

THE INTERACTIONS OF ANTIBIOTICS WITH  
EUKARYOTIC RIBOSOMES

by

Adrian N. Hobden

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at Leicester University

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Except where indicated all experiments reported in this dissertation were done by myself alone. The purification of [ $^3\text{H}$ ] anisomycin was, however, performed in collaboration with R. Skinner.

Some of the results have already been published:

Hobden, A.N. and Cundliffe, E. (1978)  
Biochem. J. 170 p.p. 57 - 61.

Since I have employed several techniques during this work I have had to consult many people. I am most indebted to Dr. Eric Cundliffe for his constant direction, advice and criticism both in the preparation of this dissertation and during my three years as a postgraduate student.

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*Adrian N. Hobden*



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ABBREVIATIONS

The following abbreviations have been used:-

DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
RNA	ribonucleic acid
RNase	ribonuclease
mRNA	messenger RNA
tRNA	transfer RNA
rRNA	ribosomal RNA
ATP	adenosine triphosphate
GTP	Guanosine triphosphate
c.p.m.	counts per minute
r.p.m.	revolutions per minute
Tris.	tris (hydroxy-methyl)-amino-methane

Purine and pyrimidine bases are specified by their initial letter. Trinucleotide codons are shown by the base initials e.g. 'AUG' stands for ApUpG.

The standard three-letter abbreviations have been used for amino acids.

Other abbreviations are defined when first used in the text.

## 1. INTRODUCTION

It is now forty years since ribosomes were first reported as sub-cellular components of living organisms (Claude, 1938). At that time they were called 'small granules' and later 'microsomes', a term used to describe a complex consisting of endoplasmic reticulum and ribosomes. Since then ribosomes from a vast range of eukaryotic and prokaryotic cells have been studied in an effort to elucidate the mechanisms of protein synthesis and the structure and function of the ribosome. The knowledge gained has been extensive but there is much more still to be gained. Clearly, a comprehensive review of so large and complicated a subject is not possible here and, therefore, only a basic outline of the history of ribosome research together with some of the strategies employed are given below.

### 1.1. A HISTORY OF RIBOSOME RESEARCH

As mentioned above ribosomal particles were first reported in 1938 but it was not until 1941 that their role in protein synthesis was hypothesised (Brachet, 1941). This hypothesis was based upon the observation that microsomes always contained RNA and that tissues which were active in protein synthesis, e.g. the glands of the silk worm were

particularly rich in RNA (Brachet 1940; Caspersson 1940; 1941).

Electron microscopy established that ribosomes from all mammalian and avian tissues studied had a diameter of 20 nm (Palade, 1955; Palade and Siekevitz, 1956). In addition, ultracentrifugal analysis of ribosomes from animal tissues revealed that they gave rise to particles of discrete sizes in the analytical ultracentrifuge having sedimentation coefficients (a sedimentation coefficient of  $1 \times 10^{-13}$  sec is a Svedberg unit, S) ranging from 40S to 76S (Petermann and Hamilton, 1952). These particles contained about 50% RNA (Petermann et al., 1954).

That ribosomes were the sites of protein synthesis was shown by Littlefield et al., (1955). After injection of radioactive amino acids into rats the ribosomes rather than the bulk of microsomal proteins became labelled maximally within a few minutes of the injection. However, only a small fraction of the amino acids in ribosomal protein were turning over, i.e. there was a steady state between formation and release of polypeptides. Subsequently, similar observations were made using cell-free extracts from rat liver (Zamecnik and Keller, 1954) and mouse ascites cells (Littlefield and Keller, 1957).

During this period, bacteria were also observed to contain ribosomes (Luria et al., 1943). The ribosomes were, however, smaller than their eukaryotic counterparts (Schachman et al., 1952) and were not proven to be



involved in protein synthesis until a pulse-labelling experiment with E.coli demonstrated this fact conclusively (McQuillen et al., 1959). Cell-free systems capable of synthesising protein were developed later from E.coli and played a vital role in the elucidation both of the steps in protein synthesis and the genetic code.

Originally ribosomes had been thought to be similar to virus particles with an RNA core surrounded by proteins. This was shown to be unlikely, however, since ribosomal particles were dissociated into subunits when the  $K^+$  and  $Mg^{2+}$  concentrations were varied. Eukaryotic ribosomes could be split into 60S and 40S subunits from the 80S parent (Chao, 1957) whilst prokaryotic ribosomes (70S) had subunits sedimenting at 50S and 30S (Tissières and Watson, 1958). In addition, the ribosomes of E.coli contained very many different proteins and those of the 50S subunit were different from those of the 30S subunit (Waller, 1964). Ribosomal proteins tend to form aggregates and it proved difficult to determine categorically the number of proteins in the E.coli ribosome. It was generally agreed (Wittmann et al., 1971) that the figure was 21 proteins in the 30S subunit and 34 proteins in the 50S subunit. More recent research

suggests, however, that the real number may be less than this since one 'protein' in the large subunit (L8) is really a complex of two other proteins, L12 and L10 (Chu et al., 1977; Petersson et al., 1976), and also that a protein found in the large subunit (L26) is identical to one (S20) previously thought to be exclusively a protein of the small subunit (Stöffler, 1974). The true total for ribosomal proteins of E.coli would appear, therefore, to be 53.

Research into the structure of the bacterial ribosome and especially that of E.coli has proceeded rapidly and the primary structures of many of the ribosomal proteins are known (for a review see Stöffler and Wittmann, 1977). Ribosomal RNA has been found to consist, in bacteria, of three distinct molecules, namely a 16S rRNA in the 30S subunit (Kurland, 1960) and both a 23S rRNA (Kurland, 1960) and a 5S rRNA (Rosset and Monier, 1963) in the 50S subunit. The nucleotide sequence of the 5S rRNA was determined rapidly (Brownlee et al., 1968) as was 95% of the nucleotide sequence of the 16S rRNA of E.coli (Ehresmann et al., 1975). The entire sequence of the 16S rRNA (H.Noller, unpublished data) and a considerable amount of the sequence of the much larger 23S rRNA (Branlant and Ebel, 1977) are known now.

Surprisingly, considering that most of the early work was done on eukaryotic ribosomes, research into their structure has lagged behind that on prokaryotic ribosomes.

The individual proteins of eukaryotic ribosomes have not been isolated, nor have they been purified or characterised. However, it is known that the small ribosomal subunit contains one molecule of 18S rRNA (Weinberg and Penman, 1970) and about 30 proteins. The large subunit contains one molecule of 28S, 5.8S and 5S rRNA (Weinberg and Penman, 1970; Udem and Warner, 1972) and about 40 proteins. These figures vary between species with, for instance, yeast ribosomes having 26S rRNA (Planta et al., 1972) and 45 proteins (Otaka and Kobata, 1978) in the large subunit.

The molecular mechanisms of protein biosynthesis are now well established for prokaryotes and eukaryotes but the questions that remain to be answered concern the function of the individual components of the ribosome. Is rRNA merely required to maintain the structure of the ribosome? Which proteins are required for which step in protein synthesis? If the ribosomes of eukaryotes perform exactly the same tasks as those of prokaryotes, why are they so much bigger? Some of these questions have been answered partially. The answers to other questions may be some years in the future.

## 1.2. MOLECULAR MECHANISMS OF PROTEIN SYNTHESIS

The outline of events in protein biosynthesis given in this Section represents the views of most workers in

the field. Only the mechanisms for protein synthesis in eukaryotes are given here. Protein synthesis in prokaryotes proceeds in a broadly similar fashion although requirements for initiation and elongation factors differ slightly.

(a) Initiation

Initiation of protein synthesis in eukaryotes is best understood in mammalian systems and in particular rabbit reticulocytes. It is only in this latter system that initiation factors have been purified and characterised. Therefore, all data given here refer to the rabbit reticulocyte.

The process whereby the ribosome, mRNA and initiator met-tRNA<sub>P</sub> assemble into an 80S initiation complex is promoted by proteins called initiation factors and is a step-wise process. Eight initiation factors have so far been reported: eIF-1\*, eIF-2, eIF-3, eIF-4A, eIF-4B, eIF-4C, eIF-4D and eIF-5 (Benne and Hershey, 1978) although other groups can only find seven factors - Staehelin's group in Basel have not reported eIF-4D (Schreier et al., 1977; Staehelin et al., 1975) whilst Anderson's group in Bethesda do not find

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\* The initiation factor nomenclature used here is that proposed at the International Symposium on Protein Synthesis, Bethesda, in 1976.

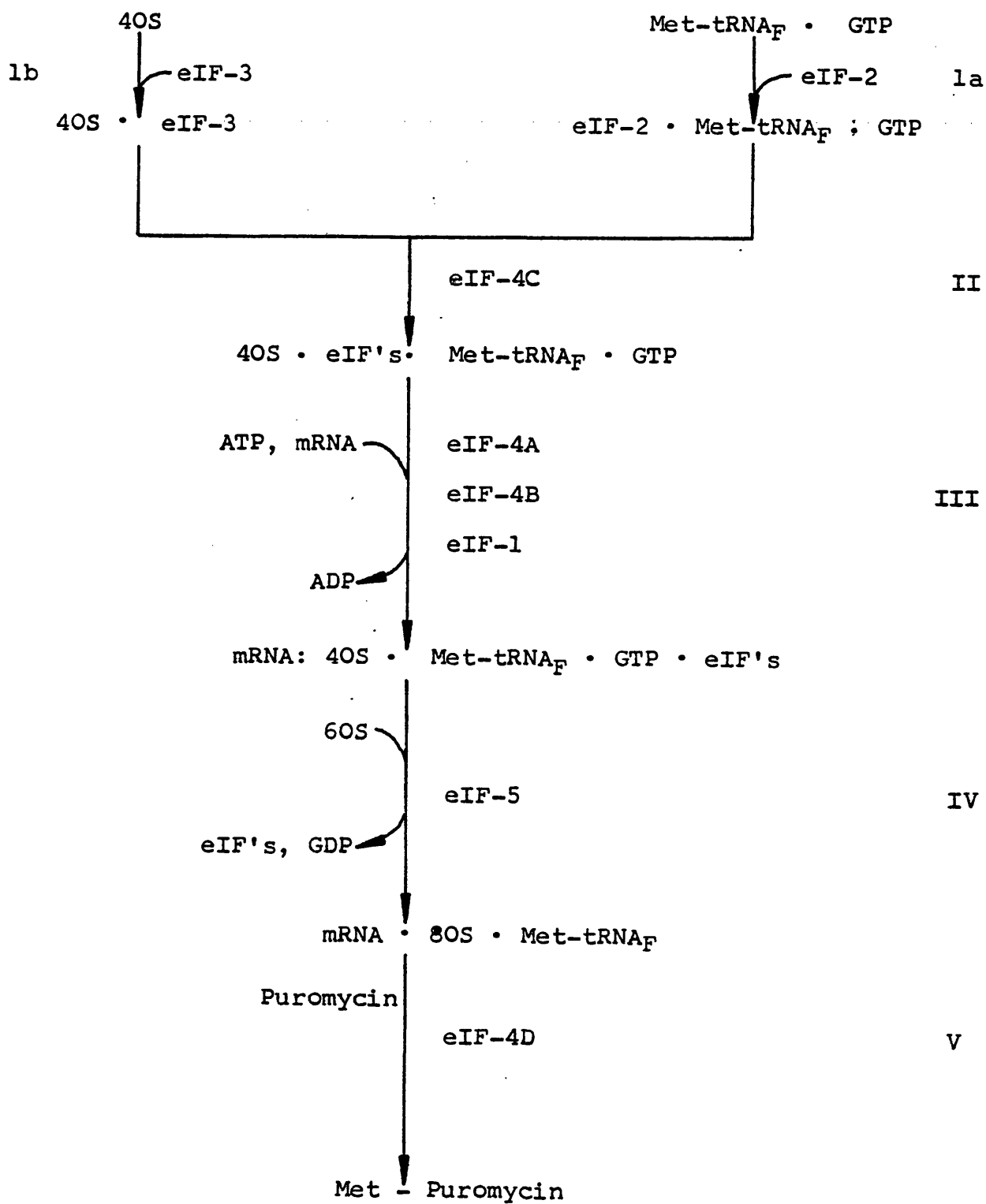


Fig. 1. 1

EVENTS IN THE INITIATION OF PROTEIN SYNTHESIS IN  
RABBIT RETICULOCYTES

eIF-1 (Merrick et al., 1975; Safer et al., 1976). Two of the initiation factors are complex proteins: eIF-2 is comprised of three polypeptides (Benne et al., 1976) and eIF-3 contains nine polypeptides (Benne and Hershey 1976). All 18 polypeptides are different from each other and are distinct from ribosomal proteins (Benne et al., 1977; 1978a).

A tentative pathway for the assembly of the 80S initiation complex is shown in Fig.1.1. Data for this figure are taken from Benne and Hershey (1978). In reaction 1b, eIF-3 binds to 40S subunits to form a stable complex which, owing to the large size of eIF-3 (724,000 daltons), may be visualised by electron microscopy (Emanuilov et al., 1978). Formation of this complex probably serves to prevent the formation of an inactive 80S 'free couple' (Thompson et al., 1977). In a parallel reaction, 1a, a ternary complex of eIF-2, mRNA and met-tRNA<sub>F</sub> forms. All three polypeptides of eIF-2 are present in the complex (Benne et al., 1976). The ternary complex then binds to the 40S - eIF-3 complex (reaction II) in the absence of mRNA, and eIF-4C stabilises the met-tRNA<sub>F</sub> - 40S product. Binding of mRNA completes the 40S initiation complex. Factors eIF-4A, eIF-4B and eIF-1 are required apparently for natural message to bind although they are not required

when the synthetic message poly AUG is used. Adenosine triphosphate is required and is hydrolysed immediately. Available evidence suggests that the ratio of eIF-4A and eIF - 4B to mRNA may be critical in determining which mRNA is to be translated. For example, the ratio of  $\alpha$ -to  $\beta$ -globin synthesised depends on the ratio of eIF -4A and eIF -4B to globin mRNA (Kabat and Chappel, 1977), and similarly, translation of EMC viral RNA in reticulocyte lysates requires higher levels of eIF -4A than does translation of globin mRNA (Erni, 1976). It is postulated (Steitz, 1978) that the mRNA is sited correctly on the 40S subunit via hydrogen bond interactions with the 18S rRNA of the small subunit. Although evidence exists for such an interaction in bacteria (Shine and Dalgarno, 1974), nucleotide sequences prior to the initiation codon in mRNAs of higher organisms appear too diverse for this to be true generally in eukaryotes (Baralle and Brownlee, 1978). However, it is possible that the initiator codon itself may be the site of interaction (Baralle, 1977).

The junction of the 60S ribosomal subunit and the 40S initiation complex (reaction IV) requires eIF-5 and the hydrolysis of GTP. At the same time, eIF-2 and eIF-3 are released (it is not known when the other initiation factors are released). The 80S initiation complex is only partially reactive, as

assayed for the formation of methionyl-puromycin. This latter step is stimulated by eIF-4D (reaction V). However, eIF-4D does appear specific to the initiation event rather than as a general activator of the peptidyltransferase.

Several of the initiation factors have been shown to be phosphorylated in vivo. Phosphorylation of the small subunit<sup>of eIF-2</sup> occurs as a result of haem deficiency in reticulocytes (Farrell et al., 1977), and causes the inhibition of protein synthesis. The factors eIF-3, eIF-4B and one of the large subunits of eIF-2 are also phosphorylated although no changes in their role in initiation have been noticed as a result of the phosphorylation (Benne et al., 1978b).

(b) Elongation

Elongation of the nascent peptide chain during protein synthesis is a cyclic process involving, in eukaryotic systems, two protein factors, EF-1 and EF-2. Ribosomes carrying peptidyl-tRNA in the ribosomal 'P' site bind aminoacyl-tRNA into the adjacent 'A' site in response to appropriate mRNA codons. Binding requires EF-1 and GTP.

The protein factor EF-1 is a heterologous combination of proteins. These forms are referred to as EF-1<sub>L</sub> which is the monomeric form and EF-1<sub>H</sub>, the multimeric form. Both forms of the factor are active



even though EF-1<sub>H</sub> is a hexamer of EF-1<sub>L</sub> in Krebs ascites cells (Nolan et al., 1974). In other systems, EF-1<sub>H</sub> consists of three subunits of differing molecular weights (Bollini et al., 1974). It is reported that EF-1<sub>H</sub> contains zinc (Kotsiopoulos and Mohr, 1975) and lipid components (Moon et al., 1973; Legocki et al., 1974).

Both EF-1<sub>L</sub> and EF-1<sub>H</sub> interact with GTP, although EF-1<sub>L</sub> binds the nucleotide three to five times more strongly (Moon et al., 1973), to form a complex of EF-1·GTP. This complex reacts with aminoacyl-tRNA to form a ternary complex.

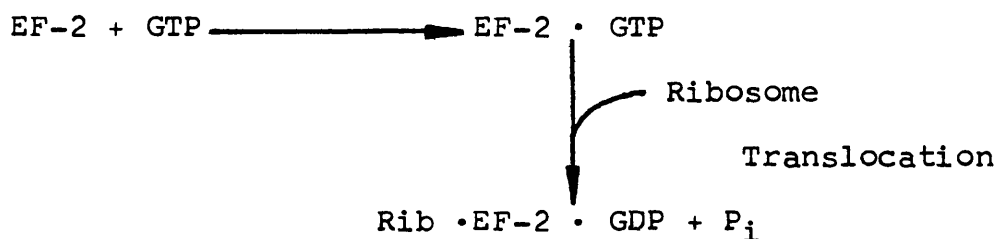


It is likely that the ternary complex only contains EF-1<sub>L</sub> since the presence of EF-1<sub>H</sub> has never been detected (Moon et al., 1972; Tarrago et al., 1973; Bollini et al., 1974). The ternary complex binds to the ribosome, GTP is hydrolysed and EF-1 · GDP released. The process whereby EF-1·GDP is recycled to EF-1 · GTP is unclear since no factors have ever been proven to affect this step. The heavy form of EF-1 may contain such factors whereas EF-1<sub>L</sub> merely functions stoichiometrically. Such a function is difficult to imagine in the case of EF-1<sub>H</sub> from Krebs ascites cells where it is a hexamer of EF-1<sub>L</sub>.

After binding, GTP hydrolysis and release of factor are completed, peptidyl -tRNA bound in the 'P' site then reacts with aminoacyl-tRNA bound in the 'A'

site in a step that requires no extra-ribosomal factor or external source of energy (Monro, 1967; Maden et al., 1968). This is a peptidyltransferase reaction and results in the elongation of the nascent peptide chain by one amino acid residue. The enzymic activity is a function of the large ribosomal subunit (Monro, 1967; Maden et al., 1968). Peptidyl-tRNA is now located in the 'A' site and must move across to the 'P' site before the next aminoacyl-tRNA can bind. Translocation of peptidyl-tRNA also involves displacement of deacylated tRNA and movement of the ribosome along the mRNA so as to position the next codon in the 'A' site. It is a step requiring GTP hydrolysis and EF-2.

In contrast to EF-1, EF-2 appears to be a single polypeptide in all the systems studied. An EF2·GTP complex has been detected in liver (Baliga and Munro, 1972), reticulocytes (Bodley and Lin, 1970) and other systems (Bermek and Matthei, 1971; Chuang and Weissbach, 1972). The EF-2·GTP complex reacts with ribosomes and GTP is hydrolysed. Presumably, EF-2 comes off the ribosome after this stage and reacts with another molecule of GTP.



After the translocation event the ribosome is ready to

bind the next aminoacyl-tRNA to the 'A' site.

(c) Termination

Peptide chain termination results in the release of the completed peptide from its ultimate tRNA. In rabbit reticulocytes, a single release factor, RF, is active with the terminator codons : UAG, UAA and UGA. Although the release factor exists as a dimer, it is not clear whether this is the active unit.

During termination, GTP is hydrolysed in an event which appears to be connected with the binding of RF to the ribosome. The nascent peptidyl-tRNA is hydrolysed to give free peptide in a reaction analogous to the peptidyltransferase. Finally, the 'terminated' ribosome falls off the mRNA and becomes available for a further round of protein biosynthesis via the initiation event.

1.3. THE ULTRASTRUCTURE OF THE RIBOSOME

Although the steps in protein biosynthesis have been identified there still remains the central question: How do 50 - 60 proteins and 3 (4 in eukaryotes) strands of RNA in the ribosome cooperate to perform these steps? At the time of writing, no single ribosomal protein or rRNA species has been shown to exhibit any catalytic activity in the absence of other ribosomal proteins. There exists, however, a great deal of evidence implicating certain ribosomal proteins in particular steps of protein synthesis. Most data has been obtained for the ribosomes of E.coli and other

prokaryotes although some research has been done with eukaryotes.

All ribosomal proteins are present in the E.coli ribosome as one copy per ribosome except the protein L7/L12 (the symbol L designates a protein of the large subunit), which is present in amounts corresponding to four copies per ribosome (Hardy, 1976). The position of these proteins relative to one another has been studied both by the use of cross-linking reagents and antibodies to individual proteins. Cross-linking experiments are designed to identify close neighbours within the ribosome but results from these experiments have proved both hard to analyse and equivocal (for a review, see Kurland, 1974a). Antibody studies of the E. coli ribosome have revealed, firstly, that every protein within the ribosome has antigenic determinants exposed on the ribosomal surface (Stöffler et al., 1973) and allowed, by the use of immunoelectron microscopy, the localisation of all the ribosomal proteins of the small subunit and many of the ribosomal proteins of the large subunit (for a review, see Stöffler and Wittmann, 1977).

Perhaps the most important fact that has been elucidated is that rRNAs are not, as had previously been supposed, merely present to provide a backbone for the ribosomal proteins. The 16S rRNA of the 30S subunit is required for the correct positioning of mRNA (Shine

and Dalgarno, 1974; Steitz and Jakes, 1975). Indeed a major function of the proteins of the 30S subunit may be to help in the correct folding of the 16S rRNA (Kurland, 1974b). Within the large subunit, the 5SrRNA has been found to bind to the T -  $\psi$  - C loop of tRNA molecules (Erdmann et al., 1973) and 23SrRNA binds strongly to the initiator tRNA<sub>f</sub><sup>Met</sup> owing to a complementary sequence of 17 nucleotides (Dahlberg et al., 1978).

Originally it was thought possible to attribute individual functions to individual ribosomal components by making use of total reconstitution of the prokaryotic ribosome - a technique developed by Traub and Nomura (1968). Simply by excluding a particular protein from the reconstitution it was hoped that it would prove possible to identify its role. Such an idea was wildly optimistic since the ribosome is an extremely complicated organelle and every protein appears to function by cooperation with other proteins and the rRNA. However, it has been possible to attribute certain ribosomal functions to certain proteins by a combination of this and other techniques. Perhaps the most information has been obtained by the use of antibiotics which are specific inhibitors of protein synthesis.

#### 1.4. ANTIBIOTICS AS TOOLS IN RIBOSOME RESEARCH

A large number of antibiotics are known which

inhibit protein synthesis in bacteria or higher organisms or both. Their use, as tools in research, is two-fold since they provide insights both into the steps in protein biosynthesis and into the structure and function of the ribosome.

The 'two-site' model for elongation of the nascent peptide (Watson, 1964) was derived as a result of studies with inhibitors of elongation since they were found to fall into three classes - namely, peptidyltransferase inhibitors, translocation inhibitors and molecules which prevented the binding of aminoacyl-tRNA (for a full discussion of this point, see Chapter 4). Additionally, the termination step in protein synthesis was known to occur as a result of peptide transfer since an inhibitor of the peptidyltransferase also blocked this step (Tate and Caskey, 1973). Evidence for the steps in initiation has been obtained by studying inhibitors of this process and determining the stage in the pathway which is inhibited. Thus, a toxin which prevents mRNA from binding to the 40S initiation complex in reticulocytes would be expected to be dominant over an inhibitor which prevents the attachment of the 60S subunit if the pathway for initiation (Section 1.2) is correct.

Other information has been obtained by raising antibiotic-resistant mutants of bacteria and higher organisms. Provided the altered gene-product can be

identified, then its wild-type counterpart is implicated as being important for binding of the particular antibiotic. Additionally, if the step in protein synthesis inhibited by the antibiotic is known, the altered gene-product (and its wild-type counterpart) must be required for this step. Similarly radioactive antibiotics may be used to determine which proteins are required for the drug to bind to the ribosome. Resistance to spectinomycin, an inhibitor of the translocation step in bacterial protein synthesis (Burns and Cundliffe, 1973) is a property of the small subunit and specifically the protein S5 of that subunit (Bollen et al., 1969; Dekio and Takata, 1969).

Such an approach has been applied to several other antibiotics: resistance to streptomycin arises from a mutation to the protein S12 (Ozaki et al., 1969), whereas reversion back to sensitivity can be effected by mutation of S4 (Apirion et al., 1969) or S5 (Stöffler, et al., 1971); kasugamycin resistance, in contrast, arises either from under-methylation of 16S rRNA of the 30S subunit (KsgA; Helser et al., 1971; 1972) or from a mutation of protein S2 (KsgC; Okuyama et al., 1974; Yoshikawa et al., 1975). Since kasugamycin inhibits a step in initiation (Okuyama et al., 1971), it is believed that S2 and 16S rRNA play a part in this process. The

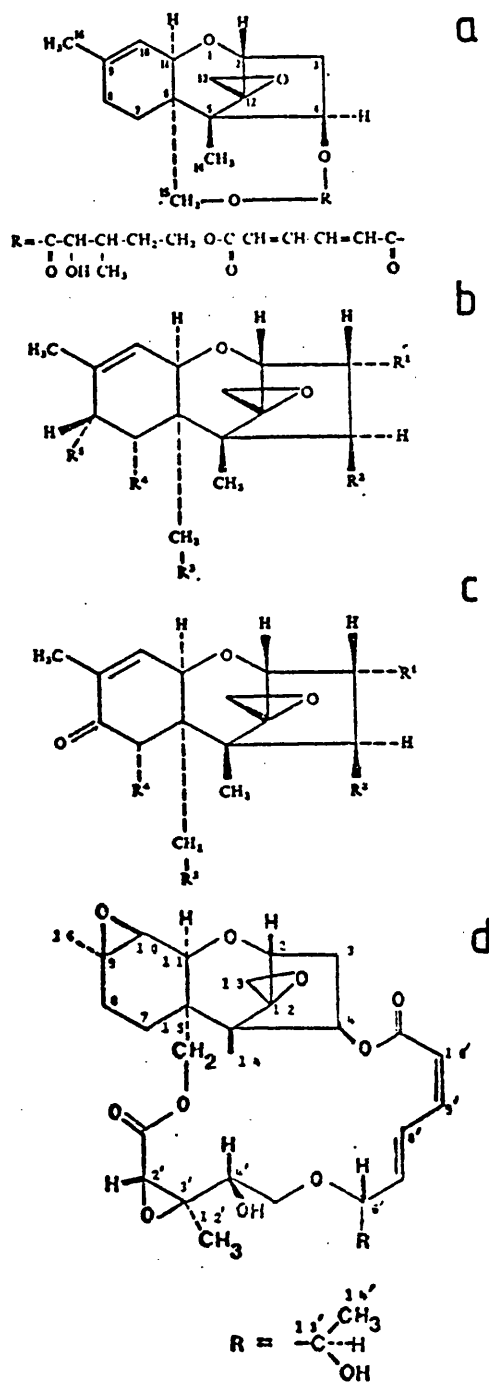
observations for streptomycin are less useful, however, because the actual step inhibited by the drug is unclear.

In this dissertation, experiments are described which seek to clarify the molecular basis of the action of certain antibiotics both by determining the steps in protein synthesis that are inhibited and by looking at organisms which are resistant to the drugs in vivo and in vitro.

#### 1.5. THE 12, 13 EPOXYTRICHOHECENES

The group of closely-related sesquiterpenoids, the 12,13 epoxytrichothecenes, are toxic metabolites of various imperfect fungi of the genera: Fusarium, Myrothecium, Trichoderma, Stachybotrys, Cephalosporium and Verticimonosporium. They are reported also to be produced by the plant species Baccharis megapotamica (Kupchan et al., 1976; 1977) although this is an isolated case and may be due to either fungal contaminants or alteration of fungal products. The toxins are associated with several mycot~~o~~xicoses in both humans and animals. Akakabi (red-mould) toxicosis in Japan (Ueno et al., 1971), mouldy corn toxicosis (Hsu et al., 1972) in the U.S.A., alimentary toxic aleukia (Bilal, 1970) in the U.S.S.R, stachybotryotoxicosis (Rodricks and Eppley, 1973) in northern Europe and several other diseases are now established to be caused by the 12,13 epoxytrichothecenes.





