BM-L11 onto the mutant ribosomes corrected this deficiency (Stark and Cundliffe, 1979 b). Accordingly, ribosomal protein BM-L11 appears to be indispensable for the production of these regulatory nucleotides.

In conclusion, the study of the mode of action and mechanisms of resistance to thiostrepton have been particularly fruitful. Not only has a detailed picture emerged at the molecular level, but it is encouraging that independent

approaches to the various problems encountered have yielded essentially-supportive conclusions.

CHAPTER 2

MATERIALS AND METHODS - GENERAL

Chapter 2

Only methodology relevant to the whole of this study will be found within this chapter. Methodology relevant to specific parts of this study will be found in the relevant chapter.

Chapter 2

1. Microbiological Methods

1.1. Microorganisms These were obtained from a variety of sources outlined in Table 2.1.

1.2. Materials All bacteriological media were obtained from Oxoid Limited except for "malt extract" which was obtained from Difco Laboratories. "Synperonic" was obtained from I.C.I. Limited.

1.3. Maintenance of Microorganisms. *Streptomyces* spores were stored in 20% (v/v) glycerol at -20°C. Routinely, *Streptomyces* were maintained on plates containing either "DYM agar" or "sporulation agar". "DYM agar" contained, per litre: D-glucose, 4g; yeast extract, 4g; malt extract, 10g; agar, 20g. The pH was adjusted to 7.3 with 1N potassium hydroxide. "Sporulation agar" contained, per litre: yeast extract, 1g; beef extract, 1g; tryptose, 2g; ferrous sulphate, trace; D-glucose, 10g; agar, 20g. The pH was adjusted to 7.2 with 1N potassium hydroxide.

Inoculated plates were incubated at 30°C for approximately one week, or until sporulation had occurred. Sub-culturing was undertaken on a monthly basis.

E. coli was maintained on plates containing nutrient agar. Inoculated plates were incubated at 37°C overnight.

1.4. Bulk Growth of Microorganisms Bulk growth of *Streptomyces* cannot always be monitered by simple, physical methods (e.g. A_{600} measurement for *Escherichia coli*). Some *Streptomyces* fragment during growth (e.g. *S. loidensis*) while others (e.g. *S. actuosus*) form large conspicuous "balls". The latter group tend to grow well from a large spore inoculum, presumably

TABLE 2.1

Sources of Microorganisms

ORGANISM	STRAIN	SOURCE
Streptomyces loidensis	ATCC 11415	E.R. Squibb and Sons Inc.
Streptomyces coelicolor	A3 (2)	Prof. D.A. Hopwood, John Innes Institute, Norwich.
Streptomyces lividans	1326	Prof. D.A. Hopwood, John Innes Institute, Norwich.
Streptomyces actuosus	NRRL 2954	NRRL
Streptomyces erythreus	NRRL 2338	NRRL
Bacillus stearothermophilus	NCIB 8924	M.R.E. Porton, Wilts.
Escherichia coli	MRE 600	M.R.E. Porton, Wilts.

because a large number of growth "nuclei" (i.e. spores) are present at the start of incubation. The method employed in this study for such organisms involved resuspending the spores from a "well-sporulated" plate in 10 ml sterile water containing 0.1% "Synperonic", using a bacteriological loop as a "scraper". The presence of detergent helped during suspension of some of the more "hydrophobic" spores. Subsequently the whole of the suspension was either poured or pipetted into one litre of growth medium and incubated at 30°C.

For "fragmenting" organisms, a loopful of spores was inoculated into 5 ml of growth medium and incubated overnight at 30°C while being agitated on a "rolling wheel" (Mickle Laboratory Engineering, Surrey). Subsequently, the inoculum was poured into one litre of growth medium and incubation continued at 30°C.

For bulk growth of Streptomyces, "PYG + BEEF" medium was used. It contained, per litre: peptone, 10g; yeast extract, 5g; D-glucose, 5g; beef extract, 1g. The pH was adjusted to 7.3 with 1N potassium hydroxide. (One litre of medium was contained in a 2l baffled conical flask). Aeration during incubation was accomplished using an orbital shaker (L.H. Engineering Co., England).

The point at which cells were harvested was necessarily qualitative rather than quantitative. Generally it was dependent upon the nature of the experiment. Ribosomes which were active in protein synthesis could be obtained from Streptomyces in most phases of growth, and hence the point at which harvesting occurred was not critical. However, to obtain active "supernatant" fractions (i.e. S100) which would support cell-free protein synthesis, cells had to be harvested at approximately mid-log phase. Generally the period of incubation was limited to 12-14 hours. The best

indication of phase of growth was the yield of cells. However, other factors (e.g. cell morphology, pigment production) were also useful indicators.

Bulk growth of E.coli was achieved by inoculating a 5 ml "starter culture" into 1 l Nutrient Broth No. 2. Incubation was continued at 37°C and cells were harvested in mid-log phase (i.e. A_{600} = 0.7 to 0.8)

Frozen B. stearothermophilus cells were purchased from M.R.E., Porton, Wilts.

Chapter 2

2. Biochemical Methods

2.1 Materials. The Radiochemical Centre, Amersham: : [U-^{14}C] Phenylalanine (513m Ci/m mol).

Sigma Chemical Co: Deoxyribonuclease I (E.C.3.1.4.5) from bovine pancreas - electrophoretically purified; pyruvate kinase, type III (E.C. 2.7.1.40), from rabbit muscle; tRNA, type XXI, from *E. coli* W; L-amino acids; 2-mercaptoethanol; HEPES; poly (U), (potassium salt);GTP, (tri-lithium salt); PEP, (potassium salt).

Boehringer Mannheim Corporation: tRNA, (phenylalanine-specific) from *E. coli* MRE 600, ATP, (di-sodium salt).

E.R. Squibb & Sons Inc: Vernamycin A and Bα

Rhône-Poulenc: Nosiheptide

General chemicals were all of A.R. quality. Bentonite was purified according to the method of Fraenkel-Conrat *et al.*,(1961).

Activated charcoal was purified according to the method of Thompson (1960).

Stock solutions of EDTA (potassium salt) and lithium chloride were treated with activated charcoal and filtered before use.

Stock solutions of sucrose were treated with DEPC (0.1% v/v) for one hour, followed by autoclaving at 10 lb/in^2 for 15 min.

Tris buffers were prepared by mixing appropriate quantities of Tris-HCl and Tris-base (Sigma).

Levigated alumina was dry-autoclaved at 180°C overnight.

Prior to use, all dialysis tubing was autoclaved in a solution of 5% (w/v) $NaHCO_3$ and 10 mM EDTA (di-sodium salt). Subsequently, the tubing was

extensively washed in Millipore Milli-Q water and stored at 4°C. For all purposes except dialysis of protein solutions, "Visking" dialysis tubing was used (molecular weight cut-off = 14,000) Otherwise "Spectrapor" No. 3 tubing was employed (molecular weight cut-off = 3,500).

Millipore Milli-Q water was used in the preparation of all solutions except media, for which glass-distilled water sufficed.

Buffers Buffers used throughout this study will be referred to in the text by shortened terminology (except where stated otherwise). The exact composition of these buffers is as follows:-

RS buffer	= 10mM Tris-HCl (pH 7.6 at 20°C); 10mM magnesium chloride; 50mM ammonium chloride; 3mM 2-mercapto-ethanol.
HS buffer	= 10mM Tris-HCl (pH 7.6 at 20°C); 30mM magnesium chloride; 3mM 2-mercaptoethanol; 1M ammonium chloride.
LM buffer	= 10mM Tris HCl (pH 7.6 at 20°C); 1mM magnesium chloride; 100mM potassium chloride; 3mM 2-mercaptoethanol.
NOM buffer	= 20mM Tris HCl (pH 7.6 at 20°C); 10mM magnesium chloride; 500mM ammonium chloride; 6mM 2-mercaptoethanol; 1.1M sucrose.
SA buffer	= 10mM Tris HCl (pH 7.6 at 20°C); 5mM magnesium chloride; 100mM sodium chloride; 3mM 2-mercaptoethanol.
$T_{30}M_{20}K_{1000}\beta_6$	= 30mM Tris HCl (pH 7.6 at 20°C); 20mM magnesium chloride; 1M potassium chloride; 6mM 2-mercaptoethanol.

2.2. Preparation of Cell Extracts Cells from bulk growth of microorganisms were harvested by centrifugation at 8,000 r.p.m. for 10 minutes in a Beckman JA-10 rotor at 4°C. Pellets were washed three times in ice-cold RS

buffer + 0.5mM EDTA. Since some Streptomyces did not pellet tightly, an additional step was usually employed - the cell suspension was poured through a Whatman No. 1 filter in vacuo.

Washed cells were broken by one of two methods, depending upon the scale of the preparation. For less than 20g of cells, grinding was preferred. Cell paste was broken by grinding with twice its weight of levigated alumina, in a pre-cooled pestle and mortar. Grinding was continued until the paste became noticeably "sticky" - efficient breakage often being characterised by "cracking/popping" noises. To reduce the viscosity of the extract, DNase was added to a final concentration of 5μg/ml and incubated at 0°C for 5 minutes; RS buffer + 0.5mM EDTA was added sparingly, where appropriate. Cell debris and alumina were removed by centrifugation (20,000 r.p.m./ 30 minutes) in a Beckman JA-21 rotor at 4°C. The supernatant, (approximately 1 ml per gram of cells) designated "S30" fraction, was either dialysed against RS buffer, frozen and stored at -70°C, or processed further. Frozen S30 was stable for several months although it lost considerable activity (e.g. 50%) upon re-freezing.

Breakage of large quantities of cells was accomplished by passage through a French pressure cell (pre-cooled on ice) at 12,000 lb/in^2. After two passages, DNase was added as above and a third passage executed. Cell debris was cleared by centrifugation either at 20,000 r.p.m. for 30 minutes in a Beckman JA-21 rotor at 4°C or at 17,000 r.p.m. for 40 minutes in an MSE 6x100 ml rotor at 4°C. The supernatant (S30) was treated as above.

2.3. Preparation of Salt-Washed Ribosomes and S100 Ribosomes and S100 fraction were always prepared from fresh, un-frozen S30 fraction. S30 was layered over 0.5 volumes of HS buffer plus 20% (w/v) sucrose. Centrifugation was at 40,000 r.p.m. for 16 hours in Beckman 75Ti or 70Ti rotors at 2°C.

The supernatant was aspirated and dialysed extensively against RS buffer. This fraction was designated S100 and contained a number of the "soluble" factors of protein synthesis. S100 was frozen rapidly in dry ice/methanol and stored at -70°C at which temperature it was stable for at least two years. Generally S100 was not re-frozen since this led to a decrease in its activity.

The ribosome pellet was resuspended in HS buffer by gentle agitation and layered over 0.5 volumes HS buffer plus 20% (w/v) sucrose. After pelleting the ribosomes (40,000 r.p.m./16 hours) the supernatant was discarded. Finally, the ribosomes were resuspended in RS buffer and layered over 0.5 volumes RS buffer plus 40% (w/v) sucrose. Centrifugation was at 40,000 r.p.m. for 18 hours at 2°C. Subsequently ribosomes were resuspended in, and dialysed against RS buffer. After rapid freezing, ribosomes were stored at -70°C. Although very stable at this temperature, and, to an extent, resistant to repeated freezing/thawing, ribosomes were aliquoted to avoid this treatment.

2.4. Preparation of Ribosomal Subunits 70S ribosomes were prepared by layering S30 fraction over 0.5 volumes of NOM buffer and centrifuging at 40,000 r.p.m. for 16 hours in Beckman 75Ti or 70Ti rotors at 2°C.

Ribosomal subunits were prepared by one of the following methods:

2.4.1. Small scale Approximately 50 A_{260} units of ribosomes (in LM buffer) were layered over 35 ml 10-30% (w/v) sucrose density gradients containing LM buffer. After centrifugation at 18,000 r.p.m. for 16.5 hours in a Beckman SW27.1 rotor at 2°C, fractions were collected by upward displacement through an Isco UA-5 absorbance monitor. Fractions containing either 30S or 50S ribosomal subunits were pooled, the magnesium ion concentration

raised to 10mM, and then centrifuged at 40,000 r.p.m. for 18 hours in a Beckman 70Ti rotor at 2°C. Ribosomal subunits were resuspended in, and dialysed against, RS buffer. 30S ribosomal subunits were heat-activated according to the procedure of Zamir et al., (1974).

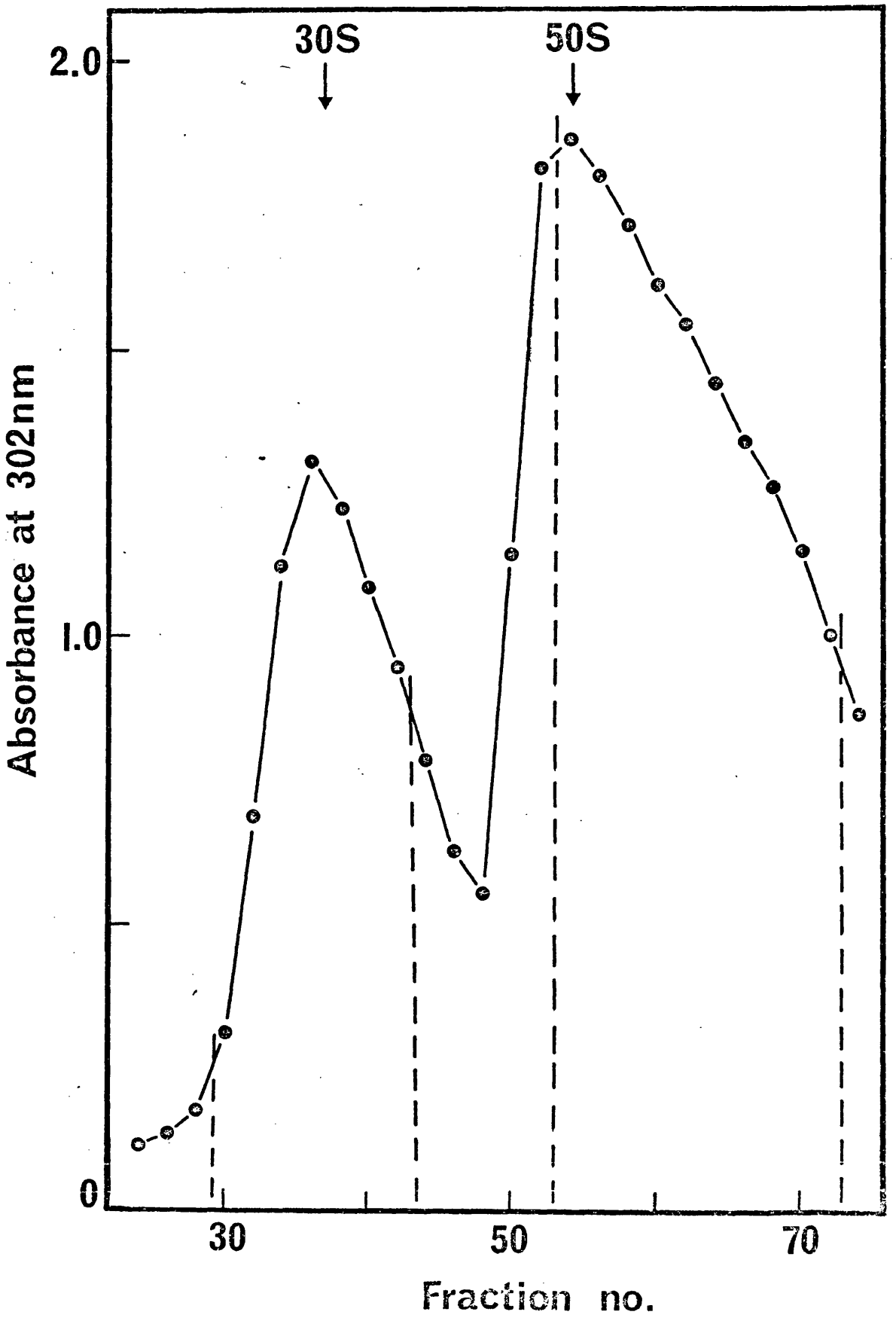
2.4.2. Large scale Preparation of large amounts of ribosomal subunits followed the procedure of Eikenberry et al., (1970). Between 10,000 and 20,000 A_{260} units of dissociated 70S ribosomes (in LM buffer) were loaded on to a hyperbolic 7.4-38% (w/w) sucrose density gradient containing LM buffer in an MSE B-XV zonal rotor. The ribosomes were loaded in an inverse concentration gradient of sucrose (zero to 7.4% w/w). The procedure was as follows: first, the rotor (at 5°C) was accelerated to 3,000 r.p.m. and 708 ml LM buffer were fed to the periphery via the central feed head. Next the ribosomes (in 100 ml of LM buffer) were pumped to the rotor periphery via a two-chambered linear-gradient maker. This was followed by the sucrose density gradient (425 ml) - generated by an automatic gradient former (MSE, model Z-100), pumped at a flow rate of approximately 20 ml/minute. Finally a cushion of 45% (w/w) sucrose (containing LM buffer) was pumped to the periphery until LM buffer emerged from the rotor core (indicating a full rotor) The rotor was accelerated to 22,500 r.p.m. and maintained for 17 h at 5°C. The gradient was fractionated by first decelerating the rotor to 3,000 r.p.m. Sucrose (60% w/w) (containing LM buffer) was pumped to the periphery of the rotor and the gradient displaced through the central core. 10 ml fractions were collected and the absorption at 302 nm measured (this wavelength was chosen in preference to A_{260} since it relieved the need to dilute ribosomal subunits unnecessarily). A typical ribosomal subunit absorption profile is shown in Fig 2.1. After fractionation, the relevant fractions were pooled and the magnesium ion concentration raised to 10mM. Ribosomal subunits were pelleted at 40,000 r.p.m. for 18 hours in a Beckman 70Ti rotor at 2°C.

Legend to Figure 2.1

Zonal density gradient centrifugation of *S. loidensis* ribosomal subunits (see Chapter 2.2.4).

Fractions (10 ml) were pooled as indicated by the dashed lines.

FIGURE 2.1



Pellets were resuspended in, and dialysed against RS buffer. 30S ribosomal subunits were heat activated. All subunits were frozen and stored at -70°C.

2.5. Assessment of Purity of Ribosomal Subunits The extent to which purified ribosomal subunits were contaminated with the corresponding ribosomal subunit was assessed on analytical sucrose density gradients.

0.5 A_{260} units of ribosomal subunits (in RS buffer) were layered over 5 ml 10-25% (w/v) sucrose density gradients containing SA buffer. After centrifugation at 45,000 r.p.m. for 2 hours in a Beckman SW50.1 rotor at 2°C, the absorbance profile of the gradient was measured by upward displacement through an Isco UA-5 absorbance monitor. No cross contamination between subunits was observed on any occasion.

2.6. Preparation of Sub-Ribosomal Components

2.6.1. Preparation of RNA and proteins from ribosomes and ribosomal subunits

Preparation of materials from ribosomes and ribosomal subunits followed essentially the method of Fahnestock *et al.*, (1974).

Ribosomes or ribosomal subunits were resuspended in RS buffer at a concentration of 300-500 A_{260} units/ml and an equal volume of 8M urea/4M lithium chloride added. This latter solution was always prepared immediately prior to use, to minimise formation of isocyanate (from the urea). As a further precaution, the solution was treated with a 0.2% (w/v) bentonite for 60 minutes at 0°C. After centrifugation at 12,000 xg for 5 minutes (to remove bentonite) the supernatant was passed through a Millipore filter (Swinnex-13 Filter unit) and was then ready for use. Extraction with urea and lithium chloride was continued for 48 hours at 0°C with occasional

mixing. The precipitated RNA, essentially 23S (+ 16S) with variable amounts of 5S RNA, was removed by centrifugation at 12,000 xg for 3 minutes and either dissolved in sterile water or extracted further (depending upon the presence of residual ribosomal proteins). The supernatant was dialysed extensively against $T_{30}M_{20}K_{1000}\beta_6$ buffer, frozen and stored at -70°C, at which temperature it was stable for at least one year.

2.6.2. Removal of residual protein(s) from RNA Following extraction with urea and lithium chloride, both *B. stearothermophilus* total ribosomal RNA (RNA 70) and *S. coelicolor* RNA 70 each contained at least one ribosomal protein (data not presented). For example, 90% of *B. stearothermophilus* ribosomal protein L3 remains RNA-bound (Fahnestock *et al.*, 1974). This can be removed by extraction at low pH thus:

RNA was dissolved in 6M urea at a concentration of 200-400 A_{260} units per ml. To this solution was added one third volume of 2M magnesium acetate (adjusted to pH 2.0 with HCl and autoclaved). After 1 hour at 0°C the precipitated RNA was collected by centrifugation at 12,000 xg for 5 minutes and dissolved in sterile water (achieved only by extensive dialysis against water). The supernatant, containing ribosomal protein, was dialysed against $T_{30}M_{20}K_{1000}\beta_6$ buffer, frozen and stored at -70°C. (The extra protein extracted from *S. coelicolor* ribosomal RNA was combined, in an equi-molar ratio, with the corresponding "TP50" fraction, frozen and stored at -70°C).

All RNA solutions were frozen and stored at -70°C at which temperature they were stable for at least one year.

It was unnecessary to perform the second extraction procedure on RNA from *S. loidensis*, *S lividans* and *S. actuosus*. Likewise, none of the RNA from *S. coelicolor* 30S ribosomal subunits required a second extraction (this

was assessed using gel techniques outlined in Chapter 5).

2.6.3. Distribution of 5S RNA Following urea/LiCl extraction of ribosomes, 5S RNA is distributed between the RNA pellet i.e. 23S (+16S) RNA and the protein fraction. For B. stearothermophilus about 30% of the 5S RNA is pelleted with the majority of the RNA, the remainder staying in solution (Fahnestock et al., 1974). For B. subtilis and B. licheniformis most of the 5S RNA remains in solution (Fahnestock, 1979). The distribution of 5S RNA in urea/LiCl extracted S. loidensis ribosomes was not established precisely. However, gel electrophoresis of dissociated material would suggest that 5S RNA cannot be allocated to one particular fraction, (data not presented). Although 5S RNA can be isolated by chromatography on DEAE-cellulose, this was not attempted.

2.7. Quantitation Of Ribosomes And Ribosomal Components

Material	p mol / A260 unit
70S ribosomes	27.2
50S ribosomal subunits	33.3
30S ribosomal subunits	66.7
RNA 70	27.0
RNA 50	46.2
RNA 30	87.0

2.8. Cell-Free Protein Synthesis One approach to determining whether an antibiotic inhibits ribosomefunction is to investigate its effect in a cell-free protein-synthesising system. Such systems can be programmed by a variety of "messages" - both natural and synthetic, the final choice often being dependent upon the mode of action of the antibiotic in question. For

example, specific inhibitors of initiation of protein synthesis (e.g. kasugamycin) do not inhibit protein-synthesising systems programmed by poly (U). The reason for this appears to be because poly (U) contains no initiation sequence (i.e. AUG or GUG). To get ribosomes to translate such a message, non-physiological concentrations of magnesium are required. Since the requirement for correct initiation has been waived, kasugamycin does not inhibit such a system. In contrast to kasugamycin, vernamycin A is a potent inhibitor of sensitive ribosomes translating poly (U). Hence, a poly (U)-directed protein-synthesising system was chosen for this present study Protein-synthesising systems programmed by poly (U) were first described by Nirenberg (1963). The system described here is derived from that of Davies and Kaesberg (1973). Generally, a total volume of 50 µl of incubation mixture was used. S30 fractions were assayed at an input of approximately 1 A_{260} unit per 50 µl mix (an accurate estimate of the ribosome concentration is difficult here due to extraneous UV-absorbing material). Ribosomes or ribosomal subunits were assayed at a concentration of 0.2-0.4 µM. Optimal inputs of S100 varied between preparations but generally a 20-30% (v/v) input was required for Streptomyces S100 fractions and a 5-10% (v/v) input for both E.coli and B. stearothermophilus S100 fractions. The rest of the assay cocktail was added as a mix, which, when added to an equal volume of biological components resulted in final concentrations thus:

Hepes/KOH (pH 7.6 at 20°C), 20mM; L-[U-^{14}C] phenylalanine, 5 µCi/ml; ATP, 2.5mM; GTP, 0.375mM; PEP, 5mM; potassium chloride, 100mM; magnesium chloride, 11mM; all amino acids except phenylalanine, 0.0375mM each; pyruvate kinase, 100 units/ml; poly (U), 0.5 mg/ml; tRNA (from E. coli W, type XXI), 1 mg/ml. (For all experiments after Chapter 3, tRNA-phenylalanine specific, was used at a final concentration of 0.5 units/ml).

Solutions of ATP, GTP and PEP were neutralised (with KOH) before use.

The order of addition of components was ribosomes, antibiotics (where relevant), S100 and assay mix. Incubation was at 30°C. Where appropriate, ribosomes and antibiotics were pre-incubated at 30°C for 10 minutes prior to addition of other components. At intervals 5 or 10 µl aliquots were delivered into 1 ml 10% (w/v) TCA. After 30 minutes at >90°C in a water bath, samples were allowed to cool and then filtered through Whatman GF/C filter papers. Filters were extensively washed four times with 3-4 ml 5% (w/v) TCA. After drying under infra-red lamps, the filters were immersed in 5 ml of 0.4% (w/v) butyl-PBD scintillant. The radioactivity was counted by liquid-scintillation spectrometry. The counting efficiency of ^{14}C-phenylalanine was determined to be ~90%.

CHAPTER 3

RESISTANCE OF STREPTOMYCES LOIDENSIS TO VERNAMYCIN A IN VIVO AND IN VITRO

Chapter 3

1. Introduction

It is beyond the scope of this introduction to discuss the effects of vernamycin A and Bα on organisms other than prokaryotes. For an extensive review of their action on eukaryotic organisms, see Cocito (1979).

1.1. Background Vernamycin A and Bα are members of the so-called "Virginiamycin (or Streptogramin) family" of antibiotics. The family, although apparently diverse, in fact represents only a small and homogenous group of antibiotics. Their interest stems primarily from the synergistic capabilities of its members, although individual components are still very potent antibiotics. Confusion in the literature has arisen from the naming of constituent members. Even so, all the antibiotics within the family can be assigned to one of two basic primary structures (Group A or Group B). Within Group A, vernamycin A is identical to ostreogrycin A, streptogramin A, virginiamycin M or M1, staphylomycin M1, synergistin A-1, PA-114 A-1, mikamycin A and pristinamycin 11A. Variations within a group result from minor modifications to the basic structure. For example, ostreogrycin A differs from ostreogrycin G due to the latter being saturated at the Δ-2,3 position, this modification being also present in both virginiamycin M2, staphylomycin M11 and pristinamycin 11B (Crooy and De Neys, 1972).

Within Group B, vernamycin Bα is identical to ostreogrycin B, streptogramin B, virginiamycin S or S1, staphylomycin S, synergistin B-1, PA-114 B-1, mikamycin B or 1A, and pristinamycin 1A. However, like Group A antibiotics, modifications to the basic structure of the molecule (e.g. alkyl or methylamino substitutions) yield a bewildering variety of derivatives. No attempt will be made here to catalogue these variants.

Group A and B antibiotics are produced by a large number of Streptomyces spp. Some (e.g. S. loidensis and S. mitakaensis) are clearly distinct morphologically, whilst others (e.g. S. olivaceous and S. pristinaespiralis) may well be similar, if not identical.

For the sake of clarity, the terminology "Group A or B" will be used throughout this introduction, in preference to other synonyms.

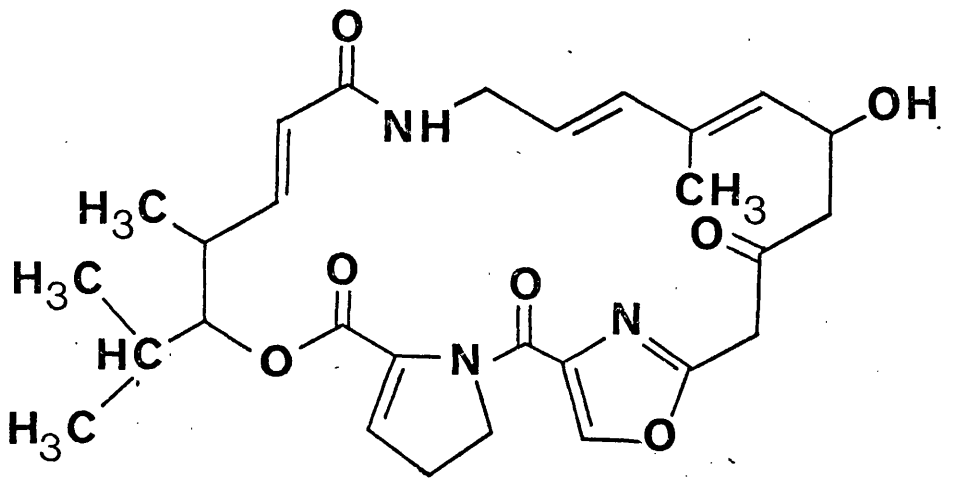
Group A antibiotics are polyunsaturated cyclic peptolides with a molecular weight of approximately 525 (Figure 3.1). In contrast, Group B antibiotics are cyclic hexadepsipeptides with a molecular weight of about 800 (Figure 3.2) (Crooy and De Neys, 1972).

1.2. Modes of Action Group A antibiotics are generally active against Gram-positive cocci whereas Group B antibiotics act mainly upon Gram-positive bacilli. Individually, neither component reduces the viability of most bacteria, unless incubation with the drug is exceedingly long. Thus, both drugs are bacteriostatic. However, in combination, both antibiotics act synergistically, potentiating antibiotic activity up to 100-fold, and exerting a bactericidal effect (Cocito, 1969).

The primary effect of both components individually, and in combination, appears to be upon polypeptide formation. Kinetics of amino acid incorporation into polypeptides in antibiotic-resistant mutants confirms this observation (Cocito and Fraselle, 1973). Secondary effects (e.g. reduction of nucleic acid biosynthesis) appear to result from the primary effect upon protein synthesis (Yamaguchi and Tanaka, 1964; Cocito, 1969).

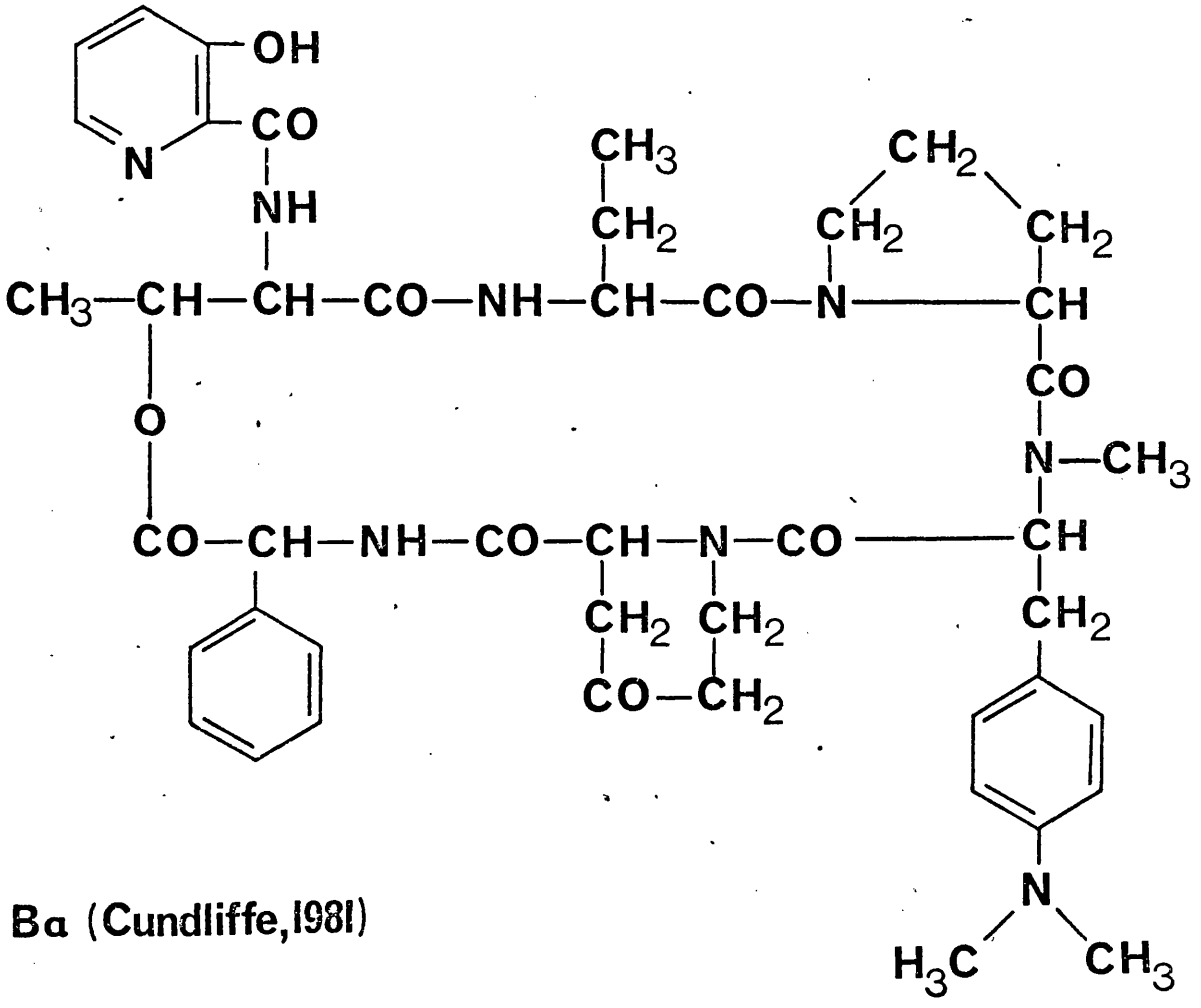
Group A antibiotics bind to both 70S ribosomes and 50S ribosomal subunits (Ennis, 1966) with a 1:1 stoichiometry (Ennis, 1971; Cocito and Di Giambattista, 1978) but not to 30S ribosomal subunits. Furthermore,

FIGURE 3.1



VERNAMYCIN A (Cundliffe, 1981)

FIGURE 3.2



VERNAMYCIN Ba (Cundliffe,1981)

the kinetics of drug binding are influenced by temperature, suggesting that binding is dependent upon ribosomal subunit conformation. However, ionic conditions under which the drug will bind to ribosomes are quite broad. Drug binding to ribosomes has been demonstrated by a variety of techniques including co-elution from Sephadex-G25 columns (Cocito and Kaji, 1971) and co-sedimentation by ethanol precipitation (Contreras and Vazquez, 1977 a; 1977 b). More recently, Cocito and Di Giambattista (1978) have described a novel method for evaluating ribosome-bound drug, based on the preferential absorption of the drug to activated charcoal. The bound drug can be removed by low-speed centrifugation, leaving ribosome-bound drug for subsequent estimation. Using this method, the association constant for the binding of Group A antibiotics to ribosomes was estimated to be $3.2 \times 10^{5} M^{-1}$. This figure differs significantly from that obtained by others. For example, Ennis (1971) determined the association constant to be $5.5 \times 10^{7} M^{-1}$, using the Millipore filtration technique. These differences may reflect the observation that Group A antibiotics attach to Millipore filters, dialysis membranes and plastic supports (Cocito and Di Giambattista, 1978). Hence, assays using such materials will probably give mis-leading results. Furthermore, estimation of association constants by Millipore filtration is hampered by the relative instability of the drug-ribosome complex. Repeated washing of filters to remove unbound drug was found to alter significantly the association/dissociation equilibrium (Cocito and Di Giambattista, 1978).

Group A antibiotics have no effect on polypeptide elongation by polysomes (Pestka, 1972), but instead induce their breakdown (Cundliffe, 1969; Ennis, 1972). Evidently, such breakdown proceeds by normal ribosomal "run-off" since certain other inhibitors of protein synthesis prevented it (Ennis, 1972). This led Pestka (1972) to repeat earlier suggestions

that the mode of action of the drug is on initiation of protein synthesis. To an extent, this has been supported by others (Tanaka, 1975; Yamaguchi and Tanaka, 1967) who found that formation of the mRNA-30S-fMet tRNA initiation complex was insensitive to drug while that of the mRNA-70S-fMet tRNA initiation complex was sensitive. Furthermore, the non-enzymatic binding of fMet tRNA to 50S ribosomal subunits and 70S ribosomes was apparently inhibited by the drug (Ennis and Duffy, 1972). This latter finding has been largely supported by Cocito et al., (1974) but only under conditions where certain initiation factors are limiting. In conclusion, whilst effects of Group A antibiotics upon initiation of protein synthesis can be observed under sub-optimal conditions, no such effects are observed under normal circumstances. Hence these drugs cannot be regarded as inhibitors of initiation of protein synthesis.

The presence of nascent peptides on polyribosomes appears to prevent the binding of Group A antibiotics, since their removal with puromycin results in drug binding (Contreras and Vazquez, 1977 b). Furthermore, binding of chloramphenicol to polyribosomes is only prevented by Group A antibiotics following removal of nascent peptides (Pestka, 1974).

Although some evidence exists to implicate the ribosomal P-site of the peptidyl transferase centre as a target for Group A antibiotics (Celma et al., 1970) most evidence suggests the ribosomal A-site as the prime target of the drug. Hence Group A antibiotics have been shown to inhibit the enzymatic binding of aminoacyl-tRNA to the A-site (Cocito and Kaji, 1971) without affecting the GTPase reaction normally associated with such binding (Modolell et al., 1971). In addition, Group A antibiotics can displace previously-bound phenylalanyl-tRNA from the ribosomal A-site (Cocito and Kaji, 1971). Furthermore, with donor substrates bound firmly into the

P-site, Group A antibiotics can still inhibit the puromycin reaction (Cocito *et al.*, 1974).

No effect upon the translocation step of protein synthesis has yet been observed with Group A antibiotics.

Finally, some evidence exists for an irreversible effect of Group A antibiotics upon ribosomes. Parfait and Cocito (1980) have shown that the binding of Group A antibiotics to ribosomes, followed by subsequent detachment yielded ribosomal particles unable to perform poly (U)-directed polyphenylalanine synthesis. Accordingly, two states of the 50S ribosomal subunit were suggested, a normal state and that induced by attachment (and subsequent detachment) of Group A antibiotic. This observation was further substantiated as a result of the inability of erythromycin to displace Group B antibiotic from 50S ribosomal subunits, either in the presence of, or following exposure to Group A antibiotic. Erythromycin ($K_{ass} = 2 \times 10^{7} M^{-1}$) would normally displace Group B antibiotic ($K_{ass} = 2.5 \times 10^{6} M^{-1}$) from ribosomes, when in the absence of Group A antibiotic (Parfait *et al.*, 1981). However, the association constant for the binding of Group B antibiotic to ribosomes (modified due to the action of Group A antibiotic) was found to be similar to that of particles incubated with a mixture of Group A+B antibiotics (i.e. $15 \times 10^{6} M^{-1}$) (Parfait and Cocito, 1980). This increase in the binding of Group B antibiotic, when in the presence of, or following exposure to Group A antibiotic may account both for the synergistic action of the pair and the inability of erythromycin to displace Group B antibiotic from the ribosome. In addition to its apparent irreversible effect upon ribosomes, Group A antibiotics have been shown to act catalytically rather than stoichiometrically. Hence Parfait and Cocito (1980) have suggested that Group A

antibiotics bind to the ribosome, exert their irreversible effect and then subsequently become detached (ready for attachment to other ribosomes).

In conclusion, the overwhelming body of evidence strongly implicates the ribosomal A-site as the target of the antibiotic. Whether inhibition of ribosome function results from the prevention of aminoacyl-tRNA binding to the A-site or by a direct effect upon the peptidyl transferase centre remains to be established. Although an irreversible effect of Group A antibiotic upon the ribosome is difficult to comprehend, results presented later (Chapter 6) do permit a rational interpretation of these observations.

Group B antibiotics bind to both 70S ribosomes and 50S ribosomal subunits but not 30S ribosomal subunits. This has been demonstrated by a variety of techniques including retention on Millipore filters (Ennis, 1974), sedimentation (Cocito, 1971), equilibrium dialysis (de Béthune and Nierhaus, 1978) and spectrofluorimetry (Parfait et al., 1978). This last technique takes advantage of the observation that solutions of Group B antibiotics fluoresce under UV light, their intensity increasing proportionately upon addition of 50S ribosomal subunits. The association constant for binding of drug to ribosomes, as determined by this technique is $2.5 \times 10^{6} M^{-1}$. This is in reasonable agreement with values obtained previously (de Béthune and Nierhaus, 1978; Contreras and Vazquez, 1977 a). Despite a synergistic effect between Group A and Group B antibiotics no evidence for a physical interaction between the pair has been observed (Parfait et al., 1978). In addition, although a 6-fold increase in the binding of Group B antibiotic to ribosomes has been observed in the presence of Group A antibiotic the converse situation has not been observed except under sub-optimal binding conditions (Ennis, 1971). Curiously, Contreras

and Vazquez (1977 a) found that Group B antibiotics hindered the binding of their Group A counterparts.

Some evidence exists for an inhibitory effect of Group B antibiotics in cell-free protein-synthesising systems translating polyadenylic acid, but not polyuridylic acid (Yamaguchi and Tanaka, 1967). Ennis (1974) observed that Group B antibiotics could be displaced from ribosomes both by erythromycin and spiramycin 111. Accordingly erythromycin and Group B antibiotics may share similar ribosome binding sites. Like erythromycin, Group B antibiotics require ribosomal protein L16 for binding to 50S ribosomal subunits (de Béthune and Nierhaus, 1978).

In conclusion, no clear indication of how Group B antibiotics inhibit protein synthesis is available at present.

1.3. Resistance to Group A and B Antibiotics

Resistance to Group A or B antibiotics can occur either through exclusion of the drug, inactivation of the drug, or modification of the target site of the drug.

A mutant of Bacillus subtilis was isolated by Ennis (1967), resistant to 25μg Group A + B drug/ml. Since the protein-synthetic machinery of the mutant was still sensitive to drug and no evidence for a drug-inactivating enzyme could be found, it was concluded that the mutant was resistant by virtue of a change in cell permeability.

Other mutants of Bacillus subtilis have been examined more extensively by Cocito and Fraselle (1973). Here the mixture of Group A + B antibiotics was lethal not only to double-sensitive strains but also to mutants resistant to only Group A antibiotic. In contrast, Group B-resistant

mutants were also resistant to the combination of the drugs. The gene conferring resistance to Group B antibiotics in Bacillus subtilis has been mapped by Ron et al., (1980). Transformation experiments indicated that the gene mapped between markers for elongation factor G and erythromycin resistance. Accordingly, it was located within the gene cluster of ribosomal proteins.

Plasmid-mediated Group A antibiotic resistance has been observed in Staphylococcus aureus (Le Goffic et al., 1977 a; 1977 b). Here the antibiotic was inactivated by O-acetylation of a hydroxyl group, by a plasmid-encoded enzyme. Although a similar mechanism has been observed by De Meester and Rondelet (1976) in a clinical isolate of S. aureus, plasmid mediation was unproven.

Resistance to Group B antibiotics is often observed in clinical isolates of bacteria carrying the so-called macrolide-lincosamide-streptogramin B-resistance phenotype (MLS-phenotype) (Yagi et al., 1978). Resistance is often inducible (Weisblum, 1975) although it is possible to isolate constitutive mutants in which the necessity for induction is abolished (Weisblum et al., 1971). Induction of MLS-resistance was found to coincide with dimethylation of 23S RNA (Lai and Weisblum, 1971) and this was later shown to be causally responsible for MLS-resistance (Lai et al, 1973). The MLS-resistance phenotype is expressed in vivo by Streptomyces erythreus, in contrast to several other MLS-producers (including Streptomyces) tested. In addition, its 23S RNA contains N^6N^6- dimethyladenine (Graham and Weisblum, 1979). Recently a causal relationship between the presence of N^6N^6- dimethyladenine and erythromycin resistance in S.erythreus has been established (R.H. Skinner, personal communication).

1.4. Resistance in Producing Organisms There is some evidence to suggest that resistance to Group B antibiotics in the producing organism Streptomyces diastaticus is inducible. Sub-inhibitory concentrations of Group B antibiotic (10 μg/ml) resulted in the apparent induction of a low level of N^6,N^6-dimethyladenine formation in 23S RNA (Fujisawa and Weisblum, 1981). This low level of dimethylation may be simply a reflection of the 23S RNA having already been methylated in vivo (due possibly to the presence of endogenous Group B antibiotic acting as an inducer). However, since neither N^6,N^6-dimethyladenine nor N^6-methyladenine were found in 23S RNA from uninduced S. diastaticus (and S. loidensis) (Graham and Weisblum, 1979), composition of growth media and time of incubation probably have a significant effect upon the onset of the induction process (if it exists).

Inactivation of Group B antibiotic has been observed in the producing organism Streptomyces mitakaensis (Kim et al., 1974). During mid-log to early stationary phase, the accumulation of Group B antibiotic in the culture medium fell, whilst an increase of its degradation product occurred simultaneously. Disappearance of Group B antibiotic was due to enzymatic hydrolysis of the lactone ring of the molecule. A similar (constitutive) enzyme has been purified from Actinoplanes missouriensis which is also capable of hydrolysing Group B antibiotics (Perlman and Hou, 1969; Hou et al., 1970).

1.5. Production of Group A and B Antibiotics Production of Group A and B antibiotics in S. mitakaensis occurred at distinct phases of growth (Kim et al., 1974). In particular, production of Group B antibiotic occurred entirely during log phase of growth. Furthermore, the content of Group B antibiotic in the culture medium was reduced rapidly as

cells entered late-log or early-stationary phase (the point at which the Group B antibiotic-degrading enzyme was most active).

In contrast, the production of Group A antibiotic occurred throughout both log and stationary phases of growth. Furthermore, no degradation of the antibiotic was observed. Hence, the producing organism is faced with the prospect of defending itself against a product (i.e. Group A antibiotic) which it cannot degrade.

The present investigation was undertaken to establish the precise nature of the mechanism of resistance to Group A antibiotics adopted by the producing organism. For this present study the vernamycin A and Bα producer Streptomyces loidensis was chosen since, in contrast to many Group A antibiotic-producing Streptomyces, the organism produces only one type of Group A antibiotic (Vazquez, 1979).

2. Materials And Methods

2.1. Purity Of Vernamycins The purity, and extent of cross-contamination of vernamycin A and Bα was established by thin-layer chromatography, according to the method of Kim *et al.*, (1974).

5μg of each antibiotic (dissolved in DMSO) was applied to an HPTLC-Alufolien Kieselgel 60F plate (5 cm x 7.5 cm). The chromatogram was run in an ascending fashion using, as solvent, butanol/acetone/water (3:1:1). After air drying, the plate was observed under UV light (Figure 3.3). Both vernamycin A and Bα were found to be essentially pure with no cross-contamination of components. Furthermore, the presence of derivatives of vernamycin A and Bα was not apparent.

The purity of each component was confirmed by a comparison of characteristic UV/visible absorption spectra (data not presented).

2.2. Determination Of Minimum Inhibitory Concentrations (MIC) Of Vernamycin A And Bα DYM agar plates containing various concentrations of vernamycin A, Bα or the combination, were prepared. This was accomplished by first dissolving the drugs in ethanol and adding this solution to liquid media (at 45-50°C). After cooling, a lawn of test organisms was spread evenly over the entire surface of the plate, and incubated for 2 days at 30°C (for *S. loidensis*) or 55°C (for *B. stearothermophilus*).

2.3. Antibiotic Sensitivity Assay Fixed quantities of drug were applied to antibiotic assay discs (Whatman AA discs) and dried under infra-red lamps. The discs were then applied to a lawn of the test organism, spread evenly over DYM agar plates. Plates were incubated for 2 days at 30°C (for *S. loidensis*) or 55°C (for *B. stearothermophilus*).

Legend to Figure 3.3

Thin-layer chromatography of vernamycin A, Bα and A plus Bα.

Vernamycin A, Bα and A+Bα (5μg each - in DMSO) were applied to an HPTLC-Alufolien Kieselgel 60F plate (5cm x 7.5cm) and the chromatogram run in ascending fashion, according to Chapter 3.2.1.

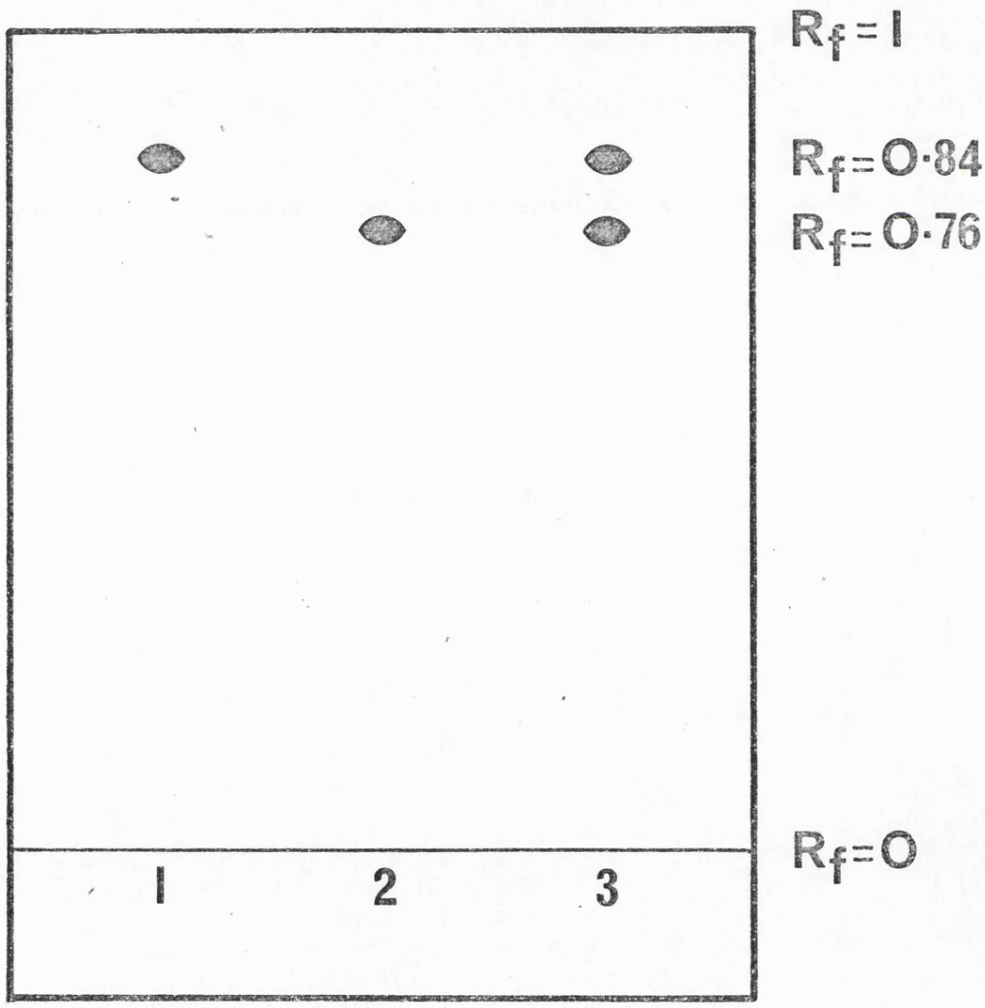
Track

1 : vernamycin A

2 : vernamycin Bα

3 : vernamycin A+Bα

FIGURE 3.3



2.4. Reconstitution Of 50S Ribosomal Subunits From Bacillus stearothermophilus Materials were prepared as in Chapter 2.2.6. In general, ribosomal RNA extracted from 70S ribosomes (RNA 70) and ribosomal proteins extracted from 50S ribosomal subunits (TP50) were found to give the highest levels of reconstitution when compared with native particles.

RNA 70 was added to a buffer such that, upon addition of TP50, the final ionic conditions during reconstitution were: 30mM Tris-HCl (pH 7.6 at 20°C); 20mM magnesium chloride; 330mM potassium chloride; 6mM 2-mercaptoethanol. 200 µl of reconstitution mix contained 120 pmol RNA 70 and 180 pmol equivalents of TP50 (+ "L3 protein"). (1 pmol equivalent of TP50 was that amount of protein obtained from 1 pmol 50S ribosomal subunits). The mixture was incubated at 60°C for 3 hours after which reconstituted particles were precipitated by the addition of 0.5 volumes of ethanol (at -20°C). After 5 minutes at 0°C, reconstituted particles were recovered by centrifugation at 12,000 xg for 2 minutes, and resuspended in RS buffer.

2.5. Reconstitution Of Hybrid 50S Ribosomal Subunits From Streptomyces loidensis RNA 70 And Bacillus stearothermophilus TP50 Reconstitution of hybrid 50S ribosomal subunits from S. loidensis RNA 70 and B. stearothermophilus TP50 essentially followed the reconstitution procedure described in the previous section (Chapter 3.2.4.) except the reconstitution mix was incubated at 55°C for 5 hours.

2.6. Estimation Of The Concentration Of Reconstituted 50S Ribosomal Subunits Absorbance due to reconstituted 50S particles constituted 65% of the total absorbance at 260 nm (See Chapter 5.3.1). Hence the concentration of reconstituted 50S particles was determined (according to Chapter 2.2.7) after this factor had been applied.

Chapter 3

3. Results

3.1. Resistance To Vernamycin A And Bα In Vivo Results from the determination of minimum inhibitory concentrations of vernamycin A and Bα are presented in Tables 3.1 and 3.2.

Since drug resistance is essentially a comparative phenomenon, *S. coelicolor* was initially chosen as a reference organism. However, *S. coelicolor* proved to be unsuitable since it was quite resistant to vernamycin A *in vivo* (data not presented). Although perhaps not a logical choice, *B. stearothermophilus* was chosen, for reasons which will be presented later.

Whilst *S. loidensis* was clearly resistant to quite high concentrations of either vernamycin A or Bα it was rather curious that the MIC was lowered slightly with a combination of both drugs (Table 3.1). In contrast, *B. stearothermophilus* was significantly more sensitive than *S. loidensis* to both vernamycin A and Bα and was fully inhibited by a combination of both drugs (Table 3.2).

Results from the antibiotic sensitivity assays are presented in Figure 3.4. Again *S. loidensis* was significantly more resistant to vernamycin A than *B. stearothermophilus* (as indicated by the absence of zones of inhibition).

3.2. Resistance To Vernamycin A In Vitro To determine whether vernamycin A resistance could be demonstrated in an *in vitro* assay system, S30 fractions were prepared from *S. loidensis* and a number of reference organisms, including *E. coli*, *B. stearothermophilus* and *S. coelicolor*.

E. coli was chosen because its ribosomes were reported to be very sensitive to vernamycin A (despite being resistant to drug *in vivo*) (Laskin and

TABLE 3.1

VERNAMYCIN CONCN. (μg/ml)	0	1	5	10	25	50	100
A	+	+	+	+	+	+	+/-
Bα	+	+	+	+	+	+	-
A+Bα	+	+	+	+	+	-	-

Legend to Table 3.1

Determination of minimum inhibitory concentrations (MIC) of vernamycin A and Bα for S. loidensis.

Vernamycin A, Bα, or a combination of both, were dissolved in ethanol and added to molten DYM agar to the concentrations indicated above (with combinations of drugs, the concentration indicated refers to each component). A lawn of S. loidensis was spread evenly over the entire surface of plates containing the agar and the plates were incubated at 30°C for 2 days (according to Chapter 3.2.2).

+ = growth

+/- = slight growth

- = no growth.

TABLE 3.2

VERNAMYCIN CONCN. (μg/ml)	0	1	5	10	25	50	100
A	+	+	+	+	-	-	-
Bα	+	+	+	-	-	-	-
A+Bα	+	-	-	-	-	-	-

Legend to Table 3.2

Determination of minimum inhibitory concentrations (MIC) of vernamycin A and Bα for B. stearothermophilus.

Vernamycin A, Bα, or a combination of both, were dissolved in ethanol and added to molten DYM agar to the concentrations indicated above (with combinations of drugs, the concentration indicated refers to each component). A lawn of B. stearothermophilus was spread evenly over the entire surface of plates containing the agar and the plates were incubated at 55°C for 2 days (according to Chapter 3.2.2).

+ = growth

- = no growth

Legend to Figure 3.4

Antibiotic sensitivity assay - response of *S. loidensis* and *B. stearothermophilus* to vernamycin A, *in vivo*.

Plates were prepared according to Chapter 3.2.3. Discs contained:

A : control (i.e. no drug)

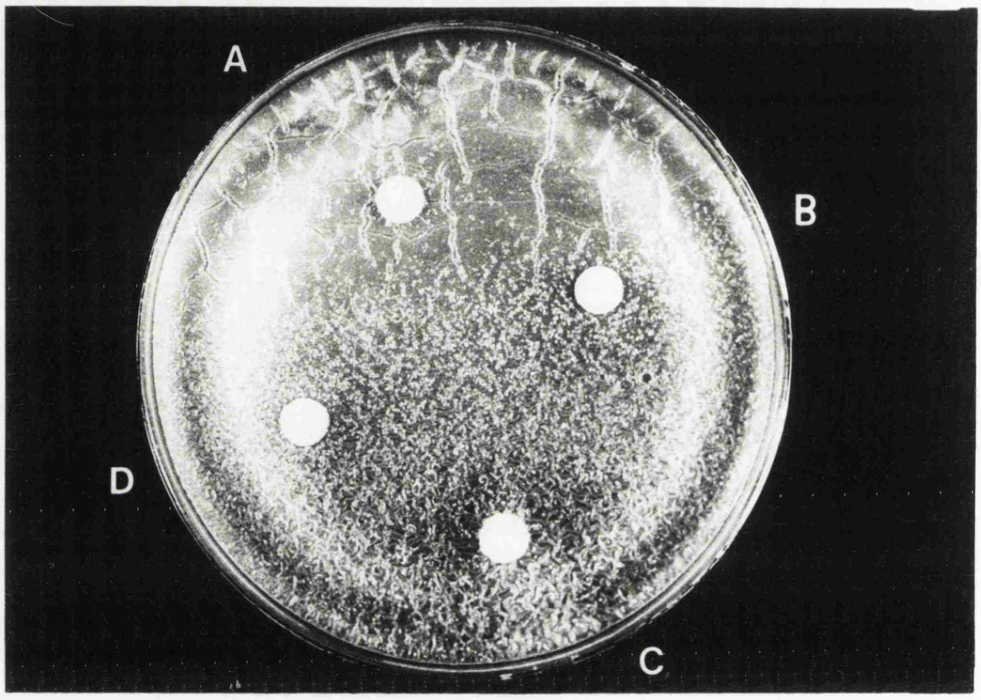
B : 25μg vernamycin A

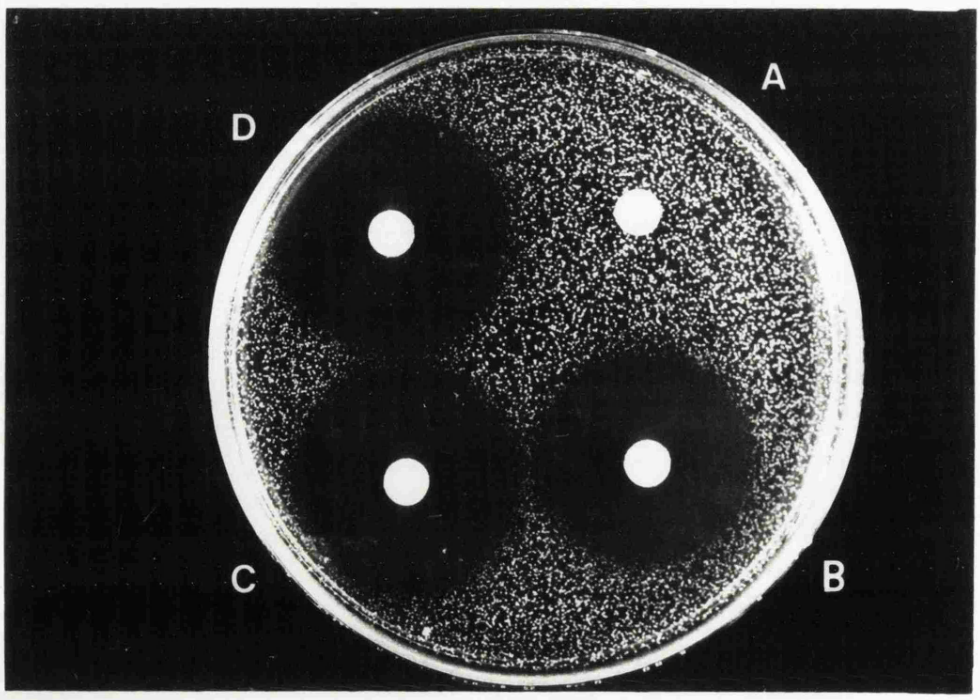
C : 50μg vernamycin A

D : 100μg vernamycin A

FIGURE 3.4

S. loidensis





B. stearothermophilus

May Chan, 1965). S. coelicolor was chosen because it was a laboratory reference streptomycete and B. stearothermophilus was chosen on the pretext that total reconstitution of ribosomes would probably be necessary at a later stage of the investigation.

S30 fractions were assayed in a poly (U)-directed protein-synthesing system in the presence and absence of vernamycin A (according to Chapter 2.2.8). The S30 fraction from S. loidensis was substantially more resistant to vernamycin A than S30 fractions from all the reference organisms (data not presented). To establish whether such resistance was the result of an antibiotic-modifying enzyme or a target site modification, S30 fractions from both S. loidensis and E. coli were separated into ribosomes and S100 fractions. (E. coli was chosen as a reference organism at this stage since its S30 fraction was very sensitive to drug). Ribosomes from both organisms were then assayed, together with S100 from E.coli, in a poly (U)-directed protein-synthesising system in the presence and absence of vernamycin A (Figure 3.5). While E. coli ribosomes were almost completely inhibited by vernamycin A, the ribosomes from S. loidensis were substantially (although not completely) resistant.

To examine the possibility that the mechanism of resistance was inducible and that the partial degree of inhibition with S. loidensis ribosomes was the result of incomplete induction, S. loidensis was grown in the presence of sub-inhibitory concentrations of vernamycin A (i.e. 10 μg/ml). Ribosomes from this batch of S. loidensis exhibited a response to vernamycin A similar to ribosomes from S. loidensis grown in the absence of added drug (data not presented). Accordingly, ribosomes from S. loidensis can be regarded as tolerant of, rather than totally resistant to, vernamycin A.

Legend to Figure 3.5

Response of 70S ribosomes from *S. loidensis* and *E. coli* to vernamycin A.

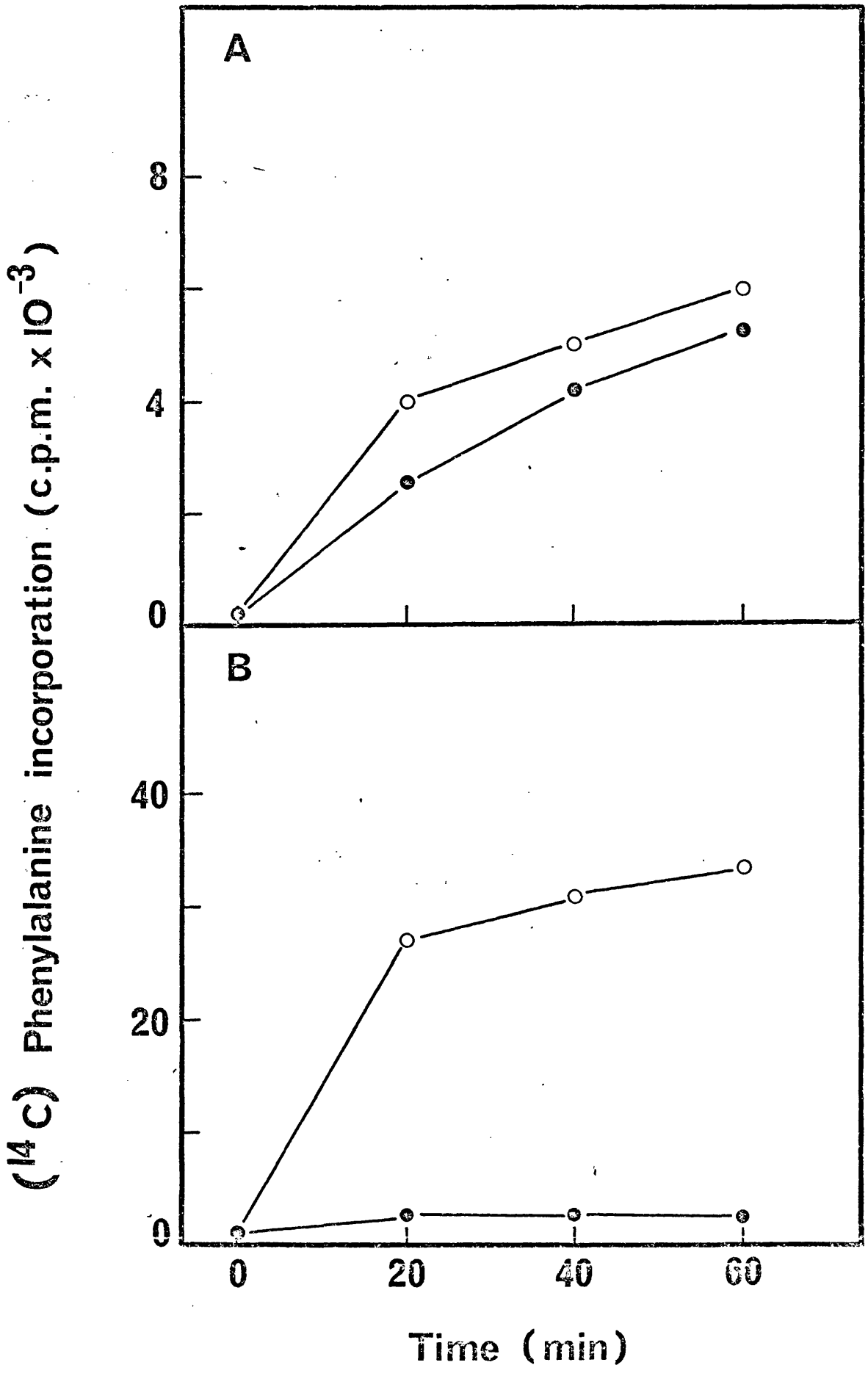
Ribosomes from *S. loidensis* and *E. coli* were supplemented with S100 from *E. coli* and assayed for their ability to synthesise polyphenylalanine in the presence (● - ●) and absence (o - o) of vernamycin A (25μg/ml).

2000 c.p.m. ≡ 1 pmol phenylalanine per pmol of ribosomes

A : *S. loidensis* 70S ribosomes

B : *E. coli* 70S ribosomes

FIGURE 3.5


A
B
8
4
0
40
20
0
0
20
40
60
Time (min)
(^{14}C) Phenylalanine incorporation (c.p.m. x 10^{-3})

To establish which ribosomal subunit conferred resistance to vernamycin A, ribosomal subunits from both S. loidensis and E. coli were recombined in all four reciprocal combinations and tested for response to vernamycin A in a poly (U)-directed protein-synthesising system (Figure 3.6). Results suggested that resistance to vernamycin A could occur only when 30S and 50S ribosomal subunits from S. loidensis were assayed together.

To establish whether similar results could be obtained using the other reference organisms, ribosomal subunits from S. coelicolor, B.stearothermophilus and S. loidensis were recombined in all nine reciprocal combinations and tested for response to vernamycin A in a poly (U)-directed protein-synthesising system (Table 3.3). Results suggested that 50S ribosomal subunits from S. loidensis were resistant to vernamycin A when paired with 30S ribosomal subunits from either of the two reference strains. Furthermore, 30S ribosomal subunits from S. loidensis could not confer any (extra) degree of vernamycin A-resistance to 50S ribosomal subunits from both S. coelicolor and B. stearothermophilus. Surprisingly, S. coelicolor 50S ribosomal subunits were found to be partially resistant to vernamycin A, irrespective of the source of the 30S ribosomal subunits. The reason for this observation is unclear, but may be purely fortuitous; for example, ribosomes from another streptomycete (i.e. S. erythreus)were fully sensitive to vernamycin A (data not presented). Hence, partial resistance to vernamycin A is not a general property of Streptomyces ribosomes.

Since resistance to vernamycin A was conferred by the 50S ribosomal subunit of S. loidensis (under certain circumstances) attempts were made to establish the nature of the vernamycin A-resistance determinant (i.e. ribosomal RNA or ribosomal proteins). Since B. stearothermophilus had been used as a reference organism in conceptually-similar experiments

Legend to Figure 3.6

Response of reciprocal combinations of ribosomal subunits from *S. loidensis* and *E. coli* to vernamycin A.

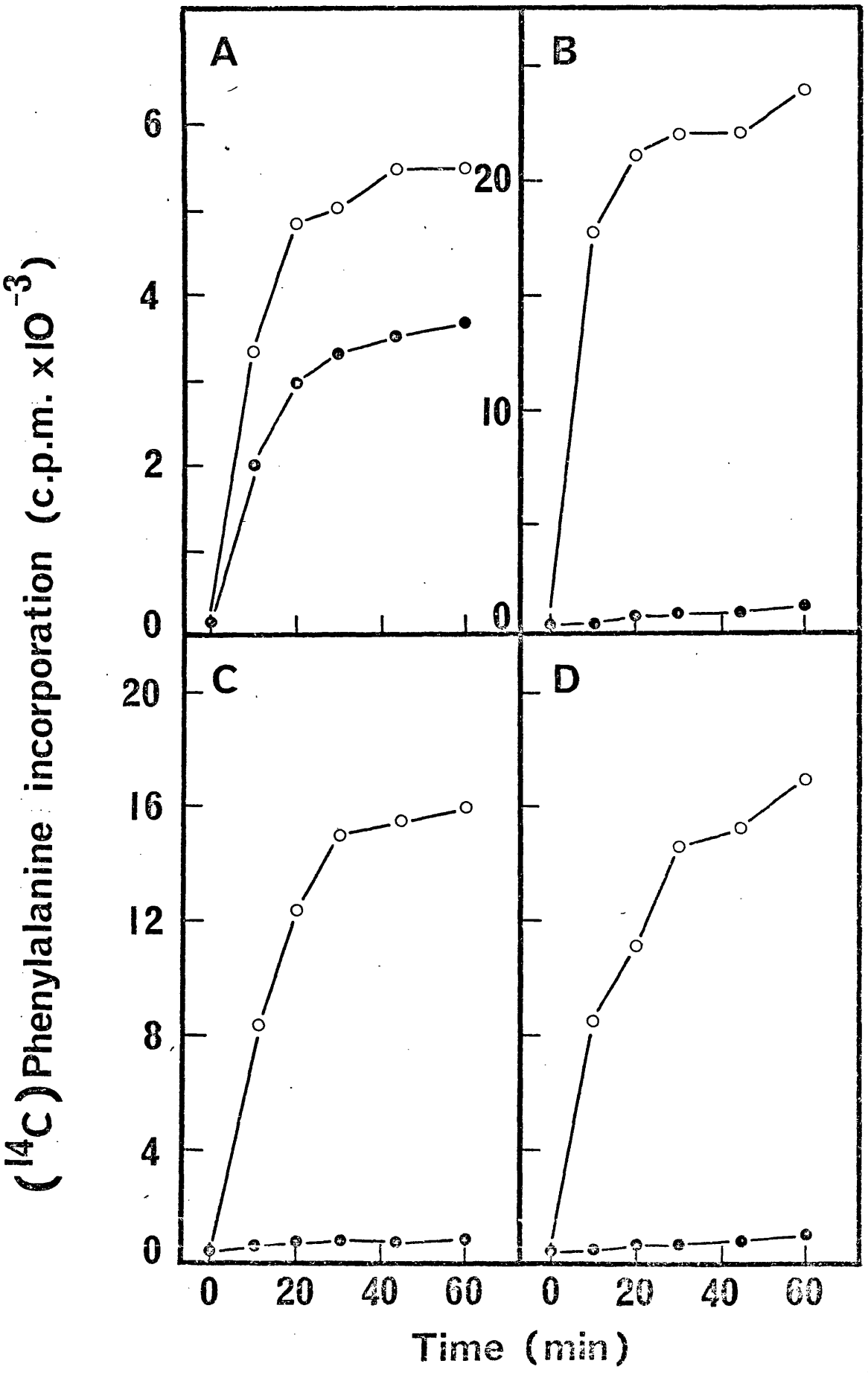
Ribosomal subunits (30S and 50S), from both organisms, were mixed in all four reciprocal combinations, supplemented with S100 from *E. coli*, and tested for the ability to synthesise polyphenylalanine, in the presence (● - ●) and absence (o - o) of vernamycin A (5μg/ml).

1000 c.p.m. ≡ 1 pmol phenylalanine per pmol of ribosomes

Source of ribosomal subunit

		30S	50S
A	:	*S. loidensis*	*S. loidensis*
B	:	*S. loidensis*	*E. coli*
C	:	*E. coli*	*S. loidensis*
D	:	*E. coli*	*E. coli*

FIGURE 3.6


A
B
C
D
6
4
2
0
20
10
0
20
16
12
8
4
0
0
20
40
60
0
20
40
60
Time (min)
(^{14}C) Phenylalanine incorporation (c.p.m. x10^{-3})

Legend to Table 3.3

Effect of vernamycin A on polyphenylalanine synthesis by reciprocal recombinations of ribosomal subunits from *S. loidensis*, *S. coelicolor* and *B. stearothermophilus*.

Ribosomal subunits (30S and 50S) from each of the above-mentioned organisms were combined, in reciprocal fashion, in a 1:1 ratio and supplemented with S100 from *B. stearothermophilus*. Subsequently, their ability to synthesise polyphenylalanine in the presence and absence of vernamycin A (5μg/ml) was tested. Results are expressed as a percentage of control incorporations which, after 15 minutes synthesis (at which time incorporation was linear) ranged from 2 to 15 pmol of phenylalanine per pmol ribosomes.

TABLE 3.3

SOURCE OF 30S RIBOSOMAL SUBUNIT	SOURCE OF 50S RIBOSOMAL SUBUNIT	INHIBITION OF POLYPHENYLALANINE SYNTHESIS (%)
S. loidensis	*S. loidensis*	30
S. loidensis	*S. coelicolor*	62
S. loidensis	*B. stearothermophilus*	75
S. coelicolor	*S. coelicolor*	50
S. coelicolor	*S. loidensis*	26
S. coelicolor	*B. stearothermophilus*	73
B. stearothermophilus	*B. stearothermophilus*	88
B. stearothermophilus	*S. coelicolor*	63
B. stearothermophilus	*S. loidensis*	30

to those envisaged (Lai et al., 1973; Cundliffe, 1978), ribosomal RNA and ribosomal proteins from both B. stearothermophilus and S. loidensis were reconstituted in all combinations of RNA 70 and TP50 according to Chapter 3.2.4. and 3.2.5. However, due presumably to the labile nature of Streptomyces ribosomal proteins, only two of the four combinations yielded active 50S particles (i.e. those containing ribosomal proteins from the thermophile). When the active reconstituted particles were tested for their response to vernamycin A in a poly (U)-directed protein-synthesising system, in the presence of B. stearothermophilus 30S ribosomal subunits and S100 fraction, both combinations (i.e. B. stearothermophilus TP 50 reconstituted with RNA 70 from either B. stearothermophilus or S. loidensis) were fully sensitive to drug (Figure 3.7). On the basis of these results, it was not possible to draw firm conclusions as to the nature of the vernamycin A-resistance determinant (i.e. ribosomal RNA or ribosomal proteins).

Legend to Figure 3.7

Effect of vernamycin A on reconstituted 50S particles from *S. loidensis* and *B. stearothermophilus*.

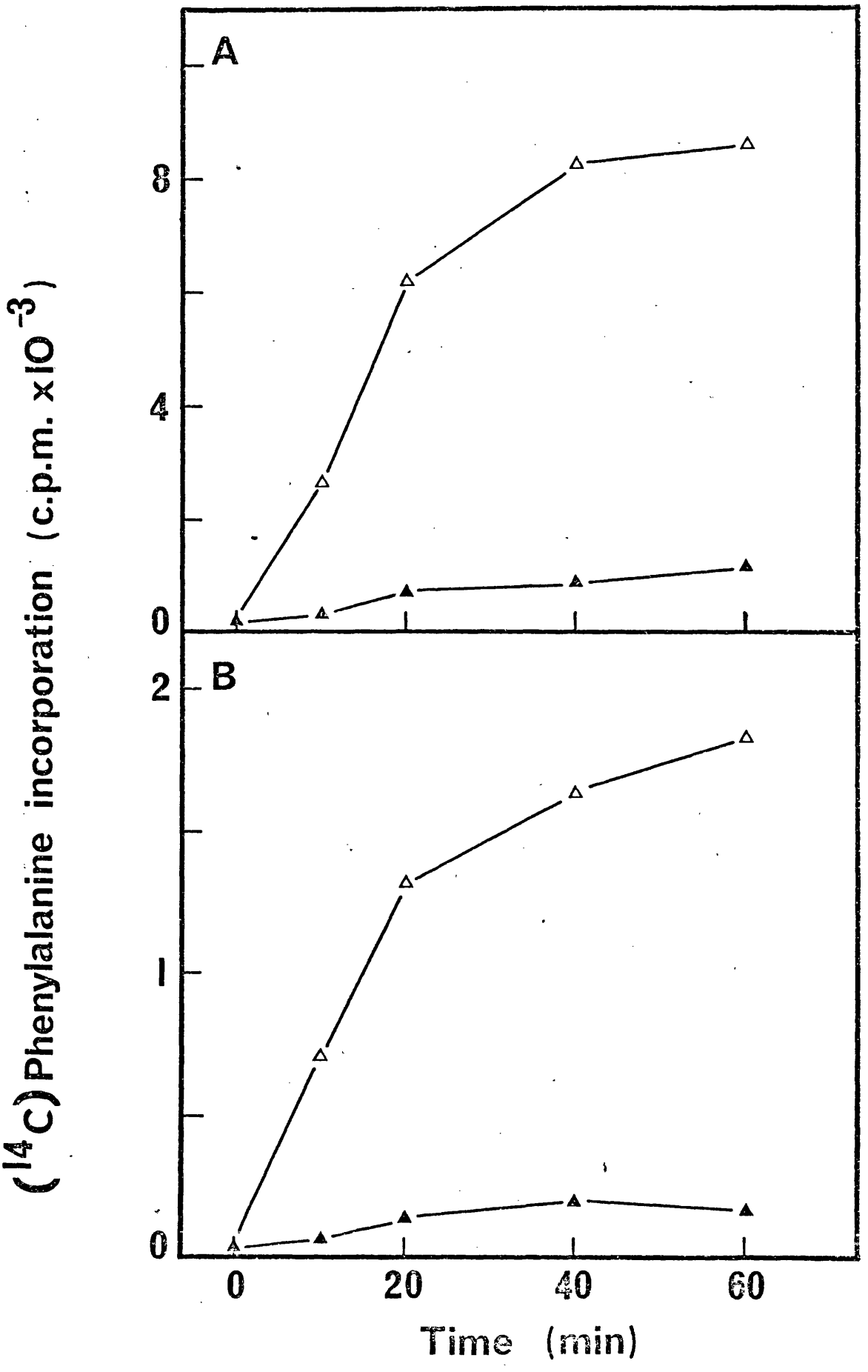
50S particles were reconstituted from RNA 70 and TP 50, according to Chapter 3.2.4. and 3.2.5. Following supplementation with 30S particles and S100 from *B. stearothermophilus*, particles were tested for the ability to synthesise polyphenylalanine in the presence (▲ - ▲) and absence (△ - △) of vernamycin A (5μg/ml).

1000 c.p.m. ≡ 1 pmol phenylalanine per pmol of ribosomes

A : 50S particles reconstituted from *B. stearothermophilus* RNA 70 and TP 50.

B : 50S particles reconstituted from *S. loidensis* RNA 70 and *B. stearothermophilus* TP 50.

FIGURE 3.7


A
B
8
4
0
2
1
0
0
20
40
60
Time (min)
(^{14}C) Phenylalanine incorporation (c.p.m. x10^{-3})

4. Discussion

The two methods used for demonstrating resistance to vernamycin A *in vivo* by *S. loidensis* are generally subject to inherent drawbacks. Accordingly, the medium upon which the organism grows could exert an effect upon the expression of resistance; a number of examples exist of antibiotic resistance determinants which are subject to various degrees of catabolite repression (de Crombrugghe *et al.*, 1973; Harwood and Smith, 1971; Tsukuda *et al.*, 1972). Furthermore, the timing of expression of resistance could have a crucial effect in the antibiotic sensitivity assay; organisms which are not constitutively resistant to an antibiotic could initially appear sensitive (revealed by a zone of inhibition) until induction of antibiotic resistance had occurred (revealed by growth over the zone of inhibition). For example, in *S. bikiniensis* (the streptomycin producer) resistance to streptomycin occurred after induction of a streptomycin-inactivating enzyme (Piwowarski and Shaw, 1979). In conclusion, despite apparent drawbacks in the methodology employed, *S. loidensis* was quite resistant to vernamycin A *in vivo*, possibly as a result of a constitutive antibiotic-resistance mechanism.

When ribosomes from *S. loidensis* were tested for response to vernamycin A, they were found to be significantly more resistant to drug than ribosomes from *E. coli*. In contrast, the S100 fraction for *S. loidensis* could not confer any degree of resistance to vernamycin A when assayed with drug-sensitive ribosomes (i.e. from *E. coli*) in a poly (U)-directed protein-synthesising system (data not presented). Accordingly, the presence of a vernamycin A-modifying enzyme in the soluble fraction of *S. loidensis* was not detected.

To establish which ribosomal subunit of *S. loidensis* conferred resistance to vernamycin A, ribosomal subunits from both *S. loidensis* and *E. coli*

were combined, in reciprocal fashion, and tested for response to vernamycin A in a poly (U)-directed protein-synthesising system. Although the homologous S. loidensis ribosomal subunits were essentially resistant to vernamycin A it was surprising to find that when 30S and 50S ribosomal subunits (from E. coli and S. loidensis respectively) were combined the resultant combination was sensitive to vernamycin A. Since vernamycin A binds only to the 50S ribosomal subunit of drug-sensitive organisms, by extension it might be expected that in a vernamycin A-resistant ribosome, the 50S ribosomal subunit would be vernamycin A-resistant. Accordingly, this observation suggested two explanations. First, that the vernamycin A-resistance mechanism in S. loidensis was "distributed" between both 30S and 50S ribosomal subunits (presumably at the subunit interface) and hence could only function when homologous ribosomal subunits were combined. Second, that differences in 30S ribosomal subunit topography might influence the response of S. loidensis 50S ribosomal subunits to vernamycin A. In support of this notion, mutations affecting E. coli 30S ribosomal proteins S5 and S12 rendered erythromycin-resistant 50S ribosomal subunits (containing an altered L4 ribosomal protein) sensitive to erythromycin (Saltzman and Apirion, 1976).

To establish whether 30S ribosomal subunits from other bacterial sources could effect a change in response to vernamycin A by S. loidensis 50S ribosomal subunits, further ribosomal subunit recombination experiments were performed. However, 30S particles from both S. coelicolor and B. stearothermophilus could not mimic the effect of E. coli 30S particles by changing the response of S. loidensis 50S particles to vernamycin A. Hence S. loidensis 50S ribosomal subunits were resistant to vernamycin A, but only when assayed with appropriate 30S ribosomal subunits. Accordingly, it is unlikely that the vernamycin A resistance determinant is distributed

between S. loidensis 30S and 50S ribosomal subunits. Although it is tempting to speculate and invoke E. coli 30S ribosomal subunit-induced changes in the conformation of S. loidensis 50S particles, there is no direct evidence to support this notion.

To establish whether ribosomal RNA or ribosomal proteins from S. loidensis 50S particles conferred resistance to vernamycin A, an attempt was made to reconstitute both native and hybrid 50S particles composed of ribosomal RNA and ribosomal proteins from S. loidensis and B. stearothermophilus. These particles could be subsequently tested for response to vernamycin A in a poly (U)-directed protein-synthesising system (in the presence of B. stearothermophilus 30S particles and S100). B. stearothermophilus was chosen as a reference organism in these experiments, since its ribosomal proteins were fairly insensitive to the extreme temperatures employed during the reconsititution procedure. Furthermore, a procedure for the successful reconstitution of B. stearothermophilus 50S ribosomal subunits had already been established. (Fahnestock et al., 1974).

Although variations in the reconstitution procedure outlined in Chapter 3.2.4. were investigated (with respect to temperature) only two combinations of ribosomal RNA and ribosomal proteins reconstituted successfully to give particles active in polyphenylalanine synthesis (when supplemented with 30S ribosomal subunits and S100). Since both combinations contained B. stearothermophilus ribosomal proteins, this suggested that the reason why the other combinations had failed to reconstitute successfully was due, at least in part, to heat inactivation of S. loidensis ribosomal proteins.

When both types of reconstituted particles (i.e. those containing B. stearothermophilus ribosomal proteins) were tested for response to

vernamycin A in a poly (U)-directed protein-synthesising system, both were found to be fully sensitive to the drug. Although it was tempting to conclude (by extension) that resistance to vernamycin A was conferred by *S. loidensis* ribosomal proteins, the possibility still existed that changes in drug response could result either from effects of the reconstitution procedure itself, or perhaps from incompatibility within the hybrid 50S ribosomal subunits. Hence, attention was focussed upon devising a reconstitution procedure for *S. loidensis* 50S ribosomal subunits, and to establish whether these reconstituted particles were still resistant to vernamycin A.

CHAPTER 4

TOTAL RECONSTITUTION OF 50S RIBOSOMAL SUBUNITS FROM STREPTOMYCES LOIDENSIS

Chapter 4

1. Introduction

1.1. Reconstitution Of Bacterial Ribosomes The reconstitution of bacterial ribosomes is necessarily a complex undertaking, requiring the sequential and, no doubt, co-operative additions of ribosomal proteins on to ribosomal RNA, with the ultimate aim of producing a particle capable of synthesising protein. Such a particle should contain one copy of each ribosomal component (Hardy, 1975) with the exception of 50S ribosomal protein L7/L12 which exists in four copies per (*E. coli*)50S ribosomal subunit (Subramanian, 1975). *In vivo*, ribosome formation can occur under relatively mild conditions (e.g. *E. coli* can grow at about 10°C), while *in vitro* far more extreme conditions have to be employed. The reasons for this discrepancy probably reflect fundamental differences between the two processes. For example, *in vivo* biosynthesis of ribosomes probably results from the sequential additions of ribosomal proteins on to precursor ribosomal RNA; there is some evidence to suggest that the RNA molecules of precursor ribosomal particles are longer than those in mature particles (Schlessinger, 1974). Spillman *et al.*,(1977) have suggested that *in vivo* assembly of ribosomal particles follows ribosomal RNA synthesis in a 5' → 3' direction; indeed this may even occur during transcription. Furthermore, the amount of a particular component (e.g. ribosomal protein) may be limited at certain stages of the assembly process by formal regulatory mechanisms. Other factors, including processing of ribosomal RNA (Schlessinger, 1974), methylation of ribosomal proteins (Alix and Hayes, 1974; Chang and Chang, 1975) and the possible presence (at least in *B. subtilis*) of specific precursor proteins not present in the mature particle (Guha *et al.*, 1975) could play an important role during ribosome biosynthesis. Finally, there is genetic evidence (from *E. coli*) to suggest that 50S ribosomal subunit assembly *in vivo* may be facilitated

by 30S ribosomal subunits or some component thereof (Nashimoto and Nomura, 1970). However, the converse situation was not observed.

In contrast, assembly of ribosomal particles *in vitro* takes place on mature (i.e. processed) ribosomal RNA, in the presence of a full complement of ribosomal proteins. However, there is some evidence for a non-ribosomal "maturation factor" which may play a role in 50S ribosomal subunit assembly both *in vivo* and *in vitro* (Bryant and Sypherd, 1974; Bryant *et al.*, 1974).

All the reconstitution techniques currently in use require the exposure of ribosomal components to extreme temperatures to induce certain conformational changes (normally rate-limiting) in the sub-ribosomal particle. Generally, these temperatures are very close to the point at which ribosomal proteins begin to denature. In an attempt to avoid this problem, Nomura and Erdmann (1970) focussed their attention on the thermophilic organism *Bacillus stearothermophilus* and were first to reconstitute successfully (at 60°C) (from ribosomal RNA and proteins) the 50S ribosomal subunit from this organism. However, this procedure was clearly inadequate for mesophilic organisms like *E. coli*; temperatures in excess of 50°C rapidly denature ribosomal proteins from this organism. The solution to this problem came with the addition of a second step to the reconstitution procedure. This presumably mimicked the *in vivo* situation by imposing certain constraints upon the ribosomal components by allowing, for example, only certain ribosomal proteins to bind to ribosomal RNA. Ultimately, this was reflected by a lowering of the reconstitution temperature to a point at which the ribosomal proteins failed to denature and the final conformational change (to an active particle) was induced. Furthermore, using the two-step procedure, the reconstituted particles were greater than 60% as active as the corresponding native particles (Dohme and Nierhaus, 1976).

Subsequently, ribosomes from othermesophilic organisms have been reconstituted successfully. Fahnestock (1979) succeeded in reconstituting 50S ribosomal subunits from both B. subtilis and B. licheniformis at 52°C using only a single-step reconstitution procedure. However, the levels of reconstitution achieved were significantly lower than those reported for E. coli. Whether this is a reflection of Bacillus ribosomal proteins being more labile than those of E. coli is unclear. Alternatively, the lower level of reconstitution could be due to the lack of a second step in the reconstitution procedure. Indeed, Dohme and Nierhaus (1976) claimed a higher level of reconstitution of B. stearothermophilus 50S ribosomal subunits using their two-step procedure than that obtained with the original single-step procedure (Nomura and Erdmann, 1970).

The reconstitution of Streptomyces ribosomes initially appeared daunting in view of their apparent labile nature; Streptomyces generally grow best at 28-30°C, often being severely inhibited by higher temperatures. However, the most logical approach to reconstituting S. loidensis ribosomes seemed initially to attempt the existing reconstitution methodology. Accordingly ribosomal RNA and ribosomal proteins were prepared both by the urea/lithium chloride extraction procedure of Fahnestock (1979) and the phenol/acetic acid-extraction procedure of Dohme and Nierhaus (1976). Reconstitutions were performed according to the respective methodology. However, none of the particles obtained were found to be active in polyphenylalanine synthesis. Similar results were obtained using the modified reconstitution procedure for E. coli 50S ribosomal subunits (Amils et al., 1978).

1.2. Rationale Theoretically reconstitution of S. loidensis 50S particles by a two-step reconstitution procedure stood a greater chance of success

than the corresponding single-step procedure. However, devising such a procedure was both technically and theoretically complex, since the critical variables of magnesium concentration, temperature and time of each step were inter-dependent (Amils *et al.*, 1978). Clearly each variable could not be optimised until a minimal level of reconstitution was achieved. Furthermore the situation was complicated by the observation of Dohme and Nierhaus (1976) that the phase of growth at which *E. coli* was harvested played a crucial role in the ability of its ribosomes to reconstitute. Although the phase of growth could be established quite easily for *E. coli* this was not the case with *Streptomyces*.

The situation was approached initially by defining the three crucial variables (magnesium concentration, temperature and time) in each step, by analogy with the reconstitution procedures for *E. coli* and taking into account the optimum growth temperature for *S. loidensis*. Each parameter was then varied independently until finally, a low level of reconstitution was obtained. Other parameters were then optimised. Ultimately, in view of the inter-dependence of the various parameters, it became necessary to optimise each one several times throughout the development of the procedure.

2. Materials And Methods

2.1. Preparation Of Ribosomal RNA And Ribosomal Proteins From Streptomyces Loidensis Preparation of materials from S. loidensis essentially followed the procedure of Fahnestock et al., (1974) and is outlined in Chapter 2.2.6.