(a) Verrucaric_A					
(b) Trichodermin	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>
	H	OAC	H	H	H
T-2 toxin	OH	OAC	OAC	H	OOCCH <sub>2</sub> OCHMe <sub>2</sub>
(c) Nivalenol	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	
	OH	OH	OH	OH	
Trichothecin	H	OOCCH =	H	H	
		CHMe			
(d) Baccharin					

FIG. 1.2. THE STRUCTURES OF SOME 12,13 EPOXYTRICOTHECENES

More than forty kinds of trichothecene are known to be produced naturally and most have been studied in order to determine their chemical structure, feasibility as anti-tumour agents and mode of action in vitro.

(a) Chemical Structure

All the sesquiterpenoids contain only carbon, hydrogen and oxygen and are derivatives of a ring system named trichothecane (Godtfredsen et al., 1967) after trichothecin, the first member of the group to be isolated (Freeman and Morrison 1948). The structure, stereochemistry and numbering system of the basic ring structure is shown in Fig.1.2. All naturally occurring toxins, with the exception of baccharin and isobaccharin, contain an olefinic bond (at 9,10) and an epoxy ring (at 12,13). Baccharin and isobaccharin should really be termed trichothecanes rather than trichothecenes since they have an epoxy ring between positions 9 and 10 (Kupchan et al., 1977).

The trichothecene ring is highly substituted in most cases and, in general the larger the substituents on the ring, the more pronounced is their biological activity. In addition, reduction of the double bond between positions 9 and 10 or removal of the epoxide ring results in a considerable loss of activity (Bamburg 1969; Wei and McLaughlin 1974), although this may not be true if an epoxy ring is substituted for the double bond

at 9,10 as in the case of baccharin. The structures of many of the 12,13 epoxytrichothecenes are given in Figs. 1.2 and 1.3.

The trichothecenes are remarkably stable to a variety of environmental conditions including variations in temperature, exposure to light and air and to moderate variations in pH. As a result the trichothecenes are not destroyed normally under conditions to which foods are exposed during preparation. (Bamburg and Strong, 1971).

(b) Biosynthesis.

The trichothecene skeleton is formed from three molecules of mevalonate via the usual pathway of lipid biosynthesis (Dawkins, 1966; Sigg et al., 1965). It is unclear, however, how the open-chain farnesyl skeleton ( $\overset{\text{C}}{\text{C}}=\text{C}-\text{C}-\overset{\text{C}}{\text{C}}=\text{C}-\text{C}-\overset{\text{C}}{\text{C}}=\text{C}-\text{C}-$ ) is cyclised.

Several details regarding sites of incorporation of labelled atoms from mevalonate precursor molecules have been established but an overall model of biosynthesis is not available. It is not clear, for instance, whether substitution on the scirpene nucleus takes place before or after the skeletal ring is formed. Oxy-substitution at position 8 appears to be subsequent to that at 4, however, since trichodermin can serve as a precursor for trichothecin (Adams and Hanson, 1970). Additionally, esterified trichothecenes are probably produced from the parent alcohols although it is possible that both forms exist in equilibrium.

(c) Biological Activity

Trichothecenes were shown, initially, to be the active constituents of metabolites of fungi of the genera Myrothecium and Fusarium. They displayed both anti-fungal and phytotoxic activity when tested in vivo, (Nespiak et al., 1961; Brian et al., 1961; Harri et al., 1962). However, it was not until 1968 that a 12,13 epoxytrichothecene, nivalenol, was shown to manifest its toxicity via an inhibition of protein synthesis (Ueno et al., 1968). Initial reports concerning the stages in protein synthesis which were inhibited by trichothecenes were contradictory apparently and gave rise to some confusion regarding the correct site(s) of action. This confusion is best illustrated by the trichothecenes Fusarenon X and Trichodermin.

Fusarenon X was reported to cause the rapid breakdown of polyribosomes in mouse L-cells (Ohtsubo et al., 1972) and was, therefore, interpreted to act like puromycin (Section 4.1). Shortly afterwards, it was shown, along with several other trichothecenes, to be an inhibitor of the peptidyltransferase - as judged by the 'fragment assay' (Carrasco et al., 1973) suggesting an effect on elongation. Additionally, and in contrast to the first report, its effect on the peptidyltransferase was apparent even when tested on yeast polyribosomes (Barbacid et al., 1975). The situation was clarified by Carter and Cannon (1978)

when they showed that Fusarenon X acted as an inhibitor of initiation at low concentrations whilst being effective against elongation at higher concentrations.

Trichodermin, on the other hand, was thought to be an inhibitor of termination. This idea was derived from data obtained by reversing a block in initiation of protein synthesis in yeast. After the block was released, the formation of polyribosomes was not inhibited by the presence of trichodermin although it was when a known inhibitor of the elongation stage was used (Stafford and McLaughlin 1973). Furthermore, trichodermin was able to inhibit a termination assay in vitro (Tate and Caskey 1973). However, since the termination step is merely a modified form of the peptidyltransferase, trichodermin was clearly an inhibitor of the peptidyltransferase as well as termination as suggested by Carrasco et al., (1973) and Tate and Caskey (1973). Trichodermin is now recognised to be an inhibitor of elongation at high concentrations (Cundliffe et al., 1974; Tscherne and Pestka, 1975) whilst showing some specificity, as judged by polyribosome profiles, for inhibition of initiation at low concentrations (Carter et al., 1976).

Whilst the 12,13 epoxytrichothecenes appear in general to be either inhibitors of elongation or initiation (Cundliffe et al., 1974; Schindler, 1974) all trichothecenes tested are capable of competing with

one another for binding to yeast ribosomes. The extent to which they compete, however, depends upon the nature of the ribosome. Thus, on ribosomes derived from polyribosomes and carrying peptidyl-tRNA, trichothecene inhibitors of initiation do not compete very successfully for binding with inhibitors of elongation (Barbacid and Vazquez, 1974a). However, if run-off ribosomes, i.e. ribosomes free of peptidyl-tRNA, are used for binding assays then all trichothecenes can compete for binding regardless of their apparent mode of action (Cannon et al., 1976). It seems, therefore, that all trichothecenes inhibit the peptidyltransferase but ~~that~~ their ability to inhibit this step is limited by the presence of peptidyl moieties upon the ribosomes (Schindler, 1974). So-called inhibitors of initiation 'pseudo-initiation inhibitors', do not inhibit the actual steps in initiation but affect the first (or so) peptidyltransferase reaction (Mizuno, 1975; Smith et al., 1975). They are prevented from affecting subsequent peptidyltransferases by the presence of peptidyl-tRNA on the ribosome.

The 12,13 epoxytrichothecenes have been classified into several groups according to their ability to inhibit the three stages in protein synthesis -

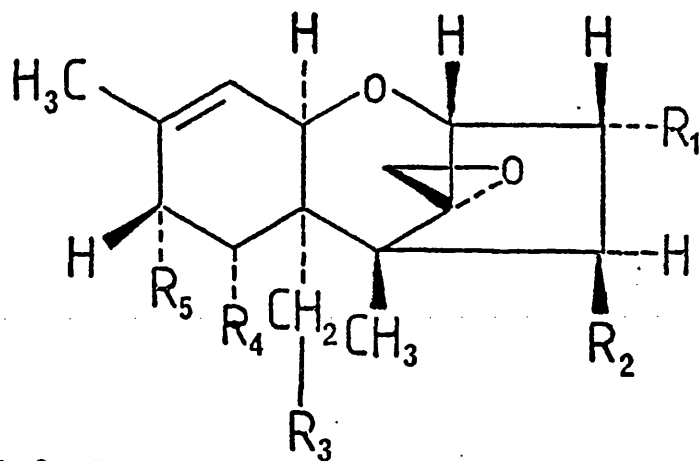
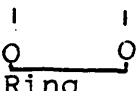


FIG. 1.3 RELATIONSHIP BETWEEN STRUCTURE AND MODE OF ACTION OF THE 12,13 EPOXYTRICHOECENES

Toxins + Stage of Inhibition	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>
(a) Termination					
Trichodermol	H	OH	H	H	H
Trichodermone	H	=O	H	H	H
Vomitoxin	H	H	H	H	H
(b) Elongation					
Trichodermin	H	OAc	H	H	H
3.15 Didesacetylcalonec- trine	OH	H	OH	H	H
15. Desacetylcalonec- trine	OAc	H	OH	H	H
(c) Initiation and Elongation					
Scirpentriol	OH	OH	OH	H	H
3. Desacetylcalonec- trine	OH	H	OAc	H	H
15. Acetoxyscirpen- diol	OH	OH	OAc	H	H
Diacetoxyscirpenol	OH	OAc	OAc	H	H
Calonec- trine	OAc	H	OAc	H	H
Fusarenon-x	OH	OAc	OH	OH	=O
(d) Initiation (I <sub>2</sub> )					
T-2 toxin	OH	OAc	OAc	H	O-CO-CH <sub>2</sub> - -CH(CH <sub>3</sub> ) <sub>2</sub>
HT-2-toxin	OH	OH	OAc	H	"
Nivalenol	OH	OH	OH	OH	=O
(e) Initiation (I <sub>1</sub> )					
Verrucarins	H			H	H

namely initiation, elongation and termination. These data are collated in Fig.1.3 along with the chemical structures of the trichothecenes. The classifications are as given by Carter and Cannon (1977) and Cundliffe and Davies (1977). In general, it seems that the larger the substituents on the trichothecene skeleton the earlier the stage in protein synthesis which is inhibited. There exists some doubt about the classification of  $I_1$  - and  $I_2$  - type inhibitors of initiation since a difference is not always observed and the cause of the differential effect is not understood. This point is discussed in detail in Chapter 5.

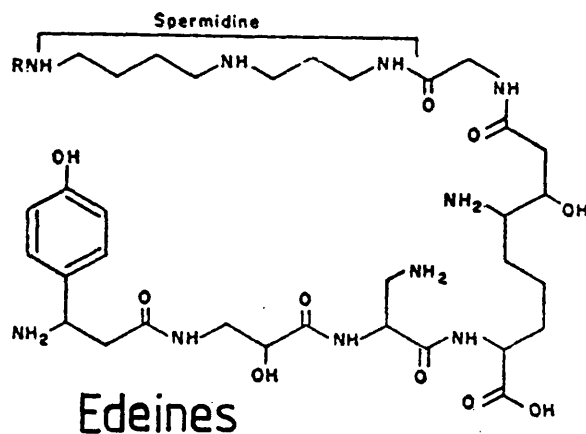
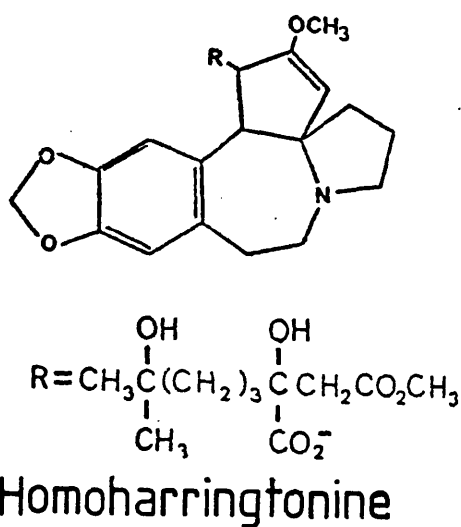
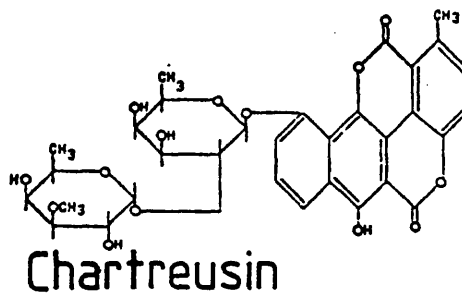
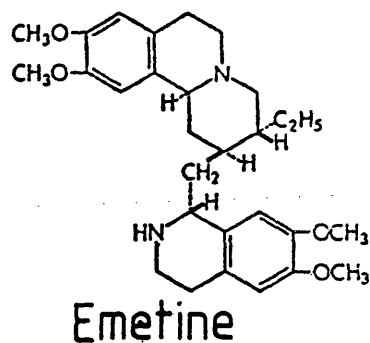
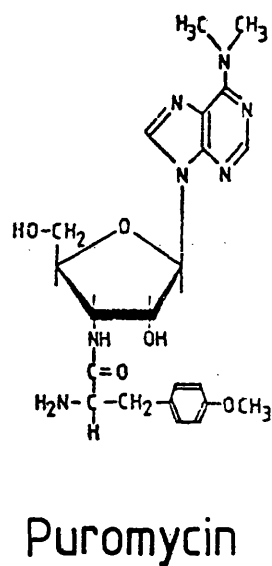
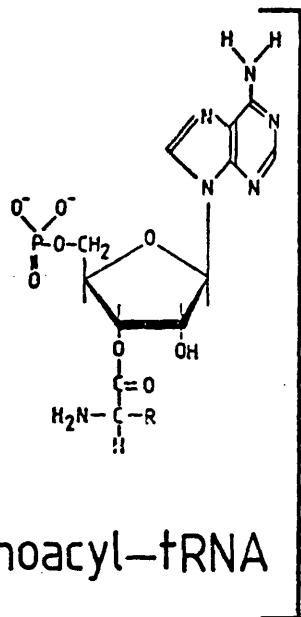
Specific 12,13 epoxytrichothecenes which were used in these studies are reviewed in greater detail elsewhere in this thesis.

#### 1.6. OTHER INHIBITORS OF PROTEIN SYNTHESIS

In addition to the 12,13 epoxytrichothecenes several other antibiotics were employed in these studies. Some of the antibiotics have well established modes of action and were used merely to prove new techniques or help in the elucidation of the modes of action of less-well studied antibiotics. The toxins used are reviewed where their use is reported in the text. The structures of these toxins (where known) are shown in Fig.1.4.

Many of the antibiotics studied here whilst being





edeine A1, R = H; edeine B1, R = C(=NH)NH2.

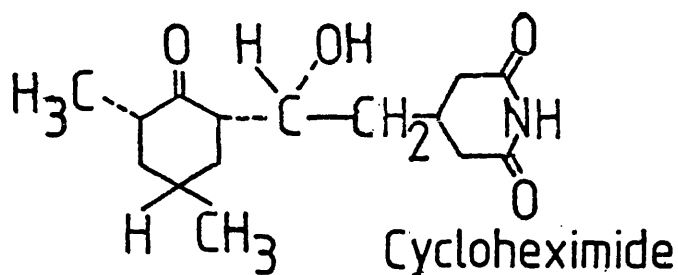
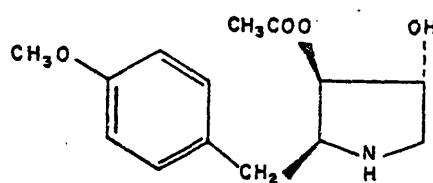
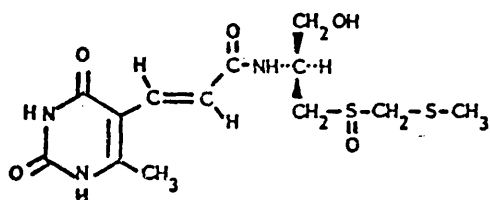


FIGURE 1.4

Chemical Structures of Various Antibiotics

potent inhibitors of protein synthesis in eukaryotes also share the property that they are produced by eukaryotes. Clearly, such producing-organisms must possess resistance to the antibiotic or risk committing suicide. One may envisage many routes by which the organism can attain resistance and these are discussed in relation to results obtained for cell-free systems of various producing-organisms (Chapters 6 and 10 ).

## 2. MICROBIOLOGICAL METHODS

### 2.1. INTRODUCTION

Several microorganisms were employed in this work for the preparation of subcellular components involved in protein synthesis.

The growth, maintenance and in vivo characterization of these organisms are described below.

### 2.2. GROWTH MEDIA

All media were prepared in distilled water.

#### (a) Solid media

(i) MES Agar	Malt Extract	2 g
	Sucrose	0.5 g
	Agar	1.5 g
	H <sub>2</sub> O to 100 ml	

(ii) PYG pH5.9 Agar - Recipe as for PYGpH5.9 liquid medium (see below) supplemented with 1.5% (<sup>W</sup>/v) Agar-agar (Davis N.Z.)

Components of solid media were autoclaved at 15 p.s.i. for 20' and allowed to cool to 50 - 60°C before addition of antibiotic (if required) and pouring into disposable plastic petri dishes (20 ml) or as slants in McCartney bottles (5 ml).

#### (b) Liquid media

(i) 50% (<sup>W</sup>/v) solutions of glucose were autoclaved at 10 p.s.i. for 15'.

(ii) PYG pH5.9 medium contained per litre

Peptone	5 g	
Yeast extract	3 g	
Glucose	5 g	
KH <sub>2</sub> PO <sub>4</sub>	12.25 g	) 0.1M phosphate buffer at pH5.9
K <sub>2</sub> H PO <sub>4</sub>	1.74 g	

If the medium was prepared as a large volume (15L), glucose was not included in the mixture for autoclaving but was added afterwards using a 50% (w/v) stock solution (see above).

(iii) Minimal amino acid medium for Saccharomyces cerevisiae, strain Y166, contained per litre:

Difco yeast nitrogen base without amino acids	6.7 g
Succinic acid	10 g
NaOH	6 g
Glucose	10 g
L-His	40 mg
L-Trp	40 mg

(pH = 5.8 unadjusted)

Ref: Udem and Warner, 1972.

Unless stated otherwise all media were autoclaved at 15 p.s.i. for 20 minutes.

TABLE 2.1  
YEAST AND FUNGAL STRAINS

<u>ORGANISM</u>	<u>STRAIN</u>	<u>SOURCE</u>
<u>Saccharomyces cerevisiae</u>	Y <sub>166</sub>	M.Cannon, Department of Biochemistry, King's College, London. Ref: Jimenez et al., 1973
<u>Saccharomyces cerevisiae</u>	TR <sub>1</sub>	M.Cannon. Ref: Jimenez et al., 1975
<u>Myrothecium verrucaria</u>	NRRL 3001	Northern Regional Research Centre, Illinois.
<u>Fusarium poae</u>	CMI 107588	Commonwealth Mycological Institute, Kew.
<u>Fusarium sporotrichiodes</u>	CMI 165537a	" " "
<u>Fusarium equiseti</u>	CMI 129002	" " "
<u>Aspergillus giganteus</u>	NRRL 2777 (MDH 18894)	Northern Regional Research Centre, Illinois.

ABBREVIATIONS:

NRRL: Northern Regional Research Laboratories  
 CMI: Commonwealth Mycological Institute  
 MDH: Michigan Department of Health

### 2.3. MAINTENANCE OF CELL LINES

Details about the microorganisms used in this work are shown in Table 2.1.

The fungi F.poae, F.sporotrichiodes, M.verrucaria and A.giganteus were plated on MES agar and grown at 30°C until sporulation. They were then stored at 4°C. F.equiseti was grown at 30°C on PYG pH5.9 agar until a considerable mat of mycelia had formed. It was then stored at 4°C.

Yeast strains Y<sub>166</sub> and TR<sub>1</sub> were streaked onto MES plates (containing 10 µg/ml trichodermin for strain TR<sub>1</sub>) and grown at 30°C for 5 - 6 days. They were then stored at 4°C. Subcultures were made every 2 - 3 months.

### 2.4. GROWTH OF CELLS

#### (a) Saccharomyces cerevisiae

For small quantities of yeast, 1 L of PYG pH5.9 medium (or minimal amino acid medium) in a 2 L Ehrlenmeyer flask was inoculated with either a single colony of strain Y<sub>166</sub> or 2ml of PYG pH5.9 medium containing 10 µg/ml trichodermin in which a single colony of strain TR<sub>1</sub> had been grown overnight. The medium was then shaken at 30°C.

Larger quantities of yeast (15 L) were grown in PYG pH5.9 medium in a 30°C constant temperature room in 20 L glass fermenting jars under forced aeration and constant stirring. A culture of 1 L of yeast in the phase of logarithmic growth in the same media was

used as an inoculum for each 15 L batch. 1 - 2ml antifoam was added to 15 L culture.

Yeast growth was halted in middle to late logarithmic growth phase at an absorbance at 650 nm ( $A_{650}$ ) of 1.2 ( $\approx 10^7$  cells/ml, Sissons, 1974) by removing the flasks to ice. The cells were then harvested by centrifugation at 2,000 r.p.m. for 10 minutes in an M.S.E. Mistral centrifuge.

Cell pellets were washed by resuspension in, and centrifugation through cold water and then washed three times in cold 'eukaryotic grinding buffer' (10 mM - Tris/acetic acid pH7.6, 90 mM - potassium acetate, 1 mM - magnesium acetate, 2mM - calcium acetate, 3mM - 2 - mercaptoethanol). Cells were stored frozen at  $-70^{\circ}\text{C}$  if not required for immediate use.

Cell yield was 3 - 4 g. wet weight/L of medium.

(b) Myrothecium verrucaria

Spores from lawns of M. verrucaria grown on M E S plates were harvested by scraping the surface of the plates under sterile water. This suspension of spores was then used to inoculate 1 L of PYG pH5.9 medium in a 2 L Ehrlenmeyer flask. After incubation for 24 - 30 hours on an orbital shaker at  $30^{\circ}\text{C}$  growth was stopped by removal of the flask to ice.

If larger quantities of M. verrucaria were required the 1 L of culture obtained, as above, was used to inoculate several more flasks of PYG pH5.9 medium (50 ml/L of medium). In this case the flasks were incubated for 12 - 14 hours before growth was stopped.

In common with some bacteria of the genera Streptomyces, M. verrucaria grows as macroscopic balls in liquid culture and, therefore, optical density measurements cannot be used to assess cell-growth. Increase in dry weight has been used as a measure of cell-growth in Streptomyces but in these studies cells were harvested when the cell density was judged to be approximately half that seen when cells were allowed to grow to stationary phase (assumed to have occurred after 72 hours at 30°C).

Cells were harvested by filtration under suction through Whatman No.1 paper in a large Buchner funnel. The cells were then resuspended in cold water and re-filtered - the process being repeated 3 or 4 times. Finally cold 'eukaryotic dialysis buffer' (10 mM - Tris/acetic acid pH7.6, 90 mM-potassium acetate, 1mM-magnesium acetate, 3mM - 2 - mercaptoethanol) was used to wash the cells which, having been allowed to re-absorb a small quantity of buffer, were scraped from the filter paper and stored at -70°C.

Yield of cells was approximately 30g/L of medium but much less if the cells were prevented from re-absorbing buffer.

(c) Growth of other fungi

Other fungi, except F. equiseti, were grown by innoculating flasks of PYG pH5.9 medium with a suspension of spores. Since F. equiseti does not produce spores when grown on PYG pH5.9 plates an innoculum was derived from a suspension of mycelia in water. The fungi were grown at 30°C and were harvested, as described for M. verrucaria, after 24 to 30 hours.



Legend to Table 2.2.

Whatmann AA discs (6mm dia.) containing antibiotics (10 µg/ml) were dried under an infra-red lamp and then placed on freshly-spread lawns of fungi on agar plates. These plates were incubated at 30°C and examined after two to three days. A clear zone around a disc was taken to indicate sensitivity (S) to that particular antibiotic; lack of a zone of inhibition indicated resistance (R). Those combinations which gave only a very small zone (<1mm) are designated R/S.

ANTIBIOTIC - SENSITIVITY OF THE MICROORGANISMS IN VIVO

TABLE 2.2

	<i>Saccharomyces cerevisiae</i> Y <sub>166</sub>	<i>Saccharomyces cerevisiae</i> TR <sub>1</sub>	<i>Aspergillus giganteus</i>	<i>Myrothecium verrucaria</i>	<i>Fusarium poae</i>	<i>Fusarium equiseti</i>	<i>Fusarium sporotrichioides</i>
Alpha Sarcin	R	R	R	R	R	R	R
Anisomycin	S	R	R	R	R	R	R
Chlortetracycline	R	R	-	-	-	-	-
Cryptopleurine	S	S	-	-	-	-	-
Cycloheximide	S	S	S	R	S/R	R	S/R
Diacetoxyscirpenol	R	R	-	-	-	-	-
Emetine	R	R	R	R	R	R	R
Fusidic Acid	R	R	-	-	-	-	-
Homoharringtonine	R	R	R	R	R	R	R
Oxytetracycline	R	R	-	-	-	-	-
Sparsomycin	R	R	R	R	R	R	R
Trichothecin	S	R	-	-	-	-	-
Trichodermin	S	R	S	R	S/R	R	S/R
Trichodermol	R	R	-	-	-	-	-
T - 2 Toxin	R	R	R	R	R	R	R
Verrucaric Acid	S	R	R	R	R	R	R
Azide	S	S	S	S	S	S	S

All cultures were checked for contaminants under a phase contrast microscope. In addition, the antibiotic-sensitivity of the yeast mutant TR<sub>1</sub> was checked on agar plates.

## 2.5. SENSITIVITY OF MICROORGANISMS TO VARIOUS ANTIBIOTICS

All the microorganisms shown in Table 2.1 were tested for their sensitivity to various inhibitors of eukaryotic protein synthesis. A wide range of antibiotics, known to inhibit protein synthesis at differing stages in peptide elongation (see Section 1.2), were tested for their effect on growth in vivo by spreading lawns of the microorganisms on agar plates and growing the microorganisms in the presence of paper discs containing antibiotic. Details of the assay and the results are given in Table 2.2.

The yeast mutant, TR<sub>1</sub>, was found to be resistant not only to trichodermin (Jimenez et al., 1975) but also to all other members of the 12, 13 epoxytrichothecene group of toxins with which it was confronted. In addition, it was resistant to the chemically - unrelated antibiotic, anisomycin. These observations are in accordance with the observation that the 12,13 epoxytrichothecenes and anisomycin have either identical or overlapping binding sites on the yeast ribosome (Barbacid and Vazquez 1974, Schindler et al., 1974; Cannon et al., 1976).

The fungi tested are all strains of genera of fungi which are reported to produce antibiotics which inhibit eukaryotic protein synthesis. However, with the exception of Aspergillus giganteus NRRL 2777 none of the strains used

TABLE 2.3  
REPORTED SITES OF INHIBITION OF PROTEIN SYNTHESIS BY ANTIBIOTICS

INITIATION OF PROTEIN SYNTHESIS		ELONGATION OF PROTEIN SYNTHESIS		
True Initiation	Pseudo Initiation (see Section 1.5c)	Pepitidyltransferase	Translocation	'A' Site Blocker
Azide	Diacetoxyscirpenol (low concentration) Homoharringtonine  T - 2 Toxin  Verrucarin A	Anisomycin  Diacetoxyscirpenol (high concentration)  Sparosomycin  Trichothecin  Trichodermin  Trichodermol	Cryptopleurine  Cycloheximide  Emetine	Alpha Sarcin  Chlortetracycline  Oxytetracycline  Emetine (see chapter 4 )  Fusidic Acid

in these experiments has ever been shown to produce any antibiotics. In order to ascertain whether they produced antibiotic when grown on agar plates, a small plug of growing mycelium was removed from plates of the fungi. These plugs were then inserted into appropriately sized holes in MES plates on which a lawn of yeast strains Y<sub>166</sub> or TR<sub>1</sub> had been spread. The plates were incubated at 30°C and scored for a zone of inhibition around the plug of fungi. Under these conditions, only Myrothecium verrucaria gave rise to a zone of inhibition. Although the zone of inhibition was present for both strains Y<sub>166</sub> and TR<sub>1</sub>, indicating the production of toxins other than trichothecenes (since strain TR<sub>1</sub> is resistant to all trichothecenes), the zone of inhibition was smaller for strain TR<sub>1</sub> and may, therefore, indicate that M. verrucaria produces both trichothecenes and other toxins. For a further discussion of these results see Chapter 10.

The reported sites of action of the antibiotics used in the antibiotic disc tests are shown in Table 2.3.

### 3. BIOCHEMICAL METHODS

#### 3.1. INTRODUCTION

The methods described here were used routinely throughout this work. More specialist techniques are described in the appropriate chapters.