2.2. Gel Electrophoresis Of RNA Under Denaturing Conditions Gel electrophoresis of RNA under denaturing conditions was performed essentially according to the method of Lehrach et al., (1977). The apparatus used is illustrated in Figure 4.1.

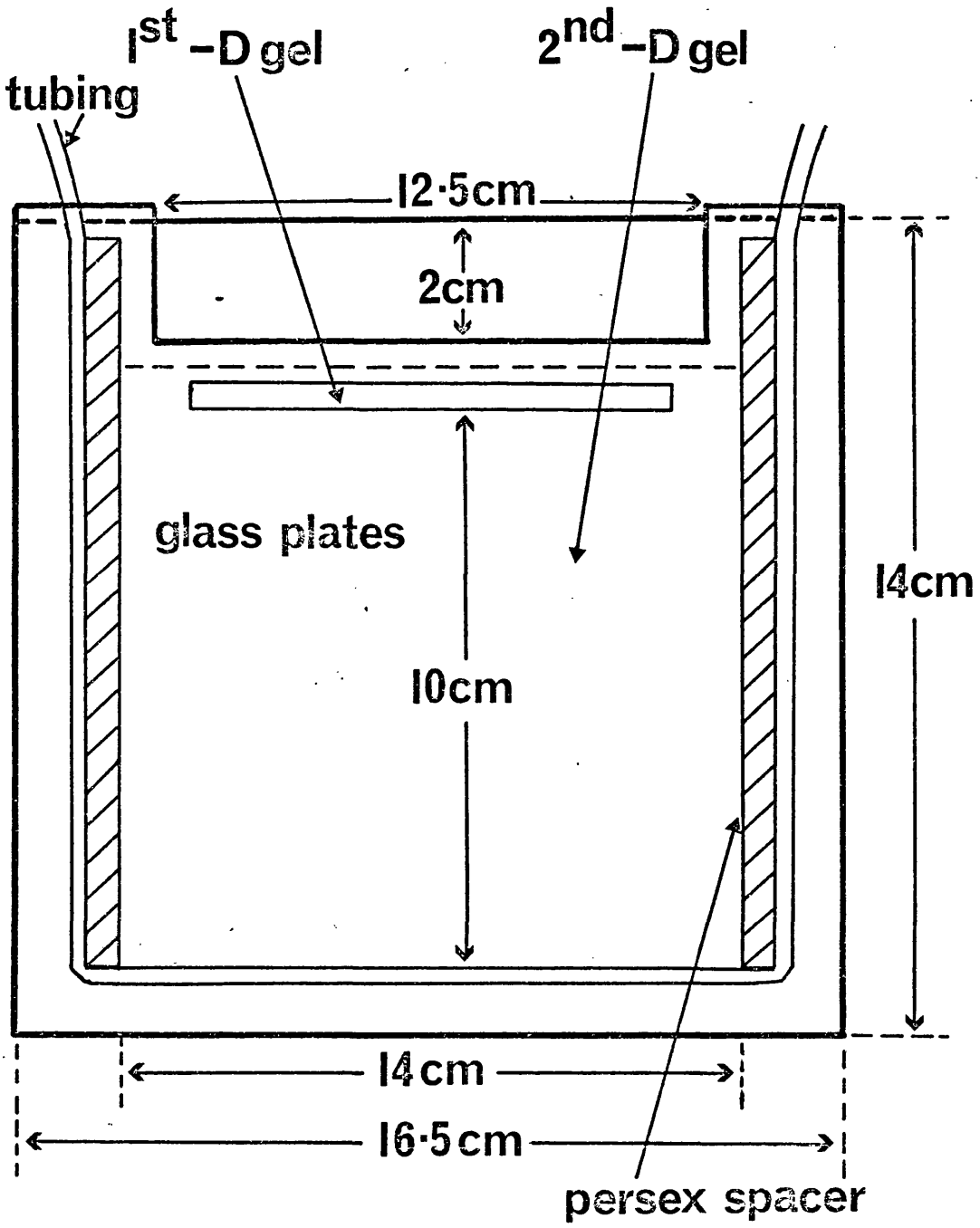
RUNNING BUFFER contained: 120 ml formaldehyde; 27 ml "PHOSPHATE" (containing 0.5 M Na_2HPO_4+ 0.055 M NaH_2PO_4); and water to 750 ml.

GEL PLUG contained: 10% (w/v) cyanogum (41), (a gelling agent) in running buffer. This was polymerised by addition (per ml) of 10 µl of fresh 10% (w/v) ammonium persulphate and 3 µl of TEMED. 5 ml of this solution were pipetted between the gel plates and allowed to polymerise.

GEL contained: 1% (w/v) agarose in "running buffer". This was conveniently prepared by first autoclaving 15 ml 2% (w/v) agarose (5 minutes at 10 p.s.i.). While still hot, 15 ml of x2 running buffer (containing 240 ml formaldehyde, 54 ml "PHOSPHATE" and water to 750 ml - all pre-warmed) was added, mixed and poured on to the gel plug. A P.T.F.E. comb was rapidly inserted at the meniscus and the gel allowed to polymerise. Following removal of the comb, sample wells were cleaned of residual agarose and rinsed with water.

RNA was dissolved in sample buffer.

FIGURE 4.1


1st -D gel
2nd -D gel
tubing
12·5cm
2cm
glass plates
10cm
14cm
14cm
16·5cm
persex spacer

SAMPLE BUFFER contained: 160 µl formaldehyde; 36 µl "PHOSPHATE"; 500 µl formamide; 200 µl glycerol; and 100 µl 0.1% (w/v) bromophenol blue.

After heating at 60°C for 3 minutes, the denatured RNA (4 µg in 5 µl sample buffer) was applied to each track. Samples were electrophoresed in "running buffer" for 2 hours at 60 V (cathode → anode). Following electrophoresis, RNA was stained with 0.4% (w/v) methylene blue in 0.2 M sodium acetate (pH 7.4) for 1 hour, and destained overnight in water.

2.3. Gel Electrophoresis Of RNA Under Non-Denaturing Conditions Gel electrophoresis of RNA under non-denaturing conditions followed essentially the method of Amils *et al.*, (1979).

A linear gradient (total volume 20 ml) of re-crystallised acrylamide (2 to 15% w/v) and *N,N'*-methylene-bis-acrylamide (0.01 to 0.075% w/v) in buffer (final pH 7.2) containing 40 mM Tris; 20 mM sodium acetate; 1 mM di-sodium EDTA; 0.027% (w/v) ammonium persulphate (fresh) and 0.056% (v/v) TEMED was poured from a two-chambered gradient maker, *via* a polystaltic pump (Buchler, U.S.A.) into the apparatus illustrated in Figure 4.1. The gel was carefully overlaid with water and allowed to polymerise. A capping gel was then poured (at approximately 50°C) over the first gel, composed of 1% (w/v) agarose in running buffer containing 40 mM Tris; 20 mM sodium acetate; and 1 mM di-sodium E.D.T.A. A P.T.F.E. comb was rapidly inserted at the meniscus and the gel allowed to polymerise. Following removal of the comb, sample wells were cleaned of residual agarose and washed with water.

RNA was dissolved in 7 M urea plus 0.1% (w/v) bromophenol blue. Samples were electrophoresed in running buffer for approximately 15 hours at 25 V (constant) (cathode to anode). Following electrophoresis, gel was

removed and stained according to Chapter 4.2.2.

2.4. Precautions Materials used in the preparation of reconstitution buffers were of at least A.R. quality. Surprisingly, certain batches of potassium chloride proved unsuitable. However, material provided by E. Merck (Darmstadt) was of consistent quality and was used throughout this study. All reconstitution buffers were autoclaved at 10 p.s.i. for 15 minutes and stored at 5°C; 2-mercaptoethanol was added immediately prior to use. All manipulations were undertaken with gloved hands.

2.5. Summary Of Reconstitution Procedure RNA 70 was added to a reconstitution buffer such that, upon addition of TP 50, the final ionic conditions during the first step of the reconstitution procedure were: 30 mM Tris-HCl (pH 7.6 at 20°C); 6 mM magnesium chloride; 425 mM potassium chloride; 6 mM 2-mercaptoethanol. After 10 minutes at 40°C, magnesium chloride was added to a final concentration of 26 mM and the temperature increased to 45°C for 3 - 5 hours. 200 µl of reconstitution mixture contained 120 pmol RNA 70 and approximately 150-180 pmol equivalents of TP·50 (1 pmol equivalent of protein is defined in Chapter 3.2.4.). Reconstituted particles were precipitated by addition of 0.25 volumes 40% (w/v) PEG (6000). After 10 minutes at 0°C particles were recovered by centrifugation at 12,000 xg for 5 minutes and suspended in RS buffer.

Although the level of reconstitution was subject to some variability, particularly between different preparations of materials, approximately 30% of the activity of control particles (i.e. native 50S ribosomal subunits) was attainable, when assayed for ability to synthesise poly-phenylalanine. Control particles were not subjected to the conditions

of the reconstitution procedure prior to their assay, although Nomura and Erdmann (1970) adopted this approach occasionally in their studies (this led to a significant reduction in control activity resulting in apparently higher levels of reconstitution). (The subject of activity of reconstituted particles is discussed in more detail in Chapter 5.)

3. Results

3.1 Stimulation Of Polyphenylalanine Synthesis By Phenylalanine-Specific Transfer RNA

To maximise the chances of observing relatively low levels of protein-synthetic activity from reconstituted particles, it was considered worthwhile to optimise fully the poly (U)-directed protein-synthesising system in which the particles were to be assayed. The only substantial modification that resulted from this exercise was the replacement of *E. coli* whole-cell tRNA with *E. coli* phenylalanine-specific tRNA. This replacement resulted in at least a two-fold stimulation in the rate of polyphenylalanine synthesis (Figure 4.2). However, the effect was observed only with systems containing S100 fractions from either *Streptomyces* or *B. stearothermophilus*. No stimulation was observed with *E. coli* S100, irrespective of the source of the ribosomes. The reason for this differential stimulatory effect of $tRNA_{Phe}$ upon polyphenylalanine synthesis is unclear but may be the result of differing rates of de-acylation of tRNA in S100 fractions - the situation with *E. coli* S100 reflecting the fastest rate of de-acylation.

3.2. Comparison Of RNA 70 With RNA 50 As Substrates For Reconstitution

Both RNA 70 and RNA 50 were suitable substrates for reconstitution. Their comparable integrity can be seen by gel electrophoresis under denaturing conditions (Figure 4.3). However, in general, RNA 70 was used in preference since its integrity (from preparation to preparation) was more consistent. Furthermore, the presence of 16S RNA does not interfere with the process of 50S ribosomal subunit reconstitution (Fahnestock *et al.*, 1974). The reason for differences of RNA integrity (particularly with RNA 50) is unclear although degradation of RNA 50 may result from ribonuclease activation during preparation of ribosomal

Legend to Figure 4.2

Stimulatory effect of phenylalanine-specific tRNA on polyphenylalanine synthesis.

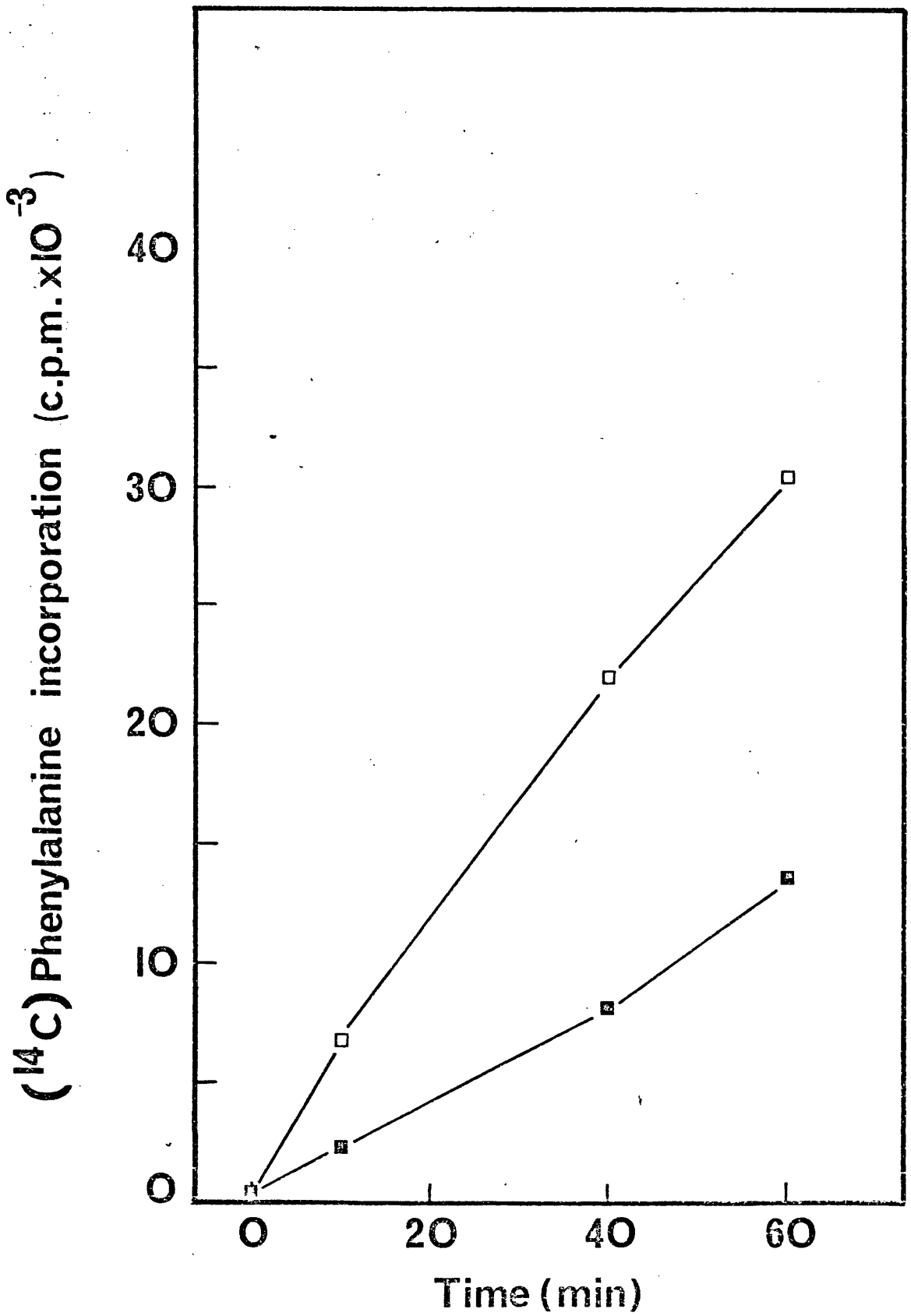
S. loidensis ribosomal subunits (30S and 50S) were mixed and supplemented with S100 (from *S. loidensis*) Subsequently, particles were assayed for the ability to synthesise polyphenylalanine using the system described in Chapter 2.2.8.

2,000 c.p.m. ≡ 1 pmol phenylalanine per pmol ribosomes

■-■ : using system containing optimal input of whole-cell tRNA (type XXI) (from *E. coli* W)

□-□ : using system containing optimal input of phenylalanine-specific tRNA.

FIGURE 4.2


(^{14}C) Phenylalanine incorporation (c.p.m. x10^{-3})
40
30
20
10
0
0
20
40
60
Time (min)

Legend to Figure 4.3

Analysis of ribosomal RNA (from *S. loidensis*)by gel electrophoresis under denaturing conditions (according to Chapter 4.2.2).

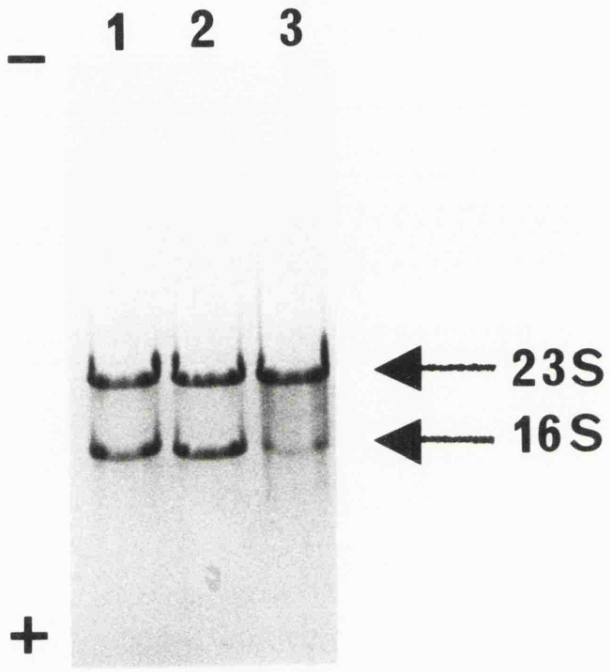
Track

1 : 4μg RNA 70 (obtained by extraction with urea/lithium chloride)

2 : 4μg RNA 70 (obtained by extraction with phenol)

3 : 4μg RNA 50 (obtained by extraction with urea/lithium chloride)

FIGURE 4.3



subunits (Ceri and Maeba, 1973). Furthermore, the integrity of both RNA 70 and RNA 50 was dependent upon the extent of storage of the ribosomes/ribosomal subunits preceeding their extraction. Surprisingly, the inherent degradation within such native ribosomal particles was not reflected in a corresponding reduction of polyphenylalanine-synthetic activity (data not presented).

3.3. Optimisation Of The Reconstitution Procedure Having achieved very low levels of polyphenylalanine-synthetic activity from *S. loidensis* reconstituted 50S ribosomal subunits under sub-optimal conditions, the procedure was optimised fully. Where appropriate, comparison will be made with conditions for the *E. coli* 50S ribosomal subunit reconstitution procedures.

The optimisation of both magnesium concentration and temperature of the first step of the reconstitution procedure is shown in Figure 4.4. Maximum activity of reconstituted particles was observed at 6 mM magnesium ion and 40°C. This contrasts with 4 mM magnesium ion and 44°C (Dohme and Nierhaus, 1976) and 7.5 mM magnesium ion and 44°C (Amils *et al.*, 1978) for reconstitution of *E. coli* 50S ribosomal subunits.

The optimal duration of the first step of the reconstitution is shown in Figure 4.5. Maximum activity was observed after only 10 minutes at 40°C. This was very quick in comparison with the conditions for reconstitution of *E. coli* 50S particles i.e. 20 minutes (Dohme and Nierhaus, 1976) and 30 minutes (Amils *et al.*, 1978) and may reflect fundamental differences in the secondary structure of ribosomal RNA prepared either by urea/lithium chloride extraction or phenol extraction. For example, Figure 4.6 shows phenol-extracted RNA to have significantly less secondary structure than the corresponding urea/lithium chloride-extracted RNA when assessed by

Legend to Figure 4.4

Optimisation of temperature and magnesium ion concentration for the first step of the reconstitution of *S. loidensis* 50S particles.

RNA 70 and TP 50 from *S. loidensis* were mixed with buffers such that the ionic conditions were 30mM Tris-HCl (pH 7.6 at 20°C); 425mM potassium chloride; 6mM 2-mercaptoethanol, and magnesium concentrations as indicated. After 10 minutes at the various temperatures indicated, magnesium was added to 26mM and reconstitution continued according to Chapter 4.2.5. The activity of the reconstituted particles was assessed (according to Chapter 2.2.8), following their prior supplementation with 30S particles and S100 from *S. loidensis*. Results are expressed as a percentage of control incorporation (i.e. by native 50S particles) which was approximately 20 pmol phenylalanine per pmol of ribosomes.

Temperature of 1st step of reconstitution procedure (°C)

△-△ : 30

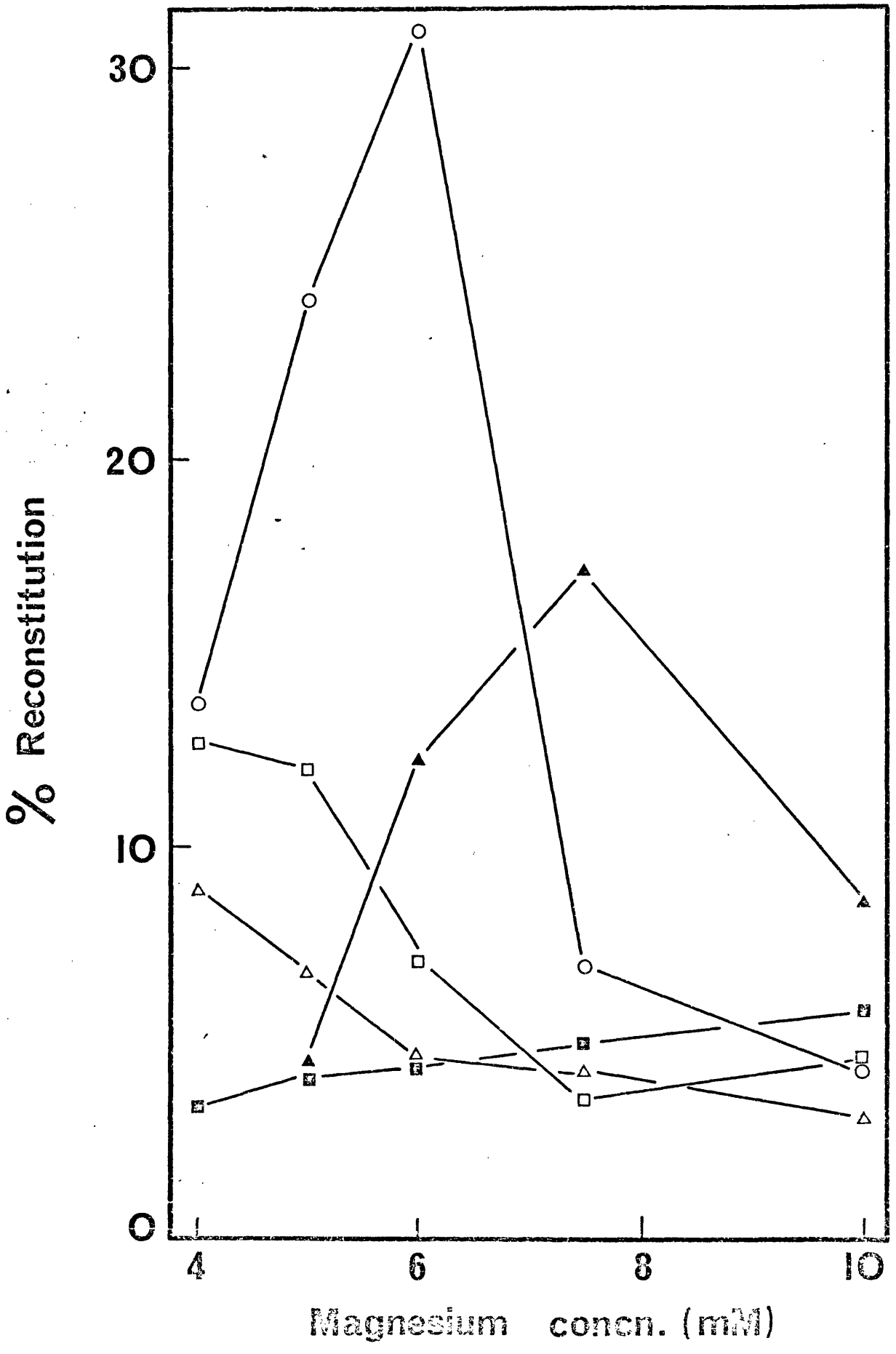
□-□ : 35

○-○ : 40

▲-▲ : 45

■-■ : 50

FIGURE 4.4

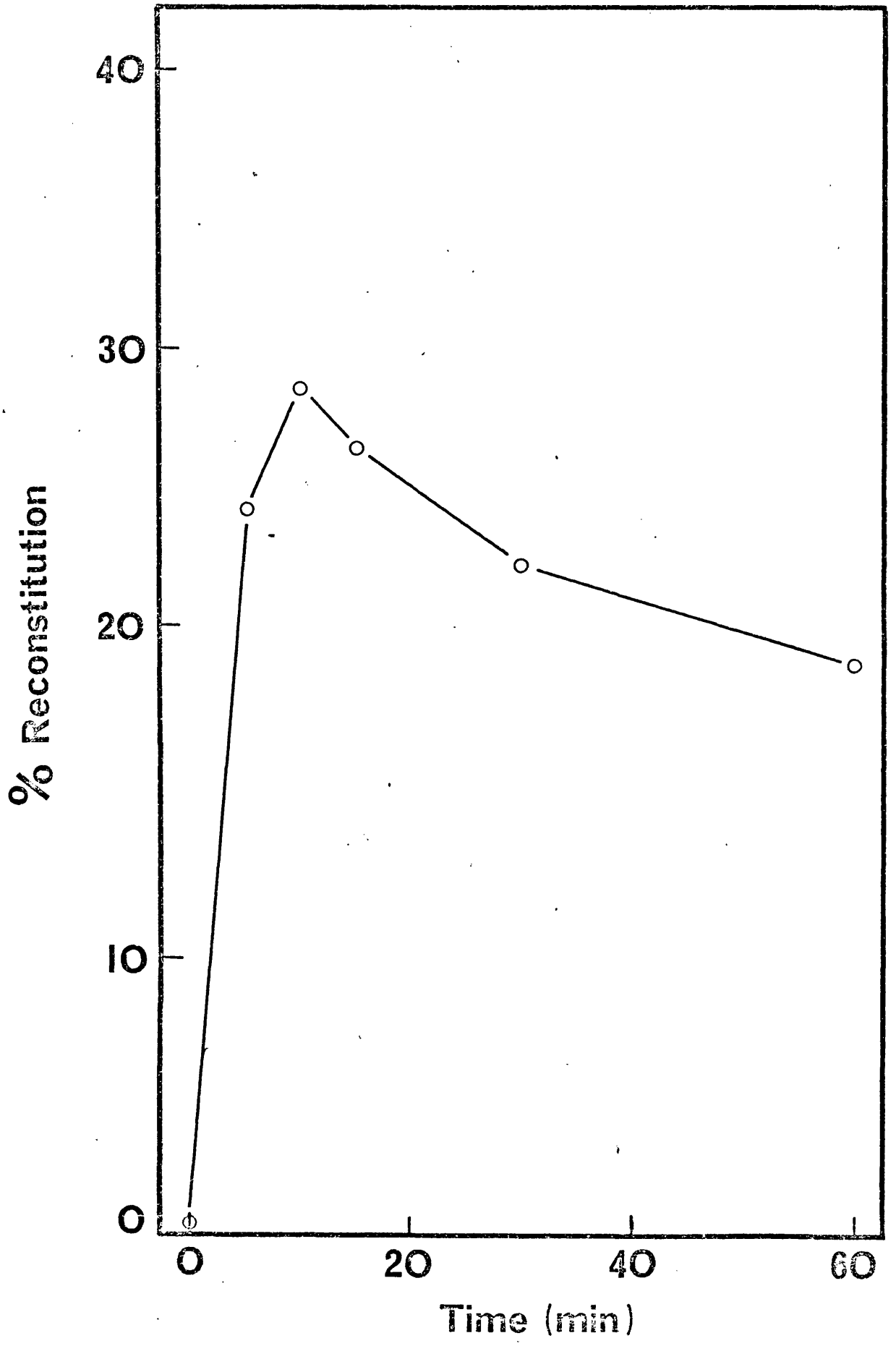


Legend to Figure 4.5

Optimisation of the duration of the first step of the reconstitution procedure for *S. loidensis* 50S particles.

RNA 70 and TP 50 from *S. loidensis* were mixed under optimal ionic conditions and incubated at 40°C for the times indicated. Subsequently, magnesium was added to 26mM and reconstitution continued according to Chapter 4.2.5. The activity of the reconstituted particles was assessed (according to Chapter 2.2.8) following their prior supplementation with 30S particles and S100 from *S. loidensis*. Results are expressed as a percentage of control incorporation (i.e. by native 50S particles) which was approximately 20 pmol phenylalanine per pmol of ribosomes.

FIGURE 4.5



Legend to Figure 4.6

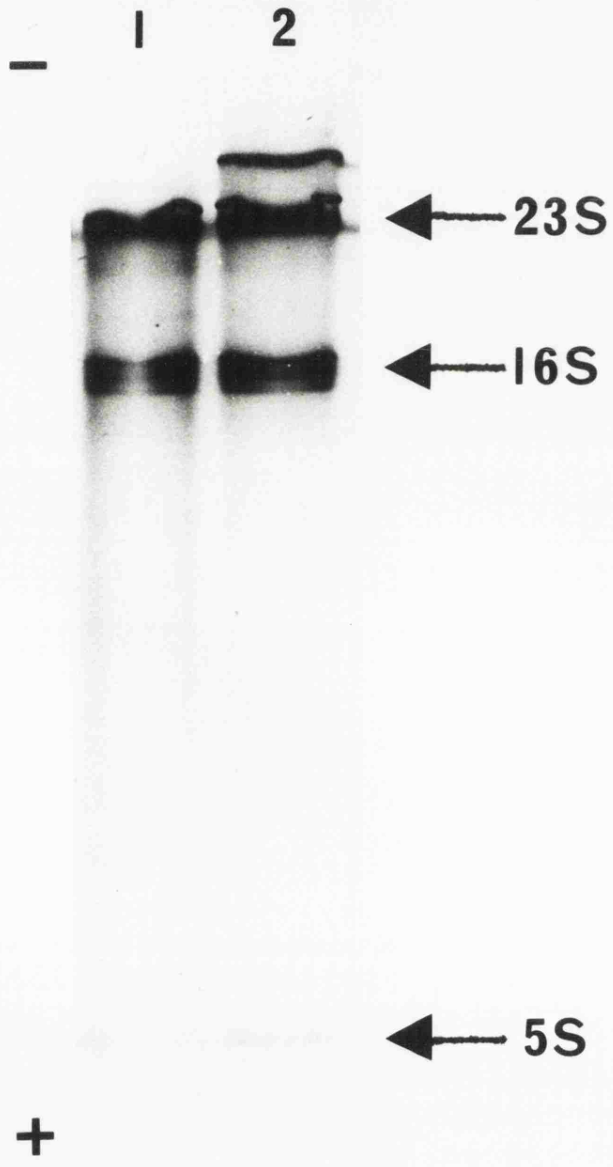
Analysis of ribosomal RNA (from *S. loidensis*) by gel electrophoresis under non-denaturing conditions (according to Chapter 4.2.3).

Track

1 : 20μg RNA 70 (obtained by extraction with urea/lithium chloride)

2 : 20μg RNA 70 (obtained by extraction with phenol)

FIGURE 4.6



non-denaturing gel-electrophoresis. Hence, it might be expected that reconstitution of 50S ribosomal subunits with RNA containing more secondary structure was faster since the RNA could perhaps adopt a conformation similar to that in native 50S particles. The decline in activity after 10 minutes was presumably due to heat inactivation of the reconstituted particles.

The effect of magnesium ion concentration on the second step of the reconstitution is shown in Figure 4.7. Not only was the optimal magnesium concentration higher than that for the reconstitution of E. coli 50S ribosomal subunits (i.e. 26 mM as against 20 mM) but the optimal range was more limited i.e. the E. coli reconstitution procedure was insensitive to magnesium in the range 14-22 mM (Amils et al., 1978). In contrast, maximum activity from S. loidensis 50S reconstituted particles was not achieved below 26 mM magnesium ion; above this concentration no further gain in activity was observed.

The reconstitution of S. loidensis 50S ribosomal subunits was essentially complete after 3 hours incubation at 45°C, although a further 2 hours incubation did increase activity marginally (Figure 4.8). Generally, the reconstitution procedure was limited to 4 hours duration. In contrast, reconstitution of 50S particles from E. coli required 90 minutes incubation at 50°C (Dohme and Nierhaus, 1976) or 2 hours incubation at 50°C (Amils et al., 1978).

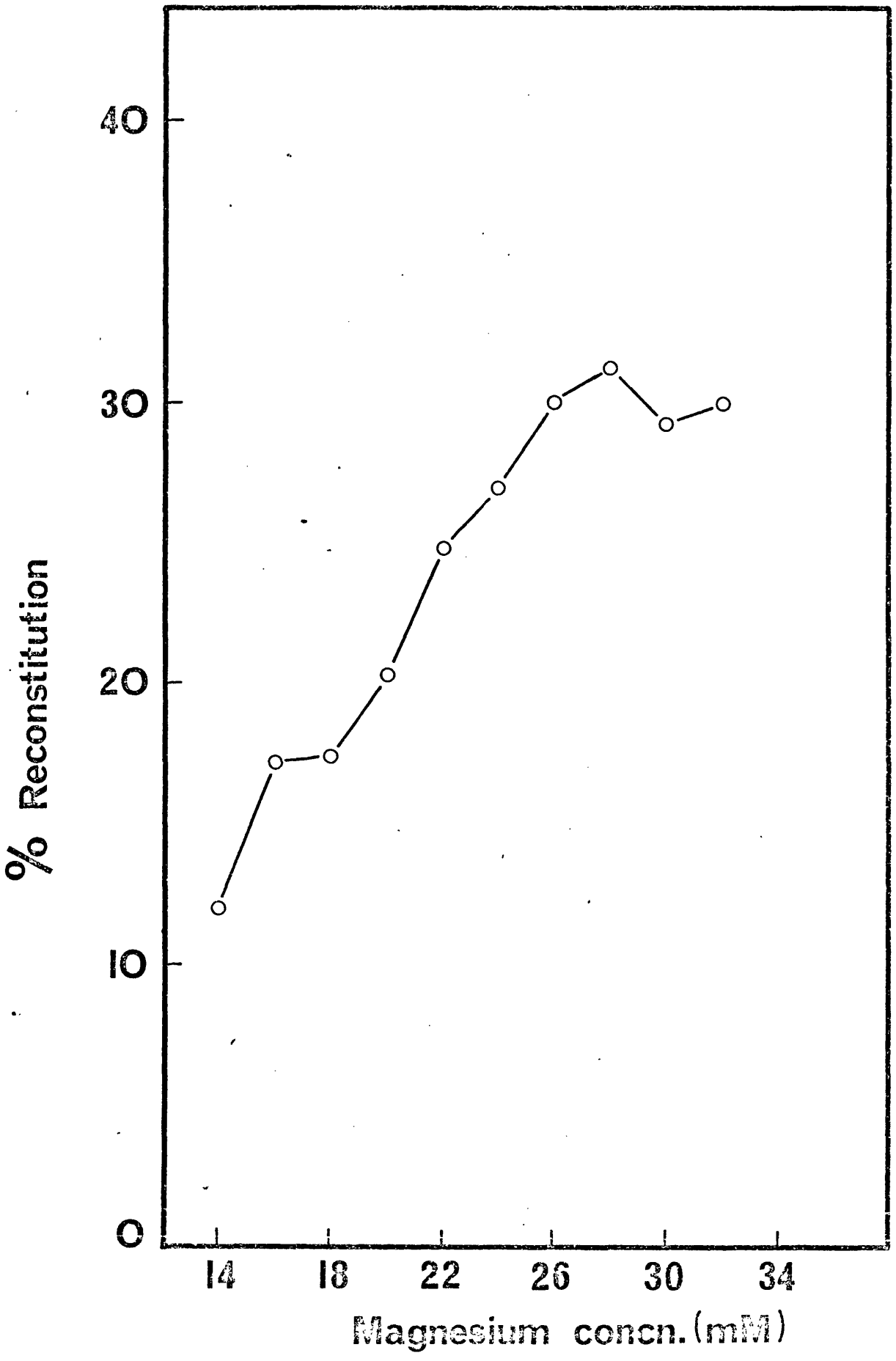
The optimisation of monovalent cation concentration is shown in Figure 4.9. The optimal concentration of potassium (i.e. 425 mM) was similar to that required for reconstitution of 50S particles from E. coli (i.e. 400 mM) and might have reflected the need to limit the number of RNA: protein, protein: protein interactions during reconstitution.

Legend to Figure 4.7

Optimisation of magnesium concentration in the second step of the reconstitution procedure for *S. loidensis* 50S particles.

RNA 70 and TP 50 from *S. loidensis* were mixed and incubated under optimal conditions for the first step of the reconstitution procedure. Magnesium was then added to the concentrations indicated and incubation continued according to Chapter 4.2.5. The activity of the reconstituted particles was assessed (according to Chapter 2.2.8) following their prior supplementation with 30S particles and S100 from *S. loidensis*. Results are expressed as a percentage of control incorporation (i.e. by native 50S particles) which was approximately 20 pmol phenylalanine per pmol of ribosomes.

FIGURE 4.7


40
30
20
10
0
% Reconstitution
14
18
22
26
30
34
Magnesium concn. (mM)

Legend to Figure 4.8

Optimisation of temperature and duration of the second step of the reconstitution procedure for *S. loidensis* 50S particles.

RNA 70 and TP 50 from *S. loidensis* were mixed and incubated under optimal conditions for the first step of the reconstitution procedure. Following the addition of magnesium to the incubation mix (to 26mM) incubation was continued at the temperatures and for the times indicated. The activity of the reconstituted particles was assessed (according to Chapter 2.2.8) following their prior supplementation with 30S particles and S100 from *S. loidensis*. Results are expressed as a percentage of control incorporation (i.e. by native 50S particles) which was approximately 20 pmol phenylalanine per pmol of ribosomes.

Temperature of 2nd step of reconstitution procedure (°C)

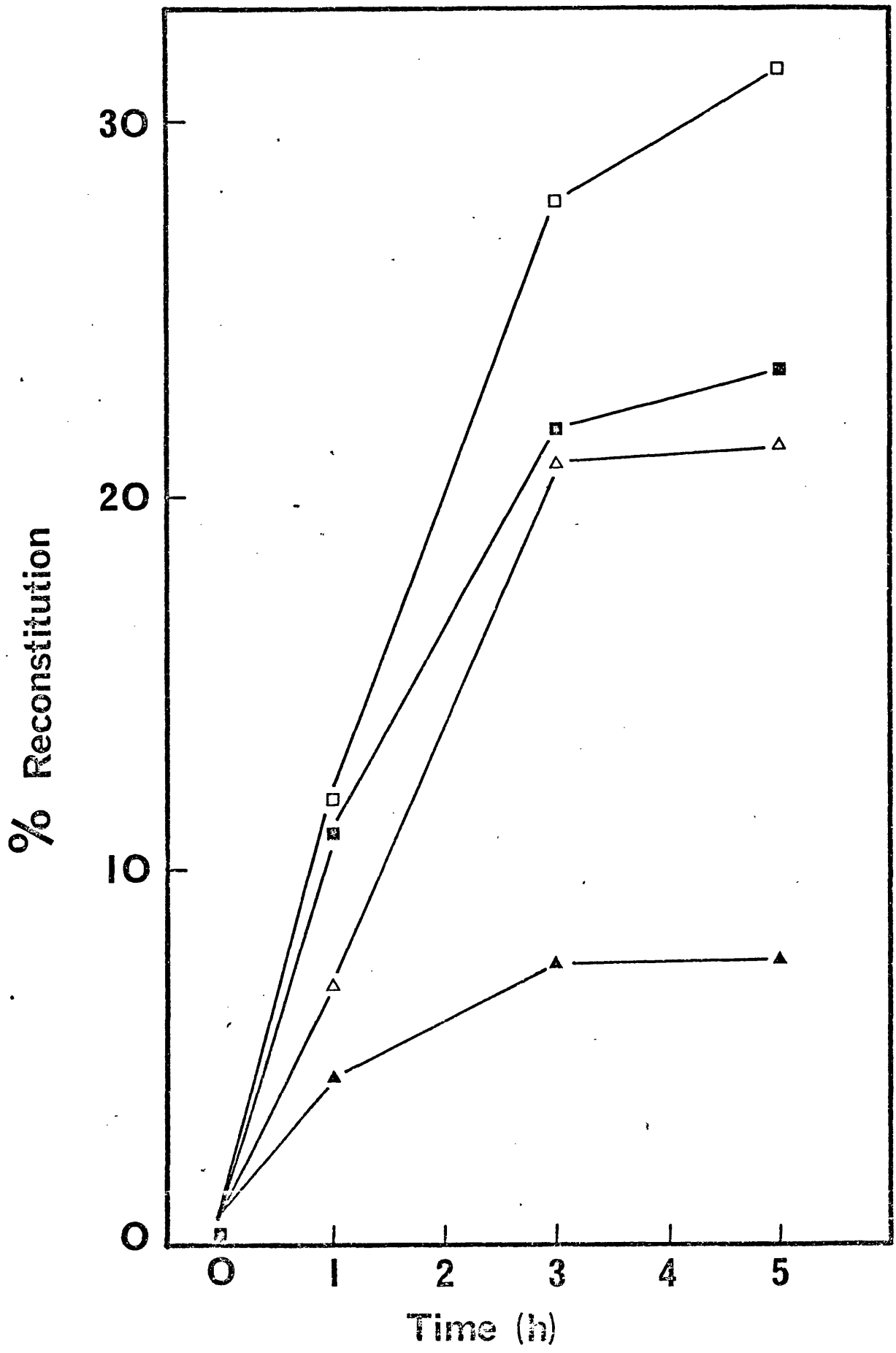
△-△ : 42

□-□ : 45

■-■ : 48

▲-▲ : 50

FIGURE 4.8

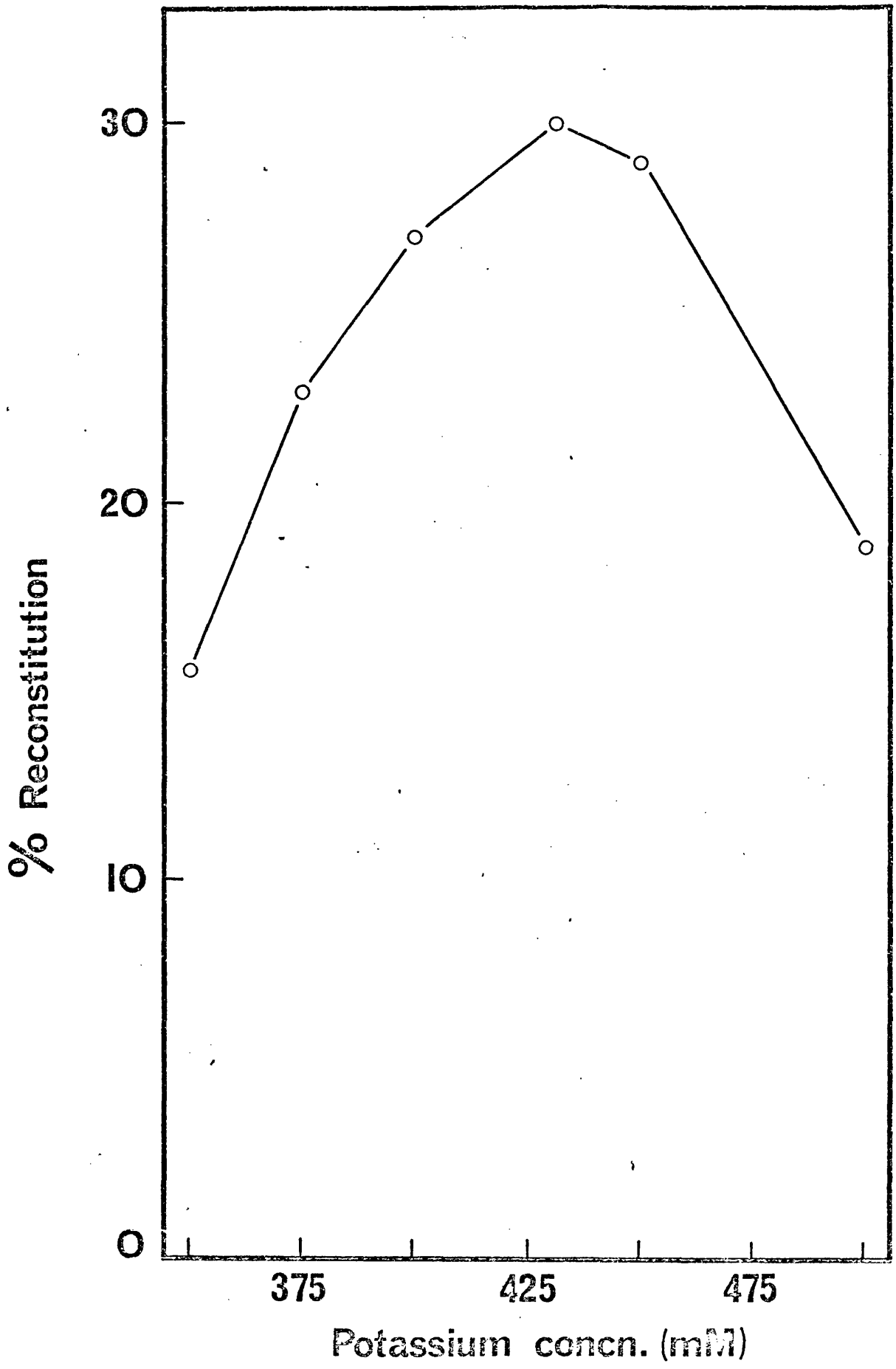


Legend to Figure 4.9

Optimisation of potassium concentration in the reconstitution procedure for *S. loidensis* 50S particles.

RNA 70 and TP 50 from *S. loidensis* were incubated under optimal conditions described in Chapter 4.2.5. except the potassium concentration (in both steps of the procedure) was varied, as indicated. The activity of the reconstituted particles was assessed (according to Chapter 2.2.8) following their prior supplementation with 30S particles and S100 from *S. loidensis*. Results are expressed as a percentage of control incorporation (i.e. by native 50S particles) which was approximately 20 pmol phenylalanine per pmol of ribosomes.

FIGURE 4.9



Finally, optimisation of the RNA 70:TP 50 input ratio is shown in Figure 4.10. This can only be a representative example of the effect since it was always necessary to optimise the relative inputs with each new batch of material. Generally, the optimal input ratio was about 1.25:1 or 1.5:1 (TP 50:RNA). Although comparable input ratios were required in the reconstitution procedure for E. coli 50S particles (Dohme and Nierhaus 1976) a much higher ratio (i.e. 2.4:1) was necessary in the modified procedure of Amils et al., (1978). This latter observation was supposedly due to the absence of a transient urea dialysis step during the preparation of the TP 50 (Nierhaus, 1980).