#### 3.2. MATERIALS

Commercial sources of materials were as follows:-  
L - [ $^4,5\text{-}^3\text{H}$ ] leucine, L - [ $^{35}\text{S}$ ] methionine,  
L - [ $^{\text{U}}\text{-}^{14}\text{C}$ ] phenylalanine were from The Radiochemical Centre, Amersham, Bucks. U.K., phosphocreatine, creatine kinase, ATP ( $\text{Na}_2$ ), GTP ( $\text{Li}_3$ ), phosphoenolpyruvate and yeast tRNA (phenylalanine specific) were from Boehringer and Soehne, Mannheim, Germany; yeast RNA, L - amino acids, Hepes, polyuridylic acid, dithiothreitol and 2 - mercaptoethanol were from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.; other chemicals were of the highest grade available - usually 'Analar'.

#### 3.3. BUFFERS

The following solutions were employed routinely. pH was adjusted at room temperature.

Saline: 0.13M-NaCl, 7.5mM-MgCl<sub>2</sub>, 5mM-KCl

Sucrose density-gradient buffer for reticulocyte

lysates: 0.5M-NaCl, 15mM-MgCl<sub>2</sub>, 10mM-Tris. HCl (pH7.6), 1mM-dithiothreitol.

Eukaryotic grinding buffer: 1mM-magnesium acetate,

90mM- potassium acetate, 2mM-calcium acetate, 3mM- 2 - mercaptoethanol.

Eukaryotic dialysis buffer: 10mM-Tris.acetic acid (pH7.6), 90mM-potassium acetate, 1mM-magnesium acetate, 3mM - 2 - mercaptoethanol.

High salt buffer: 10mM-Tris. acetic acid (pH7.6), 1M - ammonium acetate, 100mM - magnesium acetate, 3mM - 2 - mercaptoethanol.

#### 3.4. PREPARATION OF RETICULOCYTE LYSATES

Lysates of rabbit reticulocytes used in these experiments were the kind gifts of Drs. R.J.Jackson and T.Hunt. They were prepared in the following way. New Zealand white rabbits weighing approximately 7 lbs. were given four successive daily injections of 30mg. of acetylphenylhydrazine dissolved in 2 ml warm saline (0.13M-NaCl, 7.5mM-MgCl<sub>2</sub>, 5mM-KCl). The animals were bled by heart puncture on the ninth day. The reticulocytes were harvested by centrifugation at 1000 g for ten minutes and were washed twice in ice-cold saline. Finally, the cells were lysed by mixing with 1.5 volumes of cold water per volume of packed cells and the debris was removed by centrifugation at 30,000 g. for 15 minutes at 4°C (Hunt et al., 1972). The lysates were stored as 1 ml aliquots under liquid nitrogen.

#### 3.5. INCUBATION CONDITIONS FOR RETICULOCYTE LYSATES

Frozen aliquots of lysates were carefully thawed so that their temperature never exceeded 5°C and were made 0.025 mM in haemin (from a stock solution of 1mM - haemin in 90% ethylene glycol, 20mM-Tris.HCl (pH8.2), 50mM-KCl). Creatine kinase was added to a final concentration of 0.05 mg/ml.

A 'standard incubation mixture' contained the following components: 0.75 ml of lysate containing haemin, 0.05ml of salts solution (2M-KCl, 10mM-MgCl<sub>2</sub>), 0.05 ml of 0.2 M-creatine phosphate, 0.1ml of radioactive amino acid solution (see figure legends for concentrations and specific activities) or water and 0.05 ml of a mixture of amino acids with the following composition: 3mM-alanine; 0.5mM-arginine; 0.5mM-asparagine; 2.0mM-aspartic acid; 0.5mM-cysteine; 0.5mM-glutamine; 2.0mM-glutamic acid; 2.0mM-glycine; 2.0mM-histidine; 0.5mM-isoleucine; 3.0mM-leucine; 2.0mM-lysine; 0.5mM-methionine; 1.5mM-phenylalanine; 1.0mM-proline; 2.0mM-serine; 1.5mM-threonine; 0.5mM-tryptophan; 0.5mM-tyrosine; 3.0mM-valine. One of these amino acids was omitted if its radioactive analogue was used as label. This mixture was brought to pH7.5 with KOH and was made 10mM in dithiothreitol.

Final concentrations of inhibitors of protein synthesis used in these incubations were as indicated in the figure legends.

### 3.6. PREPARATION OF SAMPLES OF RETICULOCYTE LYSATES FOR FURTHER ANALYSIS.

Protein synthesis was stopped by taking 5  $\mu$ l samples of incubation mixture in 0.5ml of distilled water or, for sucrose density-gradients, 50  $\mu$ l were taken into 0.25 ml of the ice-cold sucrose density-gradient buffer (see Section 3.7).

In order to assay total protein synthesis by a method involving liquid-scintillation spectrometry it was necessary both to bleach samples to prevent 'quenching' of liquid-



scintillators and also to remove aminoacyl-tRNA. This was achieved by the addition of 0.5ml of 1M-NaOH containing approximately 1mg/ml of appropriate unlabelled amino acid and 0.1ml of H<sub>2</sub>O<sub>2</sub> (20 volumes). The mixture was then incubated at 30°C for several minutes until the solution was completely bleached and 0.5 ml of 50% (W/v) trichloroacetic acid (TCA) added in order to precipitate peptide chains. The precipitates were collected on Whatman glass-fibre filters, washed three times with 5% (W/v) TCA and the filters dried under an infra-red lamp for 15 minutes. Radioactivity on filters was estimated by liquid-scintillation (Section 3.16).

To distinguish, in samples of the incubation mixture, the peptidyl-tRNA from free peptide and aminoacyl-tRNA, 1 ml of 2% (W/v) cetyltrimethylammonium bromide (CTAB) solution was added followed by 1 ml of 0.5M sodium acetate/acetic acid (pH5.4) containing 0.5mg/ml yeast RNA as carrier. The precipitate from this process, which contains peptidyl-tRNA, aminoacyl-tRNA and other negatively charged macromolecules (Darnbrough et al., 1973; T.Hunt, personal communication) was allowed to aggregate before being collected on Whatman glass-fibre filters and then washed three times with water.

The aminoacyl-tRNA was solubilised by heating the filters in 10%(w/v) TCA for thirty minutes at 90°C. Finally, the filters were washed three times with 5% (w/v) TCA, dried and their radioactivity estimated by liquid-scintillation (Section 3.16).

### 3.7. SUCROSE DENSITY-GRADIENT ANALYSIS OF RETICULOCYTE RIBOSOMES.

For analysis of ribosome distribution 5ml sucrose density-gradients were used. Gradients were linear from 15% to 30% or 40% (w/v) (see figure legends) in a buffer containing 0.5M - NaCl, 15 mM-MgCl<sub>2</sub>, 10mM-Tris.Hcl (pH7.6) and 1mM-dithiothreitol. The gradients were centrifuged at 2°C in a Spinco SW 50.1 rotor for various times and at 40,000 - 50,000 r.p.m. (see figure legends). After centrifugation, ultra-violet absorbance of materials in gradients was monitored continuously as the gradient solution was pumped through an ISCO UA-5 absorbance monitor set at 254 n.m. For gradients containing radioactive amino acid, sequential fractions were collected from the gradients and precipitated with CTAB as above. The precipitates were collected on Whatman glass-fibre filters, washed with water, dried and their radio-activity estimated.

### 3.8. CALCULATION OF RIBOSOME CONCENTRATIONS IN RETICULOCYTE LYSATES

A value of 60.2 A<sub>260</sub> units\* per n.mol of 80 S

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\* An A<sub>260</sub> unit of ribosomes is the amount of ribosomes which in 1 ml gives an absorbance at 260 n.m. of 1.0.

ribosomes was taken on the basis of the molecular weight and extinction coefficients for reticulocyte ribosomes (Reboud et al., 1969; Hamilton et al., 1971). Ribosome concentration was estimated by planimetry of the 80S ribosome peak detected after centrifugation of a ribonuclease-treated incubation mixture on a sucrose density-gradient. Fifty microlitres of incubation mixture contained 10 p moles ribosomes.

### 3.9. PREPARATION OF YEAST 30,000 g SUPERNATANT (S-30)

A pellet of either freshly-prepared or frozen yeast cells (see Section 2.4) was placed in a mortar which had been cooled previously to  $-20^{\circ}\text{C}$ . An equal weight of levigated alumina was added to the mortar and the mixture ground vigorously in the cold until the pellet had thawed. More alumina (approximately half the original quantity) was then added and the grinding continued until the mixture was viscous. Further grinding beyond this point did not contribute significantly to the yield of broken cells. At best, approximately 70 - 80% of the yeast cells were broken by this procedure.

The ground paste was washed from the mortar with 2 volumes (assuming 1 mg of yeast cells and alumina occupy a volume of 1 ml) of 'eukaryotic grinding buffer' (1mM-magnesium acetate, 90mM-potassium acetate, 2mM-calcium acetate, 3mM- 2 - mercaptoethanol) and centrifuged at 30,000 g for 20

minutes. Stock solutions of Tris-acetic acid pH7.6 (1M) and magnesium acetate (1M) were added to the supernatant, designated the S-30 extract (Nirenberg and Matthei, 1961) to make it 10mM in Tris, acetic acid and 1mM in magnesium acetate. Occasionally, deoxyribonuclease (DNase) to a final concentration of 2 µg/ml was added to the supernatant in order to reduce its viscosity but this treatment was not found to alter the synthetic activity of the S-30. The S-30 was dialysed at 0°C against 'eukaryotic dialysis buffer' (10mM-Tris, acetic acid pH7.6, 90mM-potassium acetate, 1mM-magnesium acetate, 3mM- 2 - mercaptoethanol) and stored as 0.2ml aliquots at -70°C. At -70°C the S-30 was found to retain its synthetic activity indefinitely whereas at -20°C the S-30 lost 75% of its synthetic activity by being frozen. Storage at -20°C further decreased the activity of the S-30 (Fig.3.1).

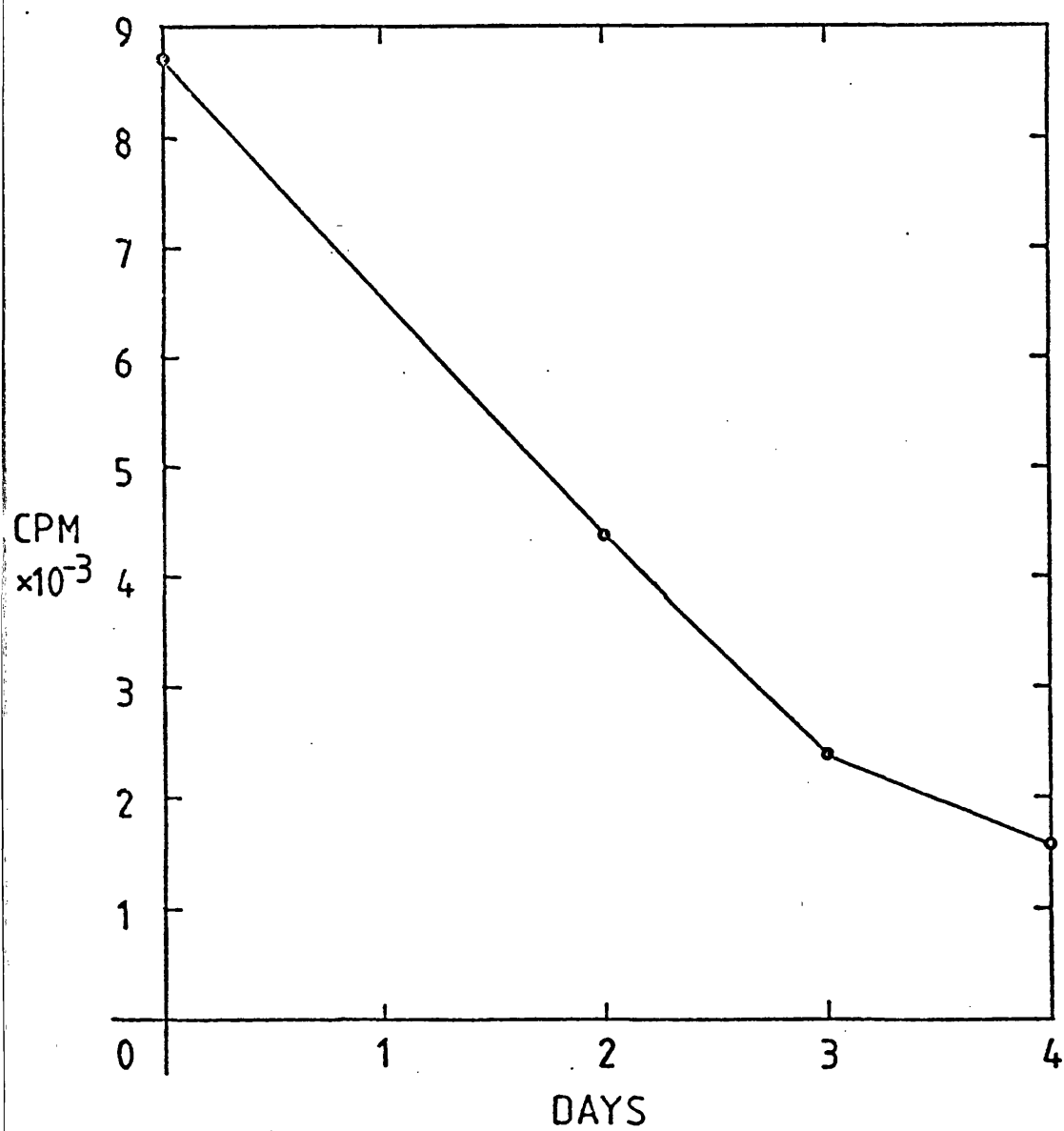
The concentration of ribosomes in the cell-free extracts (S-30s) was estimated by measuring the absorbance of the extracts at 260 n.m. (A<sub>260</sub>). Since the S-30s contained material, other than ribosomes, which absorbed at 260 n.m., 1 mg of ribosomes was taken to be equivalent to 29.4 A<sub>260</sub> units (Sissons, 1974).

### 3.10 PREPARATION OF YEAST RIBOSOMES AND 100,000 g SUPERNATANT

Yeast S-30 (5ml), prepared as described above,

FIGURE 3.1

The Effect of Storage at  $-20^{\circ}\text{C}$  upon Synthetic Activity of Yeast S-30 Extracts



### Legend to Figure 3.1

The effect of storage at  $-20^{\circ}\text{C}$  upon synthetic activity of yeast S-30 extracts.

Mixtures (Section 3.14) containing yeast S-30 extracts ( $A_{260}=25$ , final concentration) which had been stored at  $-20^{\circ}\text{C}$  for various periods of time were incubated at  $25^{\circ}\text{C}$  for 60 minutes and 5  $\mu\text{l}$  samples were precipitated with TCA. Radioactivity in the precipitates was estimated by liquid-scintillation counting (Section 3.16).

was layered onto 2ml 30% (<sup>W</sup>/v) sucrose containing high-salt (10mM-Tris.acetic acid pH7.6, 1M-ammonium acetate, 100 mM-magnesium acetate, 3mM- 2 -mercaptoethanol) and centrifuged for four hours at 50,000 r.p.m. and 2°C. The supernatant, designated S100, was taken and dialysed against 'eukaryotic dialysis buffer' (10mM-Tris.acetic acid pH7.6, 90mM-potassium acetate, 1mM-magnesium acetate, 3mM- 2-mercaptoethanol) for several hours at 0°C and stored as 0.5ml aliquots at -70°C. The ribosome pellet was resuspended in 'high-salt buffer' (10mM-Tris.acetic acid pH7.6, 1M-ammonium acetate, 100mM-magnesium acetate, 3mM- 2 - mercaptoethanol) and, following a low speed clearing spin, the supernatant was recentrifuged as above - the process being repeated up to three times in all.

Finally, the ribosomes were either dialysed into 'eukaryotic dialysis buffer' or centrifuged through a 30% (<sup>W</sup>/v) sucrose 'shelf' containing 'eukaryotic dialysis buffer' and resuspended in this buffer. Ribosomes prepared by this method appeared to be clean because they were totally inactive in polyuridylic (polyU) - directed synthesis of polyphenylalanine when no yeast S100 was present in the incubation mixture (Fig.6.5)

When ribosomes were required for the preparation of subunits, they were obtained by

layering 5ml yeast S-30 over 2ml 30% (<sup>w</sup>/v) sucrose containing 'eukaryotic grinding buffer' and centrifuging for four hours at 50,000 r.p.m. and 2°C. The ribosomes were resuspended in a small quantity of 'eukaryotic dialysis buffer' and given a low speed clearing spin. Ribosomes prepared in this manner retained considerable activity in poly U-directed synthesis of polyphenylalanine even when incubated in the absence of S100 (Fig.6.5 ).

All preparations of ribosomes were stored as small aliquots at -70°C. At this temperature, no loss of synthetic activity was found to occur with time of storage.

An extinction coefficient of 1mg of ribosomes = 13 A260 units was assumed in order to estimate ribosome concentrations (Sissons, 1974). Although the molecular weight of the yeast ribosome has been reported to be  $3.4 \times 10^6$  daltons (Bruening, 1965), most workers have assumed a molecular weight of  $4 \times 10^6$  daltons (Van der Zeijst et al., 1973; Barbacid and Vazquez, 1974; Cannon et al., 1976). Therefore, in order to compare these results with the work of others, 1mg of ribosomes was taken to be equivalent to 250 p moles.

The ratio of the absorbance at 260 n.m. (A260) to the absorbance at 280 n.m. (A280) was taken as an estimate for the 'purity' of the ribosome



preparation. Ribosomes washed extensively in 'high-salt' had an A260/A280 ratio of 1.89 - 1.90 whereas a 'crude' ribosome preparation, i.e. ribosomes prepared by centrifugation through 'eukaryotic dialysis buffer' had a ratio of 1.6 - 1.7.

### 3.11. PREPARATION OF MYROTHECIUM VERRUCARIA S-30

A pellet of Myrothecium verrucaria cells, either freshly prepared or derived from cells which had been stored at -70°C, was disrupted in a French pressure cell at 0°C. No buffer was added to the cells at this stage since they were found to have sufficient buffer trapped within the pellet. The cells were pressed twice - once at 5,000 psi and finally at 10,000 psi. An S-30 was derived from the cell paste exactly as described for the preparation of a yeast S-30 (Section 3.9). The M.verrucaria S-30 was stored as small aliquots at -70°C.

Ribosome concentration in the S-30 was estimated by measuring the absorbance at 260 n.m. of the S-30. It was assumed that 1mg of ribosomes was equivalent to 29.4 A260 units c.f. yeast S-30 (Section 3.9).

### 3.12. PREPARATION OF M.VERRUCARIA RIBOSOMES AND S100

High-salt washed ribosomes, S100 and ribosomes which were required for subunit

preparation were derived from M. verrucaria S-30s exactly as described for the preparation of yeast ribosomes. It was frequently noticed that the 'ribosome' pellet from the first high-salt wash was, in fact, a ribosome pellet with an overlayer of DNA. Pre-treatment of the S-30 with DNase prevented formation of the DNA pellet. However, the DNA pellet was very easily separated from the ribosome pellet and, therefore DNase treatment could be discarded.

An assessment of ribosome concentration was made by assuming 1mg of ribosomes to be equivalent to 13 A260 units.

Ribosomes which were extensively washed with 'high-salt' had an A260/A280 of 1.89 to 1.90 whereas 'crude' ribosome preparations had an A260/A280 of 1.6 - 1.7.

### 3.13. PREPARATION OF OTHER S-30s.

Low speed (30,000 g) supernatant extracts were prepared from the fungi Aspergillus giganteus, Fusarium poae, Fusarium sporotrichiodes and Fusarium equiseti exactly as described for the preparation of M. verrucaria S-30. It was assumed that 1mg of ribosomes = 29.4 A260 units.

### 3.14. INCUBATION CONDITIONS FOR YEAST AND FUNGAL S-30s

A standard incubation mixture for the poly U - directed synthesis of polyphenylalanine was

derived as follows:

HKM	10 $\mu$ L	
'Energy plus amino acids mix'	10 $\mu$ L	
L-[ <sup>14</sup> C]-7 Phe	10 $\mu$ L	530 mCi/mmol <del>See figure legends for</del> specific activity
Polyuridylic acid (Poly U)	5 $\mu$ L	(10mg/ml)
Magnesium acetate	5 $\mu$ L	(200 mM)
tRNA	5 $\mu$ L	(2 mg/ml yeast, Phe-specific)
Water	<u>5 <math>\mu</math>L</u> 50 $\mu$ L	
Ribosomes + S100 or S-30	<u>50 <math>\mu</math>L</u> 100 $\mu$ L	See figure legends for ribosome concentrations used.
<u>HKM</u> 200 mM - Hepes/KOH pH7.5	)	
500 mM - potassium acetate	)	10 x working
35 mM - magnesium acetate	)	concentration

Energy plus amino acids mix

3.75 mM - GTP (Li <sub>3</sub> )	)	
25 mM - ATP (Na <sub>2</sub> )	)	10 x working
50 mM - PEP	)	concentration.
0.375 mM - amino acids minus Phe	)	Adjust to pH7.0
	)	with KOH

Final ionic conditions for the assay were, therefore, 5mM-Tris/acetic acid pH7.6, 20mM-Hepes/KOH pH7.5, 95mM-potassium acetate and 14mM-magnesium acetate. These ionic conditions were sometimes altered (see figure legends). In addition, 1mM-spermidine and 8mM-glutathione were occasionally added to the incubation mixtures (see figure legends).

Concentration of inhibitors of protein synthesis used in these incubations were as indicated in the figure legends.

3.15. PREPARATION OF YEAST AND FUNGAL SAMPLES FOR ESTIMATION OF THEIR RADIOACTIVITY

Five or ten microlitre samples of the incubation mixtures were removed into 1ml of 10% (W/v) TCA. The TCA was then heated at 90° C for thirty minutes in order to hydrolyse aminoacyl-tRNA. Precipitates were collected on Whatman glass-fibre filters, washed three times with 5% (W/v) TCA and the filters dried under an infra-red lamp for at least fifteen minutes. Radioactivity on the filters was estimated by liquid - scintillation (Section 3.16).

3.16. LIQUID - SCINTILLATION COUNTING

Dried filters were estimated for their radioactivity in a scintillant containing 4 g 2-(4' - tert-butylphenyl) -5-(4" - biphenyl) -1,3,4 - oxadiazole/litre of toluene (butyl-PBD scintillant)

Radioactivity in liquid samples was estimated in a scintillant of the following composition:

Toluene	5 L	)	
Butyl-PBD	30 g	)	
Emulsifier mix		)	
No.1 (Fisons)	2.5 L	)	Mixed in this
Distilled water	750 ml	)	order

All samples were counted in Packard liquid-scintillation spectrometers at the following counting efficiencies:

( <sup>3</sup> H) on dried filters	26%
( <sup>3</sup> H) in liquid	24%

( <sup>14</sup> C) or ( <sup>35</sup> S) on filters	90%
( <sup>14</sup> C) or ( <sup>35</sup> S) in liquid	88%

Data are presented with backgrounds subtracted but an indication of background levels is given in the figure legends.

4. INHIBITION OF POLYPEPTIDE-CHAIN ELONGATION IN  
RETICULOCYTE LYSATES

4.1. INTRODUCTION

An agent which inhibits polypeptide-chain elongation may do so by affecting any one of the three component reactions outlined in Chapter 1, namely the binding of aminoacyl-tRNA to the ribosomal 'A' site, the peptidyltransferase reaction or translocation. Whether or not such an agent inhibits the puromycin reaction depends on which of these processes is inhibited.

Puromycin is an analogue of the 3'- end of aminoacyl-tRNA (Fig.1.4) and accepts the transferred peptide in the peptidyltransferase reaction. This so-called 'puromycin reaction' does not require factors or GTP and results in the formation of peptidyl-puromycin, which detaches from the ribosome (Traut and Monro, 1964).

Various drugs which block the ribosomal 'A' site do not prevent the puromycin reaction (Cundliffe and McQuillen, 1967; Cundliffe, 1971), evidently because puromycin has access to the 'A' site under conditions where aminoacyl-tRNA does not. In contrast, inhibitors of either translocation or peptidyltransferase stop the puromycin reaction in systems synthesizing protein, although it is possible to distinguish between such

inhibitors on other grounds.

Since the puromycin reaction is a measure of the peptide-transfer reaction, any drug which inhibits the peptidyltransferase will inhibit the puromycin reaction. In addition, inhibitors of translocation stop the puromycin reaction in systems synthesizing protein by halting the elongation cycle so as to leave peptidyl-tRNA in the ribosomal 'A' site, where it is unable to react <sup>with</sup> ~~in~~ the peptidyltransferase. Clearly, if there is no aminoacyl-tRNA in the 'A' site because the site is blocked by an antibiotic, an inhibitor of translocation will no longer be capable of inhibiting the puromycin reaction since peptidyl-tRNA will be trapped in the 'P' site, i.e. after the translocation step, until puromycin is added. An inhibitor of the peptidyltransferase will, however, still be capable of blocking the puromycin reaction under such conditions.

Clearly, it is important to follow the puromycin reaction in order to establish the mode of action of an antibiotic. The methods commonly used are:

(a) Sucrose density-gradient analysis

A control system making protein is set up with a radioactive amino acid label present. Once the

polyribosomes are fully charged with radioactive peptidyl-tRNA, the inhibitor is added followed by puromycin. The mixture is centrifuged through a sucrose density-gradient to separate polyribosomes of different sizes and the distribution of the radioactivity in the gradient is determined. If the puromycin reaction has taken place there will be little or no radioactivity remaining on the polyribosomes.

This procedure is lengthy and does not provide any information about the time-course of the puromycin reaction. If many different inhibitors are to be studied another technique is often employed.

(b) Use of radio-labelled puromycin.

In order to detect the puromycin reaction in this case, the presence of peptidyl-puromycin is assayed by the incorporation of radioactive puromycin into trichloroacetic acid -(TCA) precipitable material. Unfortunately this technique gives very variable results in rabbit reticulocytes due, apparently, to cleavage of puromycin from the peptidyl-puromycin molecule (R.J.Jackson, personal communication). Presumably the enzyme responsible for removing puromycin is the same as that which removes tRNA from free peptidyl-tRNA i.e. peptidyl-tRNA hydrolase.

In this work another technique was used for



detecting the puromycin reaction. Peptidyl-tRNA levels (in samples of the incubation mixture) were measured by using a radioactive amino acid as label and precipitating all RNA with cetyltrimethylammonium bromide (CTAB; Darnbrough et al., 1973). The puromycin reaction was then detected by a decrease in peptidyl-tRNA concentrations i.e. a decrease in CTAB-precipitable material.

This technique allows a rapid examination of the effects of other antibiotics on the puromycin reaction.

Rabbit reticulocyte lysates were used in these experiments since they are very active in protein synthesis over long periods. In addition, their ribosomes will perform all the necessary steps in protein synthesis: initiation, elongation and termination. Reticulocyte extracts are, therefore, convenient systems in which to study inhibitors of the various steps in protein synthesis.

#### 4.2. INCORPORATION OF LABEL INTO PEPTIDES

Reticulocyte lysates incorporated L- $\alpha$ - $^3\text{H}$ -leucine into protein (as judged by trichloroacetic acid precipitation) linearly with time for over twenty minutes (Fig.4.1). Conversely, incorporation of L- $\alpha$ - $^3\text{H}$ -leucine into peptidyl-tRNA (as judged by cetyltrimethylammonium bromide precipitation) was linear for the first four minutes and during that time was equivalent to the total protein synthesized.

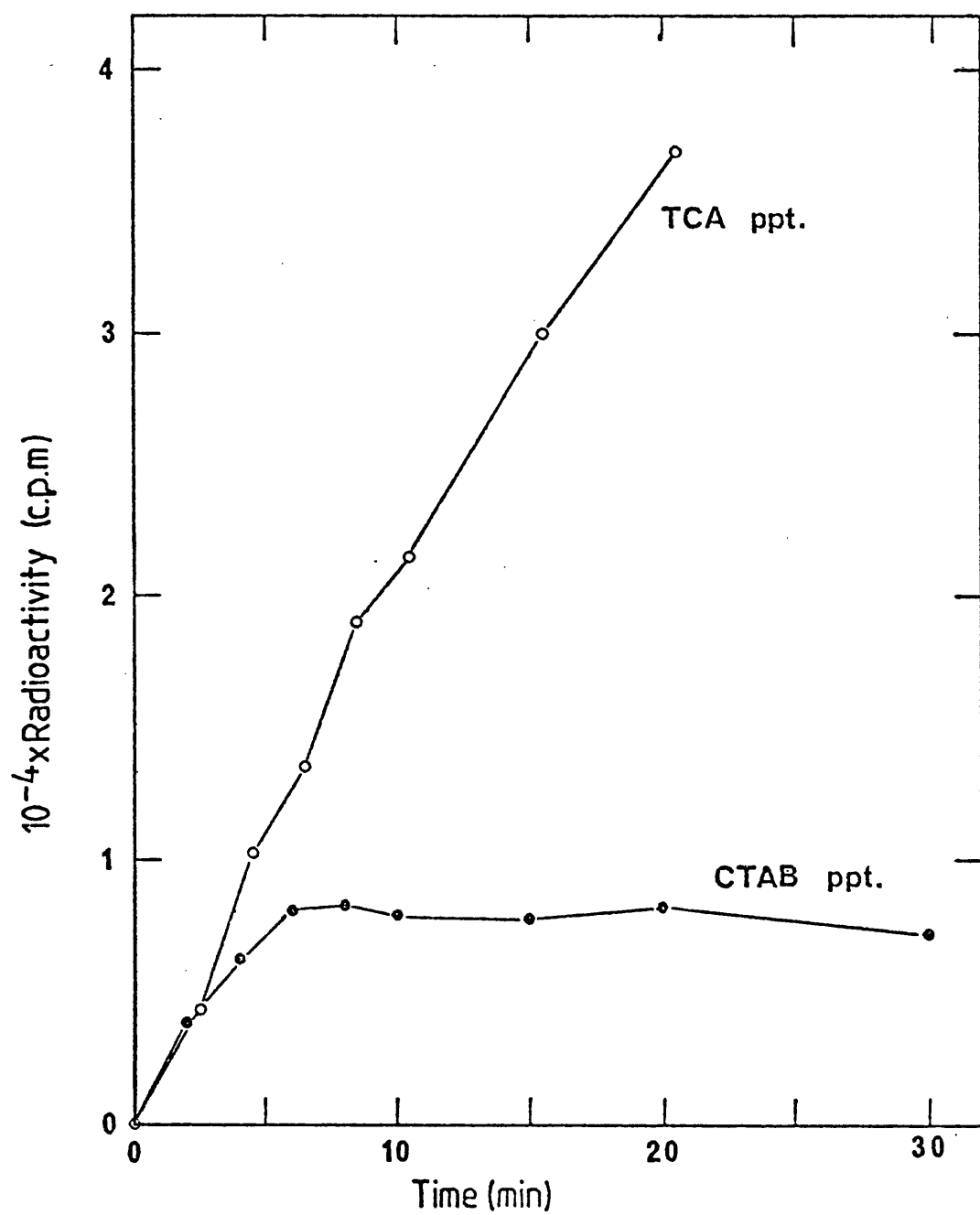


FIGURE 4.1 Incorporation of L-[<sup>3</sup>H]leucine into Peptide

Legend to Figure 4.1.

Incorporation of L- $^3\text{H}$ -leucine into Peptide.

A mixture (Section 3.5) containing L- $^3\text{H}$ -leucine (51 Ci/mmol) in 0.3 mM-L-leucine was incubated at 30°C and 5  $\mu\text{l}$  samples were removed into water at various times. These samples were precipitated with either trichloroacetic acid (O) or cetyltrimethylammonium bromide (o) and radioactivity in the precipitates was measured by liquid-scintillation counting (Section 3.16).

Shortly thereafter (Fig.4.1), incorporation into peptidyl-tRNA slowed and soon reached a plateau level where, presumably, there was no net production of peptidyl-tRNA. This implies that a steady state was reached after 6 - 10 min in which initiation of synthesis of new peptide chains on ribosomes was balanced by release of completed ones from other ribosomes (see Section 5.2 for further support for this interpretation).

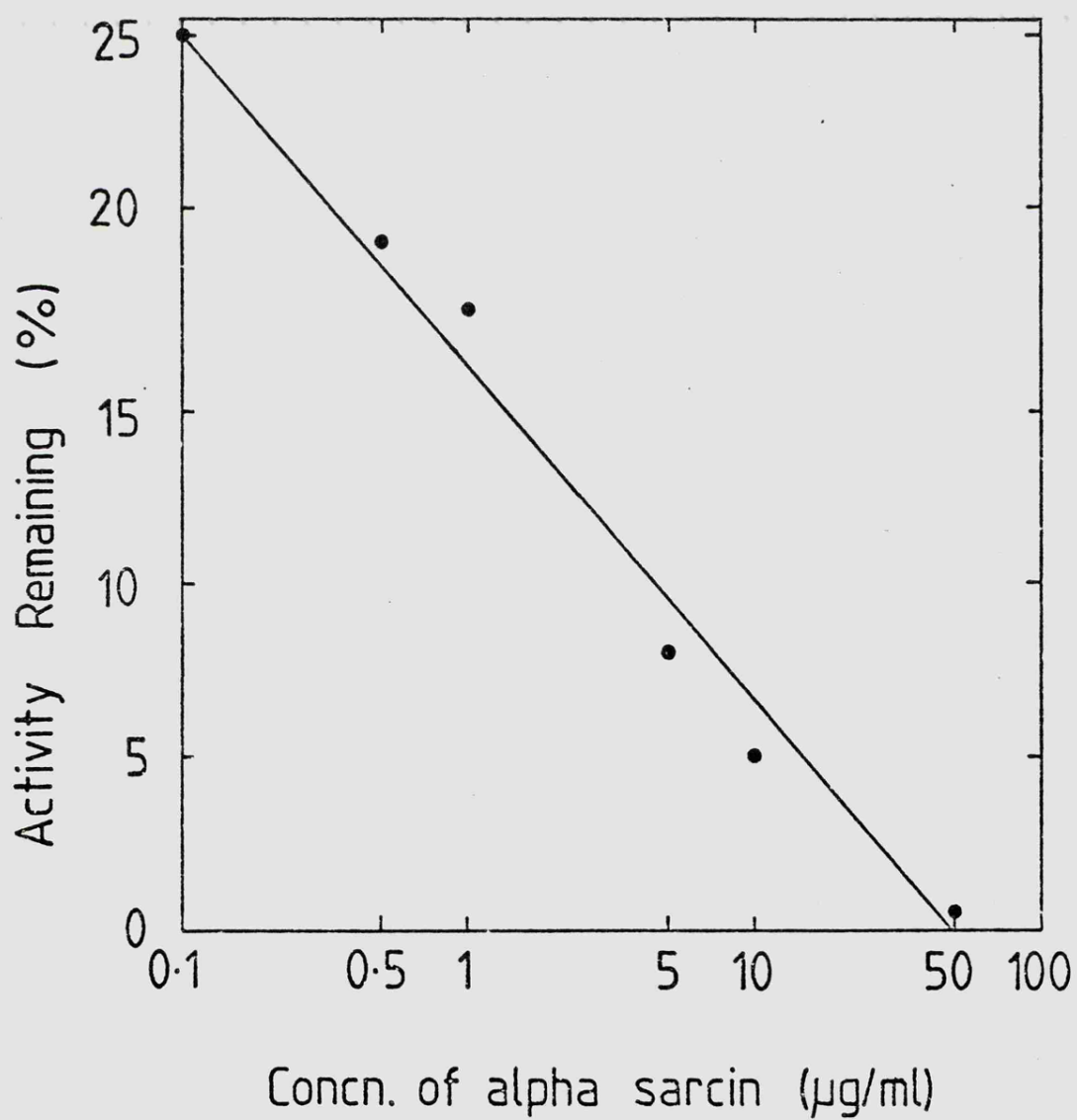
The reticulocyte lysates' abilities to incorporate L-[ $^3\text{H}$ ] leucine into protein or peptidyl-tRNA were found to vary considerably between preparations.

#### 4.3. INHIBITION OF PROTEIN SYNTHESIS BY ALPHA SARCIN

Alpha sarcin is a polypeptide of mol.wt.16,000 isolated from the mould Aspergillus giganteus (Olson, 1963; Schuurmans, et al., 1964). It inhibits several induced tumours, including sarcoma 180 and carcinoma 755, but shows no anti-bacterial or anti-fungal activity in vivo ( Olson and Goerner 1965; Olson et al., 1965).

The drug has been shown to be a potent inhibitor of protein synthesis in extracts of wheat germ, yeast, rat liver and Erlich ascites cells and to be weakly inhibitory to E.coli extracts. This inhibition apparently is caused, in eukaryotic systems, by alpha sarcin blocking the elongation factor 1 (EF-1)-dependent binding of aminoacyl-tRNA to the ribosome (D.Schindler,

FIGURE 4.2    Inhibition of Protein Synthesis  
by Alpha Sarcin



Legend to Figure 4.2

Inhibition of Protein Synthesis by  
Alpha Sarcin

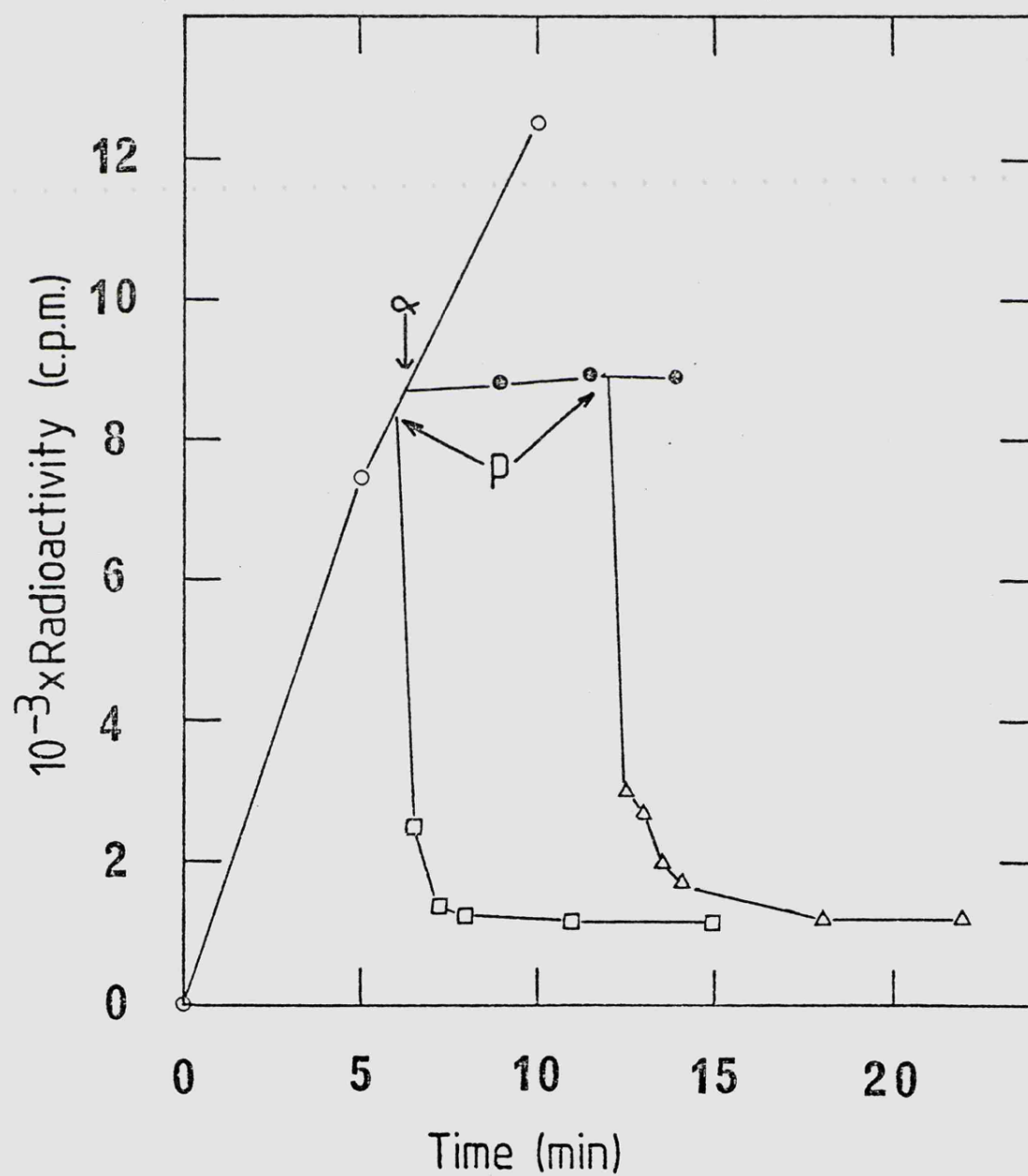
Mixtures (Section 3.5) containing L- $^{-3}\text{H}$ -leucine (51 Ci/mmol) were incubated at 30°C. Alpha sarcin at various final concentrations was added before the incubation. After 20 minutes, samples (5  $\mu\text{l}$ ) were precipitated with TCA and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).

R.D.Nolan and J.E.Davies, unpublished observations).

In addition, alpha sarcin has been shown to catalyse the specific cleavage of rRNA of the large subunit of yeast, wheat germ and E.coli ribosomes (Schindler and Davies 1977). However, this cleavage is only noticed at concentrations much higher than those required to inhibit protein synthesis so its significance is uncertain.

In reticulocyte lysates, alpha sarcin was found to inhibit protein synthesis by 95% at 10 µg/ml (3 molecules of alpha sarcin per ribosome) but total inhibition was not observed until alpha sarcin was present at 50 µg/ml (15 molecules of alpha sarcin per ribosome, Fig.4.2). Although it has been suggested that alpha sarcin may act as an enzyme, the kinetics of inhibition by alpha sarcin do not support this hypothesis since the degree of inhibition did not increase with the time of incubation with alpha sarcin (data not given). In addition, although there was considerable inhibition of protein synthesis at very low concentrations of alpha sarcin (less than 1 molecule of alpha sarcin per ribosome) this result does not, of necessity, imply that alpha sarcin acts as an enzyme. It has been argued previously (Cundliffe, 1967; 1972) that only one molecule of antibiotic is required per polyribosome in order to cause complete inhibition of protein synthesis and, presumably, less

FIGURE 4.3 Assay of the Puromycin Reaction in Control Lysates or Lysates Inhibited by Alpha Sarcin





### Legend to Figure 4.3

#### Assay of the puromycin reaction in control lysates or lysates inhibited by Alpha Sarcin

A mixture (Section 3.5) containing L- $^3\text{H}$  leucine (51 Ci/mmol) was incubated at 30°C. After six minutes portions of the control lysate were removed either into puromycin (P; 40 µg/ml final concn.) or into alpha sarcin ( $\alpha$ ; 100 µg/ml final concn.). At 13 minutes a further portion of the lysate inhibited by alpha sarcin was removed into puromycin (40 µg/ml final concn.). Samples (5 µl) were precipitated with CTAB (Section 3.6) and radioactivity in the precipitates was determined by liquid-scintillation counting (Section 3.16). o, Uninhibited control; ●, plus alpha sarcin; □, plus puromycin; Δ, plus alpha sarcin and puromycin.

than complete inhibition can be brought about by even lower concentrations of antibiotic.

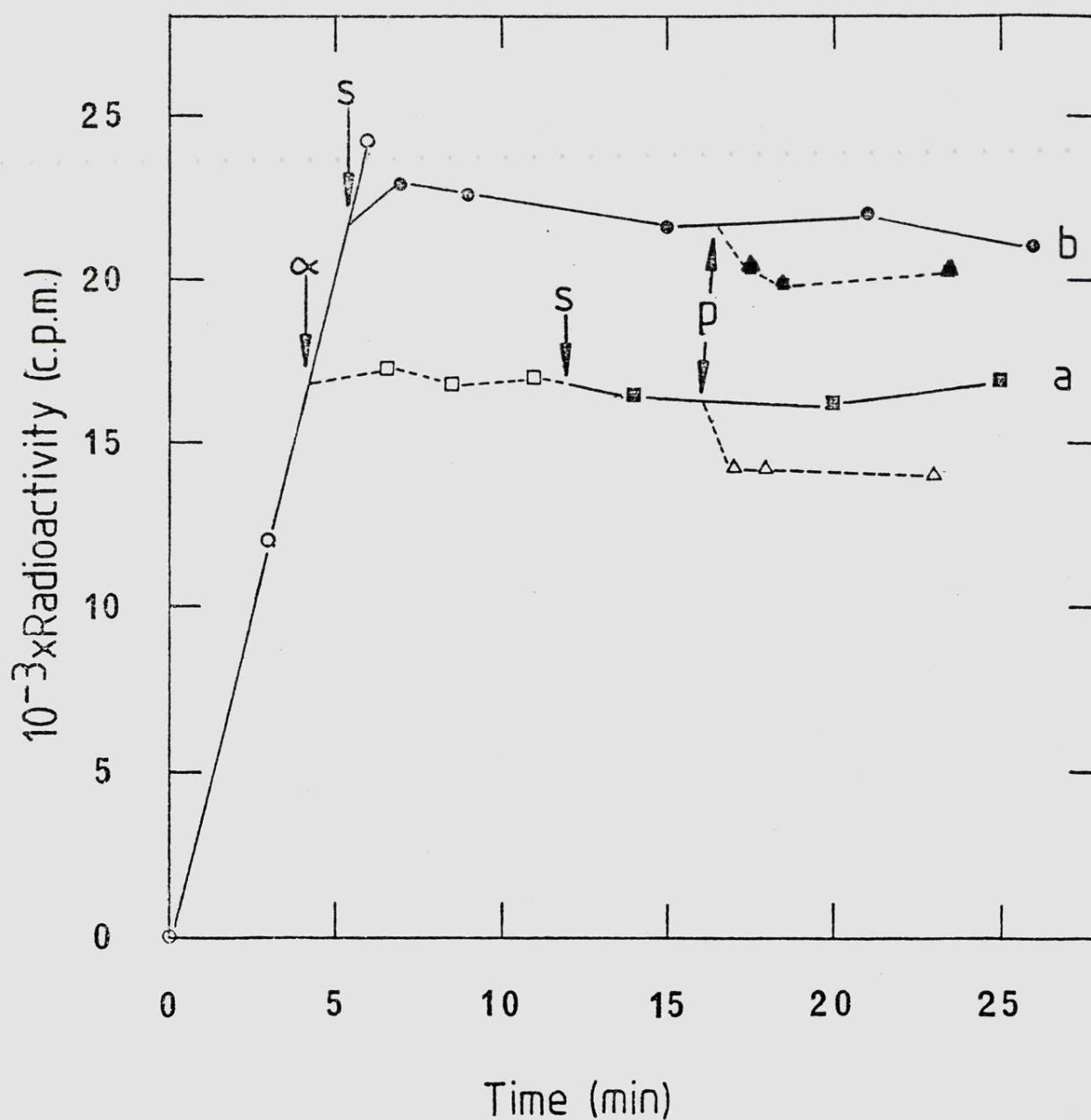
Alpha sarcin was used at 100  $\mu\text{g/ml}$  (final concentration) in all the other experiments referred to here. At this concentration it inhibited protein synthesis totally and immediately (Fig.4.2 and 4.3). Under these latter conditions, however, alpha sarcin did not prevent the rapid and almost quantitative release of nascent peptide chains from ribosomes in the presence of puromycin (Fig.4.3).

The effect of adding puromycin at the same concentration (40  $\mu\text{g/ml}$ ) to an otherwise uninhibited lysate is shown in Fig.4.3. Again there was a rapid release of nascent peptide chains from ribosomes.

The effects of sparsomycin and cycloheximide on peptidyl-tRNA synthesis in reticulocyte lysates are shown in Figs. 4.4. and 4.5, respectively. Here those antibiotics were again used at high concentrations so that protein synthesis would be totally inhibited. Their effects on the puromycin reaction are also shown in Figs. 4.4. and 4.5. Sparsomycin almost completely prevented the decrease in the concentration of peptidyl-tRNA by puromycin, whereas cycloheximide (although strongly inhibitory) caused a slow decrease.

In a further series of experiments, sparsomycin or cycloheximide was added to reticulocyte lysates in which protein synthesis had already been stopped by alpha sarcin,

FIGURE 4.4 Inhibition of Protein Synthesis and the Puromycin Reaction by Sparsomycin

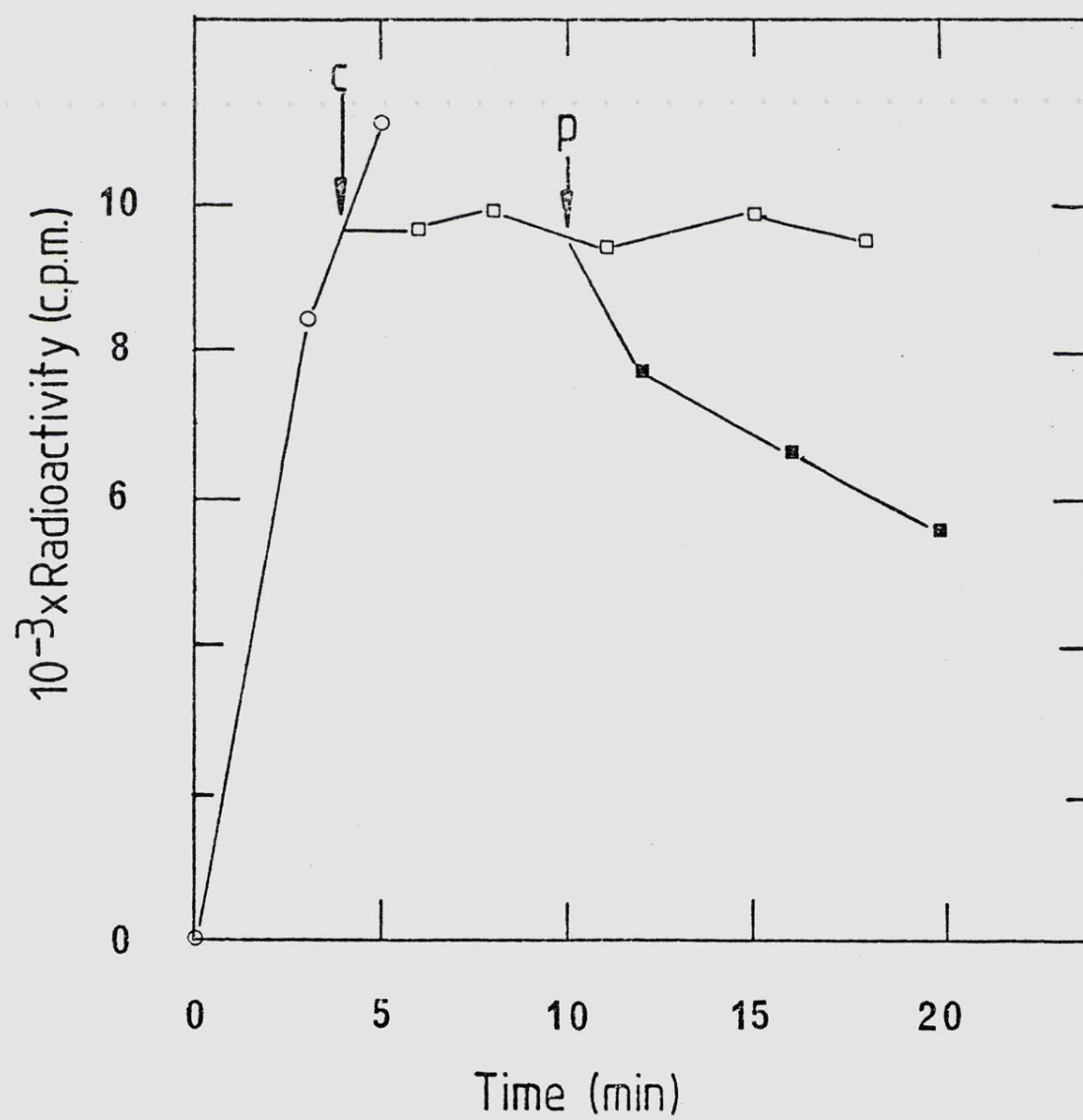


#### Legend to Figure 4.4

##### Inhibition of Protein Synthesis and the Puromycin Reaction by Sparsomycin

A mixture (Section 3.5) containing L- $^{-3}\text{H}$ -leucine (51 Ci/mmol) was incubated at 30°C. As indicated, portions were removed either into (a) alpha sarcin (  $\alpha$  ; 100  $\mu\text{g/ml}$  final concn.) or (b) sparsomycin (S; 40  $\mu\text{g/ml}$  final concn.). Sparsomycin (S, 40  $\mu\text{g/ml}$  final concn.) was added to (a) at 12 minutes, and, finally, portions of (a) and (b) were removed into puromycin (P; 40  $\mu\text{g/ml}$  final concn.) at 16 minutes. Samples (5  $\mu\text{l}$ ) were precipitated with CTAB (Section 3.6) and radioactivity in the precipitates was determined by liquid-scintillation counting. O, Uninhibited control; ●, plus sparsomycin; ▲ , plus sparsomycin and puromycin; □ , plus alpha sarcin; ■ , plus alpha sarcin and sparsomycin; Δ , plus alpha sarcin, sparsomycin and puromycin.

FIGURE 4.5      Effect of Cycloheximide on the Puromycin Reaction



# Legend to Figure 4.5

## Effect of Cycloheximide on the Puromycin

### Reaction

A mixture (Section 3.5) containing L- $^3\text{H}$ -leucine (51 Ci/mmol) was incubated at 30°C. As indicated, a portion was removed into cycloheximide (C; 200 µg/ml final concn.) and finally a portion of the inhibited lysate was removed into puromycin (P; 40 µg/ml final concn.). Samples (5 µl) were precipitated with CTAB (Section 3.6) and radioactivity in the precipitates was determined by liquid-scintillation counting. o, control; □, plus cycloheximide; ■, plus cycloheximide and puromycin.

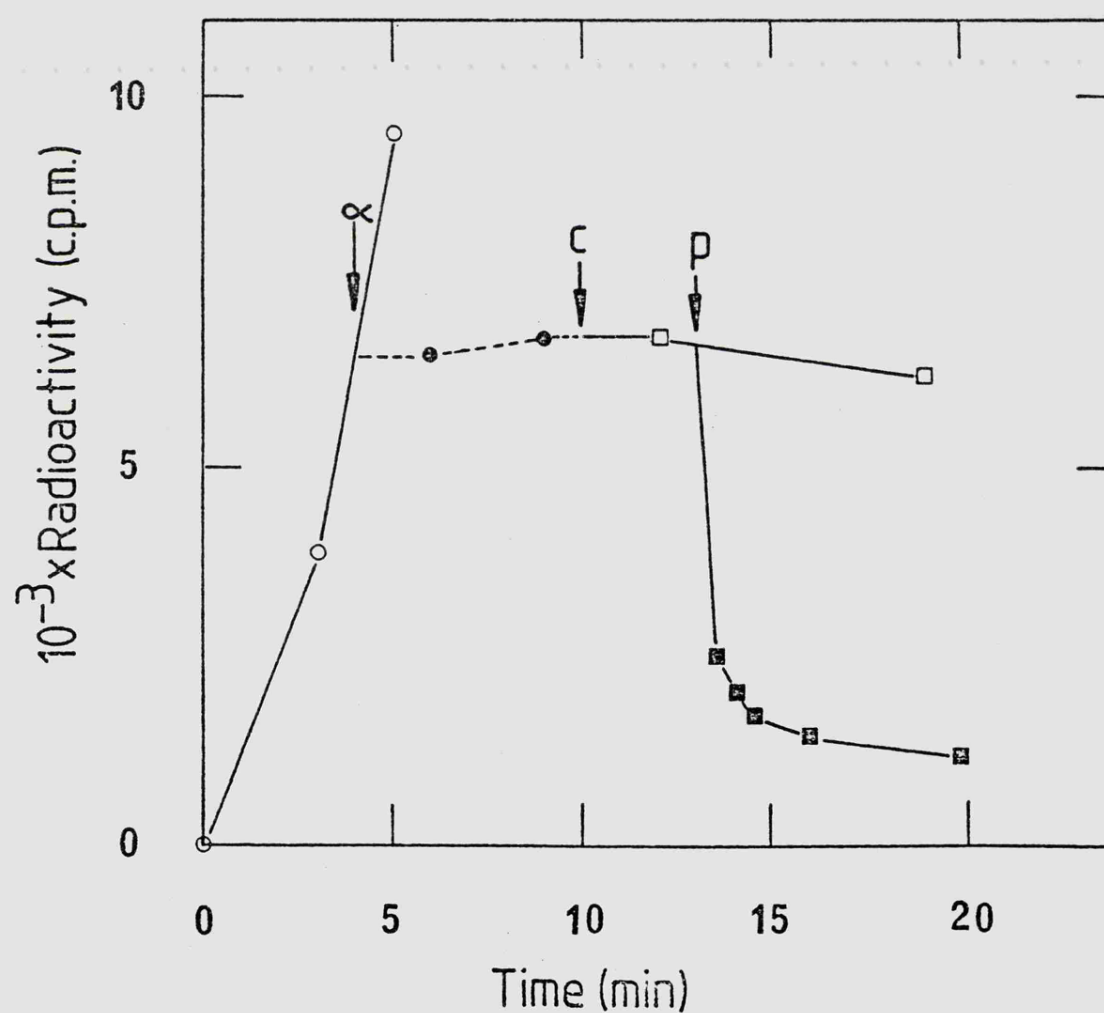
puromycin added finally and peptidyl-tRNA concentrations monitored throughout. The results are shown in Figs.4.4, and 4.6. The effect of sparsomycin, namely almost total inhibition, was unchanged by the prior addition of alpha sarcin (Fig.4.4), whereas cycloheximide now failed to inhibit release (Fig.4.6.)