3.4. Preparation Of Materials By Other Methods

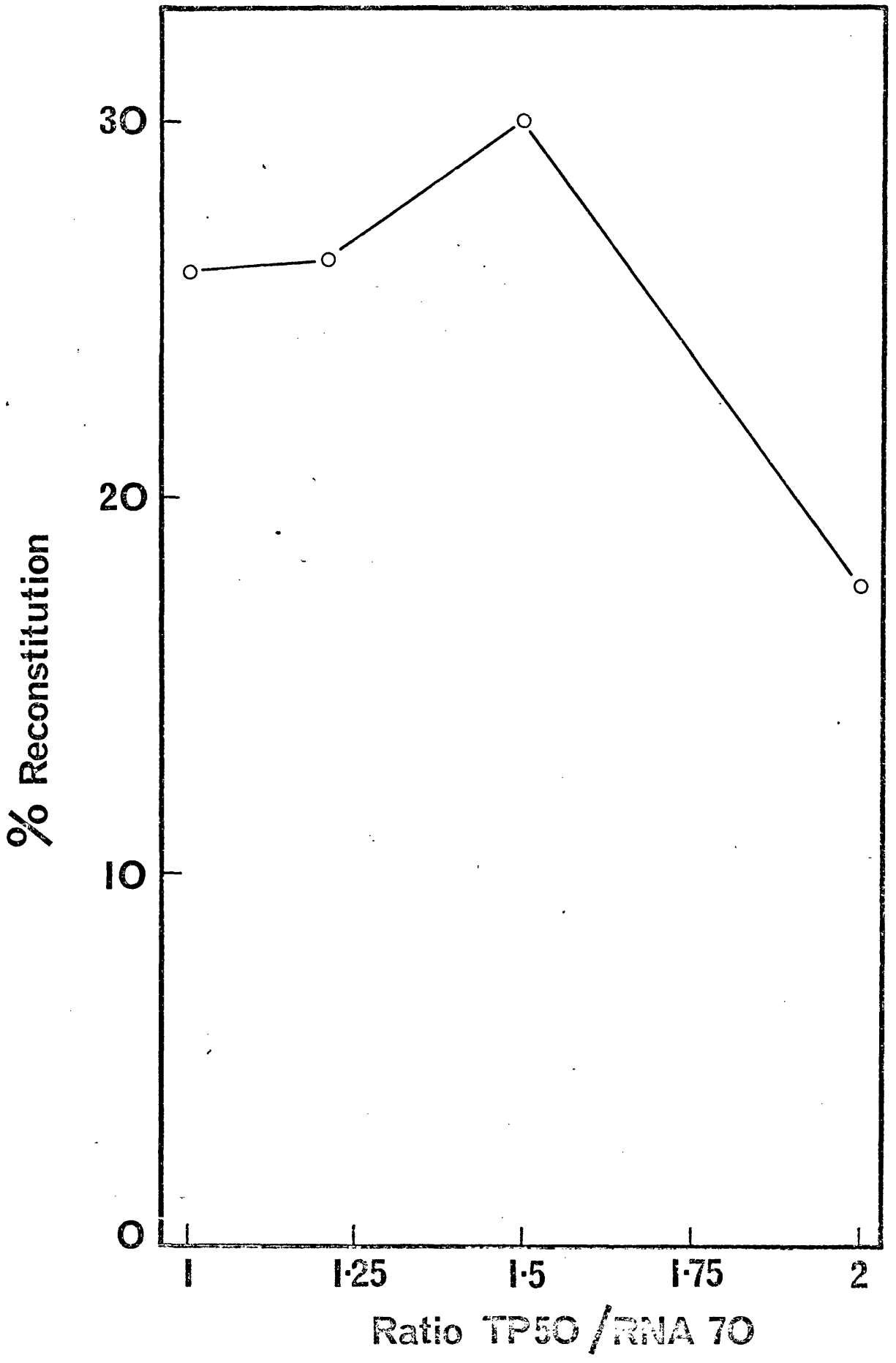
3.4.1. Phenol extraction of RNA Phenol extraction of ribosomal RNA, adopted by Amils et al., (1978) and following the method of Traub et al., (1971) seemed preferable to that of urea/lithium chloride extraction, not least because of the speed of extraction (i.e. 2 to 3 hours as opposed to 48 hours), thereby minimising any effect of ribonuclease action. Although the integrity of phenol-extracted RNA was indistinguishable from that of urea/lithium chloride-extracted RNA when examined by gel electrophoresis under denaturing conditions (Figure 4.3), the phenol-extracted RNA did not act as a substrate for reconstitution (data not presented). One fundamental reason for this arises from differences in RNA secondary structure reflected by gel electrophoresis under non-denaturing conditions (Figure 4.6). An alternative explanation could be that phenol-extracted RNA is a better substrate for ribonuclease action. Indeed, Ceri and Maeba (1973) found that agents disrupting RNA conformation (e.g. heat, urea, polyvalent anions) markedly enhanced ribonuclease activity in E. coli.

Legend to Figure 4.10

Optimisation of input ratio of RNA 70 : TP 50 in the reconstitution procedure for *S. loidensis* 50S particles.

RNA 70 and TP 50 were mixed in the molar ratios indicated, and 50S particles reconstituted according to Chapter 4.2.5. The activity of the reconstituted particles was assessed (according to Chapter 2.2.8) following their prior supplementation with 30S particles and S100 from *S. loidensis.* Results are expressed as a percentage of control incorporation (i.e. by native 50S particles) which was approximately 20 pmol phenylalanine per pmol of ribosomes.

FIGURE 4.10



3.4.2. Lithium chloride - washing of ribosomal material prior to extraction Ribonuclease action upon ribosomal RNA is probably the single most important factor deciding whether or not a reconstitution will be successful. Its presence on the ribosomes of *E. coli* has been implicated by Dohme and Nierhaus (1976) who described a washing procedure (in 0.6 M lithium chloride) for its (apparent) removal. This precise procedure could not be employed for washing *S. loidensis* ribosomes since certain ribosomal proteins were removed during the procedure. However, a ribosome wash in 0.4 M lithium chloride was examined. Despite the washing procedure, materials prepared from ribosomes washed in lithium chloride did not reconstitute to give higher levels of activity than reconstituted particles prepared from materials isolated from unwashed ribosomes. This suggested that ribonuclease, if present on the ribosomes of *S. loidensis*, was tightly bound. Ceri and Maeba (1973) found that both urea/lithium chloride extraction and phenol extraction failed to remove ribonuclease activity from *E. coli* 23S RNA, although salt extraction from a column of DEAE cellulose was successful. Since reasonable levels of reconstitution (sufficient for the types of experiment envisaged in this present study) were already attainable, the removal of ribonuclease was not investigated further.

4. Discussion

Certain criteria have to be fulfilled so that ribosomes can be reconstituted from sub-ribosomal components. First, and most important, the RNA has to be intact. This has been established by gel electrophoresis under strongly-denaturing conditions (non-denaturing gel systems are unsuitable since scissions in the RNA could be disguised by secondary structure). Although phenol-extraction of RNA might seem preferable to urea/lithium chloride extraction, only the latter method provided RNA suitable for subsequent reconstitution into active 50S particles, presumably due to a requirement (during the reconstitution procedure) for a certain degree of RNA secondary structure. In general terms urea/lithium chloride extraction of ribosomes yields RNA of indeterminate protein content. Furthermore, 5S RNA tends to be distributed between phases, during extraction. However, RNA extracted by urea and lithium chloride from S. loidensis ribosomes was found to be free of ribosomal protein contamination (data not presented).

Consequences of the action of RNase upon the reconstitution were not investigated systematically. Although precautionary measures were taken on occasions (i.e. washing of ribosomes in lithium chloride), this was not reflected in a significant increase in the level of activity of 50S reconstituted particles. This suggested that ribonuclease, if present, was tightly bound to ribosomes. Furthermore, its location (i.e. RNA or TP 50) following urea/lithium chloride extraction of ribosomes was not established. Simple experiments involving incubation of ribosomal RNA with and without ribosomal proteins, under reconstitution conditions, followed by gel electrophoresis, would have established the extent of ribonuclease action. Alternatively, the levels of reconstitution could have been a simple reflection of the compromises that were required to

reconstitute 50S ribosomal subunits from an organism with such a low optimal growth temperature.

Urea/lithium chloride extraction of ribosomal proteins was chosen in preference to the acetic acid extraction procedure of Dohme and Nierhaus (1976) since the latter methodology was subject to considerable variability, particularly during the final dialysis steps of the procedure. In contrast to other Gram-positive organisms (i.e. B. subtilis and B. licheniformis), 50S particles from S. loidensis cannot be reconstituted in a single-step procedure. Perhaps this reflects, in part, the inability of Streptomyces ribosomal proteins to bind to ribosomal RNA in the required order, under the defined ionic conditions (i.e. binding of ribosomal proteins may occur non-specifically). The reconstitution procedure is analogous to that described for E. coli 50S particles. However, the differences between the two methods are significant to the extent that material from S.loidensis will not reconstitute under the conditions of the published methods applicable to E. coli components.

Successful reconstitution of Streptomyces 50S ribosomal subunits is essentially a compromise between surmounting various rate-limiting steps, whilst still maintaining ribosomal proteins in an active state. That the conditions of in vitro ribosomal subunit assembly barely resemble the in vivo situation is clearly obvious. However, it might be considered surprising that any active ribosomal particles could be reconstituted in view of the extreme conditions to which ribosomes are subjected, both prior to, and during, the reconstitution procedure.

CHAPTER 5

ANALYSIS OF RECONSTITUTED 50S PARTICLES FROM STREPTOMYCES LOIDENSIS

Chapter 5

1. Introduction

The ability of reconstituted 50S particles to translate poly (U) is one of the more stringent assays of ribosome reconstitution conceivable. Although *S. loidensis* reconstituted 50S particles were only one third as active as native particles, quite in what fundamental respect (s) they differed from native particles was unclear. Accordingly, reconstituted 50S particles were subjected to exhaustive analysis, by a variety of techniques including sedimentation behaviour, ribosomal protein and RNA analysis.

1.1 Sedimentation Behaviour Of Ribosomal Particles

Sedimentation behaviour of reconstituted particles was examined since, for *E. coli* 50S particles, there appeared to be a relationship between sedimentation coefficient and activity. Dohme and Nierhaus (1976) found that the 50S ribosomal subunit of *E. coli* reconstituted *via* a number of discrete intermediates, each sedimenting at a different rate. Accordingly, during the first-step incubation (44°C, 4mM magnesium ion), three distinct particles were observed. The first formed upon mixing of RNA and TP 50 at 0°C and sedimented at 33S; this was designated $RI_{50}(1)$. Upon heating, the 33S peak diminished with the appearance of an intermediate (41S) peak, designated $RI^*_{50}(1)$. Finally, this peak diminished with the appearance of a third (48S) peak ($RI_{50}(2)$). All these particles were found to be inactive with respect to peptide bond formation. However, upon incubation under second-step reconstitution conditions (50°C, 20mM magnesium ion) the inactive 48S particles were converted into active 50S particles. This last step involved only a conformational change in the particle, since the presence of (additional) ribosomal proteins was unnecessary. Accordingly, comparative sedimentation data of native versus reconstituted particles could yield potentially valuable information regarding ribosomal subunit activity.

1.2 Ribosomal Subunit Association Other studies with E. coli have established that the ability of 30S and 50S ribosomal subunits to form vacant 70S ribosome couples at 6mM magnesium ion concentration is a stringent condition for activity in the translation of natural messenger RNA (i.e. R17). (Noll et al., 1973 a; 1973 b; Noll and Noll, 1974). In particular, vacant ribosome couples appear to fall into two classes -"tight" couples and "loose" couples, each differing in relative affinity for the corresponding ribosomal subunit. These two types can be differentiated between by sucrose density gradient centrifugation. Accordingly, at 6mM magnesium ion and 30,000 r.p.m., tight couples sedimented at 70S whereas loose couples remained as dissociated 30S and 50S ribosomal subunits (Hapke and Noll, 1976). Reduced affinity between ribosomal subunits can result from inactivation of either 30S or 50S particles. However, whereas 30S particles can generally be reactivated by heat (Zamir et al., 1974), inactivation of 50S particles is often irreversible. Hence, a comparative study of ribosomal subunit association (i.e. native versus reconstituted particles) should result in a good indication of potential activity of reconstituted 50S particles (despite the extra degree of stringency required for ribosomes to translate natural messenger RNA).

1.3 Analysis Of Ribosomal Proteins Ribosomal protein analysis of reconstituted particles was chosen to complement the sedimentation data. For E. coli not only were ribosomal reconstitution intermediates found to sediment at different rates, but some contained different complements of ribosomal proteins (Dohme and Nierhaus, 1976). Accordingly, 17-18 ribosomal proteins bound RNA at $0^{o}C$ to form $Rl_{50}(1)$ particles; conversion to $RI^{*}_{50}(1)$ (upon heating) occurred with no ribosomal protein additions. Continued heating under first-step conditions resulted in a further 8

proteins binding to give $RI_{50}(2)$ particles. Further heating (under second-step conditions) resulted in a conformational change (with no further additions of ribosomal proteins) to yield active 50S particles (6 proteins could not be assigned unequivocally to any stage). Hence, should reconstitution of S. loidensis 50S ribosomal subunits have resulted in particles of variable ribosomal protein content (and hence, variable activity) this could be reflected in the reduction (or absence) of certain ribosomal proteins. Furthermore, any changes in ribosomal protein mobility (as a result of the reconstitution procedure) should be clearly apparent.

Early studies on the separation of ribosomal proteins utilised starch gels (Waller and Harris, 1961; Waller, 1964). However, starch gels never attained the level of flexibility that polyacrylamide offered (i.e. varying porosity size by changing the ratio of acrylamide to N,N'-methylene-bis-acrylamide). The separation of ribosomal proteins from E. coli is potentially difficult; not only are they of similar size (Wittman, 1974) but most are basic proteins with isoelectric points of pH 10 or above (Wittman and Wittman-Liebold, 1974). Successful separation of all the ribosomal proteins of E. coli has been achieved using a two-dimensional procedure (Kaltschmidt and Wittman, 1970 a; 1970 b). In principle, ribosomal proteins are electrophoresed at pH 8.6 in the first dimension and at pH 4.6 in the second. Hence, separation is based almost entirely on charge (although there is some molecular sieving action). Later modifications by Howard and Traut (1973; 1974) allowed analysis of smaller amounts of ribosomal proteins, in a shorter time. Other modifications, incorporating SDS into the second dimension gel (Shapiro et al., 1967) combined charge separation with that of separation according to size. Such systems have been used by Martini

and Gould (1971) Hultin and Sjöqvist (1972) and, more recently, Mets and Bogorad (1974). The latter methodology has been adapted by Subramanian (1974) and Kyriakopoulos and Subramanian (1977) to identify ribosomal proteins from E. coli and to correlate their nomenclature with that originally established by Wittman et al., (1971).

Initial studies on the examination of ribosomal proteins from S. loidensis utilised one-dimensional gradient-polyacrylamide gel electrophoresis in the presence of SDS. Potentially, this technique offered both simplicity and resolving capacity, the latter resulting from band sharpening as a consequence of electrophoresis under conditions of limiting pore size. The procedure outlined below was derived from methodology used to observe variations in ribosomal proteins from cycloheximide-resistant mutants of yeast. Such variations were apparently not observed when analysis by conventional gel-electrophoretic techniques was utilised (Prof. J.E. Davies - personal communication). However, to complement the one-dimensional procedure, the two-dimensional electrophoretic method of Mets and Bogorad (1974) was chosen, primarily because this system is particularly sensitive to small changes in net charge on ribosomal proteins, because at the pH of the first dimension (pH 5.0) all ionisable amino acid side chains are fully charged. However, it does suffer from the drawback that E. coli ribosomal protein L31 is not resolved, presumably because it runs out of the gel, the result of its very basic character (Kaltschmidt and Wittman 1970 a).

1.4 Analysis Of Ribosomal RNA RNA analysis of both native and reconstituted 50S particles was performed to establish the extent of inherent degradation, since this could be reflected as differences of activity in vitro. Analysis was conducted under denaturing conditions since this gave the most realistic estimate of RNA integrity.

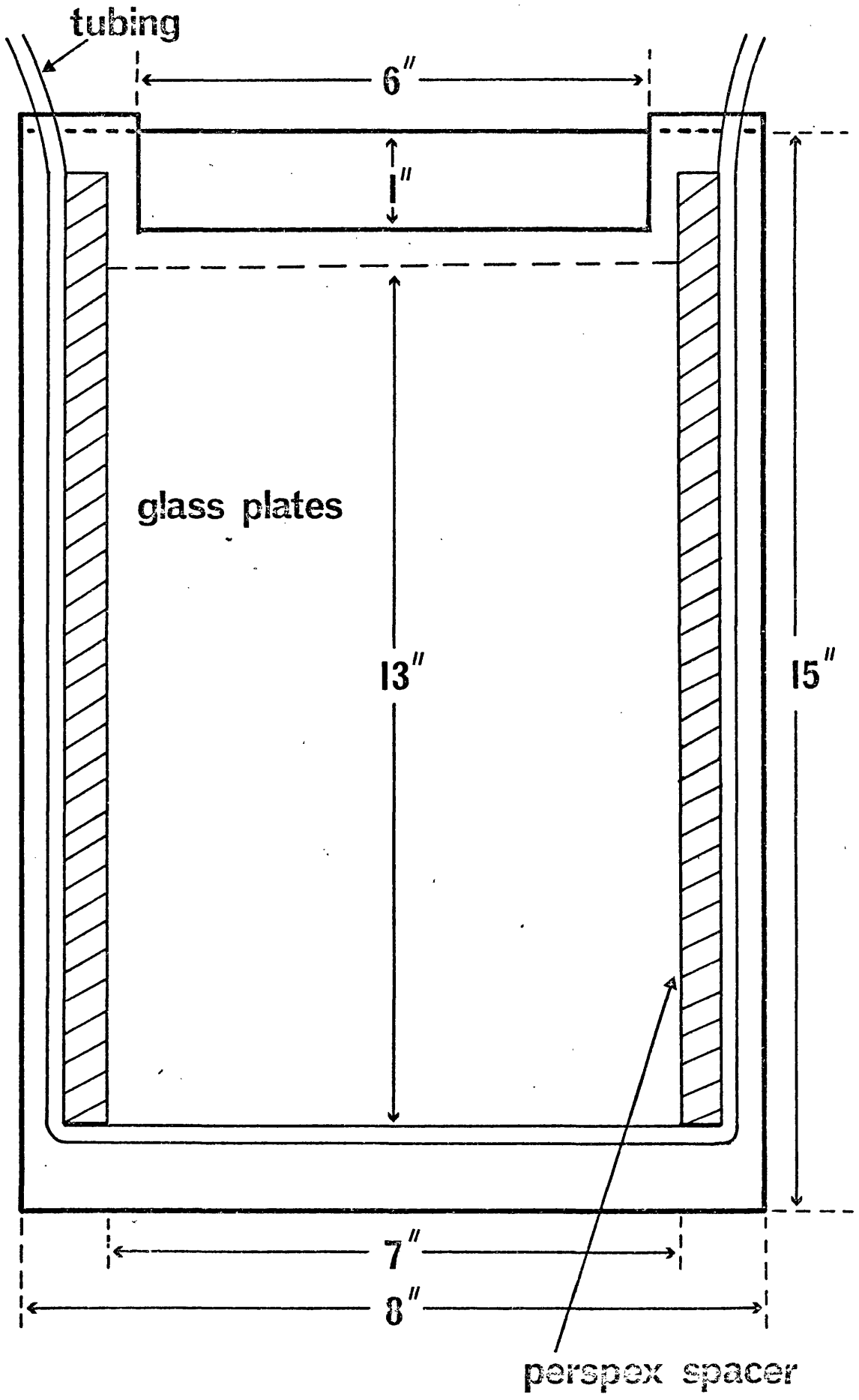
2. Materials And Methods

2.1 Analytical Sucrose Density Gradients Sucrose density gradient analysis of ribosomal particles was performed either in the presence of sodium (according to Chapter 2.2.5) or in the presence of buffer containing 10mM Tris (pH 7.6 at 20°C), 100mM potassium chloride, 3mM 2-mercaptoethanol, and concentrations of magnesium chloride indicated in the relevant Figure.

2.2 Analytical Ultracentrifugation Analytical ultracentrifugation was performed on an MSE Centriscan 75 using a 6-place rotor. Migration of ribosomal particles was monitored by Schlieren optics at 554nm. Knife-edge optics were positioned at 69° relative to the light path. Centrifugation was at 20,000 r.p.m. and 5°C. Data was accumulated *via* a multiplexing unit into a double-disc drive Apple II Plus micro-computer. The programme (devised by P. Nott) was contained within a 64K Apple Pascal System (Version 1.1).

2.3 One-Dimensional Gradient-Polyacrylamide Gel Electrophoresis, In The Presence Of SDS A linear gradient (total volume 85ml) of acrylamide (12-20% w/v), *N*,*N*'-methylene-bis-acrylamide (0.32-0.53% w/v) and glycerol (zero-5% v/v) in buffer containing 375mM Tris (pH 8.7 at 20°C); 0.1% (w/v) SDS; 0.022% (w/v) fresh ammonium persulphate; 0.006%(v/v)TEMED, was poured from a two-chambered gradient maker, *via* a polystaltic pump (Buchler, U.S.A.) into the casting apparatus illustrated in Figure 5.1. This was accomplished by inserting a narrow bore stainless steel tube to the base of the casting apparatus. The concentrated "end" of the gradient was poured first (*via* the tube) and the tube raised accordingly so that its outlet was always just above the meniscus of the gel solution. After

FIGURE 5.1



pouring, the gel was overlaid with water-saturated butanol, until polymerisation was complete. Although not strictly necessary, a stacking gel was poured over the gradient gel. This eliminated variations in overall gel height, unavoidable in view of the large volumes of gel solution involved. Stacking gel contained 5.75% (w/v) acrylamide; 0.15% (w/v) N,N'-methylene-bis-acrylamide, in buffer containing 143mM Tris (pH 6.8 at 20°C); 0.1% (w/v) SDS; 0.057% (w/v) fresh ammonium persulphate, and 0.057% (v/v) TEMED. After washing the surface of the gradient gel with water, stacking gel was poured over the surface and a perspex comb inserted at the meniscus. This was over-layered with a little water-saturated butanol. After polymerisation was complete, the comb was removed and the surface of the gel washed with water. Tubing was removed and the gel plates inserted into apparatus analogous to that described by Reid and Bieleski (1968) except this version was necessarily larger.

Running buffer (upper and lower reservoir) contained (per litre): 28.8g glycine; 6g Tris; 1g SDS.

Sample buffer contained 25mM Tris (pH 6.8 at 20°C); 10% (v/v) glycerol; 2% (w/v) SDS; 1.54% (w/v) DTT; 0.001% (w/v) bromophenol blue.

Preparation of ribosomal proteins Ribosomal proteins were extracted from ribosomal subunits according to the method of Fraenkel-Conrat (1957) as modified by Hardy *et al.*, (1969). This method removes all ribosomal proteins from both *E. coli* and *B. stearothermophilus* ribosomes or ribosomal subunits (Wittman, 1974).

Reconstituted particles were centrifuged through a 40% (w/v) sucrose shelf containing RS buffer (for 18 hours at 40,000 r.p.m.) prior to

extraction of ribosomal proteins. This ensured that only tightly bound proteins would remain on the particles (Dohme and Nierhaus, 1976).

Ribosomal subunits were resuspended in RS buffer at a concentration of 10mg/ml and magnesium chloride was added to 100mM final concentration. Two volumes of cold, glacial acetic acid were added and extraction continued for 1 hour on ice, with frequent agitation. Precipitated RNA was removed by centrifugation at 12,000 xg for 5 minutes. Five volumes of cold acetone were added to the supernatant and the proteins allowed to precipitate for 1 hour at -20°C. Proteins were recovered by centrifugation at 12,000xg for 5 minutes. The supernatant was discarded and the protein precipitate dried in vacuo. Proteins were stored at -20°C as dry precipitates. Immediately prior to use acetone precipitates were dissolved in sample buffer.

Conditions of electrophoresis 0.4-0.8 A_{230} units protein (in 50µl sample buffer) were applied to each gel track. Electrophoresis was conducted at 150 V (constant) (cathode to anode) until the marker dye reached the bottom of the gel. Following electrophoresis, the gel was stained with 0.1% (w/v) PAGE Blue 83 (B.D.H. Chemicals Ltd.) in 50% (v/v) methanol plus 10% (v/v) acetic acid, for 16 hours. Destaining was performed in 10% (v/v) methanol plus 7% (v/v) acetic acid.

2.4 Two-Dimensional Gel Electrophoresis The method described below is essentially that of Mets and Bogorad (1974).

First dimension

Separation gel (pH 5.0) contained 48g urea; 4g acrylamide; 0.1g N,N'-methylene-bis-acrylamide; 1.192g Bis-Tris; water and acetic acid to

100ml and pH 5.0.

Gel solution was de-gassed and polymerised by addition (per ml) of 5µl fresh 10% (w/v) ammonium persulphate and 5µl TEMED.

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

Running buffer (lower reservoir) pH 4.5 contained 17.5g potassium acetate; water and acetic acid to 1 l and pH 4.5.

Conditions of electrophoresis Electrophoresis was conducted at 60 V (constant) (anode to cathode) for 16 hours at 4°C. Basic fuschin was used as tracker dye.

Second dimension

Separation gel (pH 6.75) contained 100g acrylamide; 2.5g N,N'-methylene-bis-acrylamide; 30g Bis-Tris; water and HCl to 1 l and pH 6.75.

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

Running buffer (upper reservoir) pH 6.5 contained 13.7g MES; 14.6g Bis-Tris; 1g SDS; 1mM DTT; water to 1 l.

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

Conditions of electrophoresis Electrophoresis was conducted at 70 V (constant) (cathode to anode) for 5 hours at ambient temperature. Gels were overlaid with 10% (v/v) glycerol containing 0.004% (w/v) bromophenol blue as tracker dye.

Procedure First dimension gel was poured to a depth of 12 cm into chromic acid-washed, siliconised glass tubes (13 cm x 0.4 cm). The gel was overlaid with water-saturated butanol and allowed to polymerise. The surface of the gel was washed with water and drained well. After fitting into a standard disc-gel apparatus (Davis, 1964) the upper section of the tube was completely immersed in running buffer (pH 4.0). Immediately prior to use, acetone precipitates, prepared according to Chapter 5.2.3 were dissolved in a fresh solution containing 8M urea; 10mM DTT; 0.1% (w/v) basic fuschin. 0.2 A_{230} units of 50S ribosomal proteins (in 10-20µl sample solution) were carefully layered over the surface of the gel. Running buffer (pH 4.5) was added to the apparatus until the lower surface of the gel tube was just covered (ensuring no air bubbles became entrapped). Following electrophoresis gel was removed by forcing water between the gel and inner surface of the tube using a syringe with fine needle. The gel was then fixed between the second dimension gel plates as illustrated in Figure 4.1.

Second dimension gel (approximately 20ml) was carefully poured between the plates until the first dimension gel was covered by 0.5cm of gel solution. Water-saturated butanol was applied to the upper surface of the gel. After the gel had polymerised, the upper surface of the gel was washed with water, the tubing removed and the plates fitted into apparatus similar to that described by Reid and Bieleski (1968). A rubber mat was fitted between the gel plate and back of the gel box to prevent leakage between reservoirs. Following electrophoresis, the gel was removed and stained according to Chapter 5.2.3.

2.5 Ribosomal RNA Analysis Analysis of both native and reconstituted particles was performed exactly according to Chapter 4.2.2. RNA was

first extracted from ribosomal particles (at a concentration 5 to 10mg/ml) by the addition of an equal volume of re-distilled phenol (water-saturated). After vigorous mixing for 2 minutes the two phases were clarified by centrifugation at 12,000xg for 5 minutes. The upper aqueous phase was removed and the lower-phase extracted with an equal volume of water. The two aqueous phases were then pooled, re-extracted with phenol and the RNA in the final aqueous phase precipitated by the addition of 3 volumes of cold ethanol. After 1 hour at -20°C, the RNA was recovered by centrifugation at 12,000xg for 5 minutes.

3. Results

3.1 Analytical Sucrose Density Gradient Centrifugation A comparison of native versus reconstituted 50S particles on analytical sucrose density gradients is shown in Figure 5.2. Essentially, the profiles of both major 50S peaks were indistinguishable. However, a second peak was apparent with the reconstituted 50S particles; this was contributed by 16S RNA (present during reconstitution as part of the RNA 70 fraction) and accounted for 35% of the absorbance (at 260nm) of PEG-precipitated particles. Presumably, the 16S RNA bound reconstituted 50S particles in a non-specific manner during the PEG-precipitation procedure and required the ionic conditions of the sucrose density gradient to promote its dissociation from the 50S particles. Dissociation could also be promoted in LM buffer (data not presented).

3.2 Analytical Ultracentrifugation Since there are limitations to the validity of results obtained using analytical sucrose density gradients, the more stringent technique of analytical ultracentrifugation was employed to establish whether differences in sedimentation rates between native and reconstituted 50S particles could be detected. A profile of the relative positions of both native and reconstituted 50S particles after a fixed period of centrifugation is shown in Figure 5.3. This shows quite dramatically the distinct differences in relative sedimentation velocity of the particles.

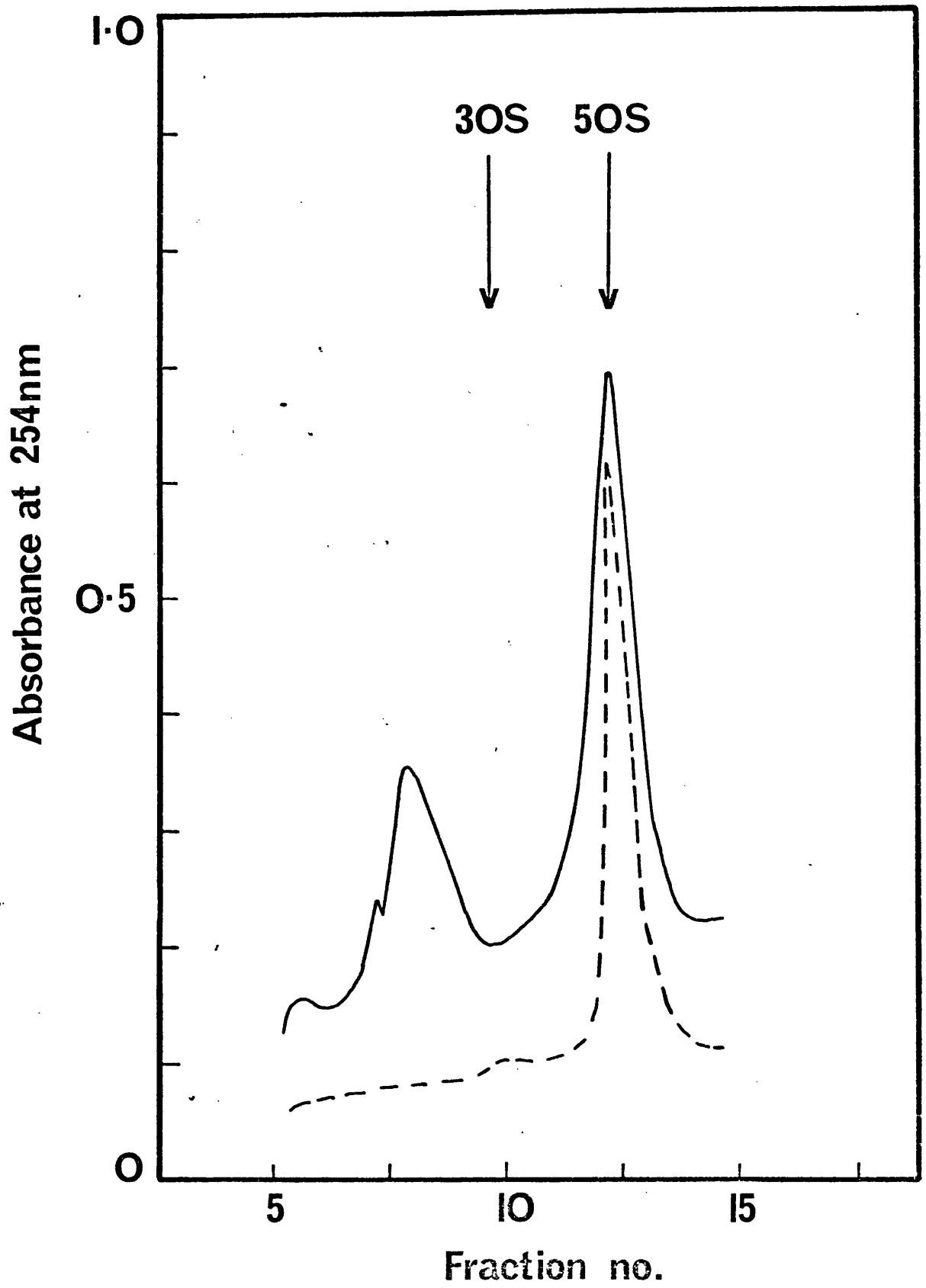
To gain an accurate estimate of the respective sedimentation coefficients, the rates of sedimentation of the particles were monitored at intervals throughout the duration of centrifugation (after the peak corresponding to reconstituted 50S particles had accelerated away from "contaminating"

Legend to Figure 5.2

Analysis of native (----) and reconstituted (——) 50S particles from *S. loidensis* in analytical sucrose density gradients.

Approximately 0.5 A_{260} units of ribosomal subunits were layered over 5ml 10-25% (w/v) sucrose density gradients containing SA buffer. After centrifugation at 45,000 r.p.m. for 2 hours in a Beckman SW 50.1 rotor at 2°C, the absorbance profile of the gradient was measured.

FIGURE 5.2

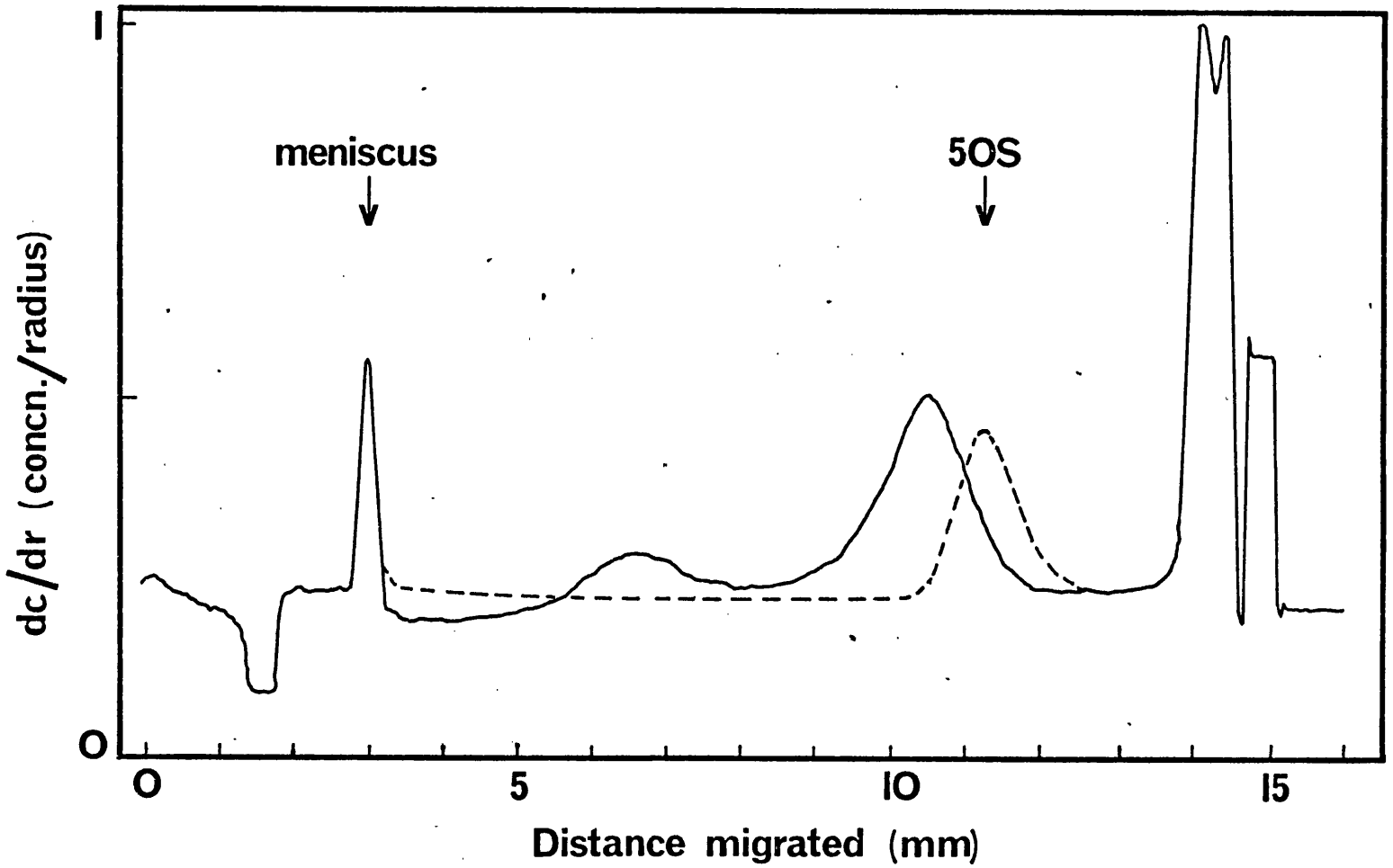


Legend to Figure 5.3

Analysis of native (----) and reconstituted (——) 50S particles from *S. loidensis* by analytical ultracentrifugation.

Approximately 400µl ribosomal subunits (at a concentration of 2mg/ml - in LM buffer) were loaded into each of the cells of a 6-place rotor (reconstituted particles were isolated from their incubation mix by prior centrifugation). Analytical ultracentrifugation was at 20,000 r.p.m. in an MSE Centriscan 75, at 5°C. After approximately 45 minutes centrifugation, ribosomal subunit boundaries were measured by Schlieren optics. Profiles were aligned according to the positions of the menisci.

FIGURE 5.3


1
0
meniscus
50S
dc/dr (concn./radius)
0
5
10
15
Distance migrated (mm)

16S RNA) and the data analysed by computer. Results from this analysis are presented in Figure 5.4. After correction for temperature and viscosity of solvent, native and reconstituted particles had sedimentation coefficients of 49.8 ± 0.2 S and 46.5 ± 0.3 S respectively.

3.3 Association Of Native 30S Particles With Either Native Or Reconstituted 50S Particles The ability of both native and reconstituted 50S particles to associate with native 30S particles in sucrose density gradients under various defined ionic conditions is shown in Figure 5.5. Whereas native 30S and 50S particles associated at 6mM magnesium ion concentration to sediment at 70S, much higher concentrations of magnesium ion (i.e. 8-10mM) were required to induce only a proportion of the population of reconstituted 50S particles to associate with native 30S particles. Hence, not only could native and reconstituted particles be differentiated between on this basis, but the results suggested that a heterogeneous population of reconstituted particles was present.

3.4 Optimisation of Magnesium Ion Concentration In Poly (U)-Directed Protein-Synthesising System Since both native and reconstituted 50S particles appeared to show different affinities for native 30S particles, it was considered worthwhile to establish whether the magnesium concentration in the poly (U)-directed protein-synthesising system favoured one particle in preference to the other (with respect to activity). Accordingly, both native and reconstituted 50S particles were mixed with native 30S particles and assayed in a poly (U)-directed protein-synthesising system containing various concentrations of magnesium ion. Results are presented in Figure 5.6. Contrary to expectation, the optimal magnesium concentration for reconstituted particles was lower than for native particles. This was a surprising observation since reconstituted 50S particles required a higher magnesium concentration to

Legend to Figure 5.4

Estimation of sedimentation coefficients of native (□-□) and reconstituted (■-■) 50S particles from *S. loidensis* by analytical ultracentrifugation.

The rate of migration of ribosomal particles during centrifugation (see Chapter 5.2.2) was monitored by Schlieren optics (at 554nm) at 12 intervals throughout centrifugation. Data was accumulated *via* a multiplexing unit into a double-disc drive Apple II Plus micro-computer.

Sedimentation coefficients were derived using the formula

$$s = \frac{dx/dt}{\omega^2 x}$$

where x = the distance of the boundary from the centre of rotation (in cm).

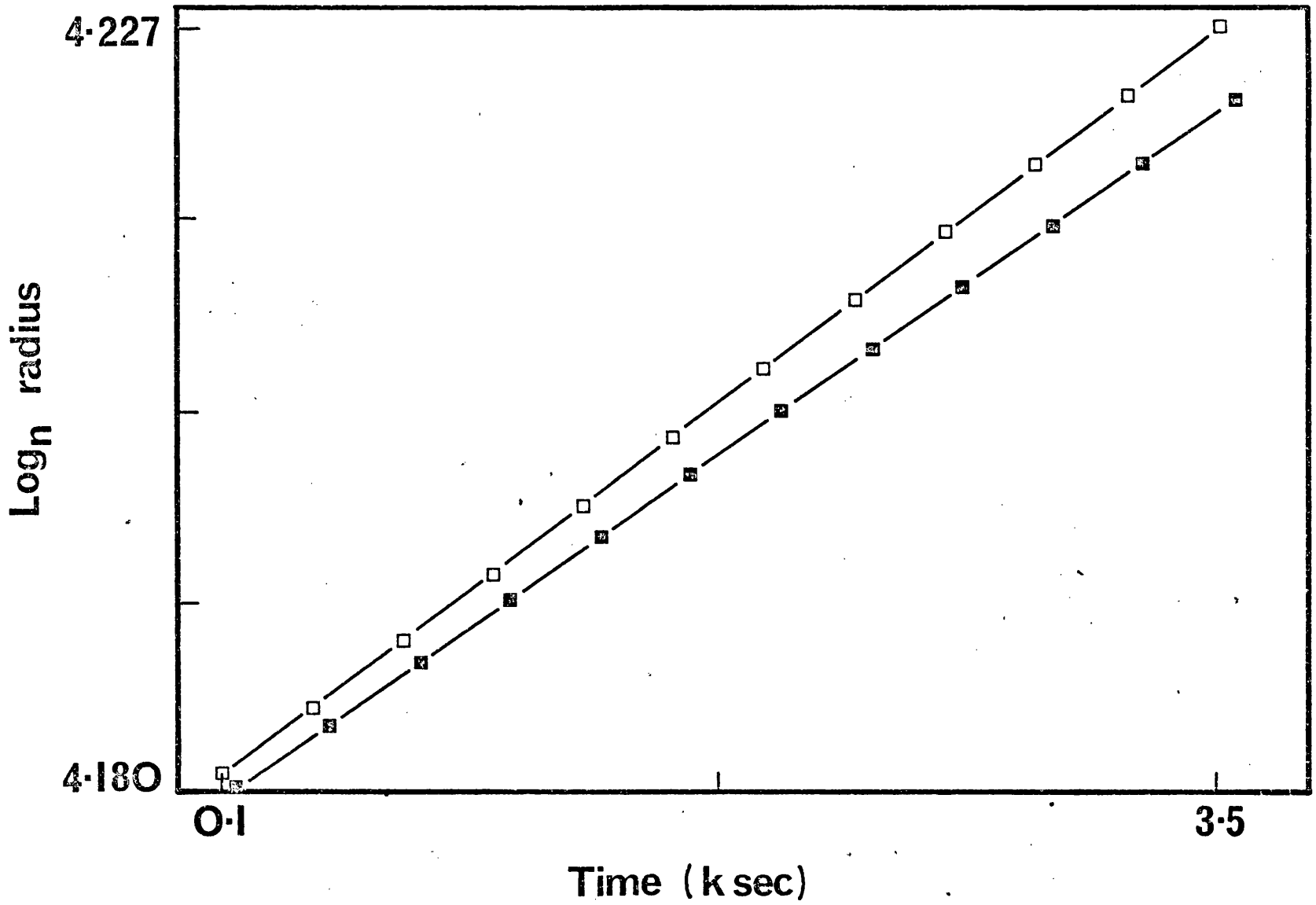
t = time (in sec.)

ω = angular velocity (radians/sec.)