These results suggest that alpha sarcin inhibits the binding of aminoacyl-tRNA to the ribosomal "A" site without preventing the binding of puromycin. It is not possible to assign a site of action either on or off the ribosome. The results of Schindler and Davies (1977) would suggest, however, that alpha sarcin causes the inhibition by binding to the ribosome.

In contrast to alpha sarcin, and in agreement with the findings of others, cycloheximide (Baliga et al., 1970) and sparsomycin (Baglioni, 1966; Goldberg and Mitsugi, 1967) both inhibited the puromycin reaction (Figs. 4.4 and 4.5). Therefore, sparsomycin and cycloheximide were inhibiting either the peptidyltransferase or translocation in the lysates.

According to my hypothesis, after the addition of alpha sarcin to reticulocyte lysates, peptidyl-tRNA was confined to the ribosomal 'P' sites, from which it was readily removable by puromycin (Fig.4.3) via the peptidyltransferase reaction. Therefore, one would expect a drug which inhibits the peptidyltransferase to prevent such puromycin reactions in the presence of

FIGURE 4.6 Effect of Cycloheximide on the Puromycin Reaction in the Presence of Alpha Sarcin





#### Legend to Figure 4.6

##### Effect of Cycloheximide on the Puromycin Reaction in the Presence of Alpha Sarcin

A mixture (Section 3.5) containing L- $^3\text{H}$ -leucine (51 Ci/mmol) was incubated at 30°C. As indicated a portion was removed into alpha sarcin (  $\alpha$  ; 100  $\mu\text{g/ml}$  final concn.) and at 10 minutes cycloheximide (C; 200  $\mu\text{g/ml}$  final concn.) was added to the inhibited lysate. Finally, a portion of the inhibited lysate was removed into puromycin (P; 40  $\mu\text{g/ml}$  final concn.). Samples (5  $\mu\text{l}$ ) were precipitated with CTAB (Section 3.6) and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).  
○, Control; ●, plus alpha sarcin; □, plus alpha sarcin and cycloheximide; ■, plus alpha sarcin, cycloheximide and puromycin.

alpha sarcin. Sparsomycin did so (Fig. 4.4), as it did in analogous experiments in a bacterial system (Cundliffe and McQuillen 1967), in accordance with its established action as a peptidyltransferase inhibitor (for reviews, see Cundliffe 1972 and Vazquez 1974). In other experiments (Fig. 4.6), cycloheximide did not inhibit the puromycin reaction after the prior addition of alpha sarcin to reticulocyte lysates. This result is consistent with an action of cycloheximide on translocation reactions, which, of course, cannot occur when peptidyl-tRNA is confined to the ribosomal 'P' site. The first report that cycloheximide inhibits translocation (McKeehan and Hardesty, 1969) has been followed by various conflicting suggestions, that the drug inhibits either this reaction or the peptidyltransferase (for reviews see Cundliffe 1972 and Vazquez 1974). Our results are consistent with the more recent general consensus that cycloheximide inhibits translocation (Barbacid et al., 1975).

These results are not, however, definitive proof that cycloheximide is an inhibitor of translocation. If the binding site for cycloheximide was identical or overlapping with that for alpha sarcin, the puromycin reaction would still not be inhibited by the combination of the two antibiotics. In addition, Fig. 4.5 shows that

cycloheximide, even at totally inhibitory concentrations, does not inhibit completely the puromycin reaction. Clearly, this latter result does not fit the predicted effect of a translocation inhibitor but the reason for the discrepancy is unclear. It may merely reflect weak binding by cycloheximide which permits ribosomes to translocate occasionally and, thus, react with puromycin

4.4. INHIBITION OF PROTEIN SYNTHESIS IN RETICULOCYTE LYSATES BY TRICHODERMIN

It is now well established that the primary mode of action of trichodermin is inhibition of the elongation stage of protein synthesis in eukaryotes (Cundliffe et al., 1974; Tscherne and Pestka, 1975; Carter et al., 1976). There are many reports that the actual step inhibited by trichodermin is the peptidyltransferase. These results are derived from three different but basically similar types of experiment using isolated ribosomes:

(a) Trichodermin was found to inhibit both the fragment assay and the formation of acetyl  $\angle^{-3}\text{H}_7$  Phe-puromycin using ribosomes isolated from human tonsils, yeast (Carrasco et al., 1973) and rat liver (Edens et al., 1975). Additionally, in an assay analogous to the termination step in protein synthesis, trichodermin was found to prevent the release of F-Met from the ribosome in response to the termination codon and release factor (RF) using rabbit reticulocyte ribosomes (Tate and Caskey 1973; Wei et al., 1974).

(b) In an assay specific for the formation of the first peptide bond during elongation, trichodermin was found to inhibit the formation of Met-puromycin from the 80S • Met-tRNA<sub>F</sub> • AUG initiation complex and puromycin (Fresno et al., 1976).

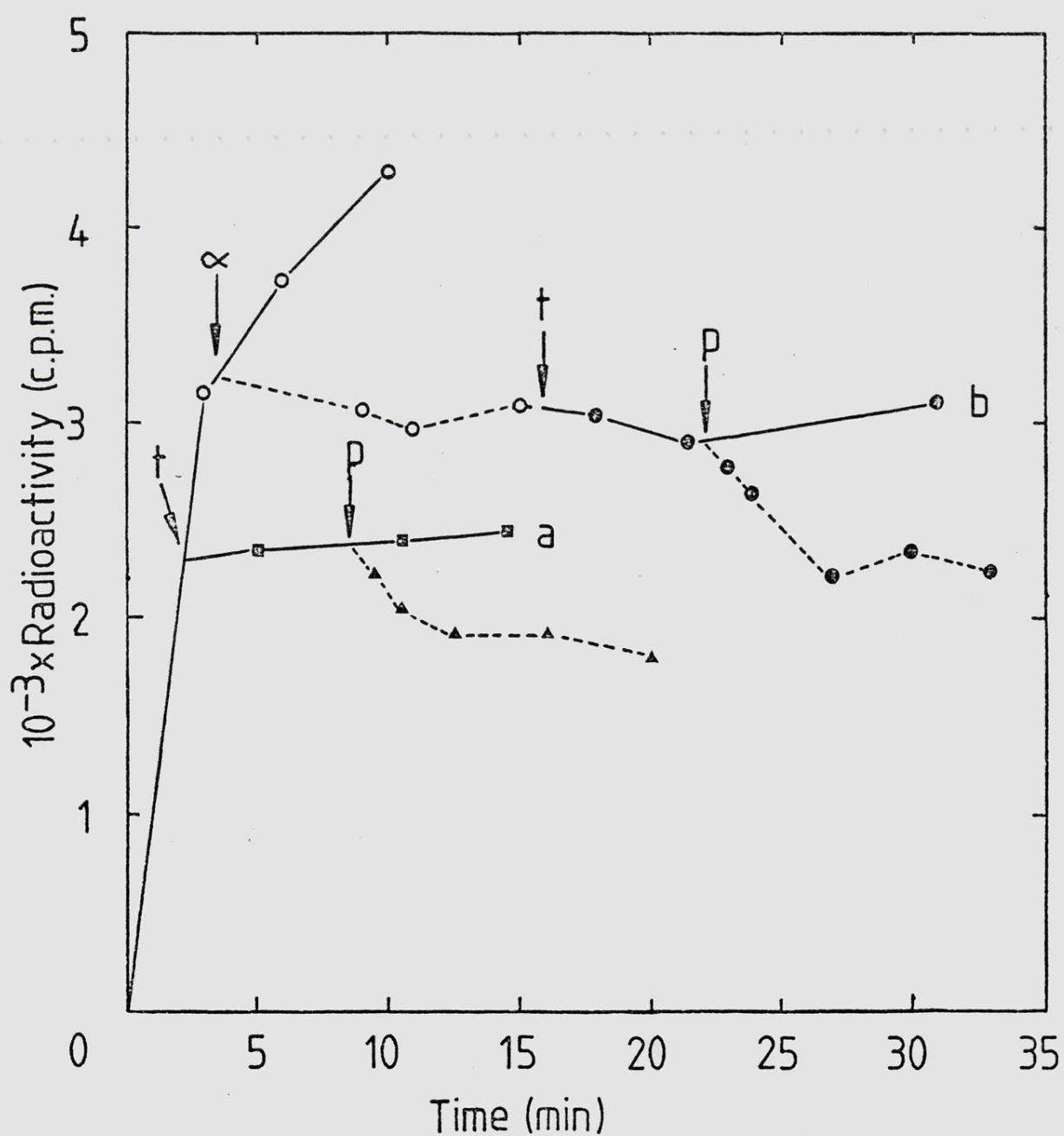
(c) Using isolated yeast polyribosomes which, when mixed with the appropriate supernatant mix, were active in all the elongation steps of protein synthesis, trichodermin totally inhibited the formation of peptidyl-(<sup>3</sup>H) puromycin. It is predicted that an inhibitor of translocation would only inhibit the formation of peptidyl-(<sup>3</sup>H) puromycin by 50% compared to an uninhibited control in this system (Barbacid et al., 1975).

Such model systems do not always give results in agreement with those obtained in vivo or in vitro using more complete systems (for a review, see Cundliffe, 1972), and even though the latter system described above has advantages over others it still requires careful interpretation of data in order to distinguish types of inhibitor.

Accordingly, it was decided to check the mode of action of trichodermin by using the CTAB assay system described in Section 4.3.

The effect of trichodermin on peptidyl-tRNA synthesis in reticulocyte lysates is shown in Fig.4.7. Although inhibition was greater than 90% at 10 µg/ml

FIGURE 4.7      Effect of Trichodermin on the  
Puromycin Reaction in the Presence  
or the Absence of Alpha Sarcin



#### Legend to Figure 4.7

##### Effect of Trichodermin on the Puromycin Reaction in the Presence or Absence of Alpha Sarcin

A mixture (Section 3.5) containing L- $^{-3}\text{H}_7$  leucine (51 Ci/mmol) was incubated at 30°C. As indicated, portions were removed either into (a) trichodermin (t; 30 µg/ml final concn.) or (b) alpha sarcin (α; 100 µg/ml final concn.). Trichodermin (t; 30 µg/ml final concn.) was added to (b) at 16 minutes and, finally, portions of (a) and (b) were removed into puromycin (P; 40 µg/ml final concn.). Samples (5 µl) were precipitated with CTAB (Section 3.6) and radioactivity in the precipitates was estimated by liquid-scintillation counting (Section 3.16). O—O, uninhibited control; O---O, plus alpha sarcin; ■ , plus trichodermin; ●—●, plus alpha sarcin and trichodermin; ●---●, plus alpha sarcin, trichodermin and puromycin; ▲ plus trichodermin and puromycin.

trichodermin (final concentration; data not given) the toxin was used at 30  $\mu\text{g/ml}$  (final concentration) when inhibition was greater than 99% (Fig.4.7). Under these latter conditions, trichodermin inhibited the release of nascent peptidyl-tRNA by puromycin (Fig.4.7) although inhibition was not absolute c.f. sparsomycin (Fig.4.4). In a further experiment, trichodermin at 30  $\mu\text{g/ml}$  (final concentration) was added to a reticulocyte lysate in which protein synthesis had already been stopped by alpha sarcin, puromycin finally added and peptidyl-tRNA levels monitored. The effect of trichodermin, namely almost total inhibition of the puromycin reaction, was unchanged by the prior addition of alpha sarcin (Fig.4.7). Clearly, therefore, and in agreement with previous observations, trichodermin is an inhibitor of the peptidyltransferase in rabbit reticulocyte lysates.

It is unclear why both sparsomycin and trichodermin allow a small amount of peptidyl-tRNA to react with puromycin in their presence. This result may reflect (i) two sites for peptidyl-tRNA within the 'P' site. The antibiotic inhibits the peptidyltransferase only when the peptidyl-tRNA is present in one of these sites: (ii) less than one molecule bound per ribosome, i.e. some ribosomes are sometimes without a bound antibiotic molecule because of weak drug binding.

#### 4.5. INHIBITION OF PROTEIN SYNTHESIS IN RETICULOCYTE LYSATES BY CHARTREUSIN AND EMETINE

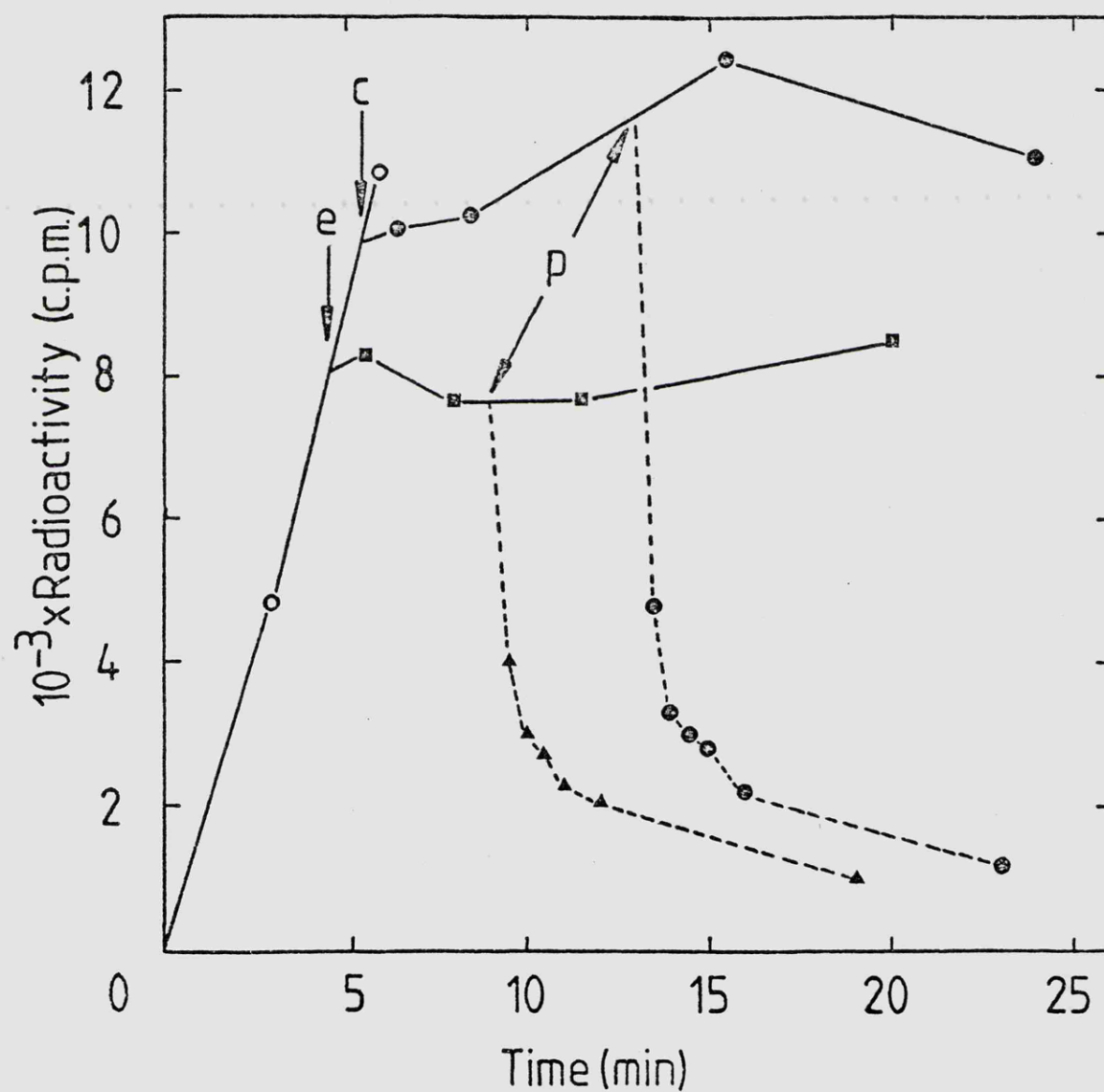
The two antibiotics, chartreusin and emetine, were tested for their ability to inhibit protein synthesis in the rabbit reticulocyte system.

Chartreusin (Fig.1.4) is a metabolite of Streptomyces chartreusis and shows significant anti-tumour activity (McGovren et al., 1977). It is reported to inhibit the elongation stage of protein synthesis in rabbit reticulocytes by preventing the elongation factor -1 (EF-1)-dependent binding of aminoacyl-tRNA to the ribosome (Gregg and Heintz, 1972) and would appear, therefore, to act in a fashion superficially similar to alpha sarcin.

Emetine (Fig.1.4) is one of a group of closely-related plant alkaloids of Ipecacuanha. It inhibits protein synthesis in mammalian, yeast and plant cells (Grollman, 1966; 1968) and supposedly has structural similarities with cycloheximide (Grollman, 1967). The site of action of the antibiotic is reported to be the translocation step in protein synthesis (Grollman, 1968; Grollman and Huang, 1973; Carrasco et al., 1976). However, whilst emetine does inhibit the puromycin reaction using yeast polyribosomes (Carrasco et al., 1976) it is unable to do so in HeLa and rat liver cell-free extracts (Grollman, 1968; Baliga et al., 1970). Clearly,



FIGURE 4.8      Effect of Chartreusin or Emetine on the Puromycin Reaction



#### Legend to Figure 4.8

##### Effect of Chartreusin or Emetine on the Puromycin Reaction

A mixture (Section 3.5) containing L- $^{-3}\text{H}$ -leucine (51 Ci/mmol) was incubated at 30°C. As indicated, portions were removed either into emetine (e; 80 µg/ml final concn.) or chartreusin (c; 320 µg/ml final concn.). Finally, portions of the inhibited lysates were removed into puromycin (P; 40 µg/ml final concn.). Samples (5 µl) were precipitated with CTAB (Section 3.6) and radioactivity in the precipitates was estimated by liquid-scintillation counting (Section 3.16). O, uninhibited control; ●—●, plus chartreusin; ●---●, plus chartreusin and puromycin; ■, plus emetine; ▲, plus emetine and puromycin.

therefore, its site of action need not be the translocation step in protein synthesis in reticulocyte lysates.

Both chartreusin and emetine inhibited protein synthesis in reticulocyte lysates. However, chartreusin was unable to inhibit by greater than 90% even at very high concentrations (320  $\mu\text{g/ml}$ ). In contrast, emetine totally inhibited protein synthesis at 80  $\mu\text{g/ml}$ , final concentration (Fig.4.8). At these concentrations, the two antibiotics were unable to inhibit the puromycin-induced decrease in peptidyl-tRNA concentrations (Fig.4.8) suggesting that they do not inhibit translocation or the peptidyltransferase. It is likely, therefore, that they both act by preventing aminoacyl-tRNA from binding to the ribosomal 'A' site.

In a further experiment, cycloheximide was added to a lysate which was already inhibited by emetine, puromycin added finally and peptidyl-tRNA levels monitored. Again, the puromycin reaction was uninhibited (data not given). Since mutants of yeast and Chinese hamster ovary (CHO) cells which are resistant to emetine in vivo and in vitro display no cross-resistance to cycloheximide (Jimenez et al., 1977; Gupta and Siminovitch, 1977) it is likely that

binding sites for these two antibiotics do not overlap and, presumably, both emetine and cycloheximide can bind to the ribosome at the same time. Therefore, the latter experiment provides further evidence that cycloheximide is an inhibitor of translocation.

No evidence is available which allows the differential effects of emetine, i.e. inhibition of translocation in yeast and prevention of aminoacyl-tRNA binding in mammalian cells to be reconciled. Whilst the reported site of action of emetine on yeast ribosomes is at variance with the results of all other experiments it need not be an artefact of the cell-free system used. Although no data are available for emetine, tubulosine which is unable to inhibit the EF-1-dependent binding of aminoacyl-tRNA to the yeast ribosome (Carrasco et al., 1976) and emetine appear to have the same or similar sites of action in vitro. They both inhibit enzymic translocation in yeast (Carrasco et al., 1976) and a yeast mutant which is resistant to tubulosine is also resistant to emetine in vivo and in vitro (Carrasco et al., 1976). The sites of action of emetine in both yeast and mammalian cells are probably closely linked, however, since resistance to emetine is a function of the 40S subunit both in yeast (Grant et al., 1974; Jimenez et al., 1977; Sanchez et al., 1977) and CHO cells (Gupta and Siminovitch, 1977). Possibly emetine

binds to the ribosome at, or near to, the ribosomal 'A' site and prevents aminoacyl-tRNA from binding to mammalian ribosomes whilst still permitting aminoacyl-tRNA to bind to the smaller yeast ribosome (and react with peptidyl-tRNA in the 'P' site). Peptidyl-tRNA in the 'A' site must then be prevented from translocating by the presence of emetine.

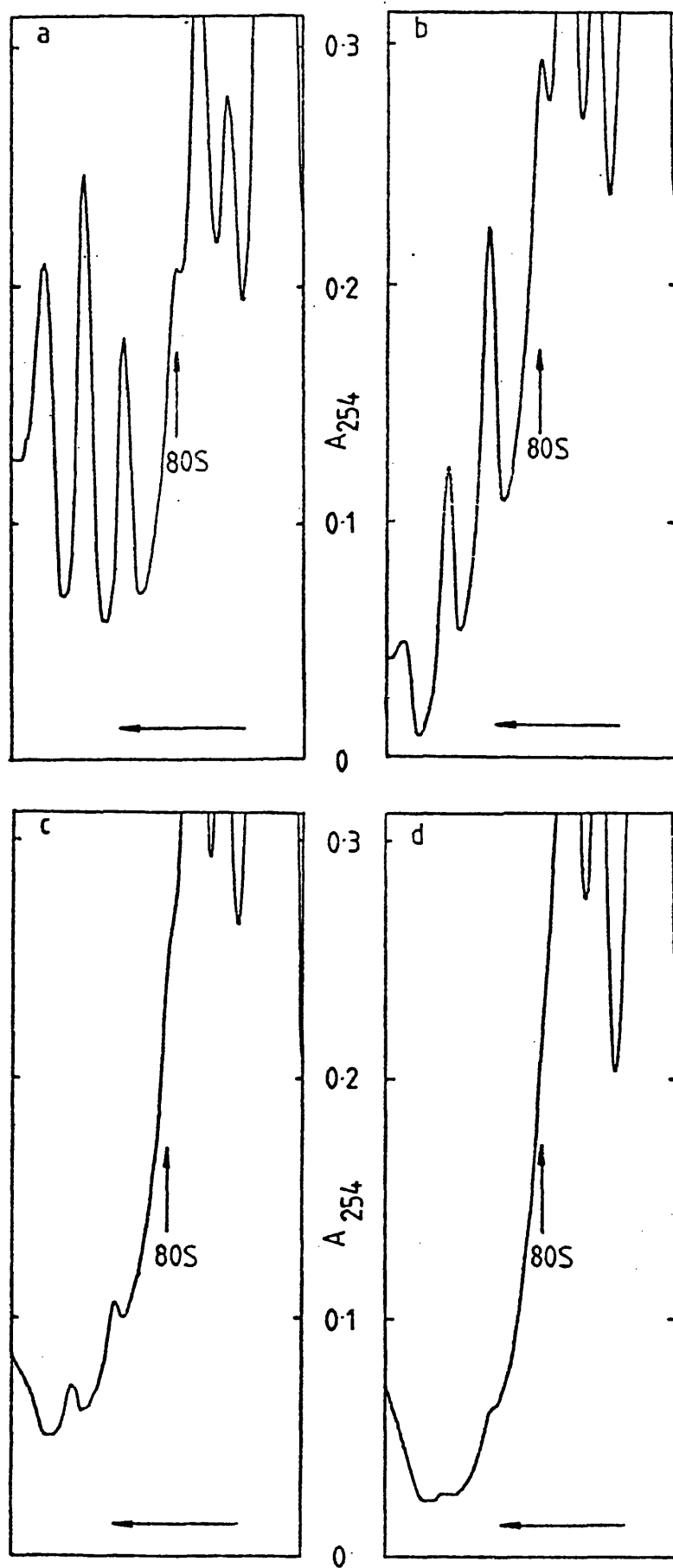
5. INHIBITION OF INITIATION OF PROTEIN SYNTHESIS IN  
RETICULOCYTE LYSATES

5.1. INTRODUCTION

Rabbit reticulocyte lysates are very active in all stages of protein synthesis and have been used extensively in studies designed to elucidate the steps in the initiation process of protein synthesis (see Chapter 1). They are, therefore, one of the best systems to use when studying putative inhibitors of initiation.

By definition, a specific inhibitor of the initiation stage of protein synthesis is incapable of inhibiting the elongation or termination stages and this provides the means by which such an inhibitor may be detected. When initiation is inhibited, ribosomes which are already on mRNA remain uninhibited and proceed along the message. On reaching the termination codon, they release a completed polypeptide, 'fall off' the message and are unable to re-initiate synthesis due to the presence of inhibitor. This process is detected by following the incorporation of radioactive label into TCA-insoluble peptide. A lag is seen between the time of addition of toxin and the inhibition of incorporation of label. In contrast, addition of an inhibitor of the elongation stage gives rise to an immediate effect upon

FIGURE 5.1 Polysome Degredation in the Presence of Edeine



## Legend to Figure 5.1

### Polysome Degradation in the Presence of Edeine

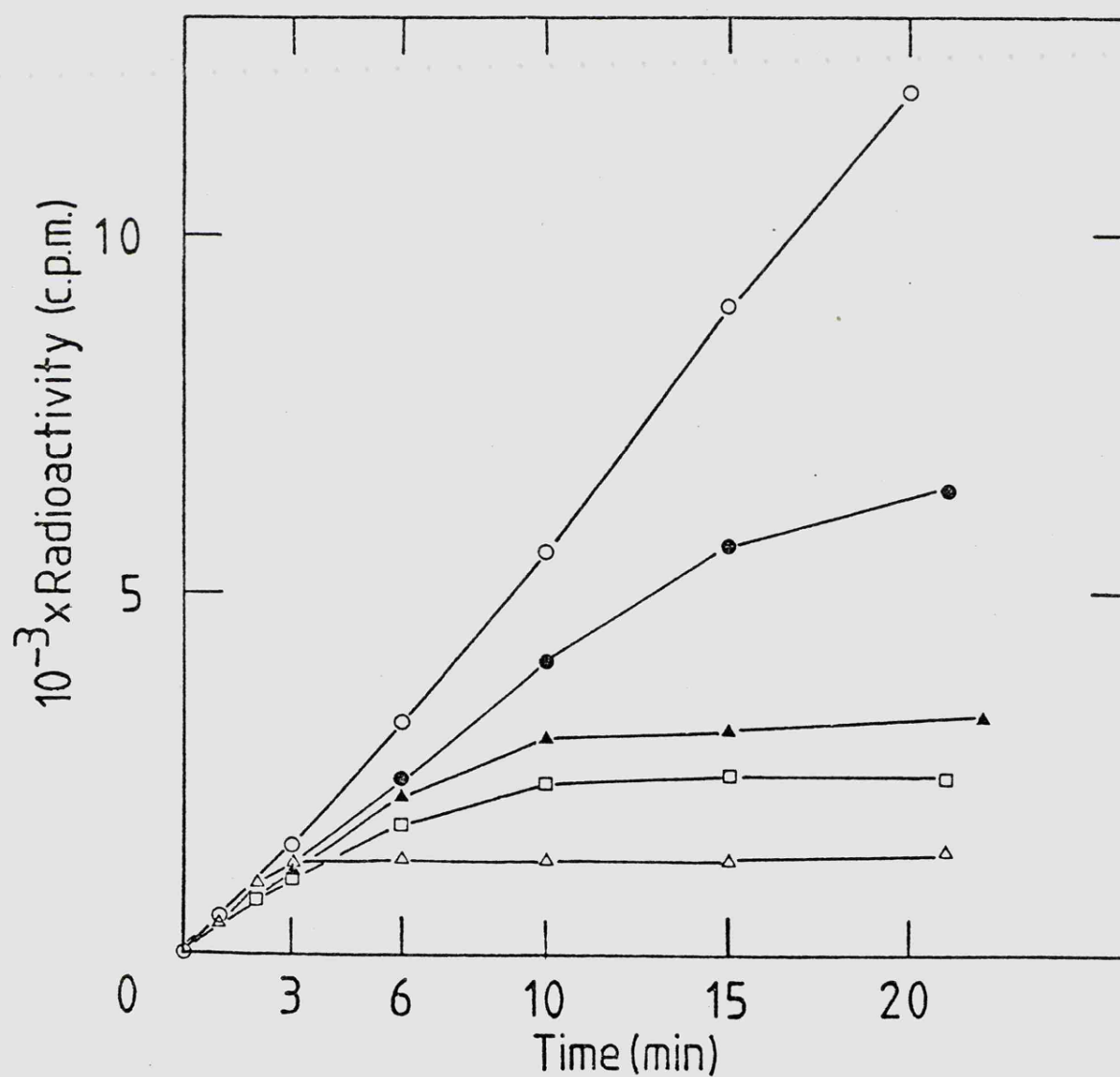
An incubation mixture of reticulocyte lysate (Section 3.5) containing no radioactive amino acid was incubated at 30°C. After ten minutes edeine acetate (10 µg/ml final concn.) was added and samples were removed to sparsomycin (40 µg/ml final concn.) after (a) 30 sec. (b) 2 min. (c) 4 min. (d) 15 min. The inhibited lysates were incubated for a further 15 min. before 50 µl samples of each mixture were removed into 0.2 ml of ice-cold buffer containing 10mM-Tris. HCl (pH7.6), 0.5M-NaCl, 15mM-MgCl<sub>2</sub> and 3mM-2-mercaptoethanol. The samples were then layered onto 15-30% (<sup>w</sup>/v) sucrose density-gradients containing the buffer given above and centrifuged at 40,000 r.p.m. for 60 min. in a Spinco SW50.1 rotor. The left side of each trace represents the bottom of the gradient and the horizontal arrows indicate the direction of sedimentation of polyribosomes.



incorporation. Also polyribosomes should disappear shortly after the addition of toxin since ribosomes 'fall off' the message and are not replaced. This process is illustrated by the polyribosome profiles of reticulocyte lysates which have been inhibited by edeine, a known inhibitor of initiation (Obrig et al., 1971). Thirty seconds after the addition of edeine there was little sign of polyribosome-breakdown (Fig.5.1) but thereafter breakdown became more obvious until by two minutes there were few tetrasomes left (Fig.5.1). Between two and four minutes after the addition of edeine most of the ribosomes traversed the mRNA and terminated protein synthesis (Fig.5.1). The ionic conditions used for the sucrose density-gradients illustrated in Fig.5.1 and elsewhere in this chapter, were chosen so as to cause the dissociation of 80S 'free couples' but retain intact all ribosomes which were attached to mRNA (Fig.5.1 and 5.5).

A further technique for detecting initiation inhibitors has been employed in this work. Peptidyl-tRNA concentrations were monitored continuously following the addition of toxin. In these circumstances a slow decline in labelled amino acids present as peptidyl-tRNA should occur due to ribosomes terminating and not re-initiating protein

FIGURE 5.2    Inhibition of Protein Synthesis by Homoharringtonine



## Legend to Figure 5.2

### Inhibition of Protein Synthesis by Homoharringtonine

Incubation mixtures (Section 3.5) of reticulocyte lysates containing L- $^{-3}\text{H}$ -leucine (51 Ci/mmol) in 3mM-L-leucine were inhibited by addition of various concentrations of homoharringtonine prior to incubation at 30°C. Samples (5  $\mu\text{l}$ ) were taken at various times, precipitated with TCA (Section 3.6) and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).

O, control; ●, plus homoharringtonine (0.07  $\mu\text{g/ml}$  final concn.); ▲, plus homoharringtonine (0.35  $\mu\text{g/ml}$  final concn.); □, plus homoharringtonine (0.7  $\mu\text{g/ml}$  final concn.); △, plus homoharringtonine (1.3  $\mu\text{g/ml}$  final concn.).

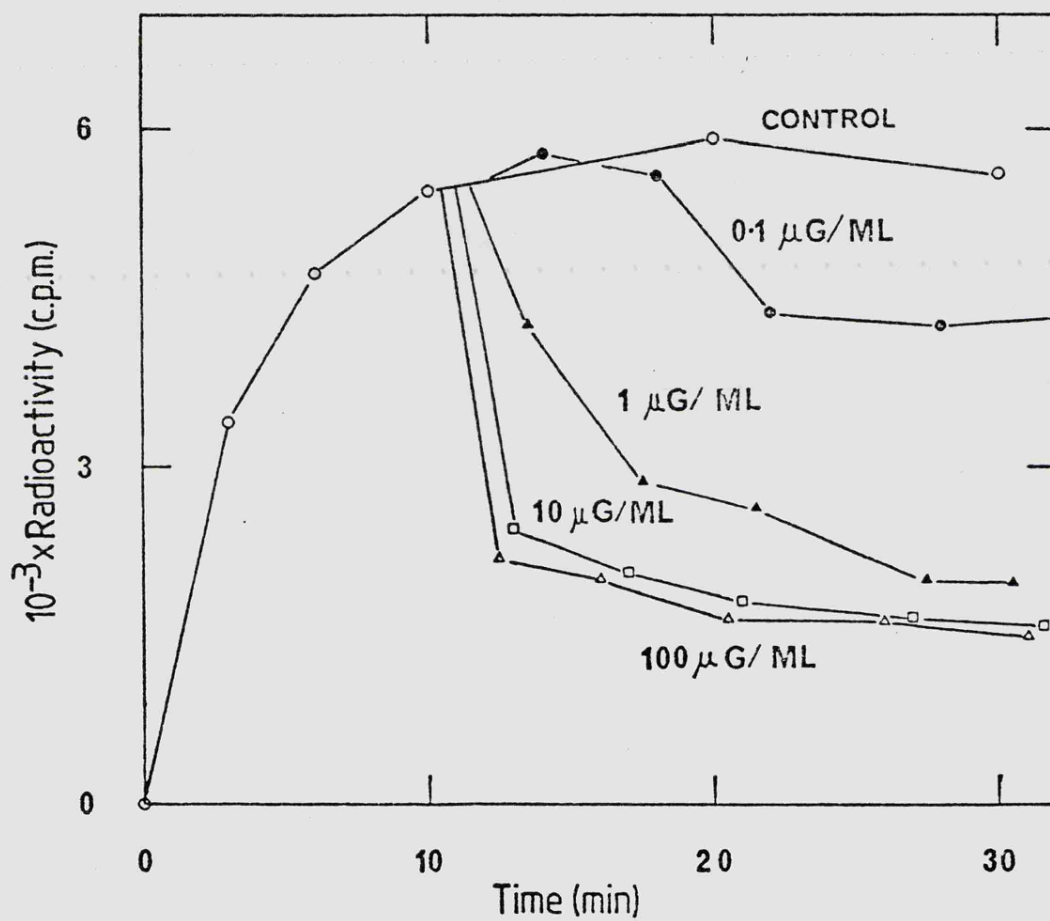


FIGURE 5.3 Effect of Homoharringtonine on the Concentration of Peptidyl-tRNA

### Legend to Figure 5.3

#### Effect of Homoharringtonine on the Concentration of Peptidyl-tRNA

An incubation mixture (Section 3.5) containing L- $[-^3\text{H}]$  leucine (51 Ci/mmol) in 0.3mM-L-leucine was incubated at 30°C. After ten minutes samples were removed into homoharringtonine at different final concentrations. Samples (5  $\mu\text{l}$ ) were precipitated with CTAB (Section 3.6) and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16). 0, control; final concentrations of homoharringtonine:

●, 0.1  $\mu\text{g/ml}$ ; ▲ , 1  $\mu\text{g/ml}$ ; □ , 10  $\mu\text{g/ml}$ ;  
△ , 100  $\mu\text{g/ml}$ .

synthesis.

## 5.2. INHIBITION OF PROTEIN SYNTHESIS BY HOMOHARRINGTONINE

Homoharringtonine is one of several related plant alkaloids isolated from Cephalotaxus harringtonia. Its structure has been established and is shown in Fig.1.4 (Powell et al., 1969). Both homoharringtonine and the related toxin harringtonine, exhibit anti-tumour activity against experimental mouse leukaemias (Powell et al., 1972) and have been employed clinically for the treatment of various human leukaemias (Cephalotaxus Research Coordinating Group, 1976). In reticulocytes and intact HeLa cells, treatment with homoharringtonine caused rapid polyribosome breakdown indicating an effect upon initiation of protein synthesis (Huang, 1975; Tscherne and Pestka, 1975).

When homoharringtonine was added to reticulocyte lysates at various final concentrations, protein synthesis did not cease immediately (Fig.5.2). Even at maximally-inhibitory drug concentrations continued synthesis was observed for 3 - 6 minutes (Fig.5.2). In contrast the concentration of peptidyl-tRNA in the incubation mixture declined immediately after the addition of homoharringtonine until 75% of the peptidyl-tRNA had been released (Fig. 5.3.). The total reduction in peptidyl-tRNA concentration (as judged by CTAB precipitation) in the

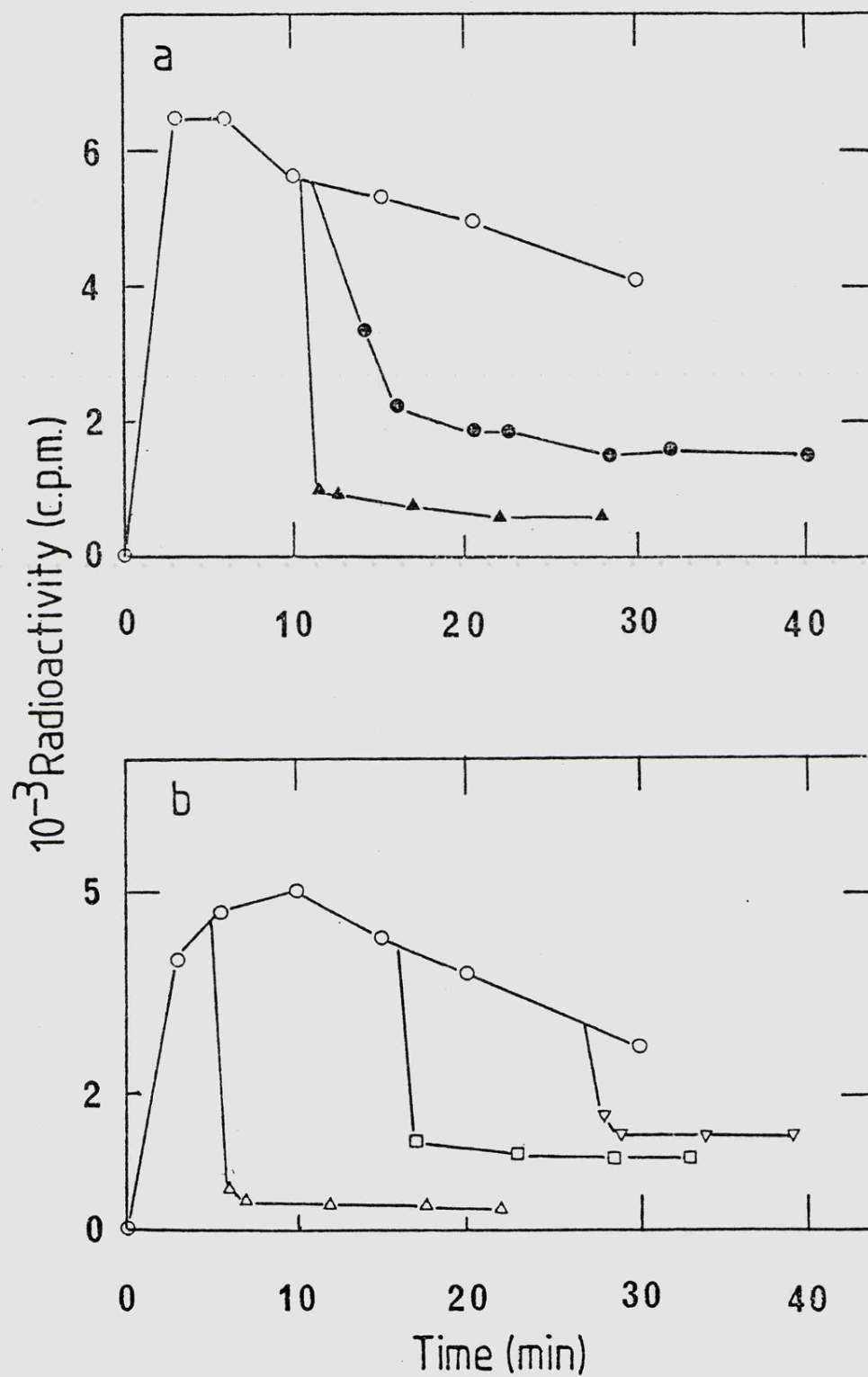


FIGURE 5.4 Decrease in Peptidyl-tRNA Concn. after Addition of Homoharringtonine or Puromycin

#### Legend to Figure 5.4

##### Decrease in Peptidyl-tRNA Concentrations after Addition of Homoharringtonine or Puromycin

Samples were removed from incubation mixtures (Section 3.5) containing L- $^3\text{H}$ -leucine (51 Ci/mmol) in 0.3mM-L-leucine into (a) either puromycin (40  $\mu\text{g/ml}$  final concn.) or homoharringtonine (10  $\mu\text{g/ml}$  final concn.); (b) puromycin (40  $\mu\text{g/ml}$  final concn.) at various times. Samples (5  $\mu\text{l}$ ) were precipitated with CTAB (Section 3.6) and radioactivity in the precipitates was estimated by liquid-scintillation counting (Section 3.16). All incubations were at 30°C. 0, control. (a)  $\blacktriangle$  , plus puromycin;  $\bullet$  , plus homoharringtonine. (b) plus puromycin at  $\Delta$  , 5 min;  $\square$  , 16 min. ;  $\nabla$  , 27 min.



incubation mixture was compared following inhibition of protein synthesis by homoharringtonine or puromycin. Addition of puromycin gave rise to a greater reduction in peptidyl-tRNA concentration than did the addition of homoharringtonine (Fig.5.4a). This unexpected observation was investigated further and puromycin was added to an otherwise uninhibited incubation mixture following various periods of synthesis. The ability of puromycin to reduce peptidyl-tRNA concentrations apparently decreased with time (Fig.5.4b) so that after 25 minutes of incubation in the absence of drug less than 50% of the peptidyl-tRNA appeared to be released by puromycin.

Therefore CTAB precipitation provides a convenient method by which inhibitors of initiation may be detected. However, it is important to look at the kinetics of the reaction rather than the absolute decrease in peptidyl-tRNA concentrations caused by the inhibitor. There is an increase in the background of CTAB-precipitable counts which are not releasable by puromycin. Presumably the background represented non-specific binding of radioactive protein to the filters which was not removed by the washing procedure. An increase in binding, therefore, reflected continued protein synthesis by the reticulocyte lysates.

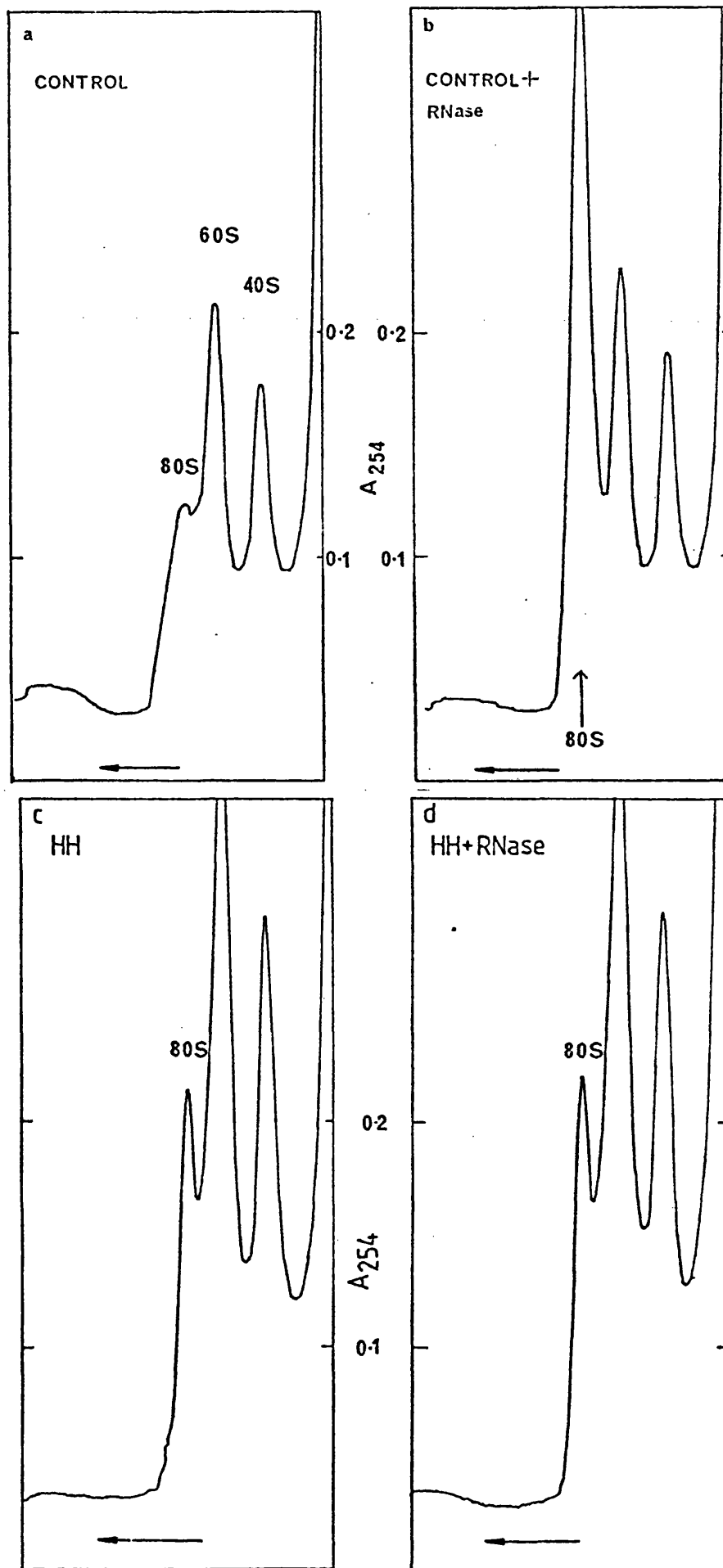
Some attempts were made to reduce the 'background' by extra washing of the filters, addition of carrier protein and siliconization (i.e. treatment with a 4% (w/v) solution of dichlorodimethylsilane in chloroform) of the filters. However, none of these procedures proved effective in reducing the 'background'.

5.3. SUCROSE DENSITY-GRADIENT ANALYSIS OF INCUBATION MIXTURES INHIBITED BY HOMOHARRINGTONINE

The experiments of Huang (1975) and Tscherne and Pestka (1975), although demonstrating that homoharringtonine inhibited initiation, failed to highlight which step(s) in initiation was/were inhibited. There was no indication whether the 80S initiation complex was formed since the ionic conditions used for the sucrose density-gradient analysis could not distinguish between 80S ribosomes bound to mRNA and 'free couples'. However, using other ionic conditions, it has been observed that inhibition of HeLa cells by homoharringtonine did not cause a complete breakdown of polyribosomes but allowed retention of a '1.5 mer' peak together with 80S monosomes (E.Cundliffe, unpublished observations). This '1.5 mer' peak, which was identical to peaks observed when HeLa cells were inhibited by I<sub>1</sub>-type 12,13 epoxytrichothecenes, consisted of mRNA to which both an 80S ribosome and a 40S subunit were bound (Cundliffe and Davies, 1977).

An unlabelled reticulocyte reaction mixture was incubated with a totally-inhibitory concentration of

FIGURE 5.5 Degradation of Polysomes in the Presence of Homoharringtonine



## Legend to Figure 5.5

### Degradation of Polysomes in the Presence of Homoharringtonine

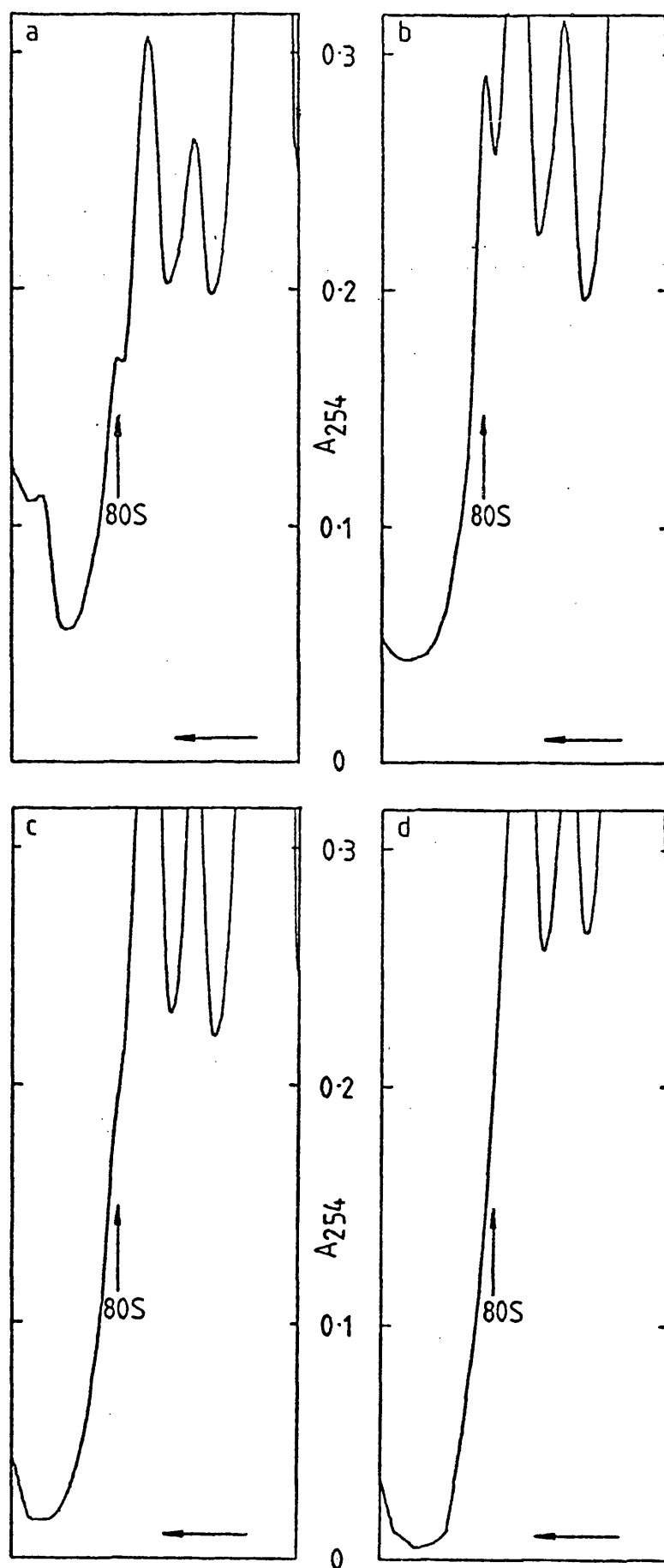
Incubation mixtures (Section 3.5) containing no radioactive amino acids were incubated at 30°C. :

(a) for 10 min. ; (b) for 10 min. and then with ribonuclease (5 µg/ml final concn.) for 30 min.; (c) with homoharringtonine (10 µg/ml final concn.) for 10 min.; (d) with homoharringtonine (10 µg/ml final concn.) for 10 min. then ribonuclease ( 5 µg/ml final concn.) for 30 min. Samples (50 µl) were taken into 0.2 ml ice-cold buffer containing 10mM-Tris.HCl (pH7.6), 0.5M-NaCl, 15mM-MgCl<sub>2</sub> and 3mM-2-mercaptoethanol, layered onto 15-40% (<sup>w</sup>/v) sucrose density-gradients and centrifuged at 45,000 r.p.m. for 70 min. The left side of each trace represents the bottom of the gradient and the direction of sedimentation of polysomes is indicated by the horizontal arrows.

homoharringtonine for ten minutes before being analysed on a sucrose density-gradient. All the polyribosomes were found to have been degraded to 80S monomers (Fig. 5.5). This result was confirmed by pre-incubating a portion of the inhibited lysate with ribonuclease prior to gradient analysis. No increase in the population of 80S monomers was then seen (Fig.5.5). If a control lysate was treated with ribonuclease before analysing on a gradient, there was found to be a large increase in the 80S population compared with untreated control lysates (Fig.5.5.), as would be expected if polyribosomes were degraded to 80S monomers by the action of the enzyme. In a reticulocyte lysate pre-treated with homoharringtonine, no evidence for the existence of '1.5 mers' or dimers was found although a peak of monomers remained (Fig.5.5) which was larger than the 80S peak in the control lysate. In contrast, inhibition of reticulocyte lysates by edeine caused the total degradation of ribosomes into their component subunits (Fig.5.1). This latter result is as predicted for a toxin which inhibits initiation by preventing the 60S ribosomal subunit from binding to the 40S-mRNA initiation complex (Kramer et al., 1976; Hunter et al., 1977).

In a further experiment, homoharringtonine and edeine were added, at the same time, to a reticulocyte

FIGURE 5.6 Effect of Homoharringtonine and Edeine on the Formation of Polysomes



## Legend to Figure 5.6

### Effect of Homoharringtonine and Edeine on the Formation of Polysomes

Incubation mixtures (Section 3.5) containing no radioactive amino acid were incubated at 30°C:

(a) for 20 min. ; (b) for 20 min. in the presence of homoharringtonine (10 µg/ml final concn.); (c) for 20 min. in the presence of homoharringtonine (10 µg/ml final concn.) and edeine (10 µg/ml final concn.); (d) for 20 mins. in the presence of edeine (10 µg/ml final concn.).

Samples (50 µl) were removed into ice-cold buffer (0.2 ml) containing 10mM-Tris. HCl (pH7.6), 0.5M-NaCl, 15mM-MgCl<sub>2</sub> and 3mM-2-mercaptoethanol and were then layered onto 15-30% (<sup>w</sup>/v) sucrose density-gradients containing the buffer described above. The gradients were centrifuged at 40K for 100 min. The left side of each trace represents the bottom of the gradient and the direction of sedimentation of polysomes is indicated by the horizontal arrows.

lysate (Fig.5.6). In this case, the 80S peak virtually disappeared when compared with a lysate which had been inhibited by homoharringtonine alone (Fig.5.6). However, there still remained a small quantity of 80S monomers which appeared as a slight shoulder on the 60S subunit peak (Fig.5.6). Therefore, homoharringtonine inhibits polypeptide synthesis at, or shortly after, formation of the 80S initiation complex.

#### 5.4. ANALYSIS OF 80S PEAKS IN SUCROSE DENSITY-GRADIENTS

Although, as described above, homoharringtonine did not cause the retention of '1.5 mers' in reticulocyte lysates, its mode of action appeared similar to the 12,13 epoxytrichothecenes of the verrucarins and roridin subgroups. Additionally, Fresno et al., (1977) reported that homoharringtonine inhibited the binding of [ $^{14}\text{C}$ ] trichodermin to yeast ribosomes. Therefore, the likely site of action for homoharringtonine was the peptidyltransferase shortly after initiation. There remained, however, some confusion as to the mechanism of formation of '1.5 mers' and why no such structures were seen in reticulocyte lysates when they were inhibited by homoharringtonine. Two possible mechanisms for '1.5 mer' formation are discussed below.