Ultimately, sedimentation coefficients were corrected for temperature (to 20°C) and viscosity (to water) to give values of $s_{20,w}$

S values:	Native 50S particles	: 49.8 ± 0.2 S
	Reconstituted 50S particles:	46.5 ± 0.3 S

FIGURE 5.4



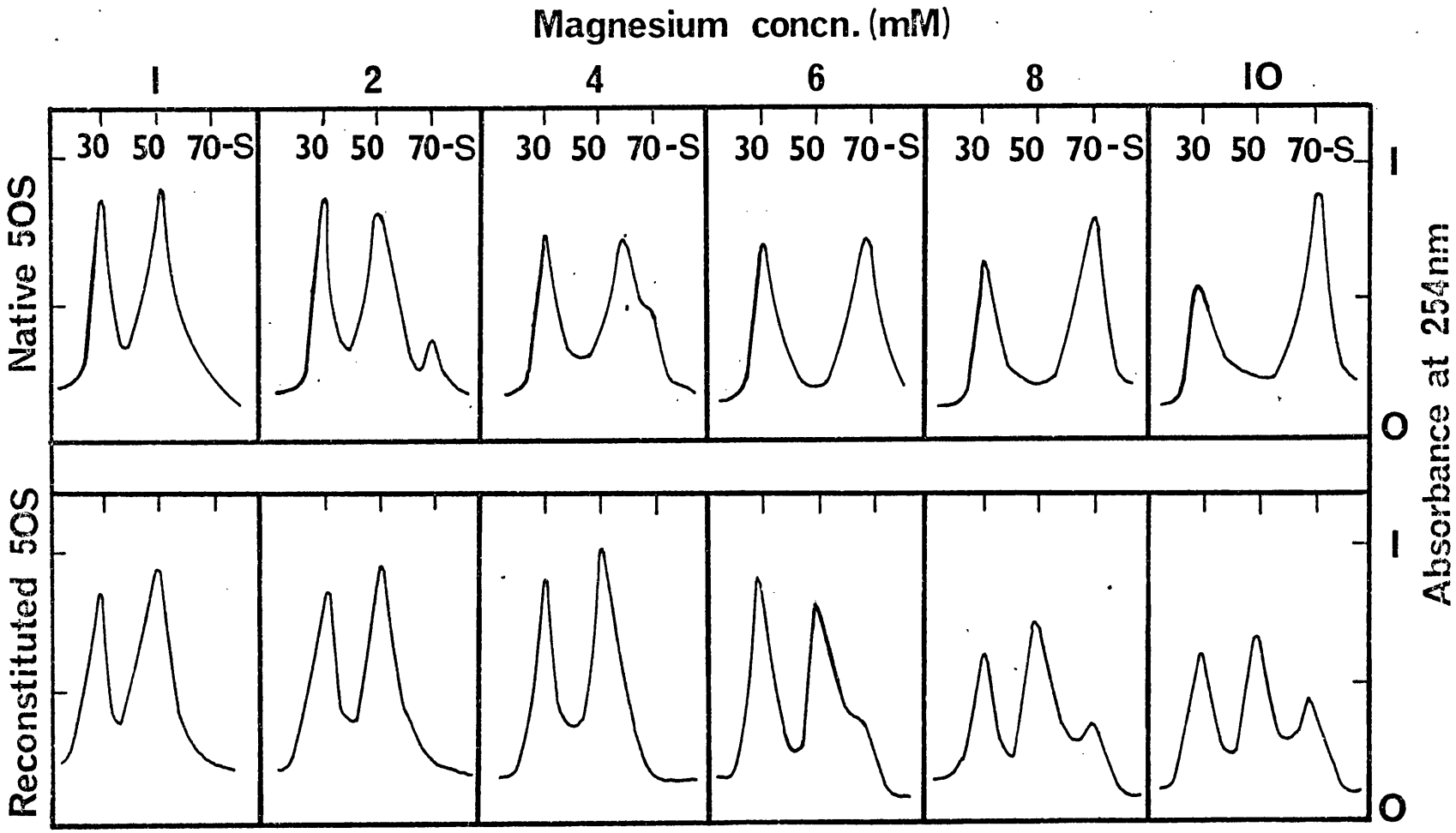
Legend to Figure 5.5

Association of native 30S particles with either native or reconstituted 50S particles from *S. loidensis*.

Either native or reconstituted 50S particles were mixed with a two-fold molar excess of native 30S particles and incubated in RS buffer for 15 minutes at 30°C.

Aliquots (50µl - containing approximately 1.5 A_{260} units of particles) were layered over 5ml 10-25% (w/v) sucrose density gradients containing 10mM Tris (pH 7.6 at 20°C); 100mM potassium chloride; 3mM 2-mercapto-ethanol, and concentrations of magnesium as indicated. After centrifugation at 45,000 r.p.m. for 90 minutes in a Beckman SW 50.1 rotor at 2°C, the absorbance profiles of the gradients were measured.

FIGURE 5.5

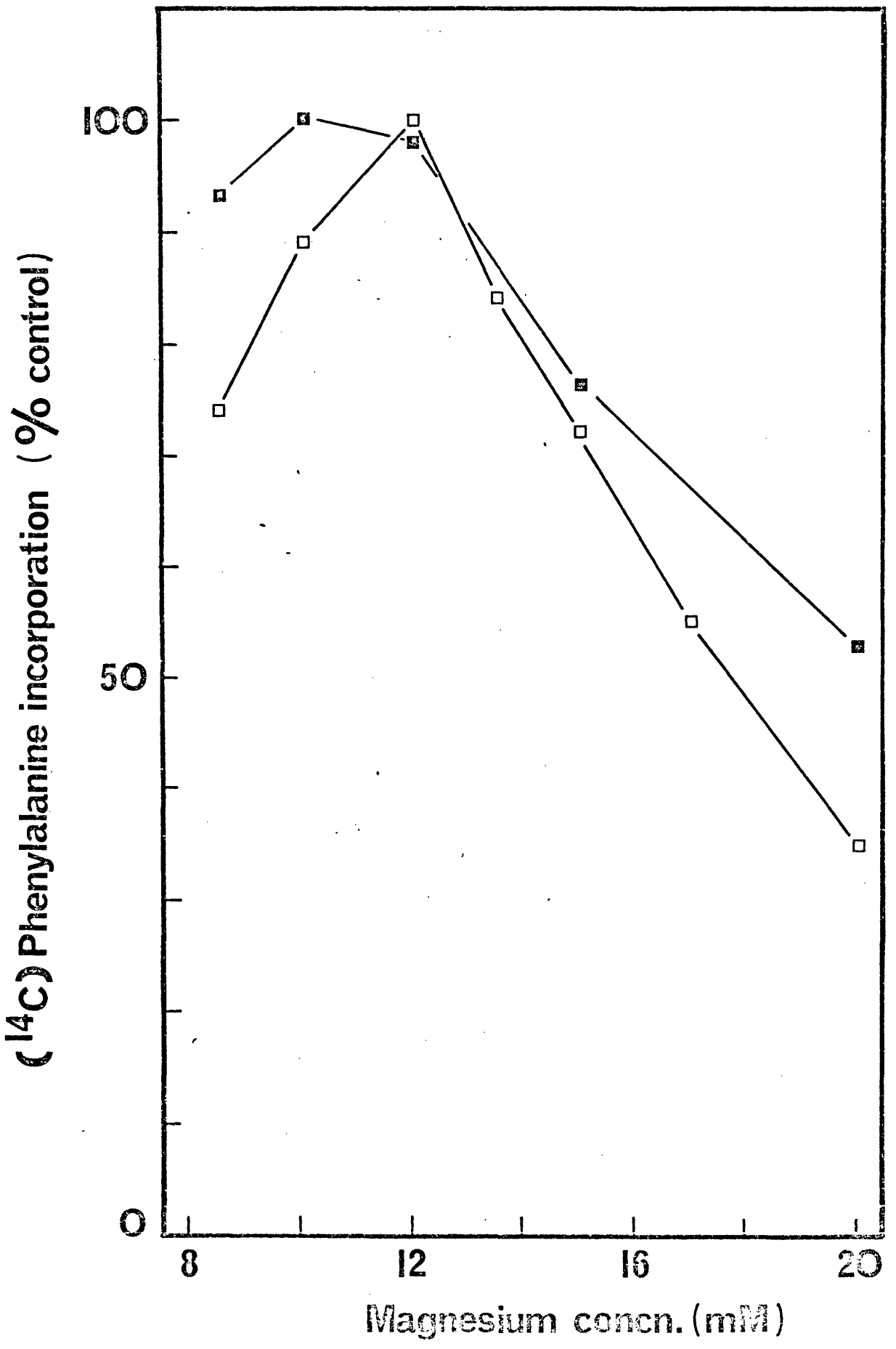

Magnesium concn. (mM)
1
2
4
6
8
10
30 50 70-S
Native 50S
Reconstituted 50S
1
0
Absorbance at 254nm

Legend to Figure 5.6

Optimisation of the magnesium concentration in the protein-synthesising system directed by poly (U).

Native (□-□) or reconstituted (■-■) *S. loidensis* 50S particles were supplemented with native 30S particles and S100, and programmed with poly (U) according to Chapter 2.2.8, except magnesium concentrations were as indicated. Results are expressed as a percentage of control incorporations (i.e. the maximum incorporation attained by each type of particle). Control incorporations ranged from 7 to 20 pmol of phenylalanine per pmol of ribosomes for reconstituted and native particles respectively.

FIGURE 5.6


100
50
0
8
12
16
20
(^{14}C) Phenylalanine incorporation (% control)
Magnesium concn. (mM)

maintain association with native 30S particles in sucrose density gradients. However, for subsequent assays, the magnesium concentration chosen was an average between the two optima (i.e. 11mM).

3.5 Estimation Of Ribosomal Subunit Activity Since ribosomal subunit association behaviour had suggested a heterogeneous population of reconstituted 50S particles, an estimate of the proportion of active particles was sought. Accordingly, the ability of both reconstituted and native 50S particles to synthesise polyphenylalanine in the presence of various proportions of native 30S particles was tested. Results are presented in Figure 5.7.

To interpret this data it was necessary to assume all 30S particles to be active. Thus, approximately 50% of the native 50S particles could participate in polyphenylalanine synthesis whereas only 30% of the reconstituted 50S particles could do so. Furthermore, a molar excess of 30S particles over 50S particles (both native and reconstituted) resulted in a significant reduction of polyphenylalanine-synthetic activity. Thus, under optimal conditions of assay, and taking into account the proportions of active 50S particles in a given population, native and reconstituted 50S particles were capable of synthesising, on average, 50 and 15-20 pmol phenylalanine per pmol ribosomes respectively.

To establish whether the population of active 50S ribosomal subunits (native or reconstituted) was homogenous or heterogeneous (with respect to activity), the data from the last experiment was plotted such that activity was expressed as a function of each 30S ribosomal subunit. Results indicated (Figure 5.8) that approximately 20-25% of native 50S particles had similar activity, the rest of the population representing particles of heterogeneous activity. In contrast, little or no

Legend to Figure 5.7

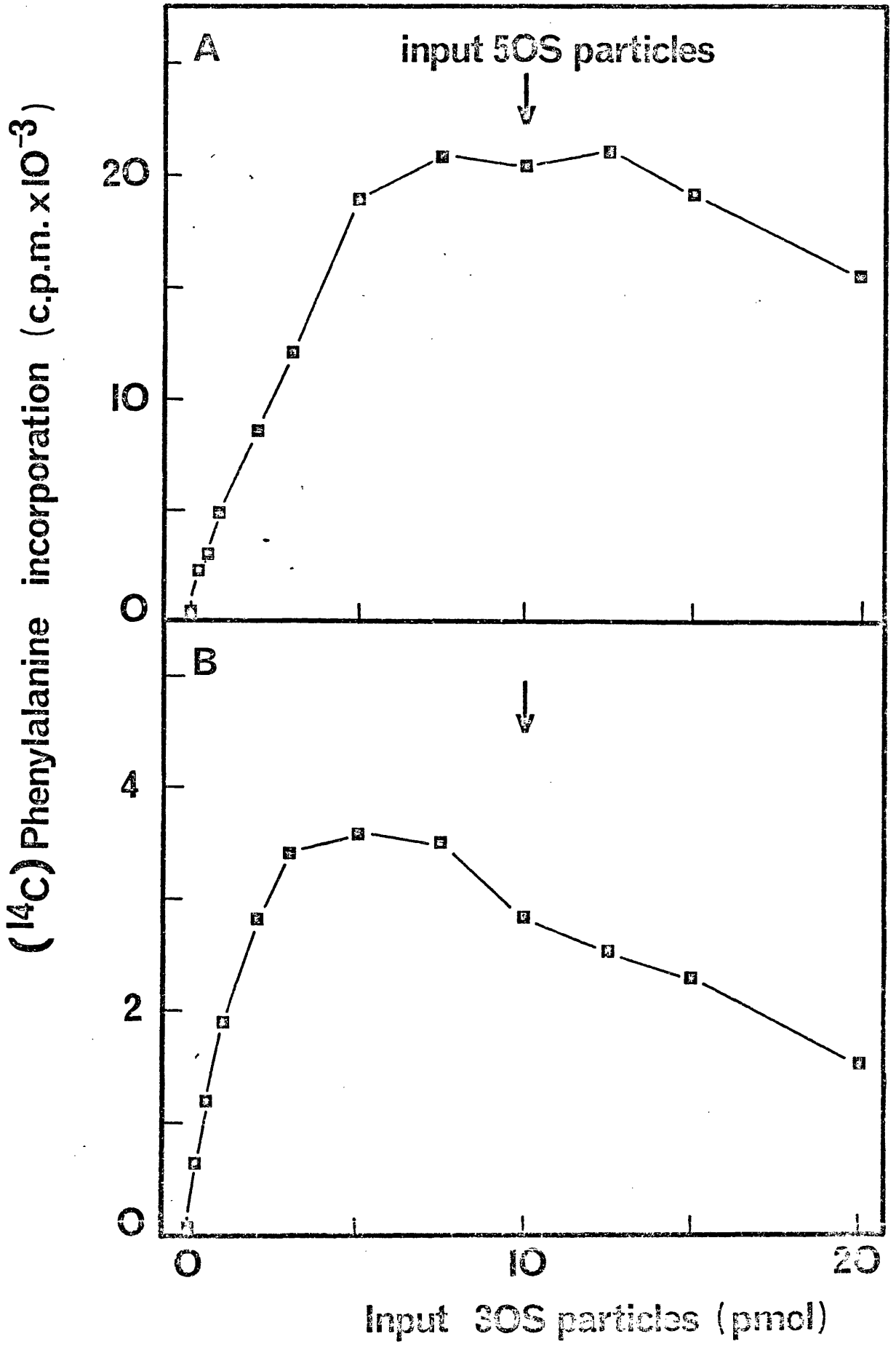
Effect of input ratio of 30S : 50S particles (from *S. loidensis*) on polyphenylalanine synthesis.

Native or reconstituted 50S particles from *S. loidensis* were mixed with increasing proportions of native 30S particles and, following supplementation with S100, were tested for their ability to synthesise polyphenylalanine, according to Chapter 2.2.8. Each point represents incorporation attained after 15 minutes incubation at 30°C.

A : native 50S particles

B : reconstituted 50S particles

FIGURE 5.7

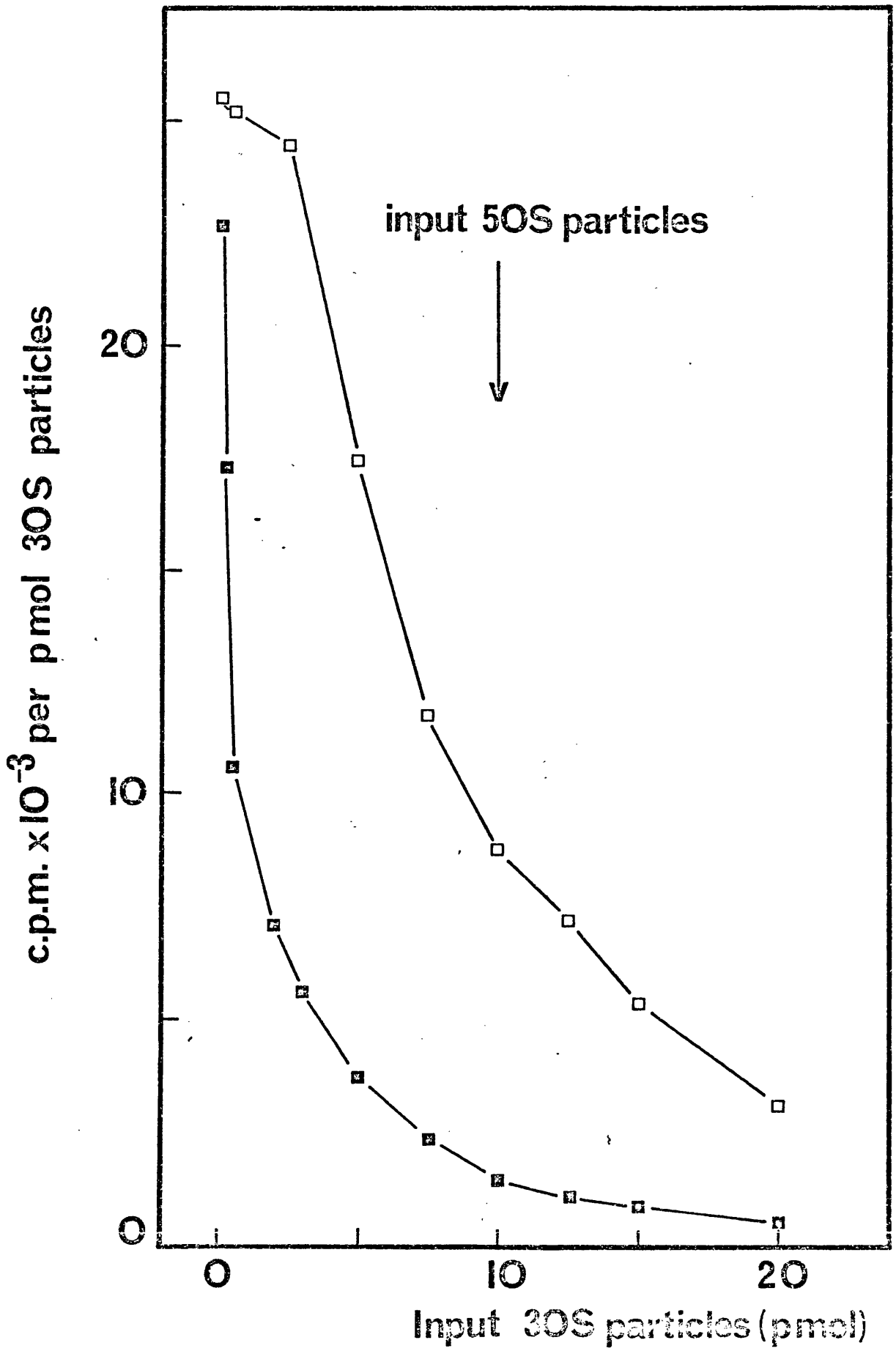

A
input 50S particles
B
20
10
0
4
2
0
0
10
20
(^{14}C) Phenylalanine incorporation (c.p.m. x 10^{-3})
Input 30S particles (pmol)

Legend to Figure 5.8

Activity of native (□-□) and reconstituted (■-■) 50S particles (from *S. loidensis*) as a function of 30S particle input.

Results derived from the last experiment were re-expressed as indicated.

FIGURE 5.8


input 50S particles
c.p.m. x 10^{-3} per p mol 30S particles
20
10
0
0
10
20
Input 30S particles (pmol)

homogeneity between particles was observed with reconstituted 50S ribosomal subunits; essentially only a very small percentage (perhaps only 2% of the total population of particles) had activity comparable with that of the most active native 50S particles.

3.6 Isolation Of Reconstituted Particles And Subsequent Activity In Vitro

To establish whether the method of isolation of reconstituted particles affected their subsequent activity *in vitro*, reconstituted particles were subjected to three different isolation procedures. First, they were isolated by the conventional procedure (i.e. PEG-precipitation). Second, reconstituted particles were isolated on a sucrose density gradient containing 1mM magnesium ion; this served to dissociate "contaminating" 16S RNA from the 50S particles. Third, reconstituted particles were mixed with native 30S particles and isolated (as 70S particles) on a sucrose density gradient containing 10mM magnesium ion, thereby (hopefully) selecting reconstituted particles of the highest activity.

Surprisingly, results presented in Figure 5.9 indicated only small differences between the activities of 50S (70S) particles isolated by the three methods. Thus, PEG-precipitation of reconstituted 50S particles from the reconstitution mixture was used routinely since, not only were the particles as active as those isolated by other techniques, but also the procedure was particularly convenient.

3.7 Ribosomal Protein Analysis

3.7.1 One-Dimensional Gradient-Polyacrylamide Gel Electrophoresis (in the Presence of SDS)

Results from the analysis of ribosomal proteins from both native and reconstituted 50S particles by gradient-polyacrylamide gel

Legend to Figure 5.9

Activity of reconstituted 50S particles (from *S. loidensis*) following prior isolation by a variety of techniques.

Reconstituted particles were isolated from the incubation mixture by one of the following procedures:-

1. Conventional PEG - precipitation (see Chapter 4.2.5).
2. Reconstituted 50S particles were mixed with a 2-fold molar excess of native 30S particles and layered over 5ml 10-25% (w/v) sucrose density gradients (5 A_{260} units per gradient - in 50µl RS buffer) containing 10mM Tris (pH 7.6 at 20°C); 10mM magnesium chloride; 100mM potassium chloride; 3mM 2-mercaptoethanol. After centrifugation at 45,000 r.p.m. for 90 minutes in a Beckman SW 50.1 rotor at 2°C, particles sedimenting at "70S" were isolated.
3. Reconstituted 50S particles were recovered from incubation mixture by precipitation with PEG, resuspended in RS buffer and layered over 5ml 10-25% (w/v) sucrose density gradients containing LM buffer (5 A_{260} units per gradient). Following centrifugation at 45,000 r.p.m. for 2 hours in a Beckman SW 50.1 rotor at 2°C, particles sedimenting at "50S" were isolated.

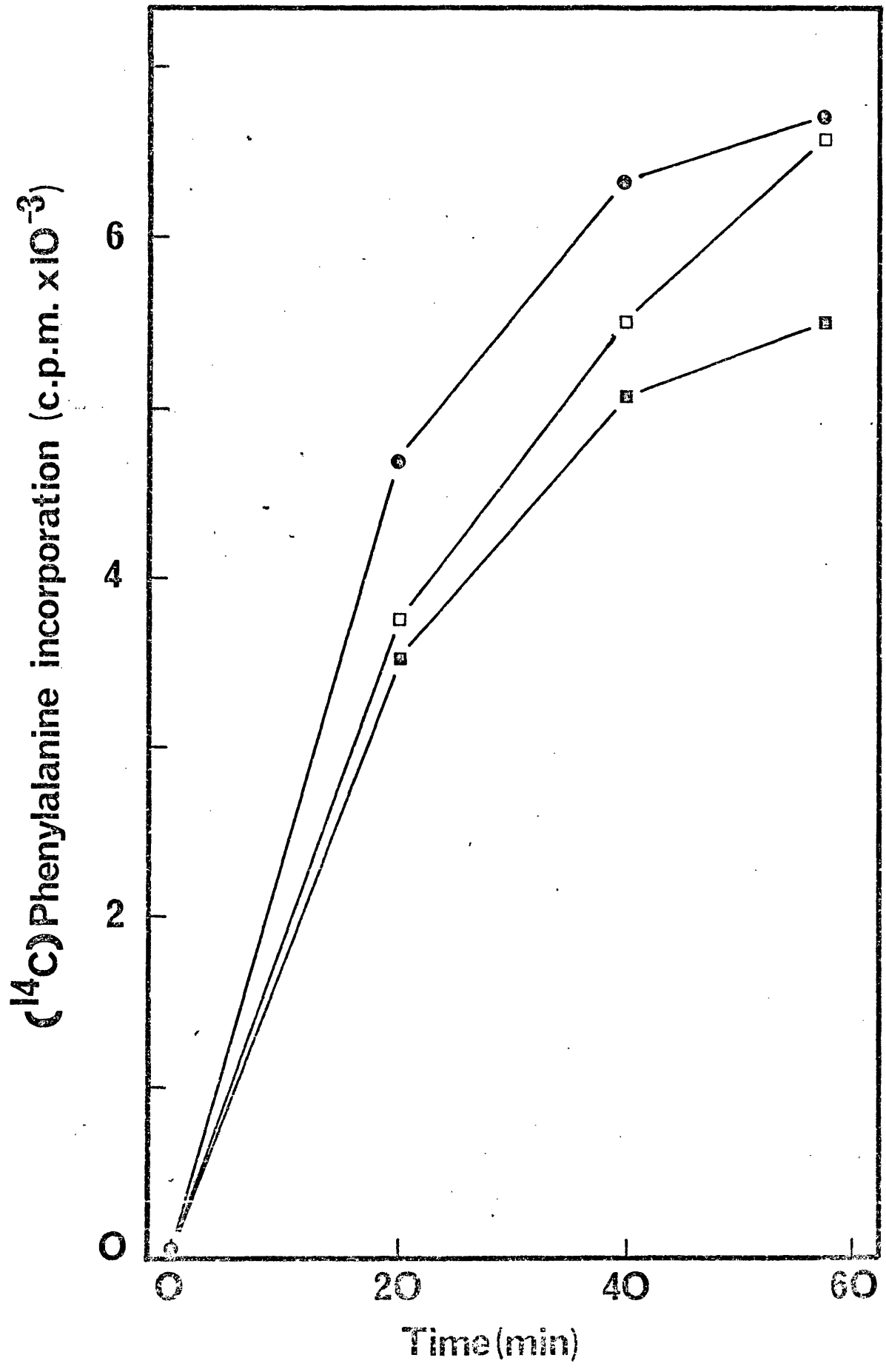
Isolated particles were mixed with equimolar amounts of native 30S particles (except for particles isolated by procedure 2) and supplemented with S100 fraction (from *S. loidensis*). 70S particles were tested for their ability to synthesise polyphenylalanine (according to Chapter 2.2.8).

● - ● : particles isolated by procedure 1

□ - □ : particles isolated by procedure 2

■ - ■ : particles isolated by procedure 3

FIGURE 5.9


(^{14}C)Phenylalanine incorporation (c.p.m. x10^{-3})
0
2
4
6
0
20
40
60
Time (min)

electrophoresis (in the presence of SDS) are presented in Figure 5.10. Since the resolution of the photograph is poor,a schematic representation of the bands is indicated to enhance legibility. While approximately thirty bands were apparent, one (i.e. that nearest to the origin) may not be a ribosomal protein, due to its very high molecular weight. In essence, the results indicated that both native and reconstituted 50S particles were indistinguishable on the basis of their ribosomal protein contents. Furthermore TP 50 contained all the ribosomal proteins present on the native 50S particles; hence the urea/lithium chloride extraction procedure removed all the ribosomal proteins from S. loidensis 50S ribosomal subunits.

3.7.2 Two-Dimensional Gel Electrophoresis The one-dimensional gradient-polyacrylamide gel system (see above) does highlight the difficulties of separating ribosomal proteins - many having very similar molecular weights. In an attempt to effect a better separation of ribosomal proteins and to establish whether any had been modified during (or prior to) the reconstitution procedure, ribosomal proteins were analysed using the two-dimensional procedure of Mets and Bogorad (1974). Results are presented in Figure 5.11. In support of evidence derived from the one-dimensional procedure, the two-dimensional procedure could not detect significant, reproducible differences between both the ribosomal protein content and relative mobilities of ribosomal proteins derived from native and reconstituted 50S particles.

3.8 Ribosomal RNA Analysis Results from the analysis of ribosomal RNA from both native and reconstituted 50S particles are presented in Figure 5.12. These clearly showed the extent of 23S RNA degradation within reconstituted particles compared with native 50S particles. However, whether the residual intact 23S RNA in reconstituted particles is derived only from those particles active in polyphenylalanine synthesis is unclear.

Legend to Figure 5.10

Analysis of ribosomal proteins by one-dimensional gradient-polyacrylamide gel electrophoresis (in the presence of SDS) (according to Chapter 5.2.3).

Track

1 : ovalbumin (10μg)

2 : *S. loidensis* TP 50 (0.4 A_{230} units)

3 : *S. loidensis* reconstituted 50S proteins (0.8 A_{230} units)

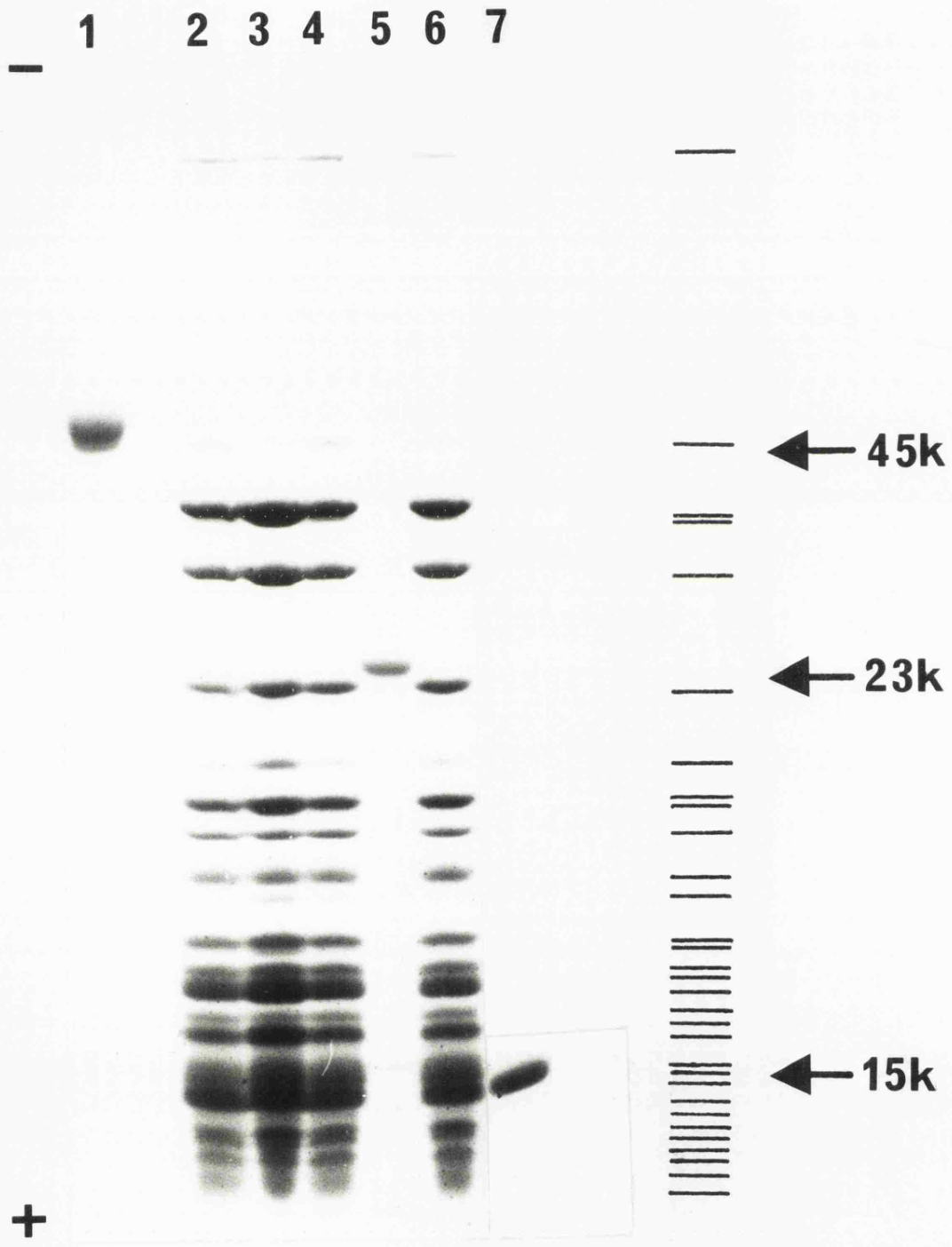
4 : *S. loidensis* native 50S proteins (0.4 A_{230} units)

5 : α-chymotrypsinogen (10μg)

6 : *S. loidensis* reconstituted 50S proteins (0.4 A_{230} units)

7 : lysozyme (10μg)

FIGURE 5.10



Legend to Figure 5.11

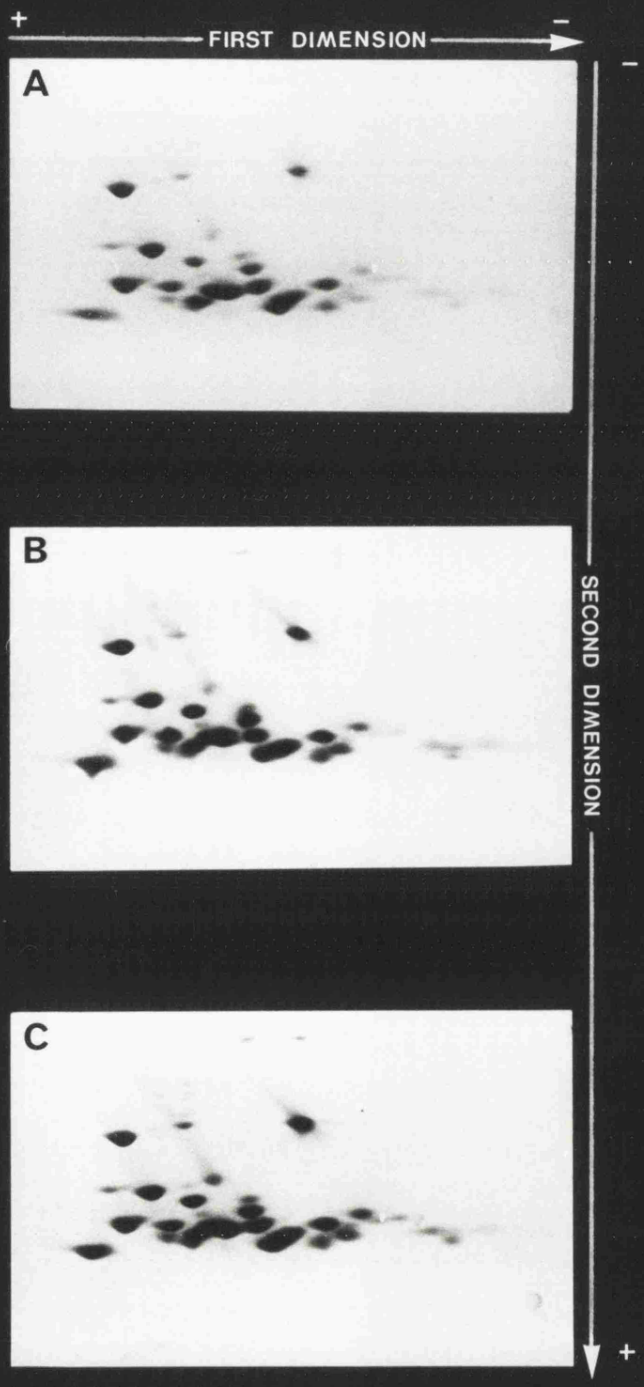
Analysis of ribosomal proteins by two-dimensional gel electrophoresis (according to Chapter 5.2.4).

A : *S. loidensis* native 50S proteins (0.2 A_{230} units)

B : *S. loidensis* TP 50 (0.2 A_{230} units)

C : *S. loidensis* reconstituted 50S proteins (0.2 A_{230} units)

FIGURE 5.11



Legend to Figure 5.12

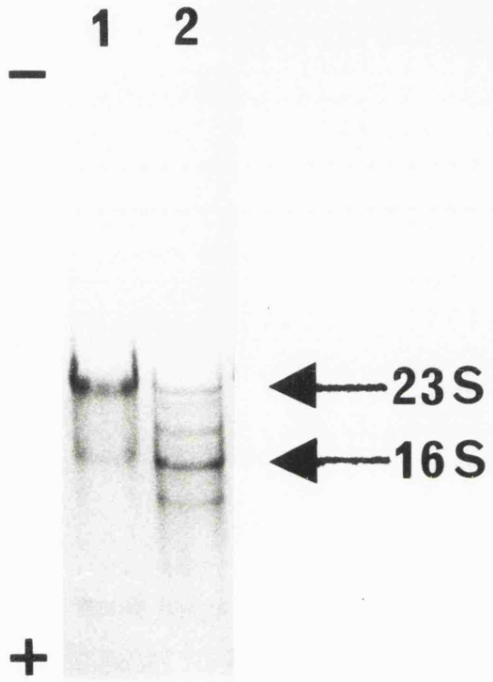
Analysis of ribosomal RNA by gel electrophoresis under denaturing conditions (according to Chapter 4.2.2).

Track

1 : RNA from *S. loidensis* native 50S particles (4μg)

2 : RNA from *S. loidensis* reconstituted 50S particles (4μg)

FIGURE 5.12



4. Discussion

As discussed in Chapter 4, the reconstitution of *S. loidensis* 50S ribosomal subunits is a compromise between inducing certain (rate-limiting) conformational changes and maintaining ribosomal proteins in an active state. This compromise is ultimately reflected by reduced levels of polyphenylalanine-synthetic activity. However, the sequence of events constituting protein synthesis on the ribosome is necessarily complex and, accordingly imposes certain constraints upon the conformation of the ribosomal particles. Hence, for *E. coli*, the difference between an active and an inactive "50S" particle is reflected by only a small change in sedimentation coefficient (i.e. 50S to 48S respectively).

A reduction of polyphenylalanine-synthetic activity by reconstituted 50S particles could conceivably result from two effects. First, from an increase in the proportion of inactive particles in the total population (e.g. by an increase in the proportion of particles sedimenting more slowly than native particles). Second, a reduction of activity could result from the whole population of particles becoming less active, but to a similar extent.

A comparison of native versus reconstituted 50S particles by analytical sucrose density gradient centrifugation did not reveal differences in sedimentation velocity. Since the technique is subject to inherent drawbacks, an attempt was made to radiolabel native 50S ribosomal subunits for comparison with reconstituted particles within the same sucrose density gradient. However, attempts to grow *S. loidensis* in media suitable for radiolabelling failed. Accordingly, the more rigorous technique of analytical ultracentrifugation was used to compare both native and reconstituted 50S particles. Differences in comparative sedimentation velocity were quite distinct. However, contrary to expectation,

analytical ultracentrifugation did not reveal a heterogeneous population of reconstituted particles (i.e. active and inactive particles sedimenting at approximately 50S and 48S respectively). Instead, the population was essentially homogenous and reconstituted particles sedimented at approximately 46.5S compared with 49.8S for native particles. It appeared therefore that S. loidensis reconstituted 50S particles could be active in polyphenylalanine synthesis, yet still sediment more slowly than native particles.

The ability of S. loidensis native 50S particles to combine with 30S particles in a sucrose density gradient containing 6mM magnesium ion, suggested that all the particles may be potentially capable of "tight" couple formation. However, in contrast, reconstituted 50S particles were unable to combine with 30S particles under similar conditions. Although increasing the magnesium ion concentration to 8-10mM promoted a net increase in 70S particle formation, their relative proportion (compared with free ribosomal subunits) was small. Hence, this strongly suggested that although the native 50S particles appeared essentially homogenous (with respect to their ability to combine with 30S particles), a heterogeneous population of reconstituted particles was apparent. The significance of this observation (in relation to polyphenylalanine synthesis) is unclear since although tight couple formation in E. coli is a prerequisite for translation of R17 messenger RNA, this is probably not the case for messages like poly (U), since ribosomal subunits unable to form tight couples were able to form a "primitive" initiation complex with poly (U), and phe-tRNA (Schreier and Noll, 1970; 1971) and to be active in polyphenylalanine synthesis. Clearly, if this were not the case, then reconstituted S. loidensis 50S particles would be totally inactive. Hapke and Noll (1976) rationalised differences in ability to

form tight couples on the basis that ribosomal subunits could exist in more than one state. Hence 30S ribosomal subunits could be inactivated by exposure to low magnesium ion concentrations and re-activated by heating (under appropriate ionic conditions). In contrast, 50S ribosomal subunits were often irreversibly inactivated by a variety of factors including exposure to low magnesium ion concentrations, high salt concentrations (i.e. 1M ammonium chloride) and prolonged storage. Such inactivation apparently resulted from an irreversible conformational change in the ribosomal subunit (Noll and Noll, 1976).

It might be expected that if high concentrations of magnesium were required for reconstituted 50S particles to associate with native 30S particles, then the optimal magnesium concentration in the poly (U)-directed protein-synthesising system in which they were assayed, would also be higher than for the corresponding native 50S particles. Contrary to expectation, the optimal magnesium concentration was higher for native than for reconstituted 50S particles. No rational explanation can be forwarded for these results.

While 30% of the population of reconstituted 50S particles were active in vitro, it was surprising to observe that only 50% of the population of native 50S particles were active, particularly in view of the observation that all the native 50S particles could associate with 30S particles in sucrose density gradients containing 6mM magnesium ion. These estimates necessarily assume all the 30S particles to be active (and this was by no means proven, even though they were routinely heat-activated). However, by increasing the ratio of 30S to 50S particles beyond 1:1 (and hence the ratio of active 30S to 50S particles) no further increase in activity could be observed. Accordingly, whilst

this may suggest that the original estimates of the proportions of active ribosomal subunits were accurate, the apparent reduction of activity *in vitro* (when 50S particles were assayed in the presence of a molar excess of 30S particles) could conceivably result from the removal of either magnesium or poly (U) from the cell-free translation system, through their binding to the excess 30S ribosomal subunits.

An estimate of the extent of homogeneity between the active ribosomal subunits of a given population (with respect to polyphenylalanine-synthetic activity) was also established. Surprisingly, both native and reconstituted 50S particles were heterogeneous but to varying extents. Accordingly while 20-25% of the population of native 50S particles were essentially homogenous, as little as 2% of the population of reconstituted 50S particles were of similar activity.

Under limiting conditions 30S ribosomal subunits appeared to bind primarily to the most active 50S particles and then to those of diminishing activity. This finding is in accordance with results for *E. coli* ribosomal subunits (Hapke and Noll, 1976).

Accordingly, the term "level of reconstitution" is meaningless unless framed in the context of the above experiments. At one extreme, under conditions where molar equivalent amounts of both 30S and 50S ribosomal subunits were present, the level of reconstitution approached 30% (compared with native particles). At the other extreme, where 50S particles were assayed under conditions of limiting 30S ribosomal subunit input, levels of reconstitution approaching 90% could be achieved.

Reconstituted particles were isolated from incubation mixture by three different methods to determine whether levels of activity of 50S particles were affected. These methods included isolation of the 50S peak from

contaminating 16S RNA in sucrose density gradients containing 1mM magnesium ion; isolation of 70S ribosomes (composed of native 30S particles and reconstituted 50S particles) in sucrose-density gradients containing 10mM magnesium ion; and finally isolation by (conventional) PEG-precipitation. Although particles isolated as 70S ribosomes were expected to be the most active, results suggested no significant differences in activity could be observed between particles isolated by the three methods.

Gel electrophoresis (and in particular 2-D gel electrophoresis) is subject to inherent variability. Hence, it is always necessary to run a number of replicates until fairly consistent results are obtained. Gel electrophoretic analysis of ribosomal proteins from both native and reconstituted particles could not reveal any consistent differences. Hence, it was not possible, for example, to implicate a lack of ribosomal protein (s) as the cause of the lower sedimentation coefficient of the reconstituted particles or the reduced level of affinity between reconstituted 50S particles and native 30S particles; for example, in E. coli, combinations of ribosomal proteins L1 and L11 or L1 and L16 were found to be essential for ribosomal subunit association (Kazemie, 1975). Furthermore, no changes in electrophoretic mobility of ribosomal proteins isolated from reconstituted 50S particles (when compared with native particles) were observed by 2-D gel electrophoresis. Hence, modification of ribosomal proteins was not observed either prior to, or during the reconstitution procedure (e.g. as the result of carbamylation).

RNA extracted from reconstituted particles was found to be extensively degraded (by comparison with fresh native 50S particles). Similar observations were made by Amils et al., (1979) despite high levels of activity from reconstituted E. coli 50S particles (i.e. 85% of the control

value). 23S RNA readily undergoes degradation into a 17S/15S pair of fragments (Hapke and Noll, 1976). However, the presence of RNA containing this "nick" in 50S particles does not necessarily correlate with the inability of that ribosomal subunit to form tight couples. Accordingly, tight couples (from E. coli) containing extensively-degraded 23S RNA have been observed (as has the converse situation). However, mild treatment of native 50S particles with ribonuclease gradually reduced the affinity of 50S ribosomal subunits for their partners until ultimately the interaction disappeared. Parallel to this effect was the reduced binding of phe-tRNA in response to poly (U). Couples formed by association of active 30S particles with ribonuclease-treated 50S particles dissociated over a broad range of ionic conditions, a situation not unlike that found with S. loidensis reconstituted 50S particles. Accordingly, it is not unreasonable to speculate and invoke ribonuclease action as a possible cause of reduced activity of reconstituted 50S particles. Indeed, integrity of ribosomal RNA could be crucial to the overall conformation of the ribosome. There is substantial evidence (from E. coli 30S ribosomal subunits) to suggest that the conformation of 16S RNA (under certain ionic conditions) is very similar to that of intact ribosomal particles (Zimmerman, 1980). Although a comparable situation has not been proven for the 50S ribosomal subunit, 23S RNA does attain a relatively unique and uniform conformation under given solute conditions which may be reflected (and indeed necessary) in the 50S ribosomal subunit.

Whereas degradation of ribosomal RNA (within ribosomal particles) is often observed upon storage, this is not usually reflected by a reduction of polyphenylalanine-synthetic activity (data not presented). Accordingly, such degradation might involve specific cleavage of 23S RNA to the 17S/15S fragments which do not manifest themselves by changes in ribosomal subunit

association (Hapke and Noll, 1976).

In conclusion, while distinct differences between native and reconstituted 50S particles are readily observable, it is not possible to define their primary cause. Unlike the situation with E. coli 50S particles, differences in activity between native and reconstituted 50S particles from S. loidensis cannot be distinguished by defined changes in sedimentation coefficient. Similarly the ability of 50 S particles to associate with native 30S particles at 6mM magnesium ion does not necessarily correlate with their ability to translate poly (U).

Reconstituted particles may adopt a "looser" conformation as a result of their inherently-degraded ribosomal RNA being unable to maintain the necessary constraints within the ribosome. Hence, the degree of heterogeneity of reconstituted 50S particles (with respect to activity) may correspond with the extent of ribosomal RNA degradation i.e. the most active particles containing intact RNA. Ultimately, the ability to remove ribonuclease contamination from ribosomal components may result in the ability to reconstitute a greater proportion of particles to higher activity.

CHAPTER 6

RESPONSE OF STREPTOMYCES LOIDENSIS RECONSTITUTED 50S PARTICLES TO VERNAMYCIN A - AN APPROACH TO THE CHANGE IN DRUG RESPONSE

Chapter 6

1. Introduction

Having both reconstituted and analysed 50S ribosomal subunits from *S. loidensis*, it was now feasible to proceed and assess the response of those particles to vernamycin A *in vitro*. This would then allow a meaningful interpretation of the findings in Chapter 3.

2. Materials And Methods

2.1 Preparation Of 50S Ribosomal Subunits Lacking Ribosomal Protein (s)

50S ribosomal subunits (native or reconstituted) were resuspended in buffer containing 10mM Tris-HCl (pH 7.6 at 20°C); 10mM magnesium chloride; 100mM potassium chloride; 3mM 2-mercaptoethanol, to a concentration of approximately 5μM, and a ten-fold molar excess of vernamycin A added. After incubation at 30°C for 30 minutes the incubation mixture was layered over an equal volume of the same buffer containing 40% (w/v) sucrose. After centrifugation at 40,000 r.p.m. in a Beckman 75 Ti rotor at 2°C, ribosomal particles were resuspended in RS buffer and extracted with acetic acid according to Chapter 5.2.3.

2.2 Analysis Of Acidic Ribosomal Proteins By One-Dimensional Gel Electrophoresis

One-dimensional gel electrophoresis of acidic ribosomal proteins was performed essentially according to the method of Kaltschmidt and Wittman (1970 a) while incorporating the modifications of Howard and Traut (1973; 1974). However, the gel was cast as a "slab" (using apparatus illustrated in Figure 4.1) rather than as a "rod".

Separation gel (pH 8.5) contained 36g urea; 4g acrylamide; 0.1g N,N'-methylene-bis-acrylamide; 0.8g EDTA (di-sodium); 3.2g boric acid; 4.86g Tris-base; water to 100 ml.

Gel was de-gassed and polymerised by the addition (per ml) of 2μl fresh 10% (w/v) ammonium persulphate and 1μl TEMED.

Running buffer (pH 8.2) (upper and lower reservoir) contained 1.2g EDTA (di-sodium); 2.4g boric acid; 3.625g Tris-base; water to 1 l.

Sample buffer contained 8M urea; 10mM DTT; 0.02% (w/v) bromophenol blue.

Conditions of electrophoresis Electrophoresis was conducted at 150 V (constant) (cathode to anode) for 5 hours at $4^{o}C$.

Following electrophoresis gels were removed from the casting apparatus and stained according to Chapter 5.2.3.

2.3 Partial Reconstitution Of 50S Ribosomal Subunits From Streptomyces loidensis Methodology suitable for the partial reconstitution of 50S ribosomal subunits has been described by Staehelin and Meselson (1966); Hosokawa et al., (1966), and Staehelin et al., (1969). The method described below was necessarily derived from the procedure for the total reconstitution of S. loidensis 50S ribosomal subunits described in Chapter 4.

Generally, the "partial" reconstitution of ribosomal subunits is technically simpler to perform than so-called "total" reconstitutions. This is presumably due to the (relatively) small number of ribosomal proteins which are required to reconstitute on to the corresponding "core" particle.

Preparation of "core" particles and "split" proteins Ribosomal core particles and split proteins were prepared essentially by the method of Nierhaus and Montejo (1973) except dissociation in the presence of lithium chloride was prolonged.

50S ribosomal subunits were resuspended (to a concentration of approximately 20 A_{260} units/ml) in buffer containing 10mM Tris-HCl (pH 7.6 at $20^{o}C$); 10mM magnesium acetate; 0.4M, 1.0M or 2.0M lithium

chloride; 3mM 2-mercaptoethanol.

After 16 hours at 0°C, core particles were pelleted by centrifugation at 55,000 r.p.m. for 5 hours in a Beckman 75 Ti rotor at 2°C, and resuspended in buffer containing 20mM Tris-HCl (pH 7.6 at 20°C); 20mM magnesium acetate; 200mM ammonium chloride; 1mM EDTA; 2mM 2-mercapto-ethanol. Core particles were frozen and stored at -70°C. The supernatant, containing "split" proteins, was dialysed against buffer containing 20mM Tris-HCl (pH 7.6 at 20°C); 20mM magnesium acetate; 400mM ammonium chloride; 1mM EDTA; 2mM 2-mercaptoethanol, and concentrated approximately 30-fold by dialysis against the same buffer containing 20% (w/v) $PEG_{20,000}$. Finally, split proteins were dialysed against buffer in the absence of $PEG_{20,000}$, frozen and stored at -70°C.

S. loidensis 50S ribosomal subunits were reconstituted from core particles and TP 50 fraction (rather than the corresponding split protein fraction). This was because reconstitution with split proteins did not result in 50S particles active in polyphenylalanine synthesis. The reason for this is unclear but may be due to ribosomal protein inactivation as a result of the concentration step (i.e. against $PEG_{20,000}$) employed during preparation of split proteins. Although alternative procedures were investigated (e.g. dialysis against Ficoll) on no occasions did reconstitution with split proteins yield particles active in polyphenylalanine synthesis. Since TP 50 was active in the reconstitution procedure, it was used in preference (the excess ribosomal proteins probably remain in solution during the step employed to precipitate reconstituted particles from incubation mixture).

Procedure Core particles were added to a reconstitution buffer such that, upon addition of TP 50, the final ionic conditions were 30mM Tris-HCl

(pH 7.6 at 20°C); 26mM divalent cation (i.e. magnesium acetate/chloride); 425mM monovalent cation (i.e. potassium/ammonium chloride); 6mM 2-mercaptoethanol. After 3 hours at 45°C, reconstituted particles were precipitated by the addition of 0.25 volumes of 40% (w/v) $PEG_{6,000}$. After 10 minutes at 0°C, particles were recovered by centrifugation at 12,000xg for 5 minutes and dissolved in RS buffer.

Incubation mix (200µl) contained 120 pmol core particles and 150 pmol equivalents of TP 50.

3. Results

3.1 Effect Of Vernamycin A On Reconstituted 50S Ribosomal Subunits From Streptomyces Loidensis

To establish whether reconstituted 50S ribosomal subunits from *S. loidensis* were resistant to vernamycin A *in vitro*, their response to the drug was tested in a poly (U)-directed protein-synthesising system. Results clearly indicated (Figure 6.1) that the 50S particles were sensitive to vernamycin A.

3.2 Displacement Of A Ribosomal Protein From Reconstituted 50S Ribosomal Subunits

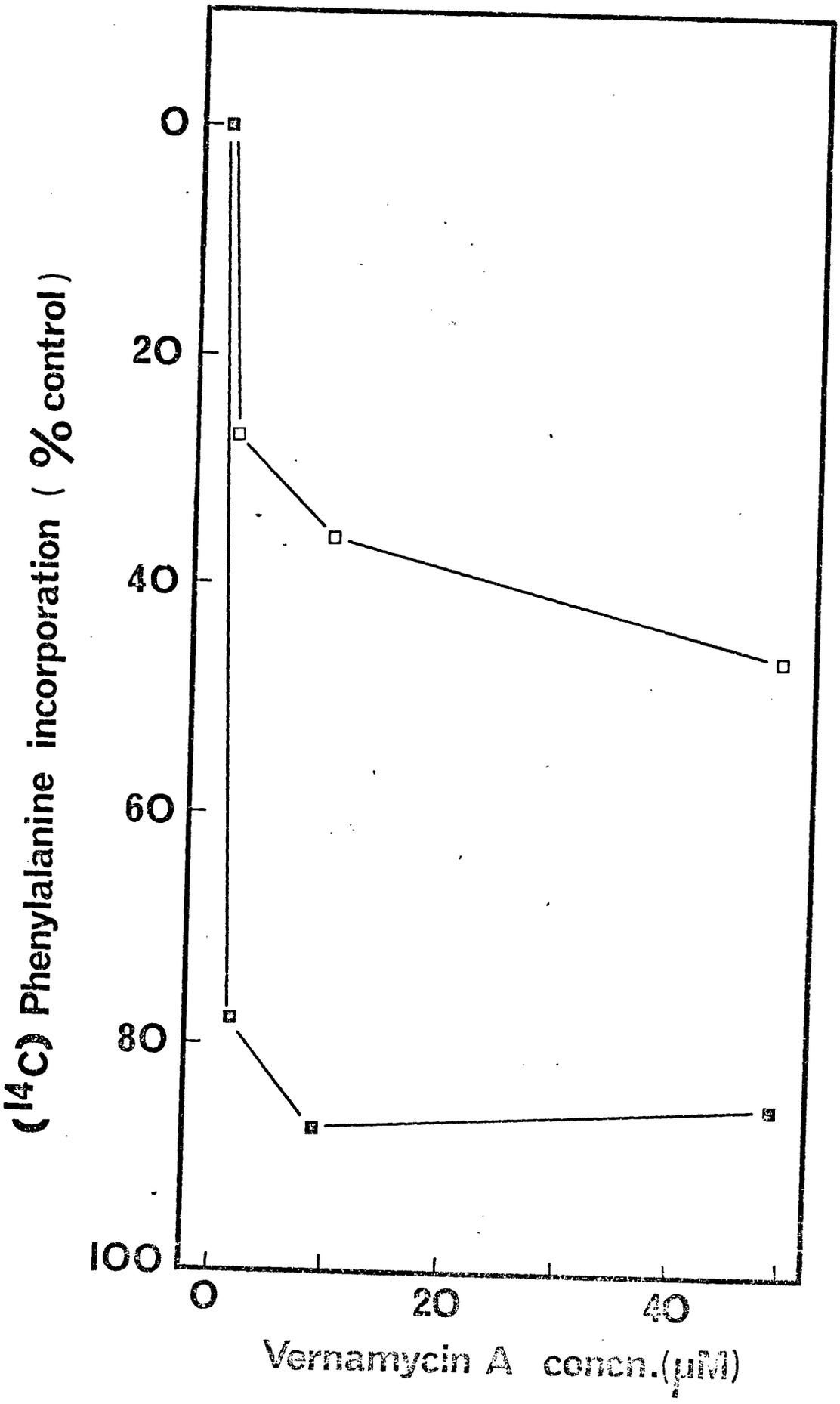
Since extensive analysis of reconstituted 50S particles (Chapter 5) had not revealed the absence/modification of ribosomal protein (s) which might otherwise have conferred vernamycin A-resistance, an alternative explanation was sought. Clearly other factors could conceivably influence the response of the reconstituted 50S particles to drug; for example, the conformation of the reconstituted particles and the integrity of their RNA. However, in view of the peculiar irreversible effect that vernamycin A was reported to exert upon 50S ribosomal subunits (Parfait and Cocito, 1980; Parfait *et al.*, 1981) an unusual approach was taken. In essence, the affinity of ribosomal proteins for their respective particles was tested in the presence and absence of vernamycin A. Accordingly, both native and reconstituted particles from *S. loidensis* were processed according to Chapter 6.2.1. Following 2-D gel electrophoresis of their respective ribosomal proteins (according to methodology outlined in Chapter 5.2.4) the results are presented in Figure 6.2. Surprisingly, these results suggested the reduction in content of a ribosomal protein from reconstituted particles exposed to vernamycin A whereas no effect was observed with native particles. This protein is the most acidic of the *S. loidensis* ribosomal proteins and hence is the one which migrates the least in the

Legend to Figure 6.1

Dose response of native (□-□) and reconstituted (■-■) 50S particles from *S. loidensis*, to vernamycin A.

Native and reconstituted 50S particles were supplemented with native 30S particles, and S100 from *B. stearothermophilus* and assayed for their ability to synthesise polyphenylalanine in the presence and absence of concentrations of vernamycin A as indicated. Results are expressed as a percentage of control incorporations which, after 20 minutes, ranged from 4 to 13 pmol phenylalanine per pmol of ribosomes (for reconstituted and native particles, respectively).

FIGURE 6.1


(^{14}C) Phenylalanine incorporation (% control)
0
20
40
60
80
100
0
20
40
Vernamycin A concn.(μM)

Legend to Figure 6.2

Effect of vernamycin A on native and reconstituted 50S particles from *S. loidensis*, as judged by two-dimensional gel electrophoresis.

Native and reconstituted 50S particles were treated with vernamycin A (according to Chapter 6.2.1). Subsequently, ribosomal proteins were extracted from the particles (according to Chapter 5.2.3) and subjected to two-dimensional gel electrophoresis (according to Chapter 5.2.4).

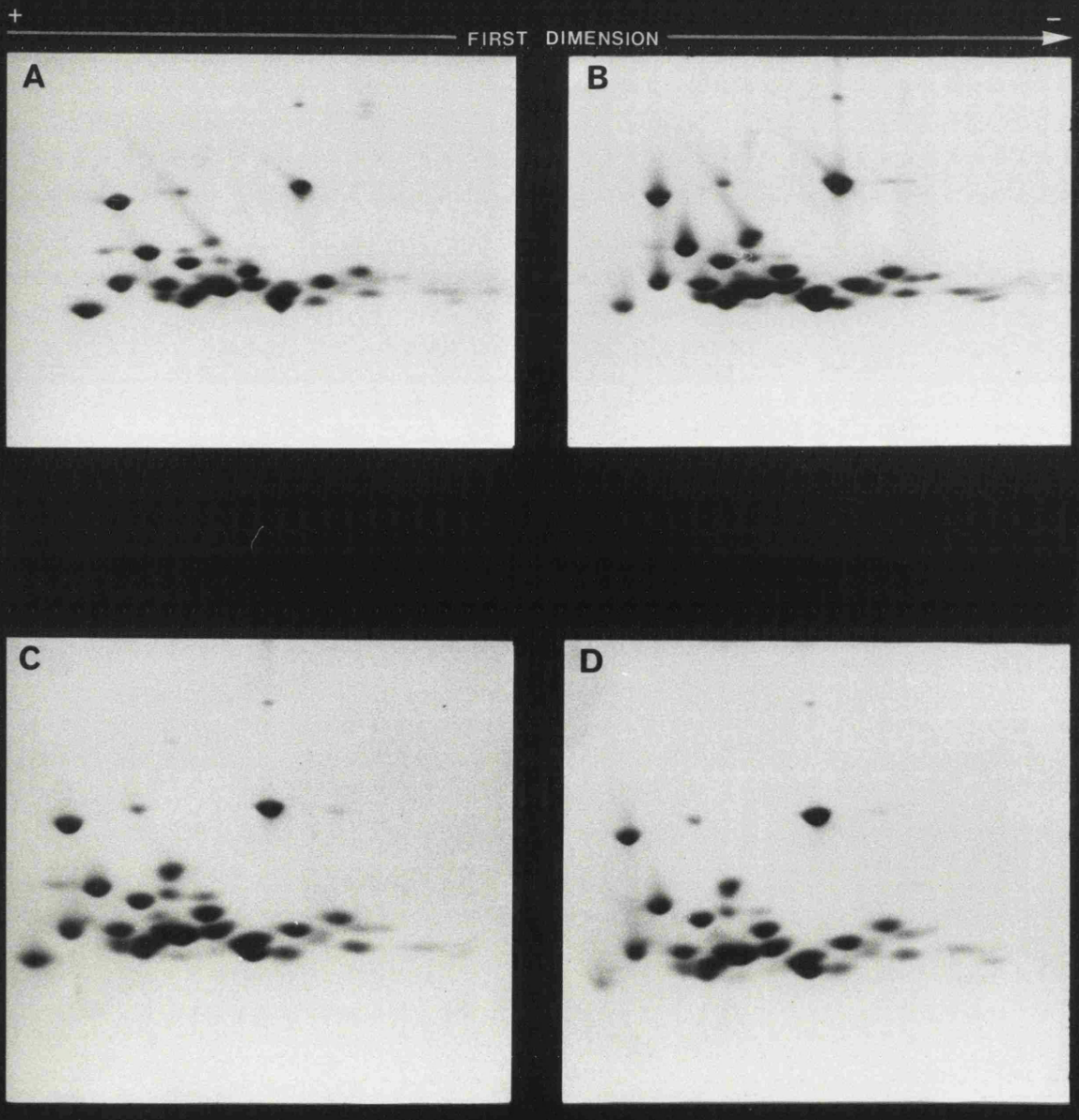
A : proteins (0.2 A_{230} units) from *S. loidensis* native 50S particles - unexposed to vernamycin A

B : proteins (0.2 A_{230} units) from *S. loidensis* native 50S particles - exposed to vernamycin A

C : proteins (0.2 A_{230} units) from *S. loidensis* reconstituted 50S particles - unexposed to vernamycin A

D : proteins (0.2 A_{230} units) from *S. loidensis* reconstituted 50S particles - exposed to vernamycin A

FIGURE 6.2



first dimension (see bottom left-hand corner of relevant photographs).

In order to quantitate the reduced level of the acidic ribosomal protein from reconstituted particles exposed to vernamycin A, 2-D gels were scanned by an automatic recording microdensitometer, Model MK 111 C (Joyce, Loebl and Co. Ltd.). To simplify a comparison of gels only three adjacent spots were scanned (as indicated in the relevant figure legend). Results presented in Figure 6.3 clearly showed the reduced content of the acidic ribosomal protein from vernamycin A-treated S. loidensis reconstituted particles.

Since the protein in question appeared to be the most acidic of the ribosomal proteins from S. loidensis, it was decided to confirm the observation that the ribosomal protein from vernamycin A-treated reconstituted particles was present in reduced quantities,by electrophoresing the ribosomal proteins in a gel system in which acidic proteins had the greatest mobility relative to basic proteins (acidic proteins move very little in the first dimension of the gel system of Mets and Bogorad). Accordingly, a slab gel electrophoresis system was devised and run according to Chapter 6.2.2. Results presented in Figure 6.4 suggested that the ribosomal protein was absent from the reconstituted particles exposed to vernamycin A. Interestingly, the corresponding ribosomal protein from unexposed particles had an electrophoretic mobility similar to purified E. coli ribosomal protein L7/L12.

3.3 Partial Reconstitution Of Streptomyces Loidensis 50S Ribosomal Subunits - Effect of Vernamycin A Since the affinity of the acidic protein for reconstituted particles was presumably reduced (by comparison with native particles) it was of interest to know whether ribosomal core particles (containing various complements of ribosomal proteins) exhibited

Legend to Figure 6.3

Densitometric analysis of two-dimensional gels.

Gels (from which photographs were taken and presented in Figure 6.2) were subjected to densitometric analysis using an automatic recording microdensitometer, Model MK 111C (Joyce, Loebl and Co. Ltd.). Four ribosomal proteins were scanned (i.e. the most acidic). Profiles 1, 2 and 3 represent proteins adjacent to the protein which appeared to be affected by vernamycin A (i.e. profile 4).Gels were scanned by a tungsten light source.

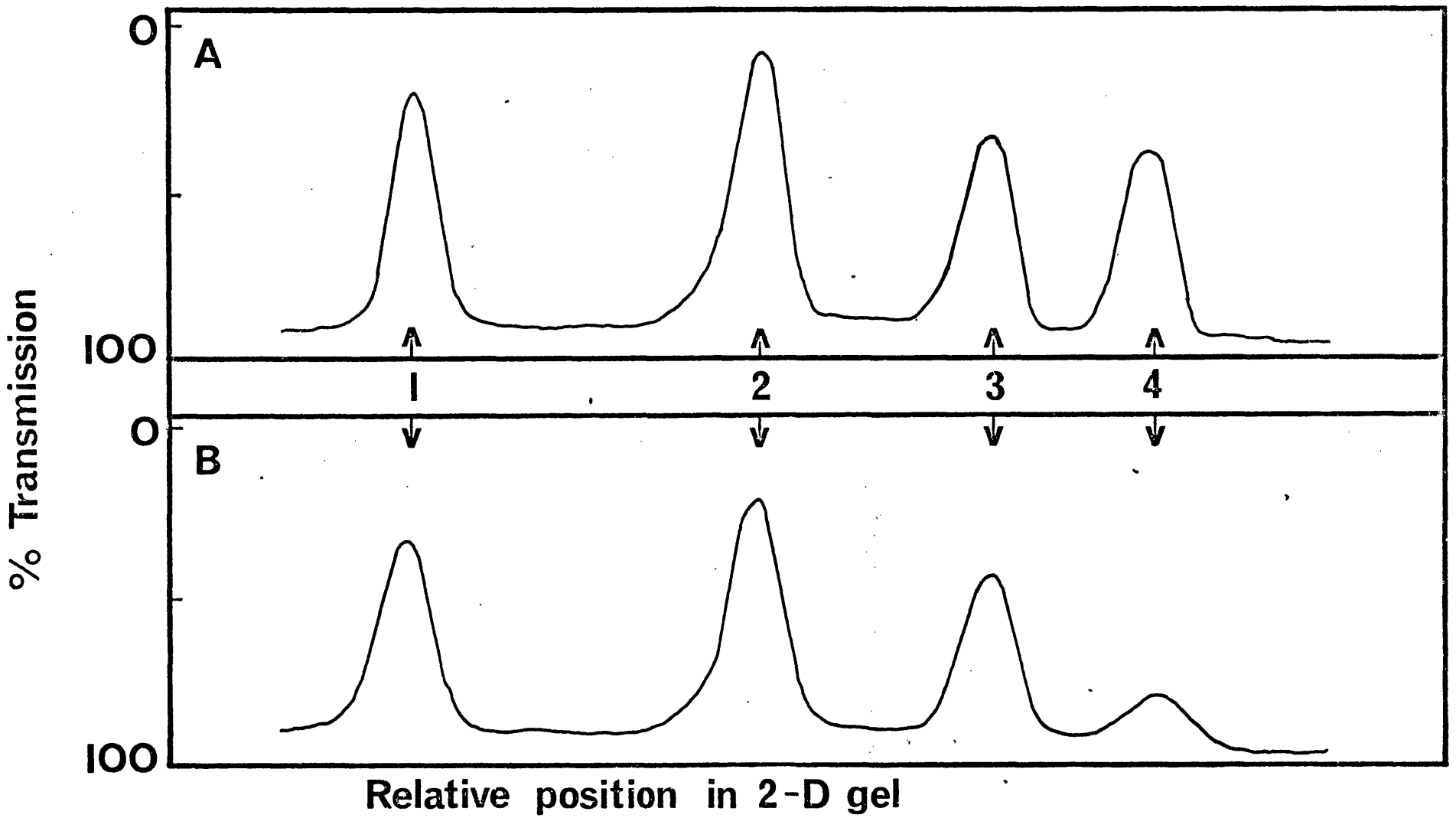
Each peak was excised, weighed and relative proportions calculated thus:

Profile	
1	0.75
2	0.88
3	1.10
4	0.38

A : *S. loidensis* reconstituted 50S particles - unexposed to vernamycin A

B : *S. loidensis* reconstituted 50S particles - exposed to vernamycin A.

FIGURE 6·3



Legend to Figure 6.4

Analysis of acidic ribosomal proteins by one-dimensional gel electrophoresis (according to Chapter 6.2.2).

Track

1 : proteins (0.4 A_{230} units) from *S. loidensis* native 50S particles

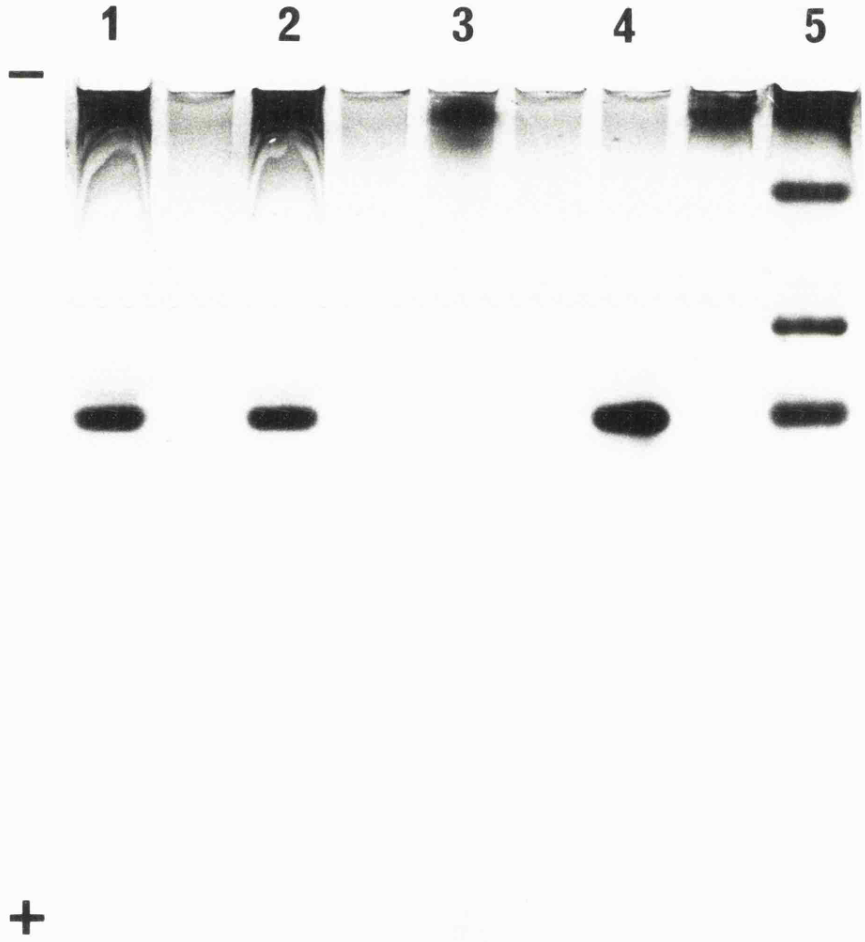
2 : proteins (0.4 A_{230} units) from *S. loidensis* reconstituted 50S particles - unexposed to vernamycin A

3 : proteins (0.4 A_{230} units) from *S. loidensis* reconstituted 50S particles - exposed to vernamycin A

4 : *E. coli* purified ribosomal protein L7/L12 (8μg)

5 : *E. coli* TP 50 (0.4 A_{230} units)

FIGURE 6.4



different levels of sensitivity to vernamycin A *in vitro* (following reconstitution with TP 50). Hence, ribosomal core particles were prepared according to Chapter 6.2.3 and reconstituted with TP 50 (prepared according to Chapter 2.2.6) according to methodology outlined in Chapter 6.2.3. Reconstituted particles were assayed in a poly (U)-directed protein-synthesising system in the presence and absence of vernamycin A. Results presented in Figure 6.5 suggested that, as more ribosomal proteins were washed from the ribosomal subunit prior to reconstitution (i.e. as the integrity of the ribosome was destroyed) then particles of increasing sensitivity to vernamycin A resulted.

3.4 Partial Loss Of Acidic Protein From Core Particles May Correspond With An Increase In Vernamycin A Sensitivity To establish whether the increase in vernamycin A-sensitivity correlated with the removal of the relevant acidic protein from core particles to the split protein fraction, the ribosomal protein content of both core particles and split protein fractions were examined by 2-D gel electrophoresis (according to Chapter 5.2.4). Results are presented in Figure 6.6.

Since no ribosomal proteins were washed from the 50S particles by 0.4M lithium chloride it was perhaps not surprising that these particles were similar to native 50S particles (regarding their response to vernamycin A *in vitro*). While a particle reconstituted from a 1.0M core plus TP 50 was more sensitive to vernamycin A than a native particle, this correlated with the removal of certain ribosomal proteins (including, to an extent, the most acidic member) from the core particles into the corresponding split protein fraction. However, it was not possible to correlate the apparent total sensitivity to vernamycin A of 50S particles reconstituted from 2M cores plus TP 50, with the complete removal of the acidic protein from the 2M core particles.

Legend to Figure 6.5

Dose response of various 50S particles from *S. loidensis* to vernamycin A.

50S particles were reconstituted from core particles (0.4, 1.0 and 2.0 M) and TP 50 (according to Chapter 6.2.3). Subsequently, particles were supplemented with native 30S particles, and S100 from *B. stearothermophilus* and assayed for their ability to synthesise polyphenylalanine in the presence and absence of concentrations of vernamycin A, as indicated. Results are expressed as a percentage of control incorporations which, after 20 minutes incubation ranged from 1 to 2 pmol phenylalanine per pmol ribosomes. For clarity, results from Figure 6.1 are re-presented.

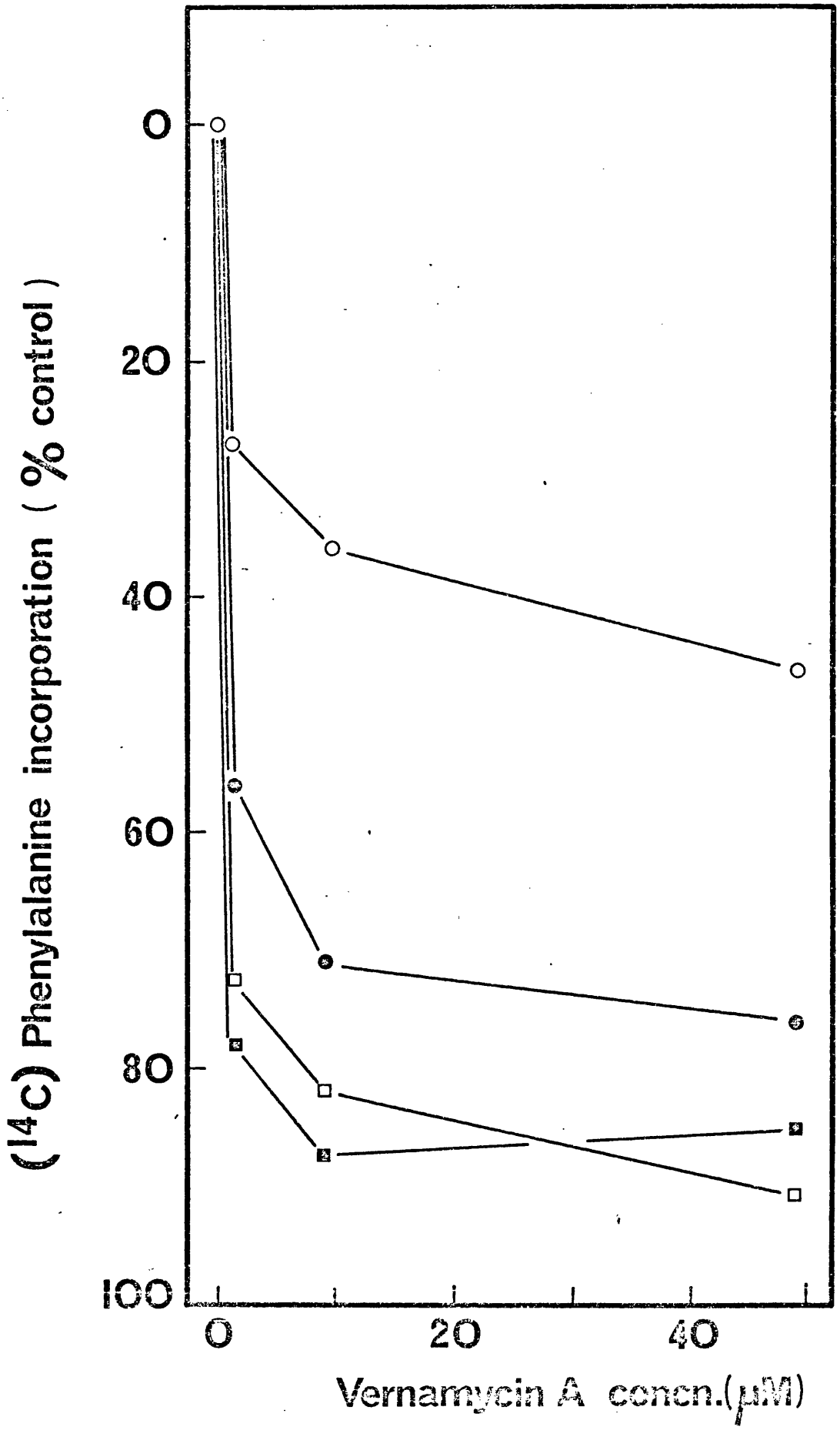
o - o : native 50S particles or particles reconstituted from 0.4M cores + TP 50

● - ● : particles reconstituted from 1M cores + TP 50

□ - □ : particles reconstituted from 2M cores + TP 50

■ - ■ : particles reconstituted from RNA 70 + TP 50

FIGURE 6.5


(14C) Phenylalanine incorporation (% control)
0
20
40
60
80
100
0
20
40
Vernamycin A concn.(μM)

Legend to Figure 6.6

Protein composition of various core particle and split protein fractions from *S. loidensis* 50S ribosomal subunits.

Core particles and split protein fractions were prepared from *S. loidensis* 50S ribosomal subunits (according to Chapter 6.2.3). Subsequently, ribosomal proteins were extracted from core particles (according to Chapter 5.2.3). Ribosomal proteins were recovered from split protein fractions by the addition of 5 volumes of cold acetone.

Ribosomal proteins were subjected to two-dimensional gel electrophoresis (according to Chapter 5.2.4).

A : proteins from 0.4M core particle (0.2 A_{230} units)

B : proteins from 0.4M split protein fraction (0.2 A_{230} units)

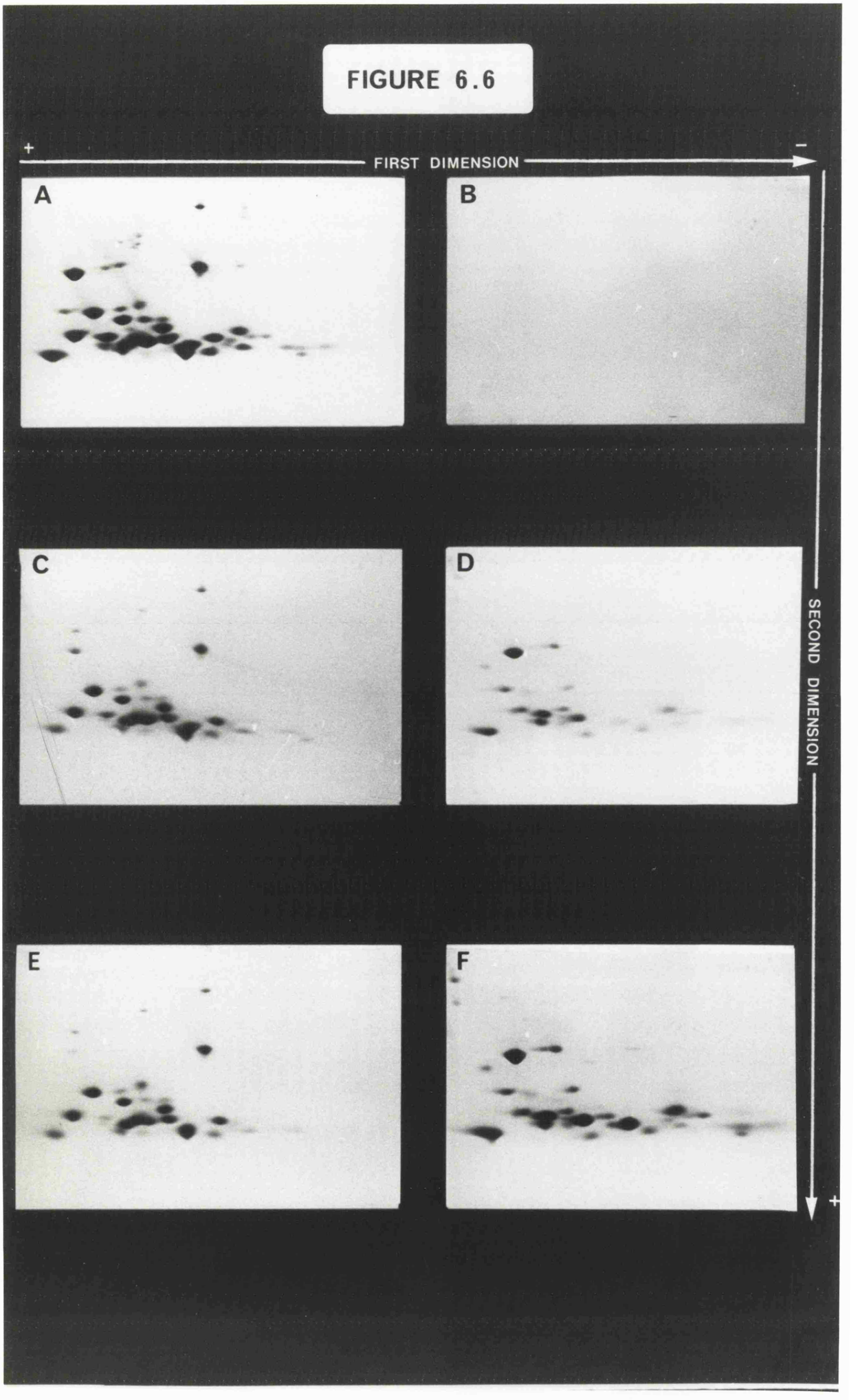
C : proteins from 1M core particle (0.2 A_{230} units)

D : proteins from 1M split protein fraction (0.2 A_{230} units)

E : proteins from 2M core particle (0.2 A_{230} units)

F : proteins from 2M split protein fraction (0.2 A_{230} units)

FIGURE 6.6



3.5 The Possible Loss Of Ribosomal Protein (s) From Vernamycin A-Sensitive 50S Ribosomal Subunits To establish whether the vernamycin A-induced removal of a ribosomal protein from reconstituted 50S ribosomal subunits was, in essence, the mode of action of the drug, 50S ribosomal subunits from both *E. coli* and *S. coelicolor* were treated according to Chapter 6.2.1. Following treatment, ribosomal proteins were electrophoresed (according to Chapter 5.2.4) and the results presented in Figure 6.7. It must be stressed at this point that these results were, to an extent, inconsistent. However, they are presented since no other rational explanation for the effects observed could be found (for example, failure of ribosomal proteins to enter the gel). Accordingly, exposure of native 50S particles to vernamycin A resulted in a reduction in the intensity of an acidic ribosomal protein from *S. coelicolor*, and a "smearing" of two acidic ribosomal proteins from *E. coli*. However, on other occasions, a smearing of the spot from *S. coelicolor* was apparent (rather than a reduction in its intensity) while the two proteins from *E. coli* were found in reduced intensity (data not presented).

Legend to Figure 6.7

Effect of vernamycin A on native 50S ribosomal subunits from *S. coelicolor* and *E. coli*, as judged by two-dimensional gel electrophoresis.

50S ribosomal subunits were treated with vernamycin A (according to Chapter 6.2.1). Subsequently, ribosomal proteins were extracted from the particles (according to Chapter 5.2.3) and subjected to two-dimensional gel electrophoresis (according to Chapter 5.2.4).

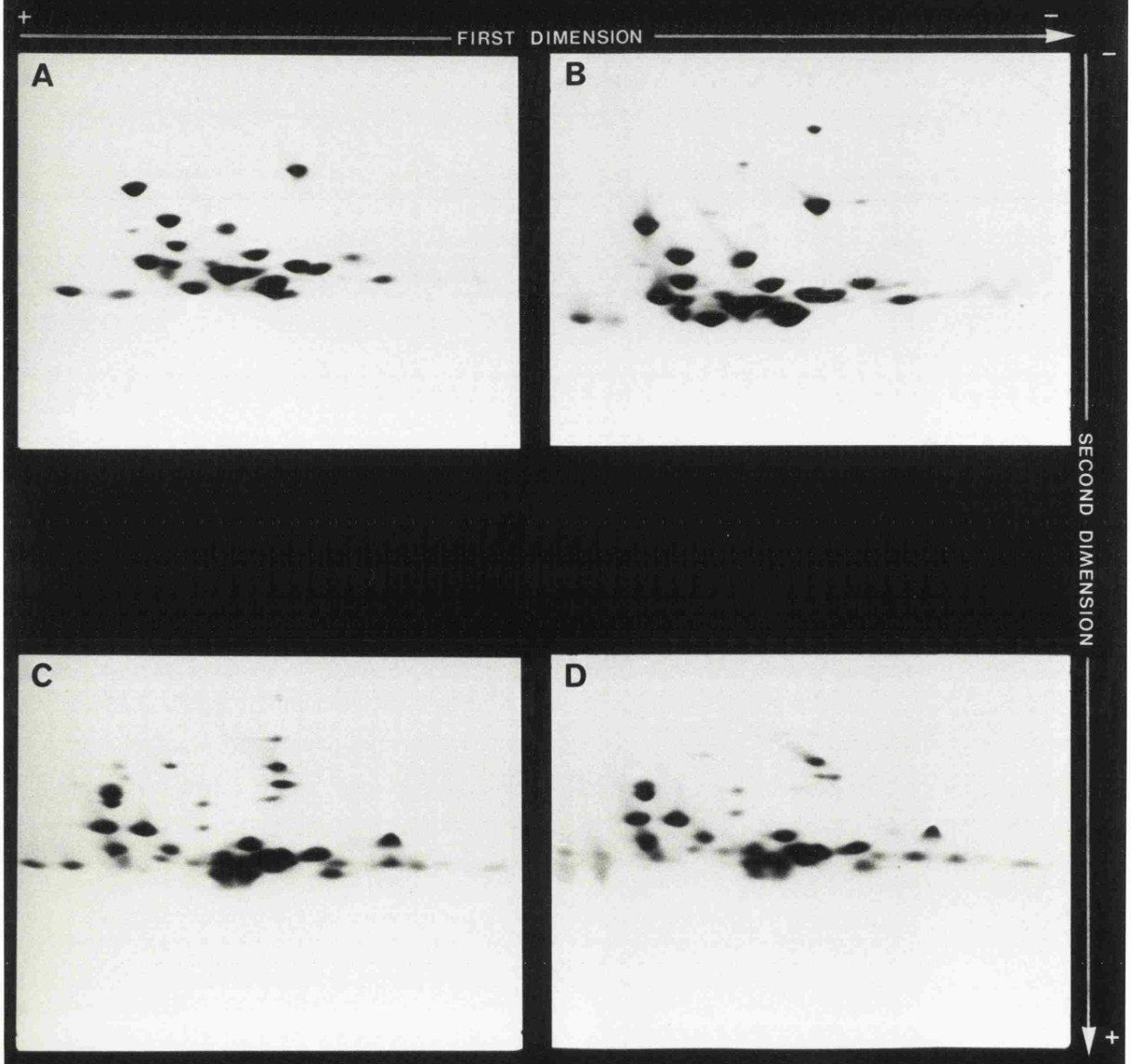
A : proteins from *S. coelicolor* 50S particles (0.2 A_{230} units) - unexposed to vernamycin A

B : proteins from *S. coelicolor* 50S particles (0.2 A_{230} units) - exposed to vernamycin A

C : proteins from *E. coli* 50S particles (0.2 A_{230} units) - unexposed to vernamycin A

D : proteins from *E. coli* 50S particles (0.2 A_{230} units) - exposed to vernamycin A.

FIGURE 6.7



4. Discussion

Contrary to expectation, reconstituted 50S ribosomal subunits from *S. loidensis* were found to be sensitive to vernamycin A *in vitro*. This was a surprising observation and implied that sensitivity to vernamycin A might be the result of either the absence of a component from the reconstituted ribosomal subunit, necessary for vernamycin A-resistance (e.g. through loss by prior dialysis of TP 50 fraction or through inability of the component to reconstitute), or alternatively, a change in ribosomal subunit conformation. Since this latter possibility was somewhat intangible, attention was focussed upon the former possibility. Accordingly, a series of experiments were designed in which either concentrated dialysates of TP 50 or ribosomal washates were added to ribosomal components during reconstitution of 50S ribosomal subunits. However, on no occasion did a change in response of reconstituted particles to vernamycin A result.

The response of ribosomes to antibiotics in *in vitro* translation systems can depend, to an extent, upon the composition of the incubation mixture. Hence, Likover and Kurland (1967) observed that ribosome-based streptomycin dependence in mutants of *E. coli* could only be observed (reproducibly) *in vitro*, when assayed in the presence of calcium ions. Accordingly, reconstituted *S. loidensis* 50S particles were assayed in the presence and absence of vernamycin A in a modified poly (U)-directed protein-synthesising system (containing calcium ions). Similarly, to test the response of reconstituted particles to drug in a cell-free system resembling (in certain respects) a natural messenger RNA-directed system, a poly (U,G)-directed protein-synthesising system was utilised. However, neither system revealed a change in response of the reconstituted particles to vernamycin A (data not presented).

Clearly even native 50S particles from S. loidensis could be rendered sensitive to vernamycin A when assayed under appropriate conditions (i.e. in the presence of native E. coli 30S particles - see Chapter 3). Accordingly, sensitivity of S. loidensis 50S particles to vernamycin A could be observed without the need for prior reconstitution.

Theoretically, for vernamycin A to inhibit ribosome function in a catalytic fashion as suggested by Parfait and Cocito (1980), it must necessarily inflict an irreversible modification to the ribosome. Logically, this could be achieved by inducing the displacement of a key ribosomal protein from the ribosome (i.e. one that is involved in ribosome function). If this ribosomal protein displacement was followed by a rapid conformational change in the ribosome then re-binding of the ribosomal protein could be prevented. Furthermore, the conformational change could also displace bound vernamycin A (so that its action could be repeated elsewhere).

To test this hypothesis, 50S ribosomal subunits from S. loidensis were exposed to vernamycin A and then centrifuged through a sucrose density shelf. Ribosomal proteins from the pelleted ribosomal subunits were then analysed by 2-D gel electrophoresis and compared with suitable controls. In support of the above hypothesis, reconstituted 50S particles were found to be deficient in one acidic ribosomal protein. Although conclusive proof of identity of the missing protein was not established (i.e. by immunological techniques) ribosomal proteins were re-analysed by gel electrophoresis at pH 8.6 (at which pH, acidic ribosomal proteins are well-resolved). Results suggested the missing protein co-electrophoresed with E. coli ribosomal protein L7/L12. Interestingly, ribosomal protein L7/L12 has been implicated in the ribosomal-binding site of initiation factor IF-2, elongation factors EF-G and EF-Tu and the release factors RF-1 and

RF-2. (Möller, 1974; Stöffler and Wittman, 1977). Accordingly, its absence would necessarily exert a profound effect upon ribosome function; for example L7/12-deficient ribosomes do not hydrolyse GTP.

It was clearly of interest to establish whether the vernamycin A-induced displacement of a ribosomal protein from reconstituted 50S ribosomal subunits was in essence, the mechanism of action of the drug. To test this hypothesis, native 50S ribosomal subunits from both E. coli and S. coelicolor were exposed to vernamycin A and processed as before. However, inconsistent results were obtained. In general terms, a vernamycin A-dependent effect upon certain acidic ribosomal proteins of both E. coli and S. coelicolor was observed on most occasions. This effect manifested itself either by the absence of one or more ribosomal proteins or alternatively by the "smearing" of the same ribosomal proteins within the gel. The reasons for such inconsistencies are unclear, but may be due in part, to the experimental method. The methodology employed appeared to weigh heavily in favour of vernamycin A interacting only poorly with 50S ribosomal subunits. For example, the methodology involved centrifugation of 50S particles exposed to vernamycin A through a sucrose density shelf; Cocito and Di Giambattista (1978) showed ultracentrifugation to alter the equilibrium between vernamycin A and ribosomes. Similarly, ribosome-bound vernamycin A could be efficiently displaced by centrifugation through a sucrose density gradient (Parfait and Cocito, 1980). Other factors, for example low temperatures, may also promote vernamycin A-displacement from 50S ribosomal subunits. Hence, conditions may not have favoured ribosomal protein displacement from 50S ribosomal subunits. That such an effect was reproducible with reconstituted 50S particles may have been simply a reflection of weaker RNA:protein or protein:protein interactions within reconstituted 50S ribosomal subunits. Accordingly, to confirm

(or otherwise) the mechanism of action of vernamycin A on native 50S ribosomal subunits, an alternative approach will probably have to be taken in the future.