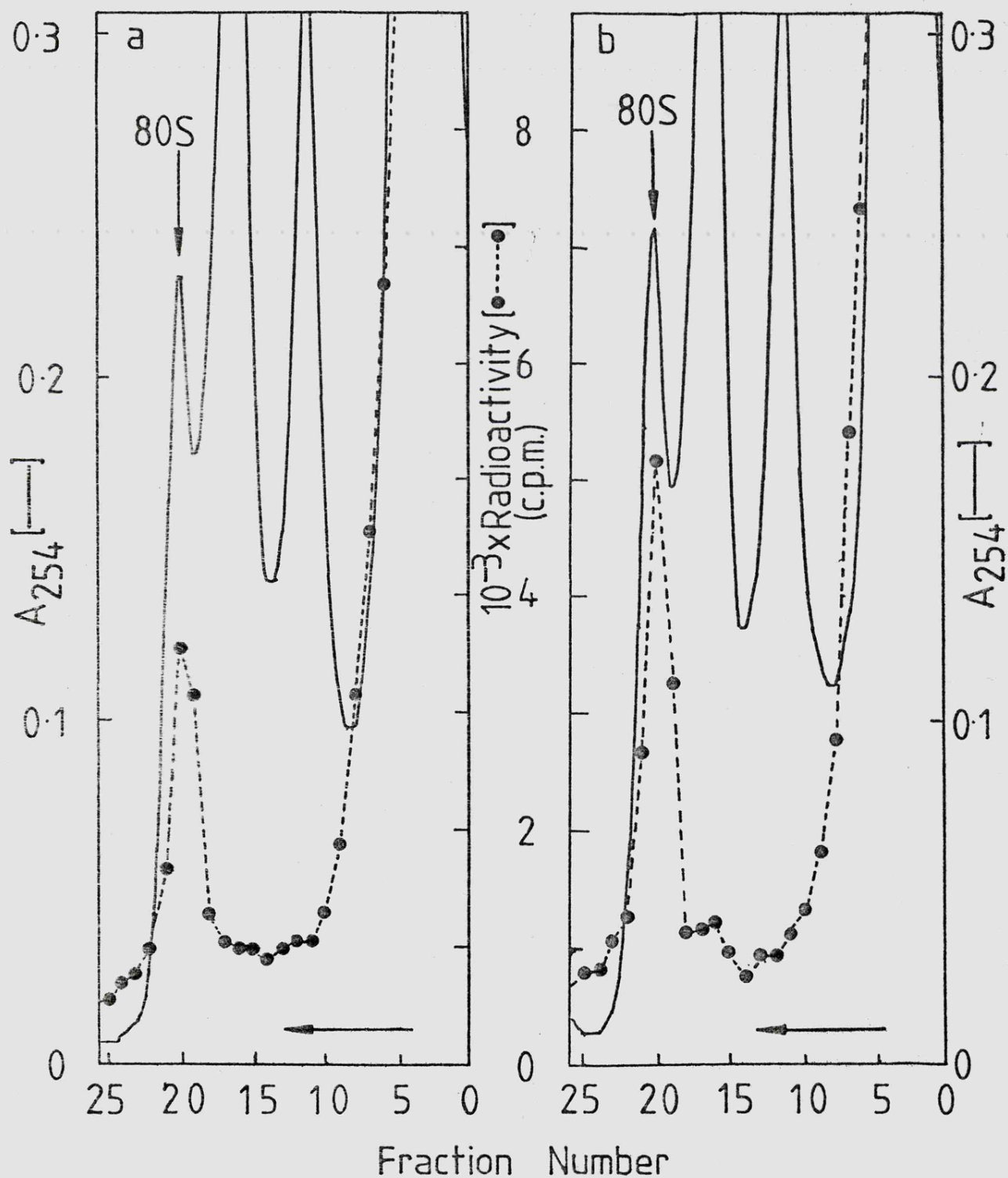
(a) The antibiotic displays a secondary effect upon formation of the 80S initiation complex by inhibiting the



association of the 60S subunit with the 40S-mRNA complex. Since trichothecenes which inhibit initiation do so by only preventing formation of either the first or second peptide bond (Smith et al., 1975; Mizuno, 1975; Carter and Cannon, 1978), it is possible that homoharringtonine also acts at these stages. The 40S initiation complex (in the '1.5 mer') would, in this case, probably be unable to bind to the AUG initiation codon because of steric hindrance with the 80S complex.

(b) The antibiotic has no direct effect upon formation of the 80S initiation complex. It does not inhibit formation of the first few peptide bonds but acts when the peptidyl-tRNA reaches a certain critical length. At this stage the ribosome will have moved along the mRNA and exposed the AUG codon and another 40S initiation complex may form. The attachment of the 60S subunit would be prevented, however, due to steric hindrance by the 80S monomer already present on the mRNA. In this case, the difference in profiles between HeLa cells and reticulocyte lysates which were inhibited by homoharringtonine would be due either to a slight change in the stage at which peptide bond formation was inhibited i.e. homoharringtonine may inhibit formation of a smaller peptide in reticulocytes and stop

FIGURE 5.7 Effect of Homoharringtonine or T-2 Toxin on the Production of Initiation Complexes



## Legend to Figure 5.7

### Effect of Homoharringtonine or T-2 Toxin on the Production of Initiation Complexes

Mixtures (Section 3.5) containing L- $^{35}\text{S}$ -methionine (295 Ci/mmol) were incubated at 30°C for 10 minutes before (a) homoharringtonine (10 µg/ml final concn.) or (b) T-2 toxin (1 µg/ml final concn.) were added. After a further 20 minutes puromycin (40 µg/ml final concn.) was added to each incubation and 10 minutes later samples (50 µl) were removed into 0.2 ml ice-cold buffer containing 10 mM-Tris. HCl (pH7.6), 0.5M-NaCl, 15mM-MgCl<sub>2</sub> and 3mM-2-mercaptoethanol. These samples were layered onto 15-30% (w/v) sucrose density-gradients containing the buffer described above and centrifuged at 40,000 r.p.m. for 120 minutes in a Spinco SW50.1 rotor. After pumping the gradients through an ISCO absorbance monitor they were fractionated and the fractions precipitated with CTAB (Section 3.6). Radioactivity in the precipitates was estimated by liquid-scintillation counting (Section 3.16). The left side of each trace represents the bottom of the gradient and the direction of sedimentation of polysomes is indicated by the horizontal arrows.

attachment of the 40S initiation complex, or there may be a different degree of packing of ribosomes on the mRNA of reticulocytes compared to HeLa cells.

The trichothecene, T-2 toxin, did not cause formation of '1.5 mers' in HeLa cells (Cundliffe and Davies, 1977) and would be expected, according to the second hypothesis, to inhibit peptide bond formation at an earlier stage than homoharringtonine. Therefore, in order to prove or disprove the second hypothesis, the constitution of the peptidyl-tRNA retained on the 80S peak following inhibition by homoharringtonine or T-2 toxin was examined and compared.

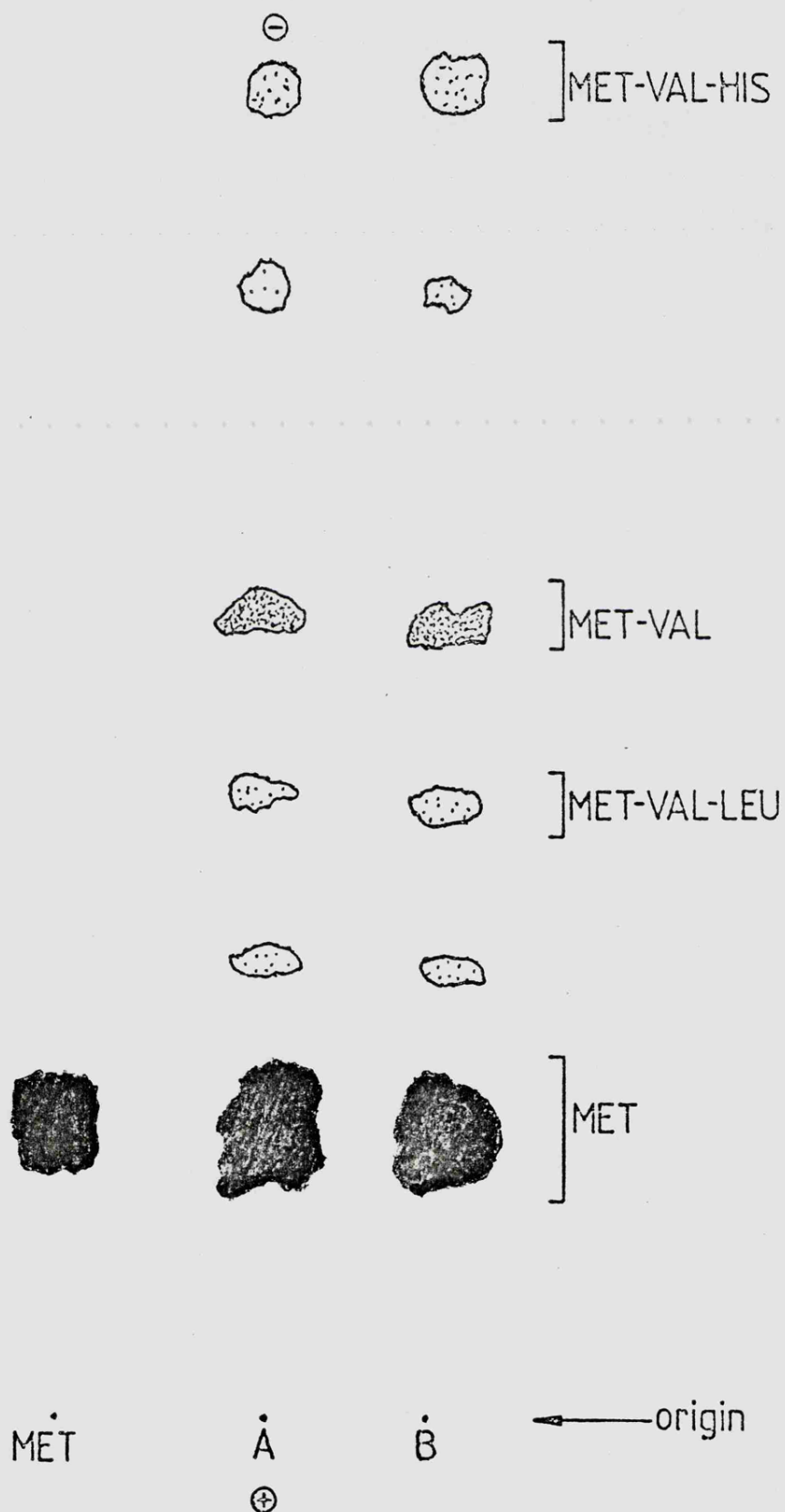
Incubation mixtures of reticulocyte lysates containing [ $^{35}\text{S}$ ] methionine as label were treated with homoharringtonine or T-2 toxin and then incubated for twenty minutes to allow complete 'run off' of polyribosomes. Compared with a control mixture, treatment with the toxins caused a large build-up in 80S monomers carrying [ $^{35}\text{S}$ ]methionine (Fig.5.7) - as would be expected for the formation of 80S initiation complexes. The N-terminal amino acid sequences of rabbit haemoglobin are,

$\alpha$  chain (Met)-Val-Leu-Ser (Von Ehrenstein, 1966)

$\beta$  chain (Met)-Val-His-Leu (Braunitzer et al., 1966)

and, therefore, further experiments were performed

FIGURE 5.8 Electrophoresis of Short Peptides  
Labelled with [ $^{35}\text{S}$ ]Methionine



## Legend to Figure 5.8

### Electrophoresis of Short Peptides Labelled with $\text{L}^{-35}\text{S}$ Methionine

Mixtures (Section 3.5) containing  $\text{L}^{-35}\text{S}$  methionine (295 Ci/mmol) were incubated for 10 minutes at  $30^{\circ}\text{C}$  before homoharringtonine (10  $\mu\text{g/ml}$  final concn.) or T-2 toxin (1  $\mu\text{g/ml}$  final concn.) were added. After a further 20 minutes puromycin (40  $\mu\text{g/ml}$  final concn.) was added to each incubation and samples (100  $\mu\text{l}$ ) were removed to ice-cold buffer containing 10mM-Tris.HCl (pH7.6), 0.5M-NaCl, 15mM-MgCl<sub>2</sub> and 3mM-2-mercaptoethanol after another 10 minutes. The samples were layered onto 15-30% (w/v) sucrose density-gradients containing the buffer described above and centrifuged for 120 minutes at 40,000 r.p.m. in a Spinco SW50.1 rotor. Gradients were fractionated and the fractions containing 80S ribosomes collected.

The ribosomes were centrifuged at 40,000 r.p.m. for 6 hours and resuspended in 0.05 ml of 1M-NH<sub>4</sub>OH. After incubating at  $37^{\circ}\text{C}$  for 30 minutes to hydrolyse tRNA the suspensions were spotted onto Whatmann '3MM' paper and the paper was electrophoresed at pH3.5 for 1 hour at 3 KV. Radioactivity was detected by autoradiography for 7 days with 'KODIREX' X-ray film. The identification of labelled material as dipeptides and tripeptides was by comparison with the results of Jackson and Hunter (1970). MET,  $\text{L}^{-35}\text{S}$  methionine only: A, material from ribosomes inhibited by homoharringtonine; B, material from ribosomes inhibited by T-2 toxin.

using  $\text{L-}^3\text{H}$  valine and  $\text{L-}^3\text{H}$  leucine instead of  $\text{L-}^{35}\text{S}$  methionine in order to determine whether any methionine on the 80S peak was present as dipeptides, tripeptides or larger peptides. Sucrose density-gradients were fractionated and radioactivity in the gradients detected by CTAB precipitation (Section 3.6). The results of these experiments proved inconclusive, however, due to background contamination from the top of the gradients (data not given).

In order to determine the constitution of the  $\text{L-}^{35}\text{S}$  methionine-labelled material, reticulocyte incubation mixtures containing  $\text{L-}^{35}\text{S}$  methionine as label were inhibited by homoharringtonine or T-2 toxin and incubated for twenty minutes. Puromycin was added finally to ensure that only ribosomes which were inhibited by homoharringtonine or T-2 toxin still carried peptidyl-tRNA. Following centrifugation of the lysates on sucrose density-gradients, the 80S material was collected and the peptides present as peptidyl-tRNA were isolated and analysed by electrophoresis at pH 3.5 (Jackson and Hunter, 1970). The  $\text{L-}^{35}\text{S}$  methionine in the 80S peaks was present mainly as methionyl-tRNA (Fig. 5.8) although there were much smaller quantities of dipeptidyl and possibly tripeptidyl-tRNA (as judged by

comparison with electrophoregrams shown in Jackson and Hunter, 1970). There was no difference in the constitution of  $\angle^{35}\text{S}_7$  methionine-labelled material on the 80S monomers of lysates inhibited by homoharringtonine or T-2 toxin (Fig. 5.8). Therefore, homoharringtonine and T-2 toxin both appear to inhibit formation of the first peptide bond. Although a direct effect of T-2 toxin and homoharringtonine on formation of other peptide bonds cannot be excluded, the results shown in Fig. 5.8 may be interpreted as 'leaky' inhibition. Sparsomycin, an established inhibitor of the peptidyltransferase, also was unable to prevent totally the formation of dipeptidyl-tRNA (Jackson and Hunter, 1970).

Unless homoharringtonine is unable to inhibit formation of the first few peptide bonds in HeLa cells (which is unlikely given the similarities in the events of initiation in eukaryotes) whilst T-2 toxin still retains this ability, these results rule out the second hypothesis for the formation of '1.5 mers'. There remain, however, many apparent contradictions which need to be explained before the first hypothesis i.e. that homoharringtonine inhibits addition of the 60S ribosomal subunit during initiation, is acceptable.



The presence of polycistronic messages in HeLa cells could explain the results for homoharringtonine and verrucarin A. However, as yet, no polycistronic messages have been reported in eukaryotes and such a mRNA would be expected to give rise to dimers when HeLa cells were treated with T-2 toxin under similar conditions. Since dimers were not observed, it is necessary to hypothesize a binding site for the 40S initiation complex which is distinct from the initiation codon and which is not present in globin mRNA. The available evidence suggests, however, that there is only one binding site on the mRNA of eukaryotes for the 40S initiation complex and this is at the AUG initiation codon (Baralle and Brownlee, 1978).

No mechanism for the formation of '1.5 mers' in HeLa is offered here, nor any explanation for the absence of '1.5 mers' in reticulocyte lysates. Such a model may only be constructed when further experiments concerning the nature of '1.5 mers' are completed. However, two further pieces of evidence should be considered before construction of any model. Firstly, although homoharringtonine and those trichothecenes which inhibit initiation do not cause the formation of '1.5 mers' in reticulocytes, such

structures are reported to be formed after inhibition of reticulocyte lysates by pactamycin (Kappen et al., 1973). Pactamycin, unlike the trichothecenes, binds to the small subunit (Macdonald and Goldberg, 1970) and inhibits formation of the 80S initiation complex (Fresno et al., 1976). Secondly, the '1.5 mer' produced by inhibition of reticulocyte lysates with pactamycin carries, on the 80S monosome, the dipeptide, Met-Val, (Kappen and Goldberg, 1973; Cheung et al., 1973) as do 80S monosomes inhibited by homoharringtonine or T-2 toxin (Fig.5.8).

6. PROTEIN SYNTHESIS BY CELL-FREE EXTRACTS FROM  
SACCHAROMYCES CEREVISIAE AND MYROTHECIUM VERRUCARIA

6.1. MATERIALS

Ribonuclease T<sub>1</sub> and E.coli tRNA were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other chemicals were obtained from the sources mentioned previously (Section 3.2).

6.2. INTRODUCTION

Although reticulocyte lysates provide a convenient and active system in which to study inhibitors of protein synthesis, they are of less value in studies designed to establish relationships between the structure and function of the ribosome because, clearly, it is impossible to select mutants which are antibiotic-resistant. Therefore, such studies have concentrated either on mammalian cells in culture or on lower eukaryotes, e.g. yeasts and fungi where selection of mutants is a relatively simple process.

The yeast Saccharomyces cerevisiae is especially useful for studies on ribosome structure in relation to antibiotic action since its biochemistry and genetics are well documented. Additionally, mutants of Saccharomyces cerevisiae which are resistant to various antibiotics have been reported already and, in some cases, the altered gene mapped. Unlike cell-free systems from other eukaryotic sources, no system has been developed from yeast which is capable of performing all the steps in protein synthesis, i.e. initiation,

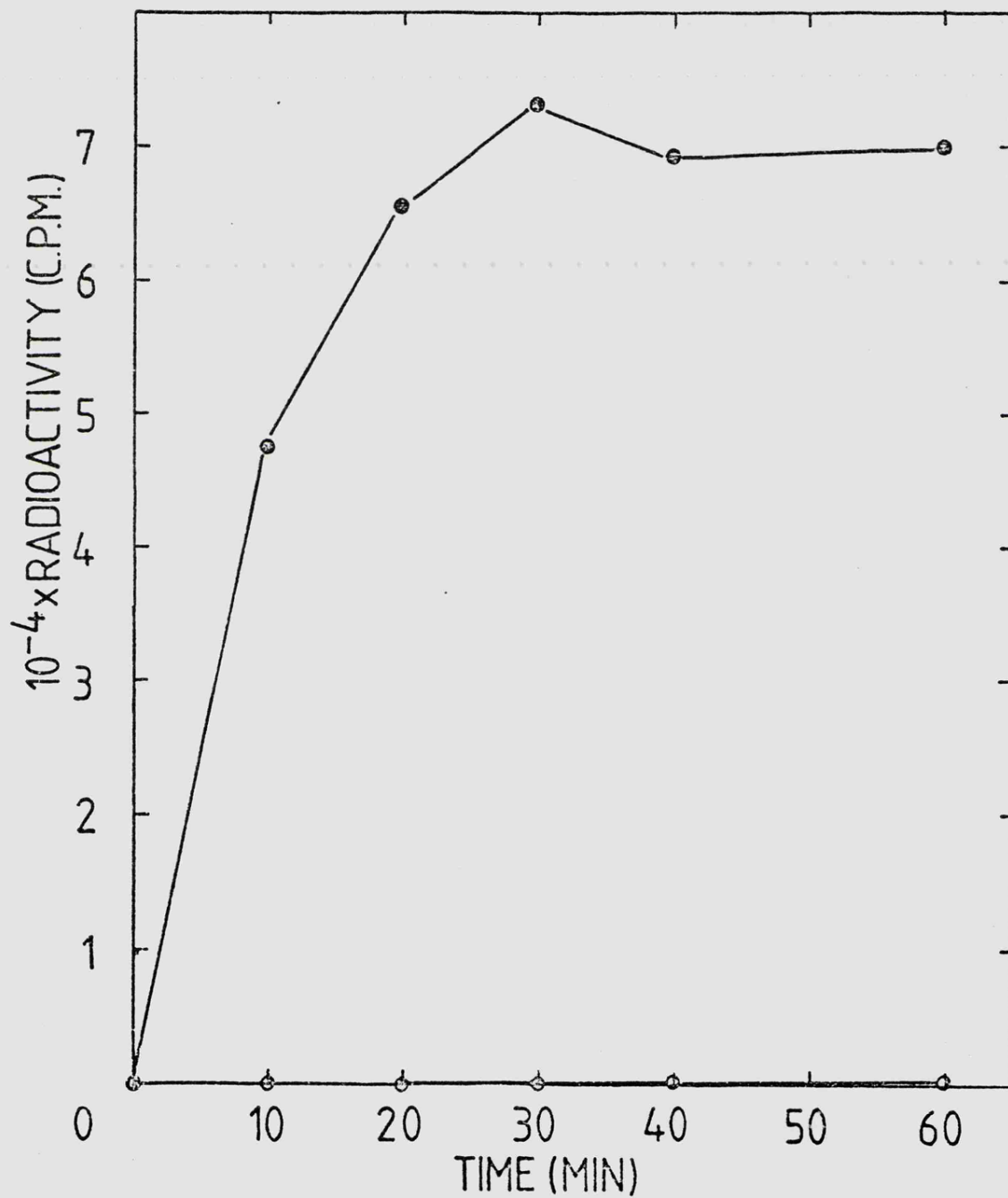
elongation and termination. However, following the paper by Nirenberg and Matthaei (1961) reporting the ability of polyuridylic acid (poly U) to direct the synthesis of polyphenylalanine from phenylalanine in a cell-free extract of Escherichia coli, many authors have reported similar systems from yeast. In most cases, these systems have been rather inactive. Here, the properties of a cell-free extract of yeast is reported. The extract (S-30) is at least twice as active as any system using S-30 from yeast reported previously. Additionally, it may be fractionated to ribosomes and high-speed supernatant (S100) which are just as active as the original S-30 when recombined. The procedure for production of yeast cell-free extracts has been applied, with only small changes (Sections 3.11 and 3.13), to the production of cell-free extracts of various fungi. These extracts are also very active in poly U-directed synthesis of polyphenylalanine.

The yeast strains used in this work were S.cerevisiae Y<sub>166</sub> ( $\alpha$  trp5 his4 MA1) and TR<sub>1</sub>. Strain TR<sub>1</sub> is an antibiotic-resistant mutant which was isolated by spreading Y<sub>166</sub> cells on agar plates containing 5  $\mu$ g/ml trichodermin (Jimenez et al., 1975). This mutation also gave rise to resistance to all other 12,13

epoxytrichothecenes and the chemically-unrelated antibiotic, anisomycin. Tetrad analysis revealed that a single mutation was responsible for all the changes in antibiotic-sensitivity (Jimenez et al., 1975) as was also the case for an independently-isolated yeast-mutant (CLP<sub>1</sub>) selected for resistance to trichodermin (Schindler et al., 1974).

A strain of the fungus Myrothecium verrucaria was also studied here. As may be seen from Table 2.2, the strain was resistant in vivo to all toxins, except azide, with which it was confronted. The imperfect fungus M. verrucaria is widespread in nature, and is found commonly on dead and dying leaves (Tullock, 1972). The occurrence of seasonal ill-health in sheep and cattle on dry autumn pastures, stachybotryotoxicoses and dendrochiotoxycosis can be ascribed, in part, to the intake of toxins produced by M. verrucaria (Mortimer et al., 1971). Various strains of the fungus have been shown to produce a wide range of 12,13 epoxytrichothecenes of the verrucaridin and roridin subgroups (Figs. 1.2 and 1.3), varying only in the nature of the bridge structure between carbons 4 and 15 of the trichothecene ring (Härri et al., 1962; Bohner et al., 1965; Traxler et al., 1970). The particular strain of M. verrucaria used here has not been shown to produce trichothecenes although it does produce anti-fungal

FIGURE 6.1 Activity of a Yeast S-30 Extract  
in Protein Synthesis



Legend to Figure 6.1

Activity of a Yeast S-30 Extract in Protein  
Synthesis

A mixture for poly U-directed protein synthesis (Section 3.14) containing yeast S-30 (A<sub>260</sub>= 42 final concn.) was incubated at 25°C in the presence (●) or absence (○) of Poly U. Samples (10 μl) were removed and precipitated with TCA. Radioactivity in the precipitates was estimated by liquid-scintillation counting (Section 3.16).

metabolites and trichothecenes may be amongst these metabolites (Section 2.5).

Cell-free extracts were prepared from M. verrucaria in order to study antibiotic activity in vitro.

### 6.3. PROTEIN SYNTHESIS BY YEAST S-30 EXTRACTS

An incubation mixture containing freshly-prepared yeast, strain Y<sub>166</sub>, S-30 incorporated 70,000 cpm of [<sup>14</sup>C] phenylalanine into TCA-precipitable material in a 10 µL sample in 20 - 30 minutes at 25°C (Fig.6.1). This represents approximately 20 molecules of phenylalanine incorporated per ribosome. There was no further incorporation after thirty minutes (Fig.6.1) which may be due either to a shortage of available [<sup>14</sup>C] phenylalanine (70% of added [<sup>14</sup>C]phenylalanine had been incorporated already into polyphenylalanine) or a shortage of energy. Although a large excess of ATP and PEP were present in the incubation mixture, it is possible that they were removed by adenosine triphosphatases (ATPases). Incorporation in the absence of poly U (endogenous synthesis) was about 500 cpm in sixty minutes (Fig.6.1).