Since the relevant acidic protein could not be displaced from native S. loidensis 50S particles by vernamycin A, on any occasion (although native E. coli 30S particles may be able to do so) the mechanism of resistance to vernamycin A could involve an unusually strong interaction of that protein with the adjacent ribosome. This would imply that as the integrity of the protein within the 50S ribosomal subunit was diminished, so a corresponding increase in sensitivity to vernamycin A (following reconstitution with TP 50) would result. To test this hypothesis, various ribosomal proteins were removed from native S. loidensis 50S ribosomal subunits by lithium chloride-extraction. Following reconstitution of 50S particles from core particles and TP 50, the response of the reconstituted 50S particles to vernamycin A was tested in a poly (U)-directed protein-synthesising system. Results suggested that as the integrity of the 50S ribosomal subunit diminished, so the particles became increasingly sensitive to vernamycin A. Furthermore, analysis of ribosomal proteins from 0.4, 1.0 and 2.0M cores and splits suggested a loss of the relevant acidic protein (and certain other ribosomal proteins) from 1.0 and 2.0M cores into corresponding split protein fractions. However, a causal relationship between the loss of the ribosomal protein and a corresponding increase in vernamycin A-sensitivity has not been established.

In conclusion, some of the results presented in this chapter are tentative and consequently open to debate. However, the possible displacement of ribosomal protein (s) from native 50S ribosomal subunits

by vernamycin A is worthy of further investigation since, if proven, could represent a totally novel mechanism of antibiotic action. Furthermore, such a mechanism could account for the irreversible effect of vernamycin A upon 50S ribosomal subunits observed by Parfait and Cocito (1980) and Parfait *et al.*, (1981). Fortunately, the presence of ribosomal protein L7/L12 on the ribosome not only appears ubiquitous (Marquis *et al.*, 1981) but is also readily apparent as a stalk-like appendage when viewed under the electron microscope. Consequently, a combination of both electron microscopic and immunological approaches may be the simplest way of solving the problem.

No firm conclusions can be drawn regarding the mechanism of resistance to vernamycin A in *S. loidensis* but it may involve an enhanced affinity of a protein, homologous with *E. coli* L7/L12, for the 50S ribosomal subunit. Clearly the resistance mechanism is subtle and the ambiguity of the results may be a direct reflection of the limitations of the reconstitution procedure - where expression of antibiotic resistance may be dependent upon the overall integrity of the 50S ribosomal subunit. Accordingly, total reconstitution procedures may be unsuitable in these types of circumstances. However, as will be shown in Chapter 7, conclusive results can be obtained from the total reconstitution procedure when examining definitive ribosomal RNA-based antibiotic-resistance mechanisms.

CHAPTER 7

RECONSTITUTION OF BOTH 30S AND 50S RIBOSOMAL SUBUNITS FROM STREPTOMYCES COELICOLOR - AN ANALYSIS OF "50S" RECONSTITUTION INTERMEDIATES AND A DEMONSTRATION OF THE EFFICACY OF THE RECONSTITUTION PROCEDURE

1. Introduction

1.1 Reconstitution Of Ribosomal Particles From Streptomyces Coelicolor

Although the 50S ribosomal subunit from S. loidensis had been reconstituted successfully, the general applicability of the procedure (i.e. to other Streptomyces ribosomes) had not been established. Accordingly, an attempt has been made to reconstitute both the 50S and 30S ribosomal subunits of S. coelicolor. This organism was chosen primarily because it has been examined extensively, particularly from a genetic viewpoint (for a review, see Hopwood et al., 1973; Hopwood and Merrick, 1977).

1.2 Correlations Between In Vivo And In Vitro Assembly

An investigation of reconstitution intermediates can yield information regarding similarities between in vivo and in vitro ribosome assembly. Accordingly, as discussed in Chapter 5, Dohme and Nierhaus (1976) found both $R1_{50}(1)$ and $R1^*_{50}(1)$ particles from E. coli to contain 17-18 ribosomal proteins. More recently, similar particles analysed by Tam and Hill (1980) were found to contain 21 ribosomal proteins, but this discrepancy may simply reflect slight differences in methodology. In vivo studies by Pichon et al., (1975) revealed two distinct groups of ribosomal proteins - those involved in early assembly of ribosomal particles and those involved in late assembly. Although some similarities between the "early" proteins and those contained in $R1_{50}(1)$ or $R1^*_{50}(1)$ particles could be observed, there were significant discrepancies. For example, ribosomal proteins L1,L8,L9 and L10 were found present in the $R1_{50}(1)$ particles but not in the "early" group of proteins. However, in contrast, an in vivo study by Nierhaus et al., (1973) did not reveal this discrepancy and suggested that both in vivo and in vitro ribosomal subunit assembly followed similar pathways (at least in the early stages). Supportive

evidence for this conclusion came from the observation that all the essential proteins required for $Rl^*_{50}(1)$ formation (i.e. L4, L13, L20, L22 and L24) could bind to the 5' end of 23S RNA (Roth and Nierhaus, 1980). Since in vivo ribosome assembly occurs in a 5'→3' direction (see Chapter 4) this strongly suggested that the early stages of assembly in vivo and in vitro were similar.

Recently a detailed assembly map of the 50S ribosomal subunit has been described (Roth and Nierhaus, 1980) and it is striking that the sequence with which ribosomal proteins assemble on to ribosomal RNA in vitro is roughly the reverse of the order in which increasing concentrations of lithium chloride displace them (Dohme and Nierhaus, 1976).

In this present study, reconstitution intermediates from S. coelicolor have been investigated by two-dimensional gel electrophoresis.

1.3 Reconstitution Of 30S Ribosomal Subunits The total in vitro reconstitution of 30S ribosomal subunits from E. coli was first achieved by Traub and Nomura (1968) and later confirmed, using purified components, by Held et al., (1973). Successful reconstitution required only a single-step reconstitution procedure.

In contrast to the in vitro assembly of 50S ribosomal subunits, reconstitution of 30S particles proceeded via only two reconstitution intermediates, designated Rl_{30} and Rl^*_{30}. Notably, Rl_{30} particles were found to be very similar to particles isolated in vivo (P_1 30S particles) with respect to both ribosomal protein content and sedimentation coefficient (Held and Nomura, 1973; Nomura and Held, 1974).

Studies of the conformation of 16S RNA in both the 21S reconstitution

intermediate and its heat-activated form (from E. coli) have revealed that as reconstitution proceeded, so the conformation of the 16S RNA alternated until ultimately the final conformation was stabilised (Dunn and Wong, 1979). In contrast, naked 16S RNA appeared to adopt a conformation in reconstitution buffer not unlike that found in native 30S ribosomal subunits (Allen and Wong, 1978). Certain ribosomal proteins also appeared to undergo discrete conformational changes of secondary structure at the optimal temperature of reconstitution (Lemieux et al., 1974). Accordingly, while the final conformation of the 30S particle may be a compromise between the various RNA: protein, protein: protein interactions, ultimately ribosomal proteins may stabilise the 16S RNA conformation (Allen and Wong, 1978). Like 50S ribosomal subunit assembly, 30S particles are probably assembled on precursor 16S RNA in vivo (Adesnik and Levinthal, 1969; Lindahl, 1973). Such RNA is undermethylated and contains excess oligonucleotides at both the 5' and 3' ends (Wireman and Sypherd, 1974). Trimming of the precursor may occur late in the assembly process (Bryant, 1973) since certain assembly-defective mutants contain precursor 16S RNA in their "30S" particles.

Surprisingly, studies of in vitro reconstitution using precursor 16S RNA as a substrate have revealed that although an homogenous group of reconstituted particles could be obtained, all were deficient in ribosomal protein S3 (Wireman and Sypherd, 1974). Such particles were unable to bind phe-tRNA and 50S ribosomal subunits, but could bind poly (U) (but were inactive in polyphenylalanine synthesis).

Evidently, all the information required for reconstitution of active 30S ribosomal subunits is contained within the primary sequence of mature 16S RNA. Total heat-denaturation of 16S RNA followed by

reconstitution with TP 30 into particles active in protein synthesis has been demonstrated by Barritault et al., (1979).

Surprisingly, mature 16S RNA lacking 160 nucleotides from the 3'-terminal end of the molecule can still act as a substrate for reconstitution and yield particles which not only contain a full complement of ribosomal proteins but which are also active in poly-phenylalanine synthesis and poly (AUG)-dependent binding of fMet-tRNA (Zagórska et al., 1980).

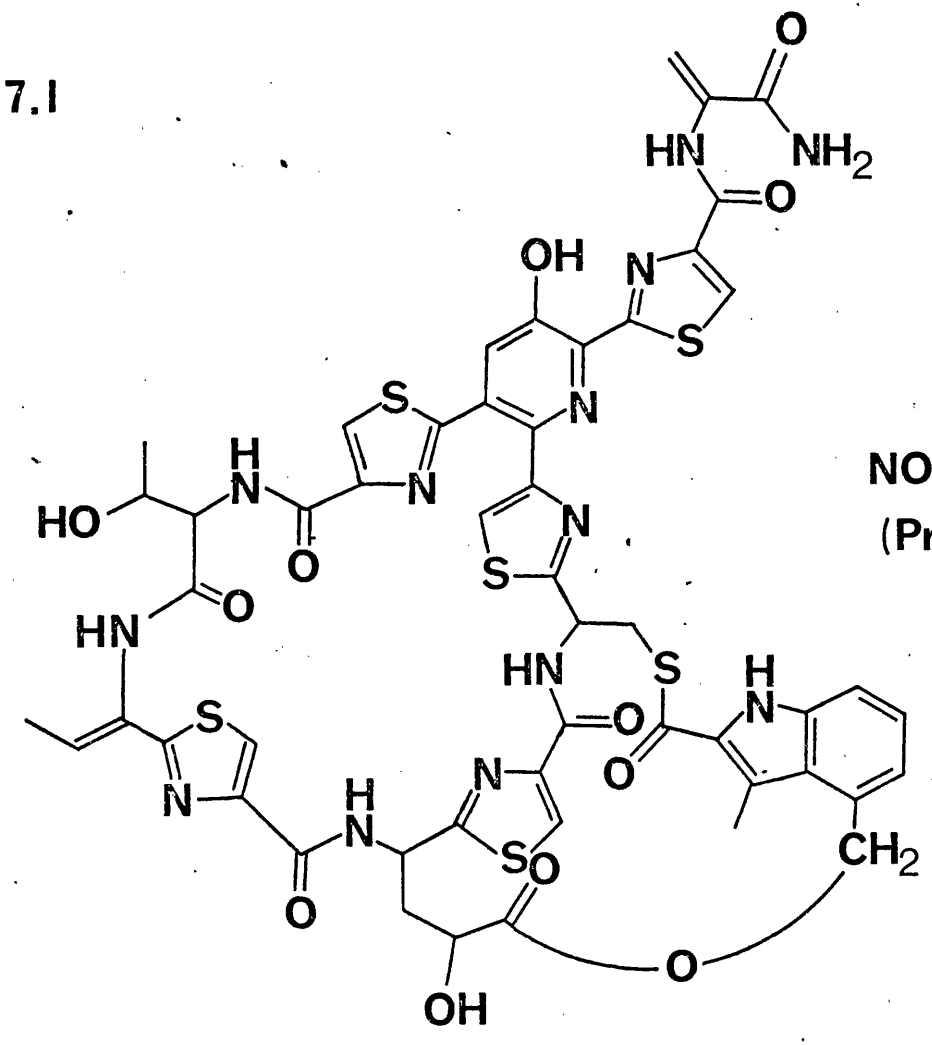
In the present study, the 30S ribosomal subunit of S. coelicolor has been reconstituted from mature 16S RNA and 30S ribosomal proteins. The methodology employed was a modification of the procedure outlined for the reconstitution of 50S ribosomal subunits of S. coelicolor.

1.4 Resistance To Nosiheptide In The Producing Organism Streptomyces actuosus The sulphur-containing antibiotic nosiheptide (multhiomycin) produced by Streptomyces actuosus inhibits protein synthesis both in vivo and in vitro (Tanaka et al., 1970). The drug partially inhibits both the enzymic binding of phe-tRNA to [ribosome-poly (U)] complexes (Tanaka et al., 1971 b; Cundliffe and Thompson, 1981) and concommitant GTP hydrolysis (Tanaka et al., 1971 b). Although the EF-G-dependent hydrolysis of GTP occurring during the translocation step of polypeptide chain elongation is only poorly inhibited by nosiheptide, formation of [ribosome-EF-G-guanine nucleotide] complexes (in the presence of fusidic acid) is potently inhibited by the drug (Cundliffe and Thompson, 1981). Finally, nosiheptide inhibits the formation of "magic spot" compounds (i.e. guanine penta- and tetraphosphates) in response to stringent factor, messenger RNA and deacylated tRNA (Cundliffe and Thompson, 1981).

The structures of both nosiheptide and thiostrepton are very similar (see Figures 7.1 and 7.2). Thiostrepton inhibits all the partial reactions of protein synthesis outlined above, but in a more potent manner than nosiheptide. Like thiostrepton, nosiheptide binds exclusively to 50S ribosomal subunits (Cundliffe and Thompson, 1981) and may share with thiostrepton similar, if not identical, binding sites. In support of this notion, both S. actuosus and S. azureus (the thiostrepton-producing organism) appear to share a common antibiotic-resistance mechanism. Accordingly, both organisms are resistant to their respective toxic products in vivo (see Figure 7.13 and Cundliffe, 1978) while their respective ribosomes are cross-resistant to both nosiheptide and thiostrepton in vitro (Cundliffe and Thompson, 1981). Since the mechanism of resistance to thiostrepton involves specific methylation of 23S RNA (Cundliffe, 1978; Cundliffe and Thompson, 1979) by a SAM-dependent methylase (Thompson and Cundliffe, 1981) the inability of 23S RNA from S. actuosus to act as a substrate for the methylase suggested a similar mechanism of drug resistance. This was confirmed by Cundliffe and Thompson (1981) who further showed that S. actuosus S100 (in the presence of SAM) could render E. coli ribosomal core particles resistant to thiostrepton (following reconstitution with split proteins). An analysis of 23S RNA from S. coelicolor following exposure to S. actuosus S100 and SAM revealed the presence of 2'-O-methyladenosine (a residue found also in S. azureus 23S RNA).

Although all the available evidence strongly suggested that methylation of ribosomal RNA conferred resistance to nosiheptide, it had not been possible to show unequivocally that rRNA from S. actuosus could confer resistance to the drug. Using the reconstitution procedure outlined in Chapter 4, the role of S. actuosus ribosomal RNA in determining resistance to nosiheptide has been established.

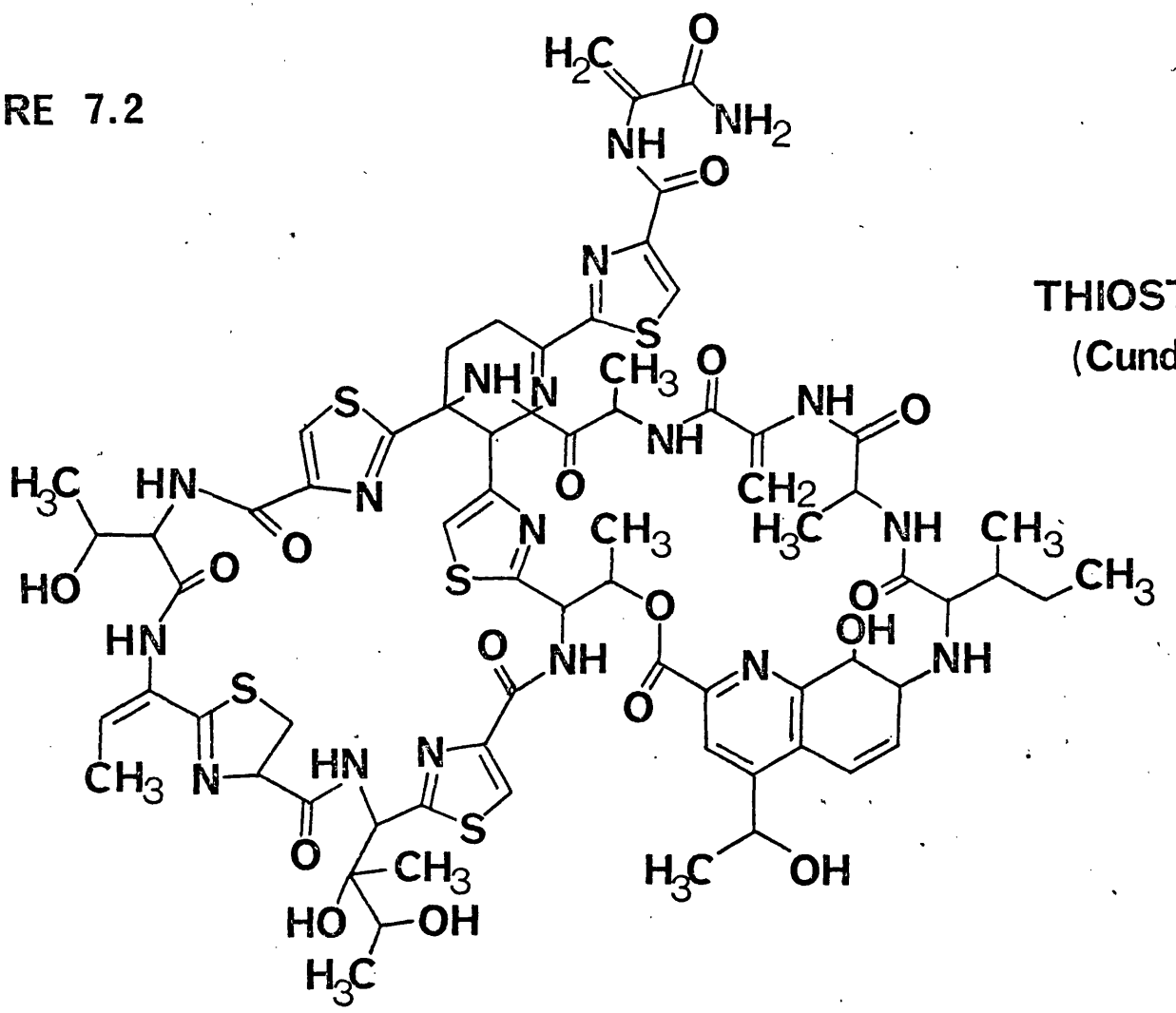
FIGURE 7.1



NOSIHEPTIDE

(Prange et al.,1977)

FIGURE 7.2



THIOSTREPTON
(Cundliffe, 1981)

2. Materials And Methods

2.1 Preparation Of Materials From S. coelicolor Ribosomes And Ribosomal Subunits Ribosomal RNA and ribosomal proteins were prepared according to Chapter 2.2.6. RNA 30 (i.e. 16S RNA) was always prepared from fresh 30S ribosomal subunits.

2.2 Summary Of The Procedure For Reconstitution Of RNA 70 And TP 50 From S. coelicolor RNA 70 was added to a buffer such that, upon addition of TP 50, the final ionic conditions during the first step of the reconstitution procedure were: 30mM Tris-HCl (pH 7.6 at 20°C); 7.5mM magnesium chloride; 425mM potassium chloride; 6mM 2-mercaptoethanol. After 10 minutes at 45°C magnesium chloride was added to a final concentration of 26mM and the temperature increased to 50°C for 3 hours.

Reconstitution mixture (200µl) contained 120 pmol RNA 70 and approximately 150 pmol equivalents of TP 50. Reconstituted particles were recovered by addition of 0.25 volumes 40% (w/v) PEG_{6000}. After 10 minutes at 0°C particles were recovered by centrifugation at 12,000 xg for 5 minutes, and suspended in RS buffer.

2.3 Preparation And Analysis Of Reconstitution Intermediates At intervals throughout the reconstitution procedure, samples of the incubation mixture were withdrawn and layered over equal volumes of 40% (w/v) sucrose containing RS buffer. After centrifugation at 40,000 r.p.m. for 20 hours in a Beckman 75 Ti rotor at 2°C, reconstitution intermediates were resuspended in RS buffer and extracted with acetic acid, according to Chapter 5.2.3. Two-dimensional gel electrophoresis of ribosomal proteins was performed by the method of Mets and Bogorad (1974) as

described in Chapter 5.2.4.

2.4 Summary Of Procedure For Reconstitution Of RNA 30 And TP 30 From *S. coelicolor* RNA 30 was added to a buffer such that, upon addition of TP 30, the final ionic conditions were: 30mM Tris-HCl (pH 7.6 at 20°C); 26mM magnesium chloride; 425mM potassium chloride; 6mM 2-mercaptoethanol. After 20 minutes at 50°C reconstituted particles were precipitated by the addition of 0.25 volumes 40% (w/v) PEG_{6000}. After 10 minutes at 0°C, particles were recovered by centrifugation at 12,000 xg for 5 minutes and resuspended in RS buffer.

Reconstitution mix (200µl) contained 120 pmol RNA 30 and approximately 360 pmol equivalents of TP 30.

2.5 Analytical Ultracentrifugation of Native And Reconstituted 30S Ribosomal Subunits Analytical ultracentrifugation of native and reconstituted 30S ribosomal subunits was performed according to Chapter 5.2.2.

2.6 Resistance Of *S. actuosus* To Nosiheptide In Vivo Antibiotic sensitivity assays were performed according to Chapter 3.2.3.

2.7 Reconstitution Of 50S Particles From Both *S. actuosus* And *S. lividans* Reconstitution of RNA 70 and TP 50 from both *S. actuosus* and *S. lividans* was performed according to methodology described in Chapter 4.2.5.

3. Results

3.1 Optimisation Of The Reconstitution Procedure Using RNA 70 And TP 50 From S. coelicolor

Certain steps of the reconstitution procedure were found to have optimal conditions very similar to those observed in the procedure for S. loidensis 50S particles. Accordingly, only conditions found to differ significantly from those already presented, will be shown and discussed below.

The optimisation of both magnesium concentration and temperature of the first step of the reconstitution procedure is presented in Figure 7.3. Maximal activity of reconstituted particles was observed following reconstitution at 7.5mM magnesium ion concentration and 45°C.

The optimal temperature of the second step of the reconstitution procedure is shown in Figure 7.4. Maximal activity of reconstituted particles was observed following reconstitution at 50°C.

The optimal input ratio of RNA 70:TP 50 was found to be 1:1.75 (Figure 7.5).

3.2 Analysis Of Protein Content Of Reconstitution Intermediates

The results from the analysis of ribosomal protein contents of various reconstitution intermediates are presented in Figure 7.6. For ease of reference, the various proteins were allocated a letter and tabulated accordingly in Table 7.1. While some proteins were clearly absent from certain reconstitution intermediates, others were present in either full or diminished amounts. Accordingly, upon mixing of RNA 70 and TP 50 at 0°C, approximately 19 ribosomal proteins bound tightly to form the first intermediate particle; 7 ribosomal proteins were completely absent from the particle while 3 ribosomal proteins were present in trace amounts.

Legend to Figure 7.3

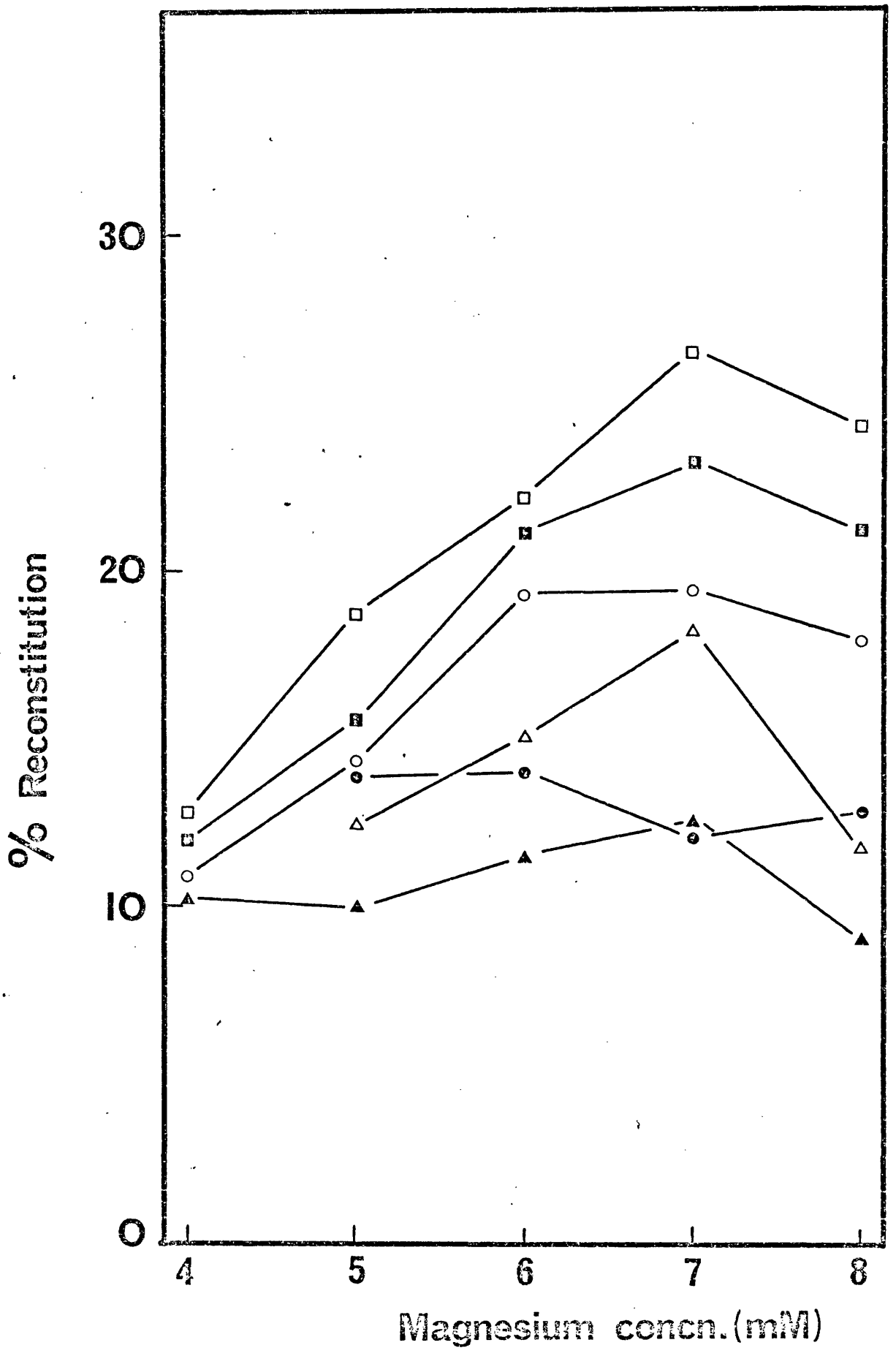
Optimisation of temperature and magnesium ion concentration for the first step of the reconstitution procedure for *S. coelicolor* 50S particles.

RNA 70 and TP 50 from *S. coelicolor* were mixed with buffers such that the ionic conditions were 30mM Tris-HCl (pH 7.6 at 20°C); 425mM potassium chloride; 6mM 2-mercaptoethanol, and magnesium concentrations as indicated. After 10 minutes at the various temperatures indicated, magnesium was added (to 26mM) and reconstitution continued (according to Chapter 7.2.2). The activity of the reconstituted particles was assessed (according to Chapter 2.2.8), following their prior supplementation with 30S particles and S100 from *S. coelicolor*. Results are expressed as a percentage of control incorporation (i.e. by native 50S particles) which was approximately 15 pmol phenylalanine per pmol ribosomes.

Temperature of 1st step of reconstitution procedure (°C)

▲ - ▲	37
○ - ○	40
■ - ■	42
□ - □	45
△ - △	48
● - ●	50

FIGURE 7.3

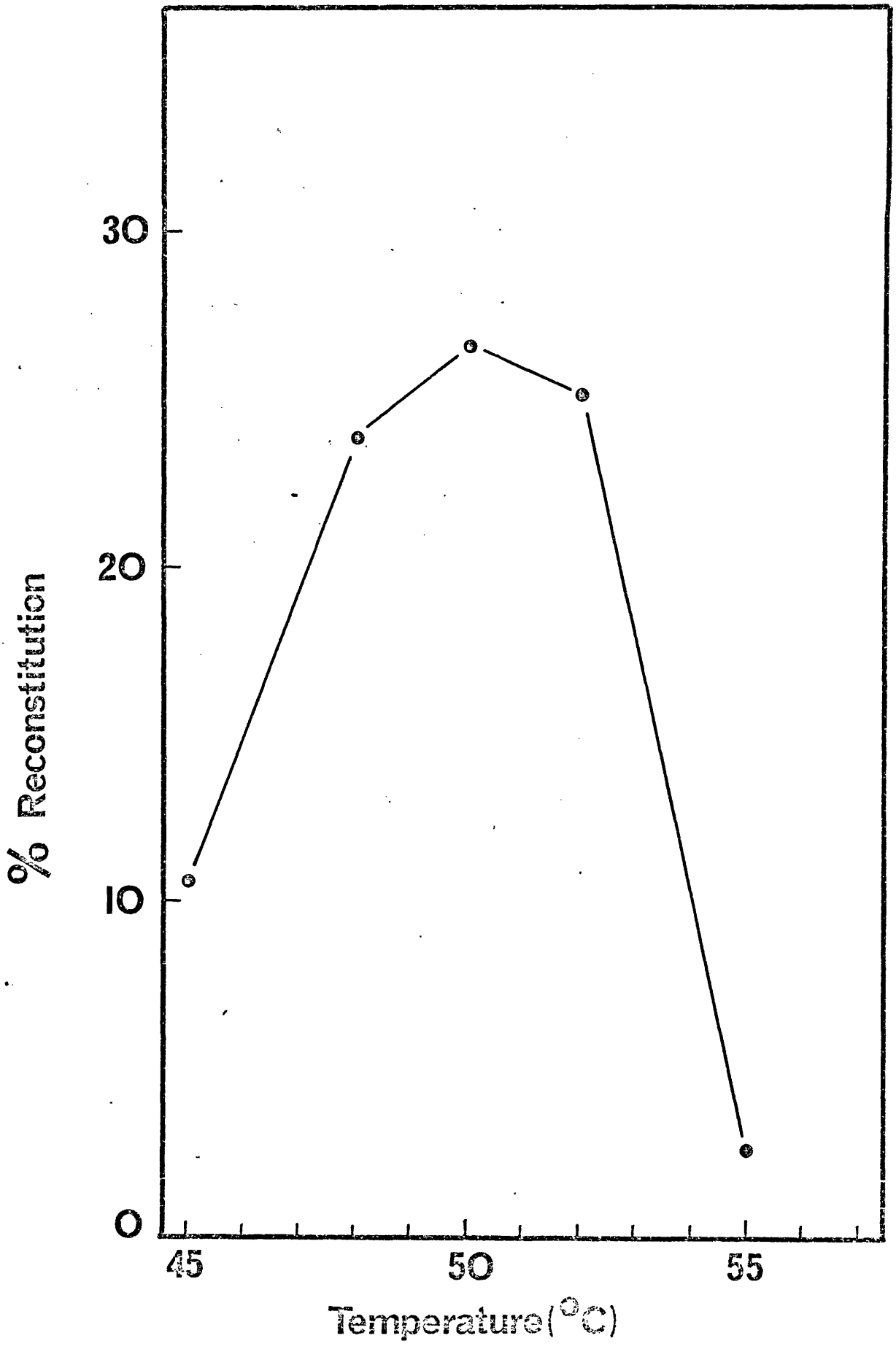


Legend to Figure 7.4

Optimisation of the temperature of the second step of the reconstitution procedure for *S. coelicolor* 50S particles.

RNA 70 and TP 50 from *S. coelicolor* were mixed and incubated under first step conditions (according to Chapter 7.2.2). Subsequently, magnesium was added (to 26mM), samples were removed and incubated for 4 hours at the temperatures indicated. The activity of the reconstituted particles was assessed (according to Chapter 2.2.8) following their prior supplementation with 30S particles and S100 from *S. coelicolor*. Results are expressed as a percentage of control incorporation (i.e. by native 50S particles) which was approximately 15 pmol of phenylalanine per pmol of ribosomes.

FIGURE 7.4

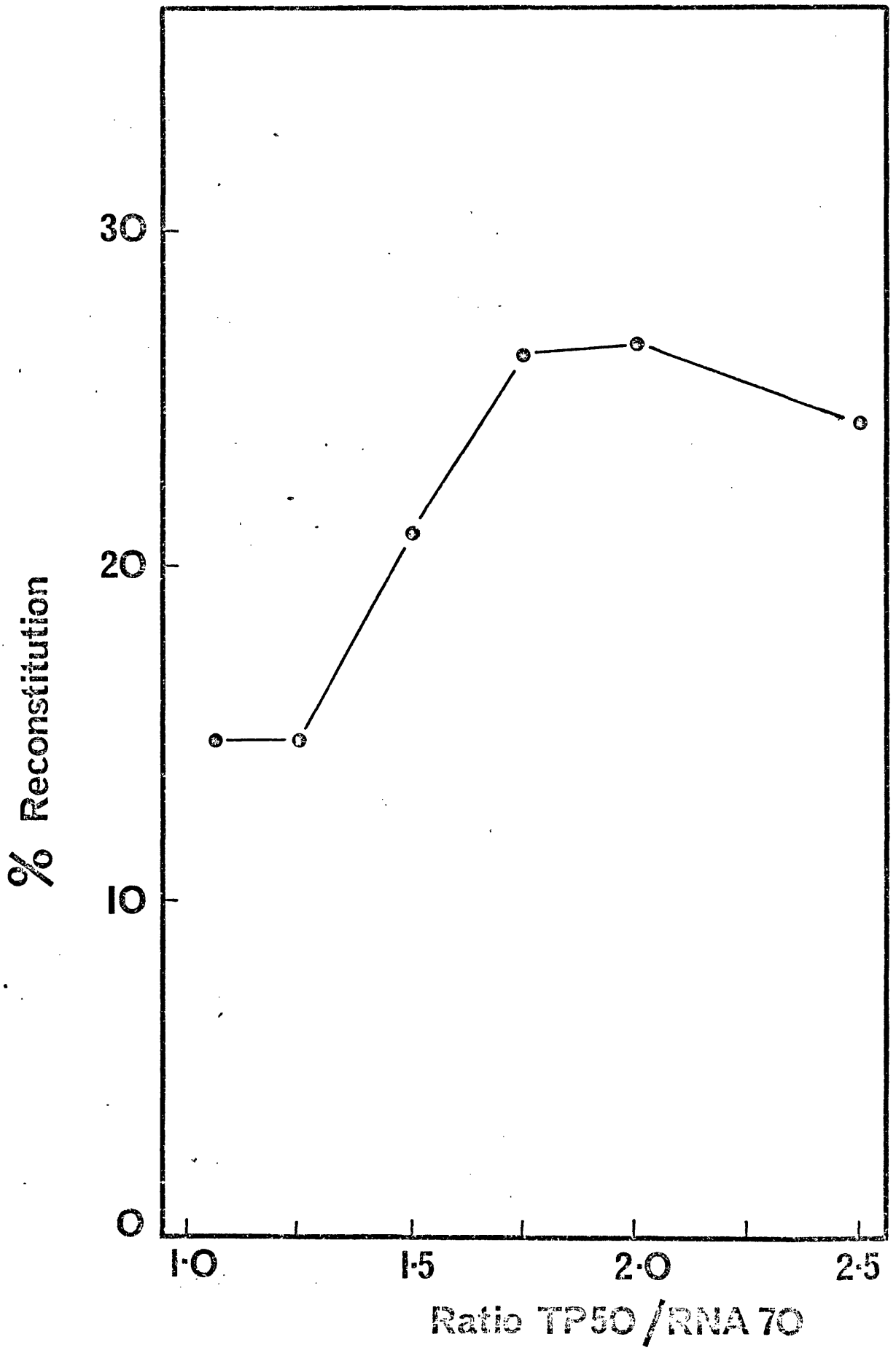


Legend to Figure 7.5

Optimisation of input ratio of RNA 70 : TP 50 in the reconstitution procedure for *S. coelicolor* 50S particles.

RNA 70 and TP 50 were mixed in the molar ratios indicated, and 50S particles reconstituted (according to Chapter 7.2.2). The activity of the reconstituted particles was assessed (according to Chapter 2.2.8) following their prior supplementation with 30S particles and S100 from *S. coelicolor*. Results are expressed as a percentage of control incorporation (i.e. by native 50S particles) which was approximately 15 pmol of phenylalanine per pmol of ribosomes.

FIGURE 7.5



Legend to Figure 7.6

Analysis of ribosomal protein contents of reconstitution intermediates from S. coelicolor, by two-dimensional gel electrophoresis.

50S reconstitution intermediates, prepared according to Chapter 7.2.3 were subjected to analysis by two-dimensional gel electrophoresis (according to Chapter 5.2.4).

A : proteins from particles formed after the mixing (at $0^{o}C$) of RNA 70 and TP 50 (0.2 A_{230} units).

B : proteins from particles formed by the end of the first step of the reconstitution procedure (0.2 A_{230} units).

C : proteins from particles formed after the addition of magnesium (to 26mM) to establish conditions for the second step of the reconstitution procedure (0.2 A_{230} units).

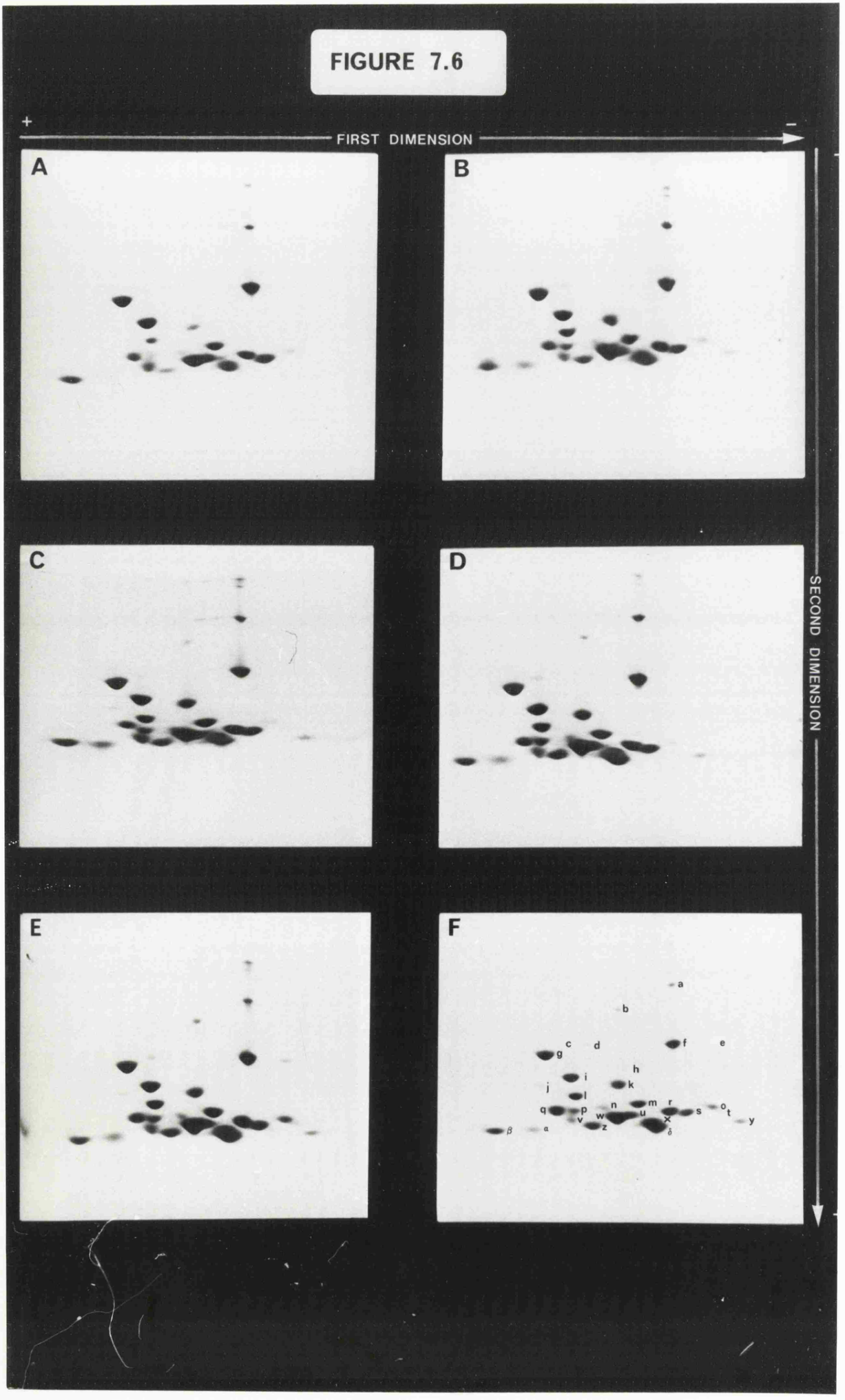
D : proteins from particles formed by the end of the reconstitution procedure (0.2 A_{230} units).

E : proteins from native S. coelicolor 50S particles (0.2 A_{230} units).

F : code letters for ribosomal proteins from S. coelicolor.

Note Certain ribosomal proteins stained only weakly and did not reproduce well in photographs; however, their position has been noted.

FIGURE 7.6


+
FIRST DIMENSION
−
SECOND DIMENSION
A
B
C
D
E
F
a
b
c
d
f
e
g
h
i
j
k
l
m
r
o
q
p
n
s
t
y
w
u
x
v
z
β
α
δ

Legend to Table 7.1

Distribution of 50S ribosomal proteins in reconstitution intermediates from *S. coelicolor*.

Ribosomal proteins, present in various reconstitution intermediates (as indicated in Figure 7.6) were tabulated according to the intensity of their respective spots (as coded in photograph F, Figure 7.6).

+ = protein present in full amount

+/- = protein present in reduced amount

- = protein absent

Track A = protein distribution after mixing of RNA 70 and TP 50 at 0°C.

Track B = protein distribution after 10 min. incubation at 45°C and 7mM magnesium ion concentration .

Track C = protein distribution after increasing the magnesium ion concentration to 26mM.

Track D = protein distribution at the end of the reconstitution procedure (i.e. after 3 hours at 50°C).

TABLE 7.1

CODE LETTER FOR RIBOSOMAL PROTEIN	A	B	C	D
a	+	+	+	+
b	-	+/-	+	+
c	+	+	+	+
d	+	+	+	+
e	+	+	+	+
f	+	+	+	+
g	+	+	+	+
h	+	+	+	+
i	+	+	+	+
j	+	+	+	+
k	+/-	+	+	+
l	+/-	+	+	+
m	+	+	+	+
n	-	-	-	-
o	-	-	-	-
p	-	+	+	+
q	+	+	+	+
r	+	+	+	+
s	+	+	+	+
t	+	+	+	+
u	+	+	+	+
v	+	+	+	+
w	+	+	+	+
x	+	+	+	+
y	-	+	+	+
z	+/-	+	+	+
α	-	+	+	+
β	+	+	+	+
ε	-	+	+	+

After 10 minutes at 45°C, a further 7 ribosomal proteins bound the intermediate tightly, three of which were present in trace amounts in the first intermediate. One ribosomal protein was found in trace amounts in the second intermediate but became fully incorporated when magnesium was added to the reconstitution mixture to establish the second step conditions. Subsequent heating (at 50°C) under second step conditions did not result in the binding of two ribosomal proteins which were present both in native 50S particles and PEG-precipitated reconstituted 50S particles. Accordingly, it must be assumed that these bound to intermediate particles only loosely and were lost during isolation of the particles.

3.3 Reconstitution Of 30S Ribosomal Subunits From S. coelicolor The optimisation of the input ratio of RNA 30:TP 30 is shown in Figure 7.7. Surprisingly, a high input ratio (i.e. 3 or 3.5:1 TP 30:RNA 30) was required for maximum activity of reconstituted particles.

At 50°C, reconstitution of 30S ribosomal subunits from RNA 30 and TP 30 was essentially complete after 20-25 minutes (Figure 7.8). While both RNA 70 and RNA 30 were equally good substrates for reconstitution with TP 30 (Figure 7.9), RNA 30 was employed on a routine basis.

The results from the analysis of ribosomal proteins from both native and reconstituted 30S particles are presented in Figure 7.10. Results indicated that not only did the urea/lithium chloride extraction procedure remove all ribosomal proteins from 30S ribosomal subunits, but that all the proteins could be found on reconstituted 30S particles.

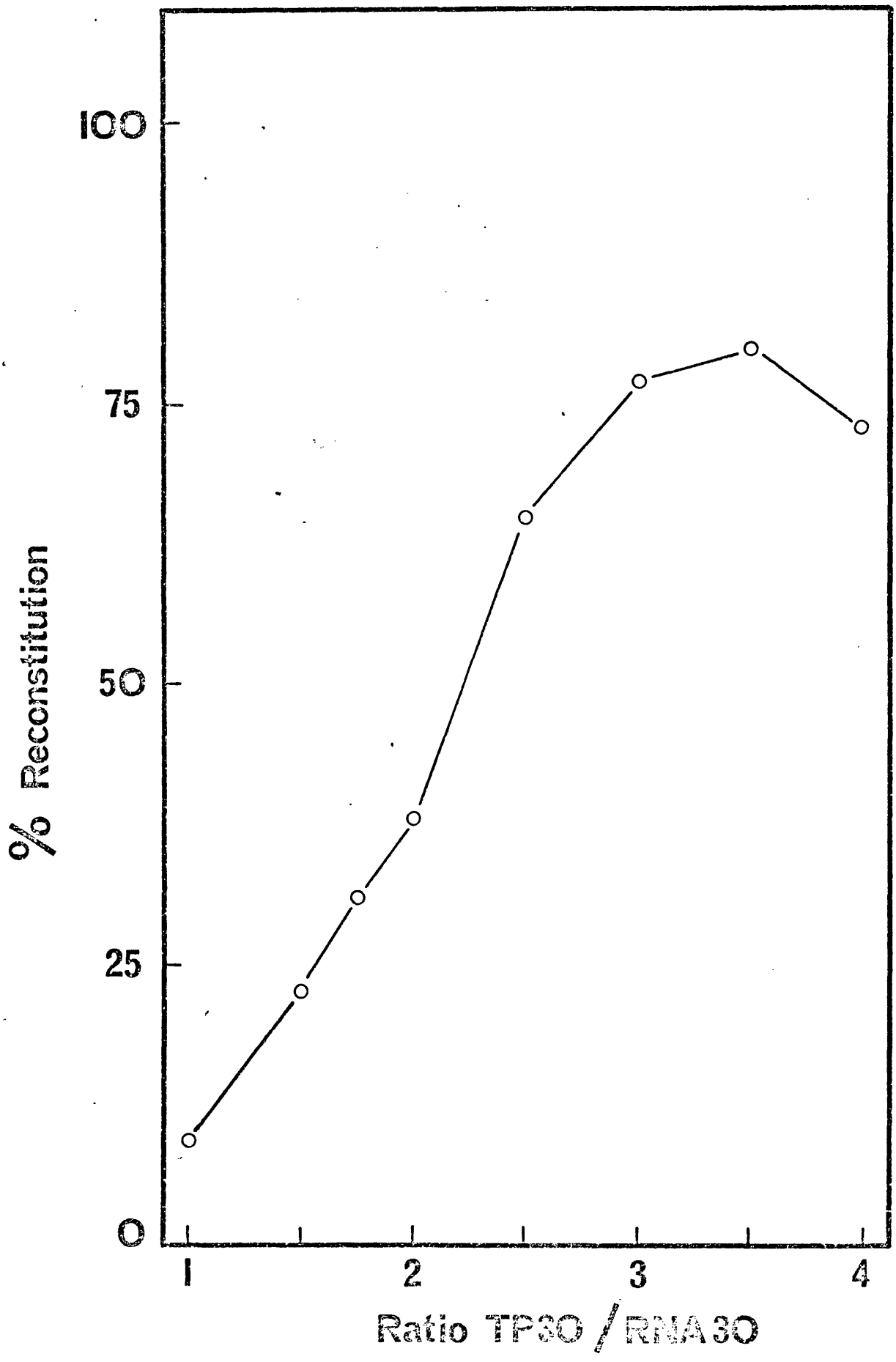
An analysis of the ribosomal RNA from both native and reconstituted 30S ribosomal subunits is presented in Figure 7.11. Although slight

Legend to Figure 7.7

Optimisation of input ratio of RNA 30 : TP 30 in the reconstitution procedure for *S. coelicolor* 30S particles.

RNA 30 and TP 30 were mixed in the molar ratios indicated, and 30S particles reconstituted (according to Chapter 7.2.4). The activity of the reconstituted particles was assessed (according to Chapter 2.2.8) following their prior supplementation with 50S particles and S100 from *S. coelicolor*. Results are expressed as a percentage of control incorporation (i.e. by native 30S particles) which was approximately 15 pmol of phenylalanine per pmol of ribosomes.

FIGURE 7.7

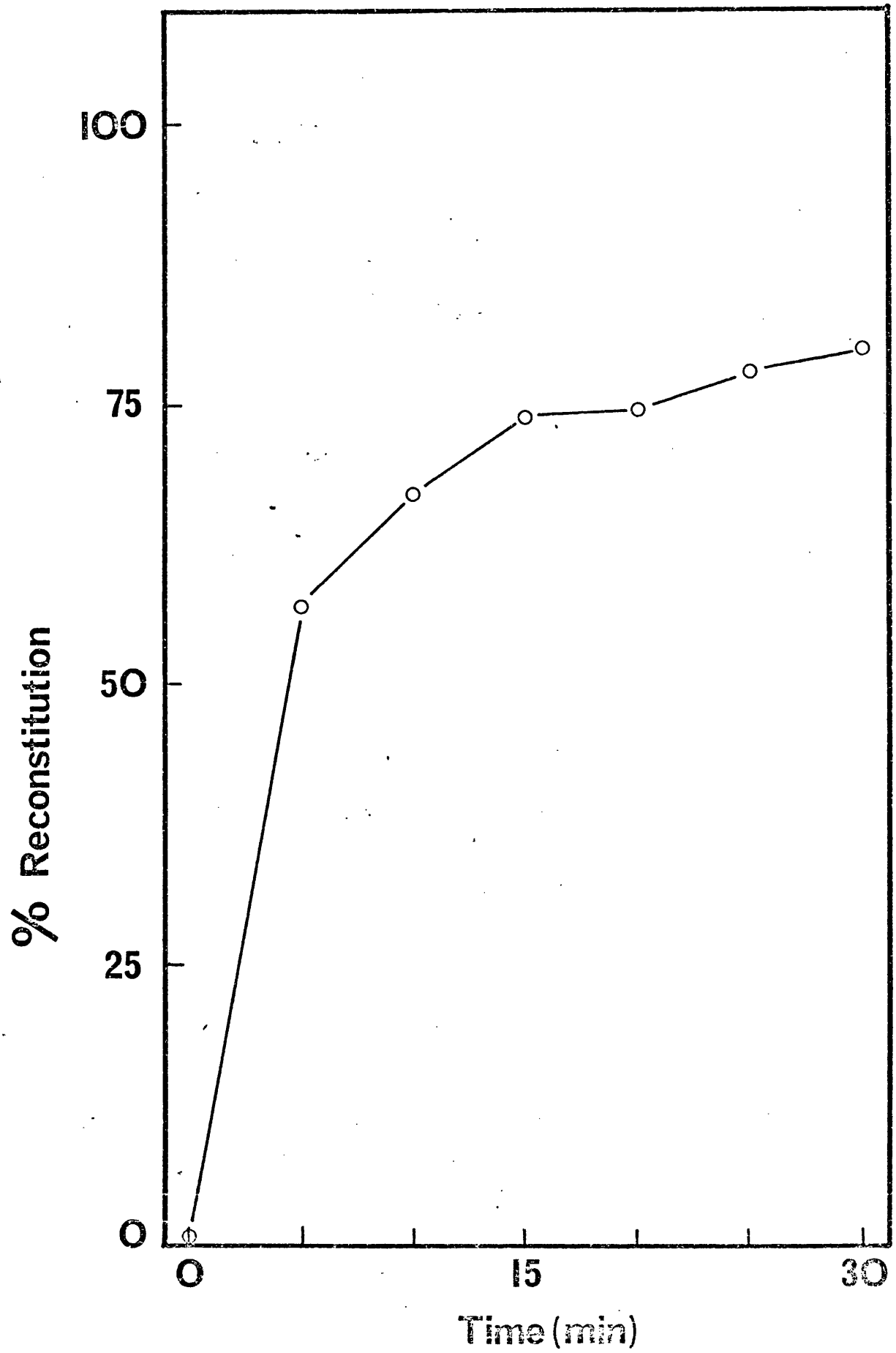


Legend to Figure 7.8

Time course of reconstitution of *S. coelicolor* 30S particles, from RNA 30 and TP 30.

RNA 30 and TP 30 were mixed under optimal ionic conditions and incubated at 50°C. Samples were withdrawn at the times indicated and the activity of the particles assessed (according to Chapter 2.2.8) following their prior supplementation with 50S particles and S100 for *S. coelicolor*. Results are expressed as a percentage of control incorporation (i.e. by native 30S particles) which was approximately 15 pmol of phenylalanine per pmol of ribosomes.

FIGURE 7.8



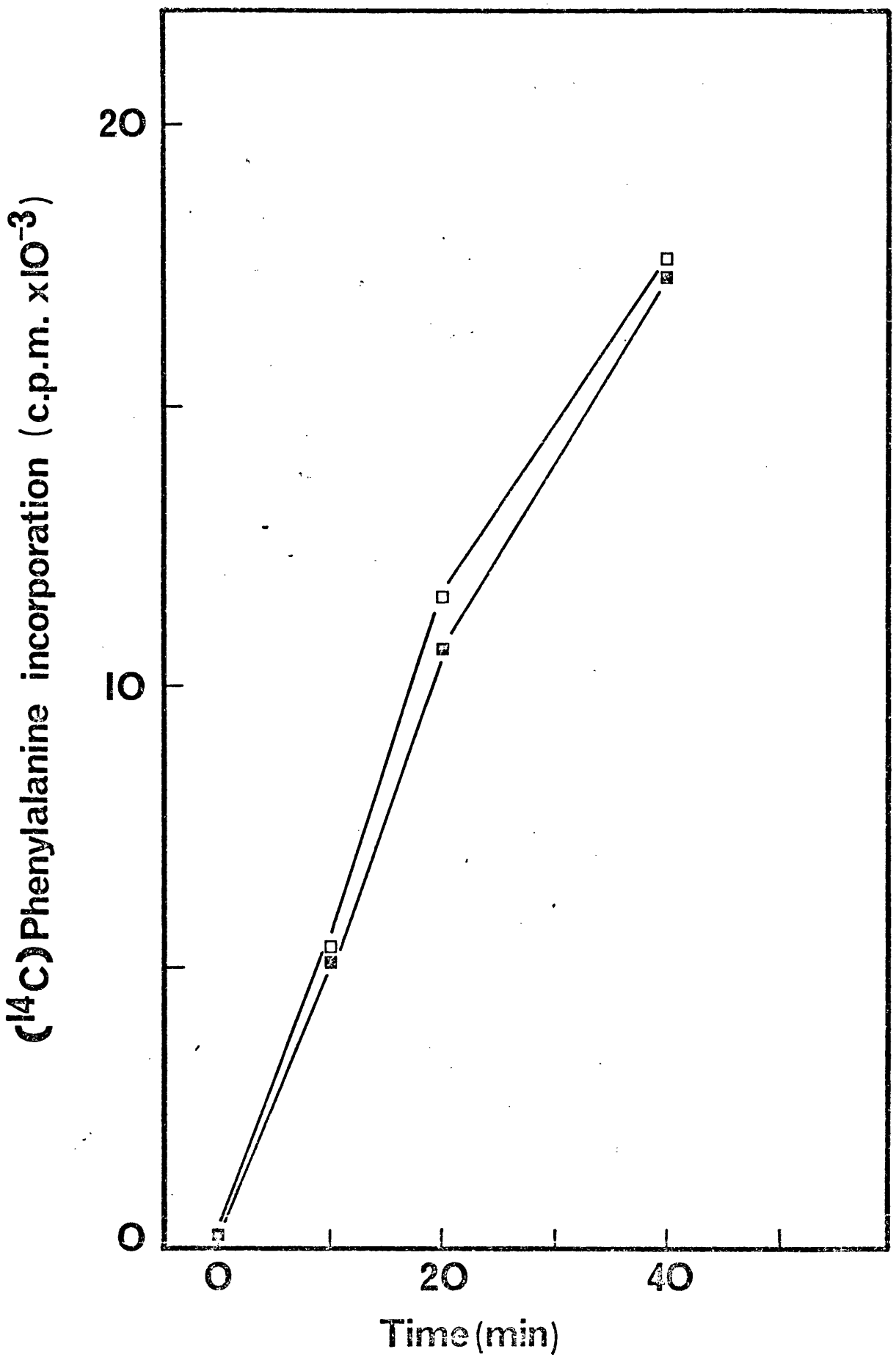
Legend to Figure 7.9

A comparison of RNA 70 (■-■) and RNA 30 (□-□) as substrates for the reconstitution of *S. coelicolor* 30S particles.

30S ribosomal subunits were reconstituted from either RNA 70 or RNA 30 (according to Chapter 7.2.4). The activity of the reconstituted particles was assessed (according to Chapter 2.2.8) following their prior supplementation with 50S particles and S100 from *S. coelicolor*.

2,000 c.p.m. ≡ 1 pmol phenylalanine per pmol of ribosomes.

FIGURE 7.9


20
10
0
0
20
40
Time (min)
(^{14}C) Phenylalanine incorporation (c.p.m. x10^{-3})

Legend to Figure 7.10

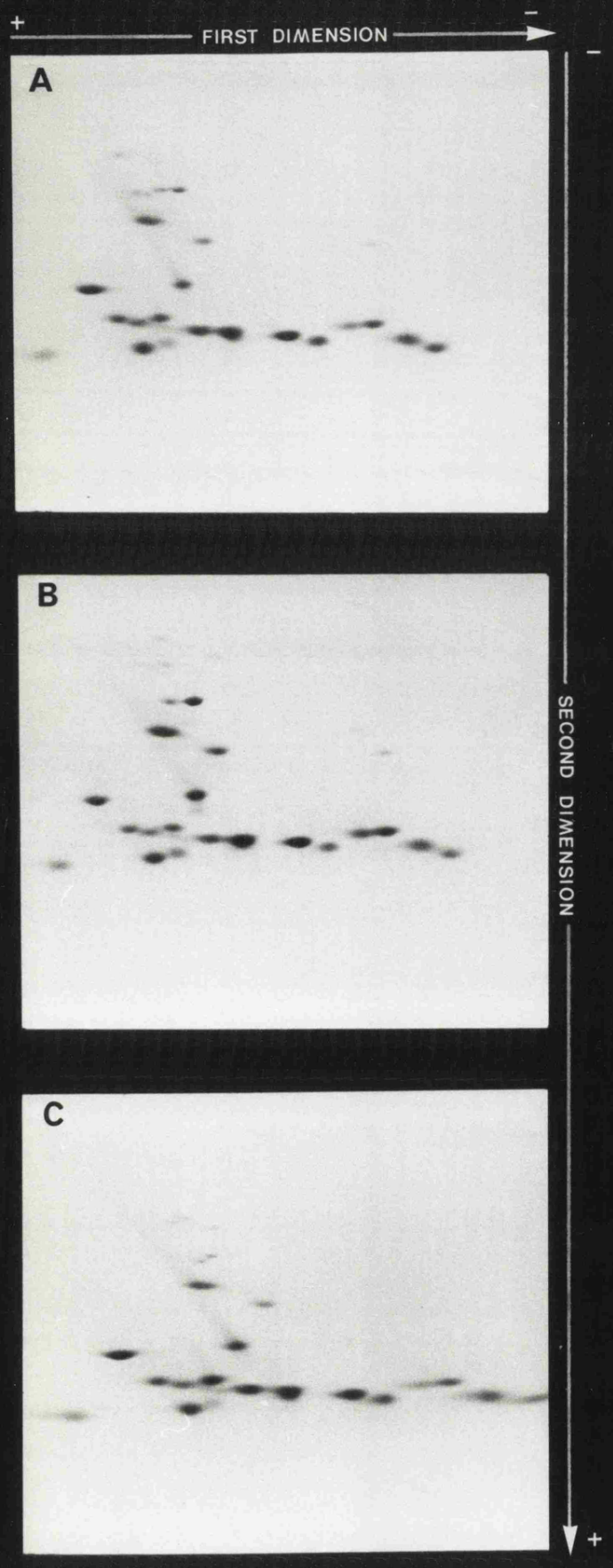
Analysis of ribosomal proteins from *S. coelicolor* 30S particles by two-dimensional gel electrophoresis (according to Chapter 5.2.4).

A : proteins from native *S. coelicolor* 30S particles (0.1 A_{230} units)

B : proteins from reconstituted *S. coelicolor* 30S particles (0.1 A_{230} units)

C : *S. coelicolor* TP 30 (0.1 A_{230} units)

FIGURE 7.10



Legend to Figure 7.11

Analysis of *S. coelicolor* ribosomal RNA by gel electrophoresis under denaturing conditions (according to Chapter 4.2.2).

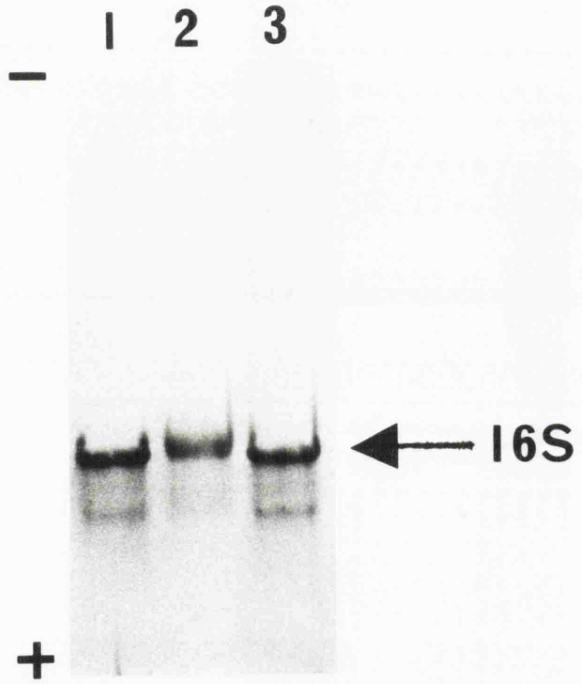
Track

1 : RNA 30 (4μg) - used as a substrate for reconstitution

2 : RNA from native 30S particles (4μg)

3 : RNA from reconstituted 30S particles (4μg)

FIGURE 7.11



degradation of the RNA 30 was apparent prior to reconstitution with TP 30, the actual procedure did not result in further breakdown. No 16S RNA degradation was apparent in native 30S particles.

When examined by analytical ultracentrifugation both native and reconstituted 30S particles sedimented at 30.8±0.2S and 30.1±0.5S respectively (Figure 7.12).

Reconstituted 30S particles were found to have 80-100% of the activity (in polyphenylalanine synthesis) of native 30S particles (when assayed with native 50S particles).

3.4 Resistance To Nosiheptide In S. actuosus The resistance of S. actuosus to nosiheptide in vivo is illustrated in Figure 7.13.

Results indicated that S. actuosus was totally resistant to nosiheptide while significant inhibition of growth of S. lividans, a reference streptomycete was apparent. To establish the nature of the mechanism of resistance to nosiheptide, ribosomes from both S. actuosus and S. lividans were assayed, together with S. lividans S100 in the presence and absence of nosiheptide (20:1, drug: ribosome) in a protein-synthesising system directed by poly (U). Results indicated (data not presented) that the ribosomes from S. actuosus were totally resistant to nosiheptide while ribosomes from S. lividans were completely inhibited.

To establish whether 50S or 30S ribosomal subunits from S. actuosus were resistant to nosiheptide, ribosomal subunits both from S. actuosus and S. lividans were combined, in reciprocal fashion, and tested for their response to nosiheptide in a protein-synthesising system directed by poly (U). Results indicated (Figure 7.14) that the 50S ribosomal subunit of S. actuosus was resistant to nosiheptide.

Legend to Figure 7.12

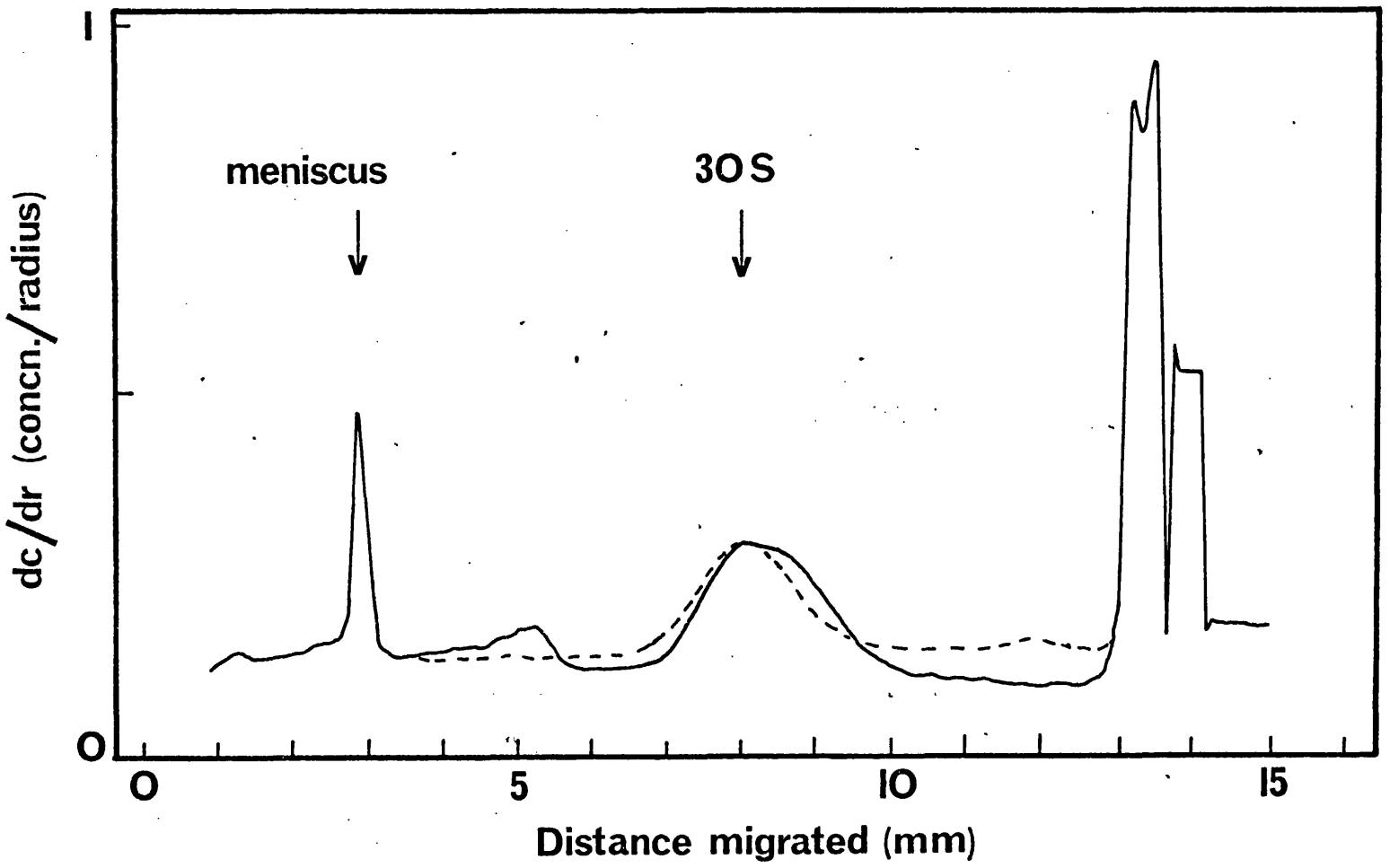
Analysis of native (———) and reconstituted (------) 30S particles from *S. coelicolor* by analytical ultracentrifugation.

Approximately 400µl ribosomal subunits (at a concentration of 2mg/ml - in LM buffer) were loaded into each of the cells of a 6-place rotor (reconstituted particles were isolated from their incubation mix by prior centrifugation). Analytical ultracentrifugation was at 20,000 r.p.m. in an MSE Centriscan 75, at 5°C. After approximately 45 minutes centrifugation, ribosomal subunits boundaries were measured by Schlieren optics. Profiles were aligned according to the positions of the menisci. Sedimentation coefficients were calculated as described in the "Legend to Figure 5.4".

S values : Native 30S particles : 30.8 ± 0.2 S

Reconstituted 30S particles : 30.1 ± 0.5S

FIGURE 7.12


1
0
dc/dr (concn./radius)
meniscus
30 S
0
5
10
15
Distance migrated (mm)

Legend to Figure 7.13

Antibiotic sensitivity assay - response of *S. actuosus* and *S. lividans* to nosiheptide *in vivo* (according to Chapter 7.2.6).

Discs contained:

A : control (i.e. no drug)

B : 1μg nosiheptide

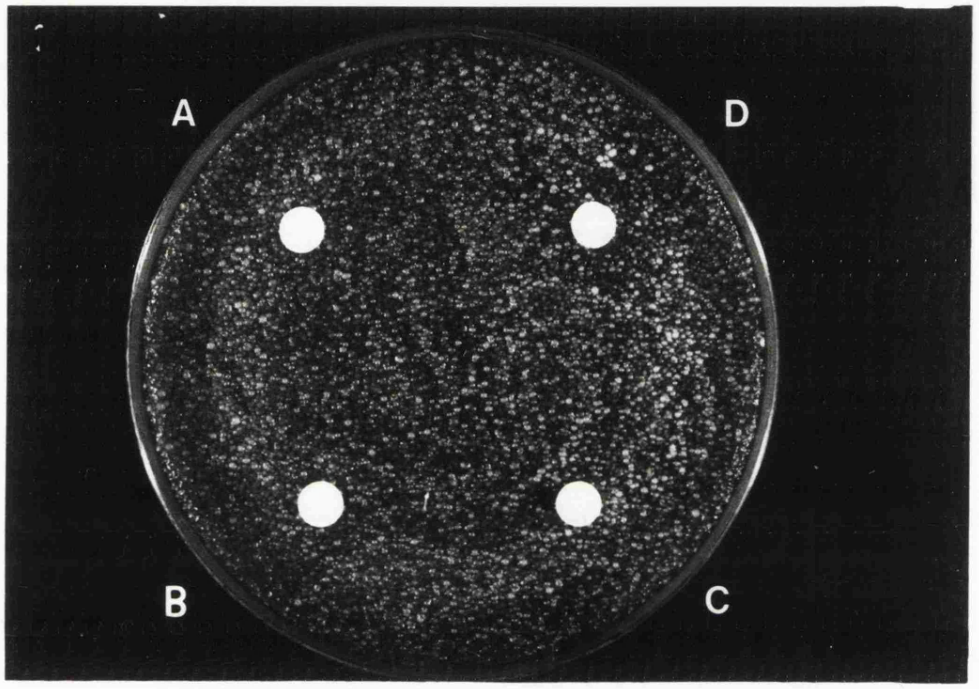
C : 50 μg nosiheptide

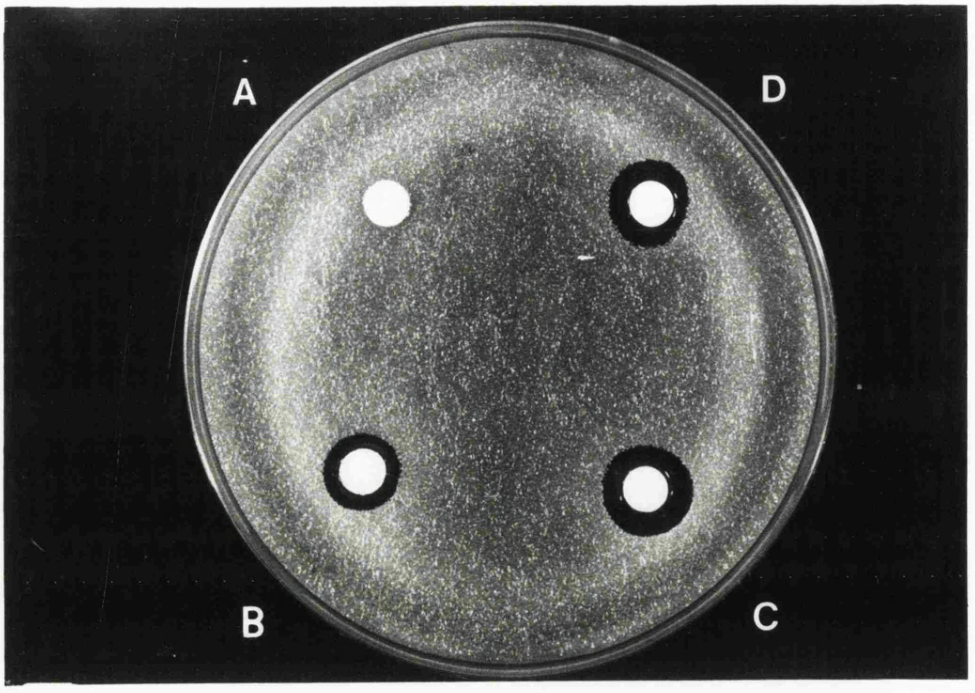
D : 200 μg nosiheptide

Plates were incubated at 30°C for 2 days.

FIGURE 7.13

S.actuosus





S.lividans

Legend to Figure 7.14

Response of reciprocal combinations of ribosomal subunits from *S. actuosus* and *S. lividans* to nosiheptide.

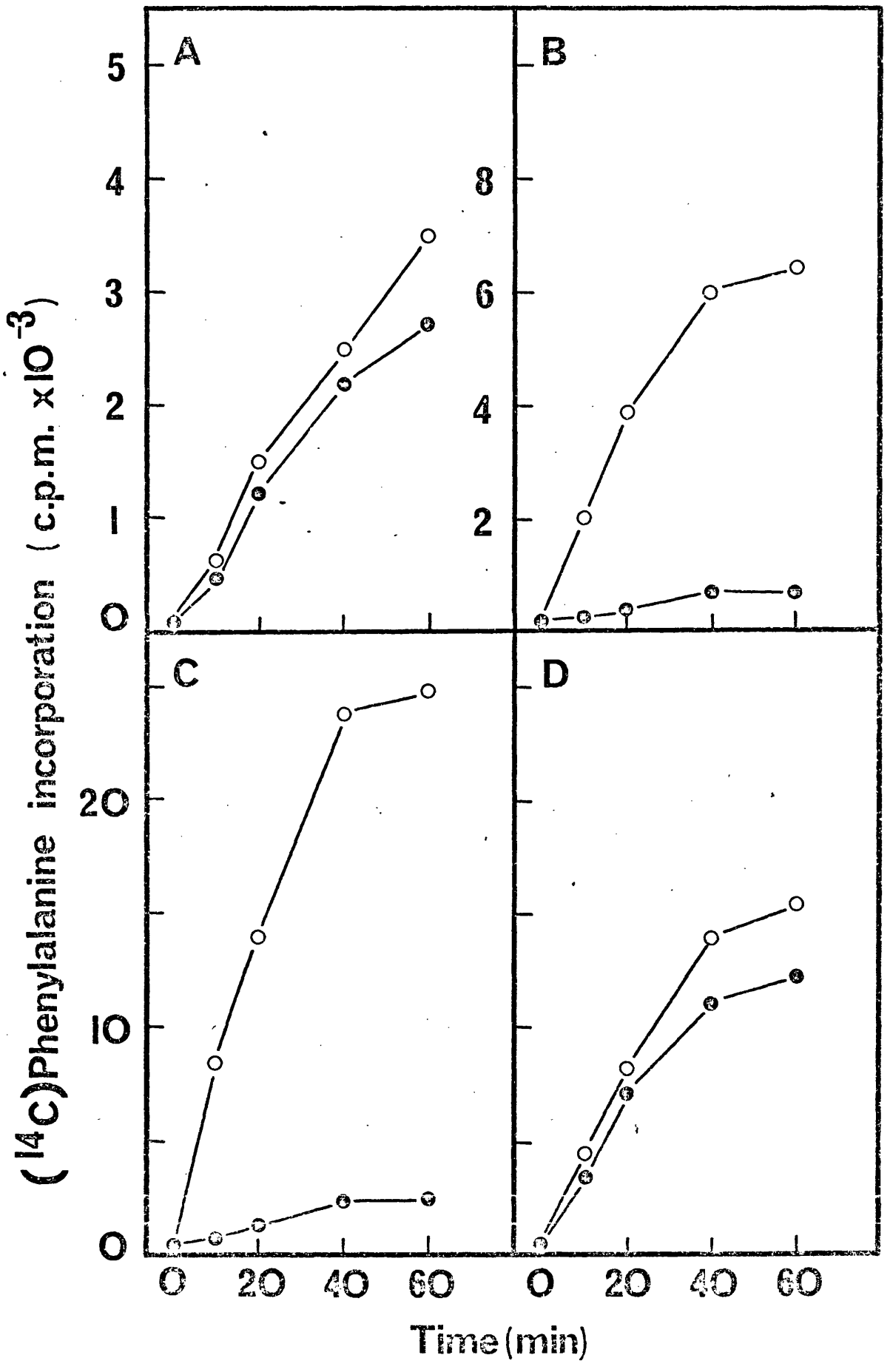
Ribosomal subunits (30S and 50S), from both organisms, were mixed in all four reciprocal combinations, supplemented with S100 from *S. lividans* and tested for the ability to synthesise polyphenylalanine, in the presence (● - ●) and absence (o - o) of nosiheptide (at a 20 : 1 molar excess over ribosomes).

1,000 c.p.m. ≡ 1 pmol phenylalanine per pmol of ribosomes

Source of ribosomal subunit

	30S	50S
A :	*S. actuosus*	*S. actuosus*
B :	*S. actuosus*	*S. lividans*
C :	*S. lividans*	*S. lividans*
D :	*S. lividans*	*S. actuosus*

FIGURE 7.14


A
B
C
D
5
4
3
2
1
0
8
6
4
2
20
10
0
0
20
40
60
0
20
40
60
Time (min)
(^{14}C)Phenylalanine incorporation (c.p.m. x10^{-3})

Finally, to determine whether ribosomal RNA or ribosomal proteins from the 50S ribosomal subunit of S. actuosus conferred resistance to nosiheptide, native and hybrid 50S particles were constructed from RNA 70 and TP 50 prepared from both S. actuosus and S. lividans. Reconstituted particles were subsequently tested, together with S. lividans 30S ribosomal subunits and S100, for their response to nosiheptide in a protein-synthesising system directed by poly (U). Results presented in Figure 7.15 clearly indicated that the ribosomal RNA from S. actuosus could confer resistance to nosiheptide.

Legend to Figure 7.15

Effect of nosiheptide on reconstituted 50S particles from *S. actuosus* and *S. lividans*.

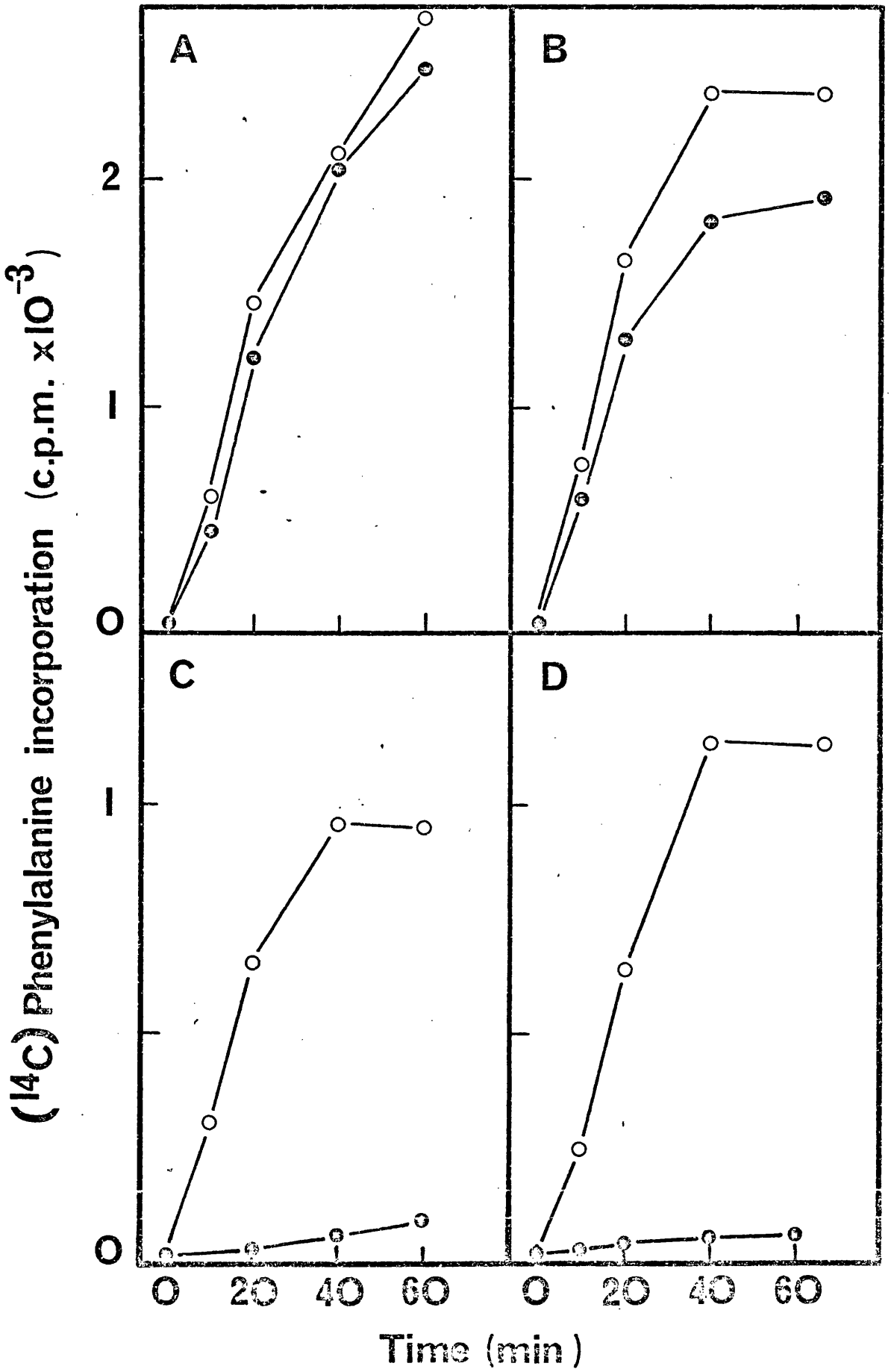
50S particles were reconstituted from RNA 70 and TP 50 (according to Chapter 7.2.7). Following supplementation with 30S particles and S100 from *S. lividans*, particles were tested for the ability to synthesise polyphenylalanine in the presence (● - ●) and absence (o - o) of nosiheptide (at a 20 : 1 molar excess over ribosomes).

1,000 c.p.m. ≡ 1 pmol phenylalanine per pmol of ribosomes

Composition of reconstituted 50S particles

	Source of ribosomal RNA	Source of ribosomal proteins
A :	*S. actuosus*	*S. actuosus*
B :	*S. actuosus*	*S. lividans*
C :	*S. lividans*	*S. lividans*
D :	*S. lividans*	*S. actuosus*

FIGURE 7.15


A
B
C
D
2
1
0
1
0
0
20
40
60
0
20
40
60
Time (min)
(^{14}C) Phenylalanine incorporation (c.p.m. x10^{-3})

4. Discussion

The reconstitution of 50S ribosomal subunits from S. coelicolor required methodology significantly different from that employed for reconstitution of S. loidensis 50S ribosomal subunits. The most significant difference between the two methods was in their respective temperatures of reconstitution, components from S. coelicolor requiring temperatures $5^{o}C$ higher than for the corresponding procedure. Hence, ribosomal proteins from S. coelicolor may be less labile than those from S. loidensis. However, it was interesting to note that low levels of reconstitution of S. coelicolor 50S particles (i.e. 10%) were achieved using optimal conditions established for S. loidensis 50S particles (data not presented). Accordingly, when devising a reconstitution procedure suitable for other Streptomyces ribosomes, it may be advisable to employ the procedure used for reconstituting S. loidensis 50S particles initially and then to optimise for other parameters subsequently. Although reconstituted 50S particles isolated by PEG-precipitation were found to contain a full complement of ribosomal proteins, a rigorous washing procedure (i.e. centrifugation through a sucrose shelf) resulted in the loss of two ribosomal proteins. This may suggest that the conformation of the reconstituted particles was perturbed to the extent that certain ribosomal proteins could no longer bind correctly; with such a large number of ribosomal proteins, some of which may be acting co-operatively, it is conceivable that certain mistakes in the order of assembly of ribosomal proteins could occur. These, in turn, could influence the subsequent binding of other ribosomal proteins, ultimately resulting in a particle with a less compact structure.

An examination of reconstitution intermediates of S. coelicolor 50S particles revealed essentially two major groups of ribosomal proteins -

those binding at 0°C and those binding during the first step of the reconstitution procedure. The ribosomal proteins of S. coelicolor have not been identified immunologically and consequently it was not possible to correlate the data presented here with those of Dohme and Nierhaus (1976). However, certain broad trends are apparent. For example, in accordance with findings using E. coli reconstitution intermediates, approximately 19 ribosomal proteins from S. coelicolor became incorporated into a reconstitution intermediate initially, while subsequent heating (under first step conditions) resulted in the binding of a further 7 ribosomal proteins. In accordance with previous observations, no ribosomal proteins were found to bind to reconstitution intermediates during the second step of the reconstitution procedure; thus, the second step is probably required only to induce conformational changes within the particles.

The reconstitution of 30S ribosomal subunits from S. coelicolor proved to be a simpler undertaking than the procedure for the corresponding 50S particles. In part this may be because the 30S particle is less complex - containing only one molecule of RNA and (nominally) 21 ribosomal proteins. Although both single- and two-step reconstitution procedures were investigated and found to be suitable, ultimately only the single-step procedure was used on a routine basis. The procedure was essentially a modification of the second-step procedure for reconstitution of S. coelicolor 50S ribosomal subunits. The major modification employed was a reduction in the time of reconstitution - 30S particles reaching full activity in approximately 20 minutes compared with at least 3 hours for 50S particles. In general, reconstituted 30S particles routinely showed 80-100% the polyphenylalanine-synthetic activity of control (i.e. native) 30S particles. In accordance with the high levels of activity of

reconstituted 30S particles, ribosomal protein analysis by two-dimensional gel electrophoresis revealed that the particles contained a full complement. Furthermore, analysis of ribosomal RNA integrity in reconstituted particles revealed little, if any, degradation. Finally, a comparison of sedimentation behaviour of both native and reconstituted 30S particles by analytical ultracentrifugation revealed only slight differences in sedimentation coefficients. In conclusion, reconstitution of 30S particles from S. coelicolor resulted in particles which were almost indistinguishable from native 30S particles.

To demonstrate both the efficacy and the general applicability of the procedure for reconstituting 50S particles, the methodology was utilised to confirm results presented by Cundliffe and Thompson (1981) regarding resistance to nosiheptide in the producing organism S. actuosus. In support of previous evidence S. actuosus was found to be resistant to nosiheptide both in vivo and in vitro. For comparative purposes S. lividans was chosen as a reference streptomycete, since, like S. coelicolor it had received considerable attention from other workers. The response of reciprocal recombinations of 30S and 50S ribosomal subunits from both S. actuosus and S. lividans to nosiheptide, implicated the 50S ribosomal subunit of S. actuosus as resistant to the drug.

When 50S particles were constructed from ribosomal RNA and TP 50, from both S. actuosus and S. lividans, a drop in polyphenylalanine-synthetic activity was observed, particularly with the hybrid particles (in comparison with native 50S particles). When the relative proportions of active 50S particles within a given population were accounted for, however, (see Chapter 5) reconstituted particles had activities ranging from approximately 10% to 40% of control (i.e. native) 50S particles (data not presented). The lower levels of activity of hybrid reconstituted

particles compared with homologous reconstituted particles was not entirely surprising when a comparison of ribosomal proteins from various Streptomyces was made (by 2-D gel electrophoresis). In essence, large variations in 2-D gel patterns were observed (data not presented); hence, a certain degree of incompatibility was to be expected and reflected by a lowering of activity. However, the response of the reconstituted 50S particles to nosiheptide, when assayed in the presence of S. lividans 30S particles and S100 was unequivocal. Ribosomal RNA from S. actuosus could confer substantial resistance to nosiheptide, irrespective of the source of ribosomal proteins.

In conclusion, the reconstitution methodology originally devised to investigate the mechanism of resistance to vernamycin A in S. loidensis, has been developed and modified so that 50S ribosomal subunits from a variety of Streptomyces spp. can now be reconstituted from component parts. This has resulted in studies of both the various intermediate stages of the reconstitution procedure, and of the reconstituted 50S particles themselves. In addition, 30S particles have been successfully reconstituted from RNA and proteins, using a modification of the second step of the reconstitution procedure (for S. coelicolor 50S particles). While it has not been possible to draw firm conclusions regarding the mechanism of resistance to vernamycin A in S. loidensis, the reconstitution procedure has enabled the source of resistance to nosiheptide in the producing organism S. actuosus to be established unequivocally.

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RESISTANCE TO VERNAMYCIN A IN STREPTOMYCES LOIDENSIS

by

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The vernamycin A (VA)-producing organism, Streptomyces loidensis, is refractory to indigenous drug in vivo. Through the use of an in vitro assay system, it has been shown that the 50S ribosomal subunit (normally the target site of VA) is resistant to drug. To determine whether ribosomal RNA (rRNA) or ribosomal proteins (r proteins) conferred resistance to VA, it became necessary to develop a procedure for reconstituting 50S particles (with the aim of performing "crossover" experiments with rRNA and r proteins from a VA-sensitive organism).

Curiously, 50S particles reconstituted from S. loidensis rRNA and r proteins, were found to exhibit a changed response to VA in vitro (i.e. they were sensitive to drug by comparison with native 50S particles). However, while a rigorous examination of reconstituted 50S particles by a variety of analytical techniques did not reveal an unequivocal reason for the change in response to VA, one novel approach did yield a plausible explanation. Thus, reconstituted 50S particles exposed to VA appeared to lose a ribosomal protein (tentatively identified as homologous with E. coli L7/L12) while no such protein was lost from native 50S particles. Hence, the mechanism of resistance to VA in S. loidensis may involve an increased affinity of the protein for the corresponding ribosome. In support of this notion, the "displacement effect" has been observed (on occasions) with native 50S particles from VA-sensitive organisms. However, it has not been possible to establish unequivocally whether this effect could be the mode of action of the drug.

The reconstitution methodology has been adapted and modified to embrace 50S ribosomal subunits from a variety of Streptomyces spp. and 30S ribosomal subunits from Streptomyces coelicolor. In addition, the methodology has been employed to demonstrate that the resistance determinant for nosiheptide, in the producing organism Streptomyces actuosus, is rRNA.