The number of molecules of phenylalanine incorporated into polyphenylalanine per ribosome is taken normally as a measure of the efficiency of yeast S-30 extracts in poly U-directed protein synthesis. By this criterion, dilution of the yeast S-30 to half its original

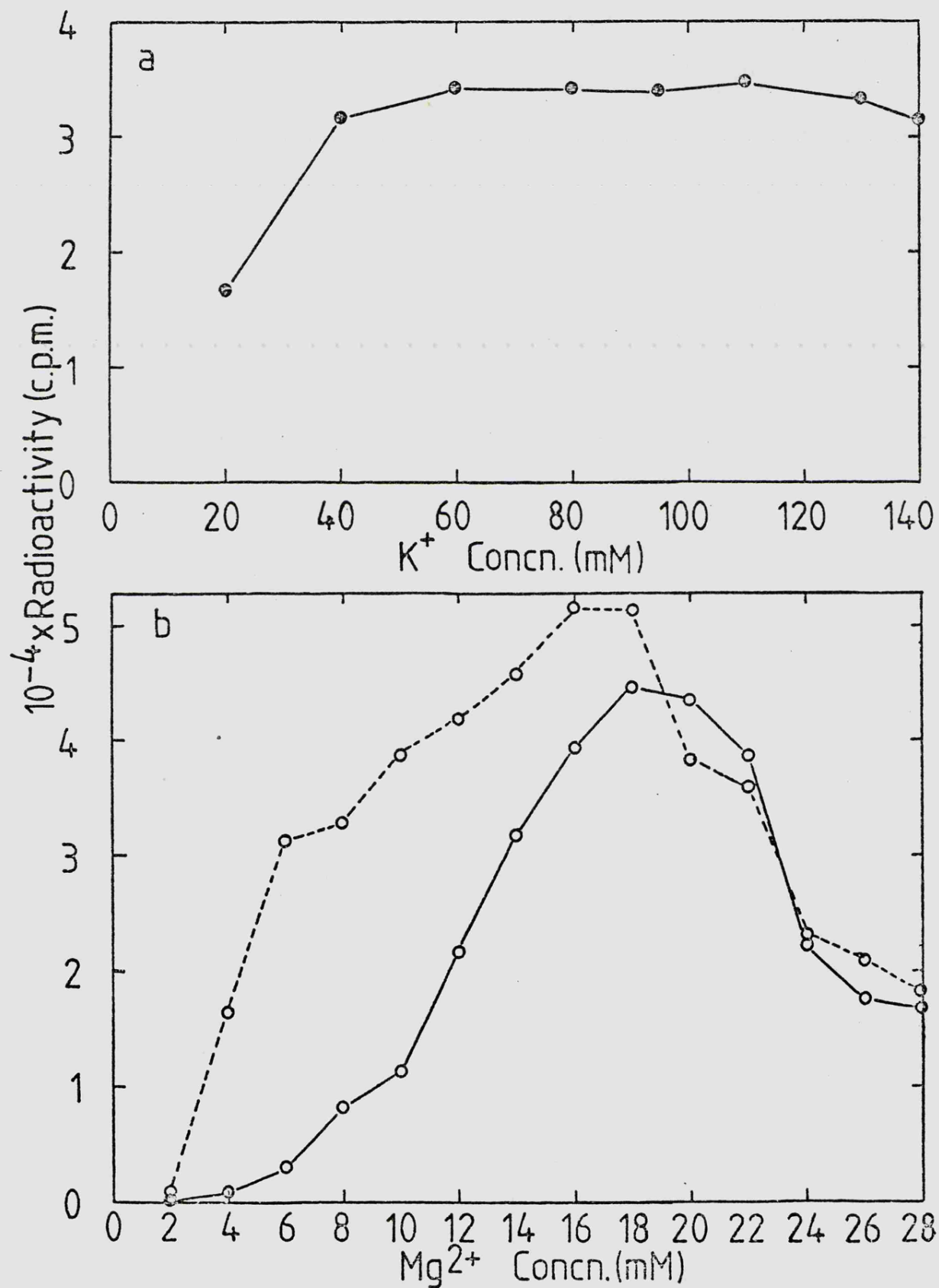


concentration increased the efficiency to approximately 1.5 times the value given above (data not given). However, overall synthesis was decreased at the same time. In all experiments reported here the final ribosome concentration of the incubation mixtures was adjusted to 1.5 - 2 mg/ml. This concentration was chosen as a compromise between efficiency of the incubation mixture and total incorporation into polyphenylalanine. In comparison with other systems, the incubation mixture with yeast S-30 was at least two times as efficient as that of Van der Zeijst et al., (1973) and four times better than that of Sissons (1974).

The data given above represent the maximum activities of yeast extracts. Continued freezing and thawing of the extracts caused a decrease in their activities. Additionally, some yeast extracts were less active than others even when freshly-prepared. Consequently, many other experiments were performed with less active extracts. However, the results, reported in this dissertation, were unaffected by the activity of the yeast extracts.

Conditions of the yeast incubation mixtures were varied in order to determine optimum ion concentrations for poly U-directed protein synthesis. There was found to be a broad optimum for  $K^+$  concentration

FIGURE 6.2 Effect of Ion Concentration on PolyU-Directed Protein Synthesis by Yeast



Legend to Figure 6.2

Effect of Ion Concentration on Poly U-Directed  
Protein Synthesis by Yeast

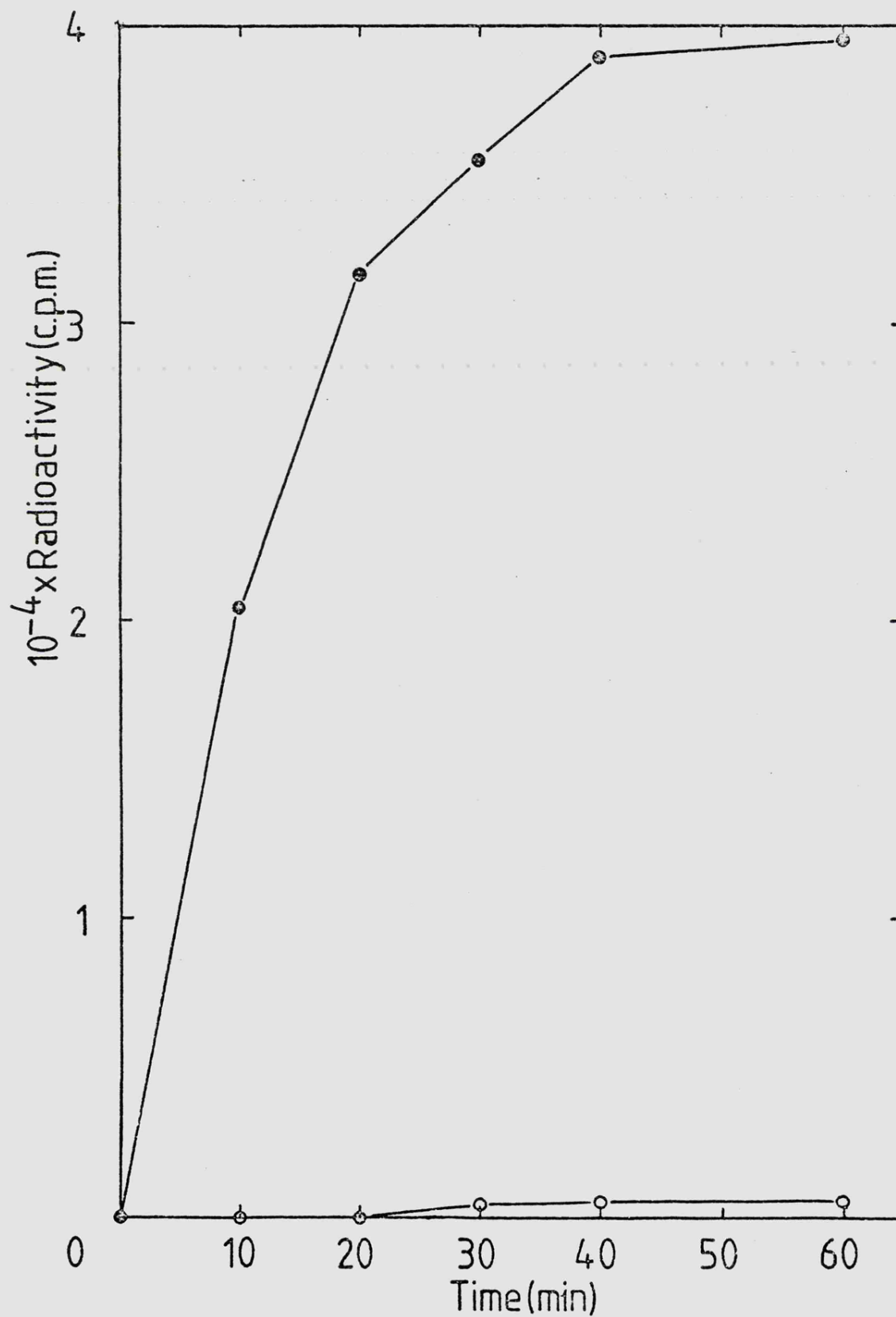
Mixtures for Poly U-directed protein synthesis (Section 3.14) containing yeast S-30 (A260 = 42 final concn.) and (a) 14mM-Mg<sup>2+</sup> but various concentrations of K<sup>+</sup> ; (b) 95mM-K<sup>+</sup> but various concentrations Mg<sup>2+</sup> (o—o) or 95mM-K<sup>+</sup>, 1mM-spermidine but various concentrations of Mg<sup>2+</sup> (o---o) were incubated at 25°C for 60 mins. Samples (10 µl) were precipitated with TCA and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).

between 60 and 130 mM  $K^+$  (Fig. 6.2a). Ammonium ions could be substituted either partially or completely for  $K^+$  without loss of activity (data not given). The optimum  $Mg^{2+}$  concentration was 20 mM although this could be lowered to 16 mM by addition of 1 mM-spermidine trichloride (Final concentration, Fig.6.2b). Addition of spermidine also stimulated maximum incorporation in the incubation mixture (Fig.6.2b). Although the optimum magnesium ion concentration was 18 - 20 mM, most incubations with yeast were done at 14 mM (e.g. Fig.6.1). This concentration does not lower the activity of the yeast incubation mixtures by very much and is much closer to the optimum for M.verrucaria incubation mixtures (Fig.6.4).

Cell-free extracts from yeast, strain TR<sub>1</sub>, were equally active in poly U-directed protein synthesis and shared the same ionic optima as the parent strain Y<sub>166</sub> (data not given).

Yeast cell-free extracts were unable to translate added Bromegrass mosaic virus RNA (BMV RNA) when incubated under the conditions employed for message-dependent wheat germ incubation mixtures (Shih and Kaesberg, 1973).

FIGURE 6.3 Activity of M. verrucaria S-30 Extract  
in Protein Synthesis

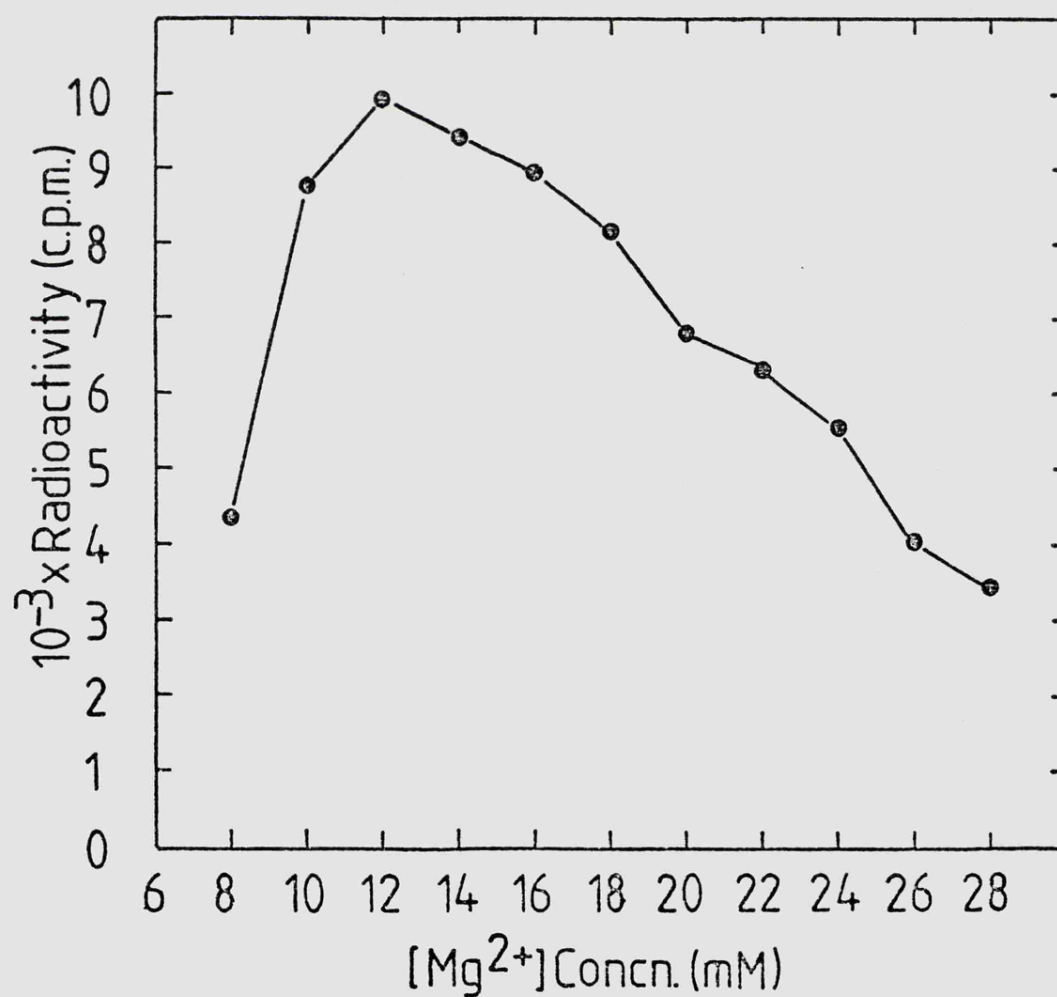


Legend to Figure 6.3

Activity of M. verrucaria S-30 Extract in  
Protein Synthesis

Mixtures for poly U-directed protein synthesis (Section 3.14) containing M. verrucaria S-30 extract (A260 = 36 final concn.) were incubated at 25°C in the presence (●) or absence (o) of Poly U. Samples (10 µl) were precipitated with TCA and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).

FIGURE 6.4 Effect of  $Mg^{2+}$  Concentration on PolyU-Directed Protein Synthesis by M. verrucaria



Legend to Figure 6.4

Effect of  $Mg^{2+}$  Concentration on Poly U-Directed  
Protein Synthesis by M. verrucaria

Mixtures for Poly U-directed protein synthesis  
(Section 3.14) containing M. verrucaria S-30 extract  
(A260 = 32 final concn.) and various concentrations  
of  $Mg^{2+}$  were incubated at 25°C for 10 minutes.  
Samples (10  $\mu$ l) were precipitated with TCA and  
radioactivity in the precipitates estimated by  
liquid-scintillation counting (Section 3.16).



#### 6.4. PROTEIN SYNTHESIS BY *M. VERRUCARIA* S-30 EXTRACTS

Extracts from *M. verrucaria* cells were very active in poly U-directed protein synthesis. An incubation mixture containing freshly-prepared *M. verrucaria* S-30 incorporated 39500 cpm of  $\angle^{-14}\text{C}\angle$  phenylalanine into TCA-precipitable material in a 10  $\mu\text{L}$  sample in 60 minutes at 25°C (Fig.6.3). This represents an incorporation of 13 molecules of  $\angle^{-14}\text{C}\angle$  phenylalanine into polyphenylalanine per ribosome. In the absence of poly U, the incubation mixture incorporated about 100 cpm (in a 10  $\mu\text{L}$  sample) into TCA-precipitable material in 60 minutes (Fig.6.3).

As a compromise between the total amount of  $\angle^{-14}\text{C}\angle$ phenylalanine incorporated into TCA-precipitable material and the 'efficiency' of incorporation, the ribosome concentration in the incubation mixtures was adjusted to 1.5 - 2 mg/ml. The activity of the extracts varied from one preparation to another.

The  $\text{K}^+$  (or  $\text{NH}_4^+$ ) concentration of the incubation mixtures was found to be optimum between 60 and 130 mM (data not given). The  $\text{Mg}^{2+}$  concentration was optimum at 12 mM (Fig.6.4) but in all other experiments reported here the  $\text{Mg}^{2+}$  concentration was 14 mM (as it was for yeast incubation mixtures).

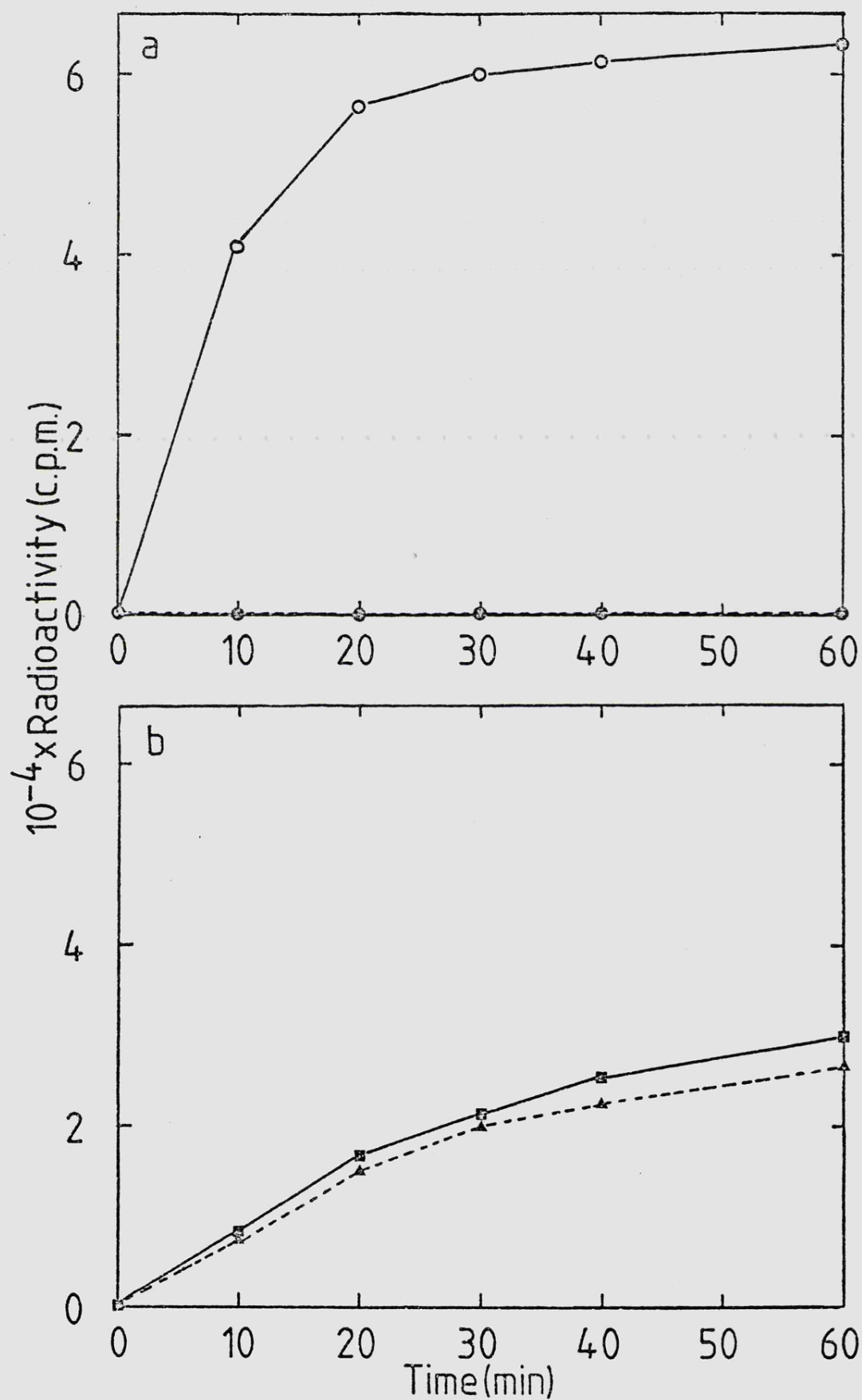
#### 6.5. FRACTIONATION OF YEAST S-30 EXTRACTS

Ribosomes and S100 were prepared from yeast S-30

by a modification of the high salt-wash procedure of Battaner and Vazquez (1971). The S-30 was centrifuged at 50,000 r.p.m. through 30% (<sup>W</sup>/v) sucrose containing 'high salt buffer' (10 mM-Tris.acetic acid (pH7.6), 1M-ammonium acetate, 100mM-magnesium acetate and 3mM-2-mercaptoethanol, c.f. 0.5M - NH<sub>4</sub>Cl for Battaner and Vazquez, 1971). The supernatant (S100) was removed and dialysed against 'eukaryotic dialysis buffer' and the rib<sup>o</sup>somes were resuspended in 'high salt buffer' for further washing (Section 3.10). After high salt-washing, membranous material on the surface of the ribosome pellet could be removed easily leaving a colourless ribosome preparation.

Neither ribosomes nor S100 prepared as above were active in poly U-directed protein synthesis unless combined together (Fig.6.5a). An incubation mixture containing both high salt-washed ribosomes and S100 incorporated 60,000 r.p.m. into a 10 μL sample in 60 minutes at 25°C (Fig.6.5a). This represents an incorporation of 16 molecules of  $\text{L}^{-14}\text{C}^-$  phenylalanine into polyphenylalanine per ribosome. An increase in the 'efficiency' of the system (as defined in Section 6.2) could be achieved by lowering the ribosome concentration in the incubation mixture but as this also resulted in a decrease in total incorporation of  $\text{L}^{-14}\text{C}^-$  phenylalanine into TCA-precipitable material the ribosome

FIGURE 6.5 Activity of Yeast Ribosomes and S-100 in Protein Synthesis



Legend to Figure 6.5

Activity of Yeast Ribosomes and S-100 in Protein  
Synthesis

Mixtures for Poly U-directed protein synthesis (Section 3.14) containing (a) high salt-washed yeast ribosomes (A260 = 19.5 final concn. ; Section 3.10) or (b) 'crude' ribosomes (A260 = 21 final concn.; Section 3.10) were incubated at 25°C in the presence or absence of yeast S100. Samples (10  $\mu$ l) were precipitated with TCA and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).

- (a)  $\bullet$ , plus ribosomes;  $\circ$ , ribosomes and yeast S100.  
(b)  $\blacksquare$ , plus ribosomes;  $\blacktriangle$ , ribosomes and yeast S100.

concentration in the incubation mixture was adjusted to 1.5 - 2 mg/ml. At best, the efficiency of the incubation mixtures was as good as that achieved by others (Jimenez et al., 1975).

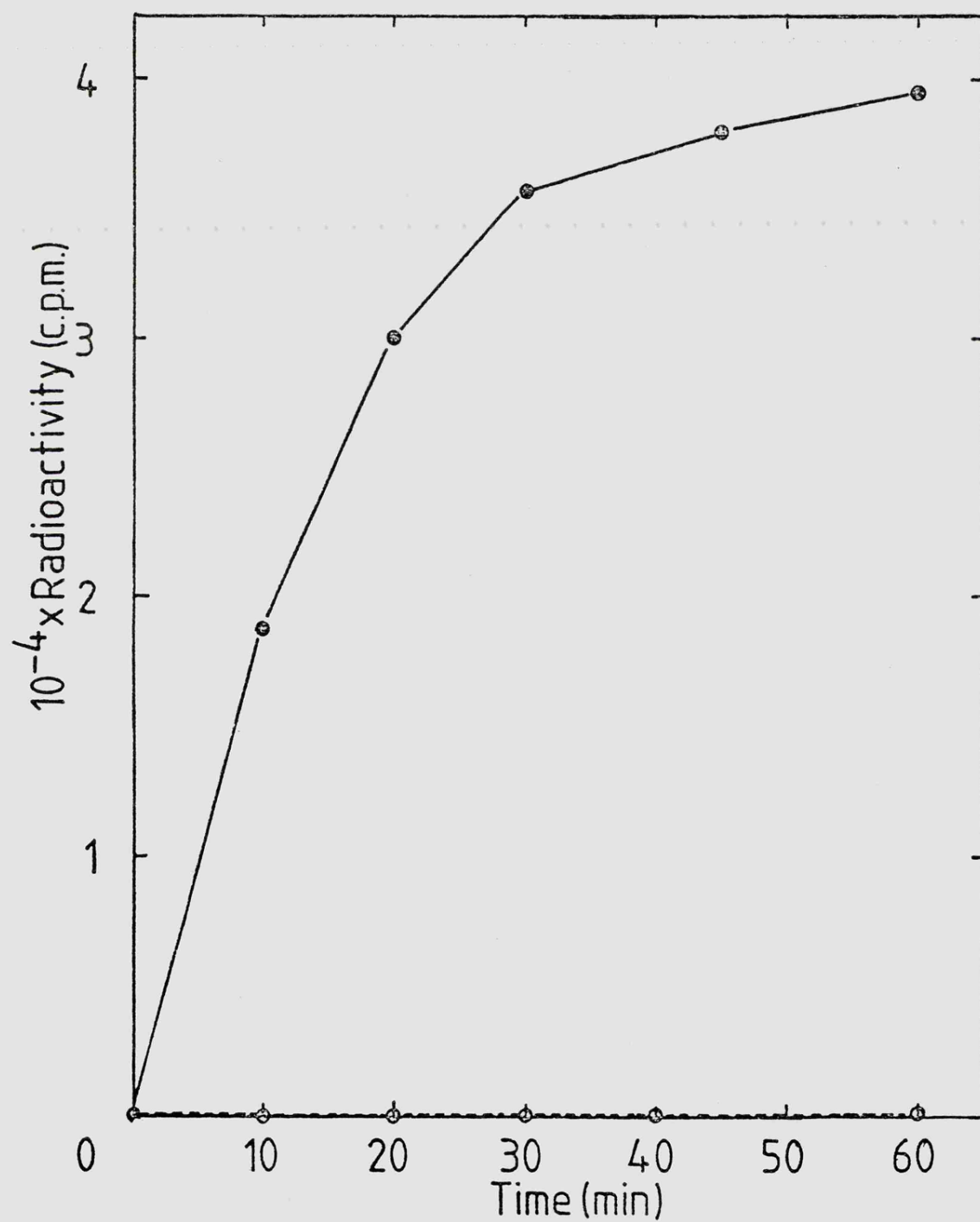
The activity of incubation mixtures was found to depend upon the batch of ribosomes and S100 used and the number of times they had been frozen and thawed prior to use. The amount of S100 added to the incubation mixtures was varied in order to obtain greatest synthetic activity. In general, best synthesis was obtained when 10  $\mu$ l of S100 were present in 50  $\mu$ l of incubation mixture (S100 had an A260 of 15-16 and 1.5 ml was obtained/gram of cells).

When ribosomes were prepared from yeast S-30 extracts by centrifugation through 30% (<sup>w</sup>/v) sucrose containing 'eukaryotic dialysis buffer', they retained considerable activity in poly U-directed protein synthesis in the absence of S100 (Fig.6.5b). In contrast, the S100 prepared by this method retained little ability to stimulate ribosomes in poly U-directed protein synthesis (data not given). Ribosomes prepared in this way could be made dependent upon added S100 by washing in high salt.

#### 6.6. FRACTIONATION OF M.VERrucARIA S-30 EXTRACTS

Ribosomes and S100 were prepared from M.verrucaria

FIGURE 6.6 Activity of M. verrucaria Ribosomes in Protein Synthesis



Legend for Figure 6.6

Activity of M. verrucaria Ribosomes in Protein  
Synthesis

A mixture for poly U-directed protein synthesis (Section 3.14) containing high salt-washed ribosomes from M. verrucaria (A<sub>260</sub> = 22 final concn.; Section 3.12) was incubated at 30°C •, plus yeast S100 or o, without yeast S100. Samples (5 µl) were precipitated with TCA and radioactivity in the samples was estimated by liquid-scintillation counting (Section 3.16).

S-30 extracts exactly as described for yeast S-30. The supernatant (S100) derived from this procedure was found to be inactive since it would not stimulate poly U-directed protein synthesis in incubation mixtures containing either high salt-washed M. verrucaria or yeast ribosomes. However, M. verrucaria ribosomes were very active in poly U-directed protein synthesis when combined with yeast S100 (prepared as in Section 6.6). The ribosomes of M. verrucaria incorporated 40,000 c.p.m. of  $\text{L-}^{14}\text{C}$  phenylalanine into TCA-precipitable material in a 5  $\mu\text{l}$  sample at 25°C (Fig. 6.6) which represents 19 molecules of phenylalanine incorporated per ribosome. Ribosome concentrations in the incubation mixtures were adjusted to 1.5 - 2 mg/ml and yeast S100 was used at 10  $\mu\text{l}$  per 50  $\mu\text{l}$  of incubation mixture.

In contrast to 'crude' yeast ribosomes, ribosomes prepared from M. verrucaria S-30 extracts without washing in high salt (Section 3.10) were unable to synthesise polyphenylalanine in the absence of yeast S100. They were also rather inactive even with added yeast S100 unless washed in high salt previously.

#### 6.7. INHIBITION OF POLY U-DIRECTED PROTEIN SYNTHESIS IN YEAST AND M. VERRUCARIA EXTRACTS.

The effects of various antibiotics on poly U-directed protein synthesis by yeast (strains Y<sub>166</sub> and TR<sub>1</sub>) and



INHIBITION OF INCUBATION MIXTURES CONTAINING EITHER  
YEAST OR M. VERRUCARIA S - 30 EXTRACTS

Antibiotic	Final Concentration μg/ml	% Inhibition of Synthesis	
		Yeast (Y <sub>166</sub> ) S - 30	M. verrucaria S - 30
Alpha Sarcin	100	99%	94%
Anisomycin	3	82%	6%
Cycloheximide	200	98%	91%
Homoharringtonine	30	62%	10%
Sparsomycin	50	93%	94%
T-2 Toxin	10	74%	7%
Verrucaridin A	30	69%	33%

TABLE 6.7

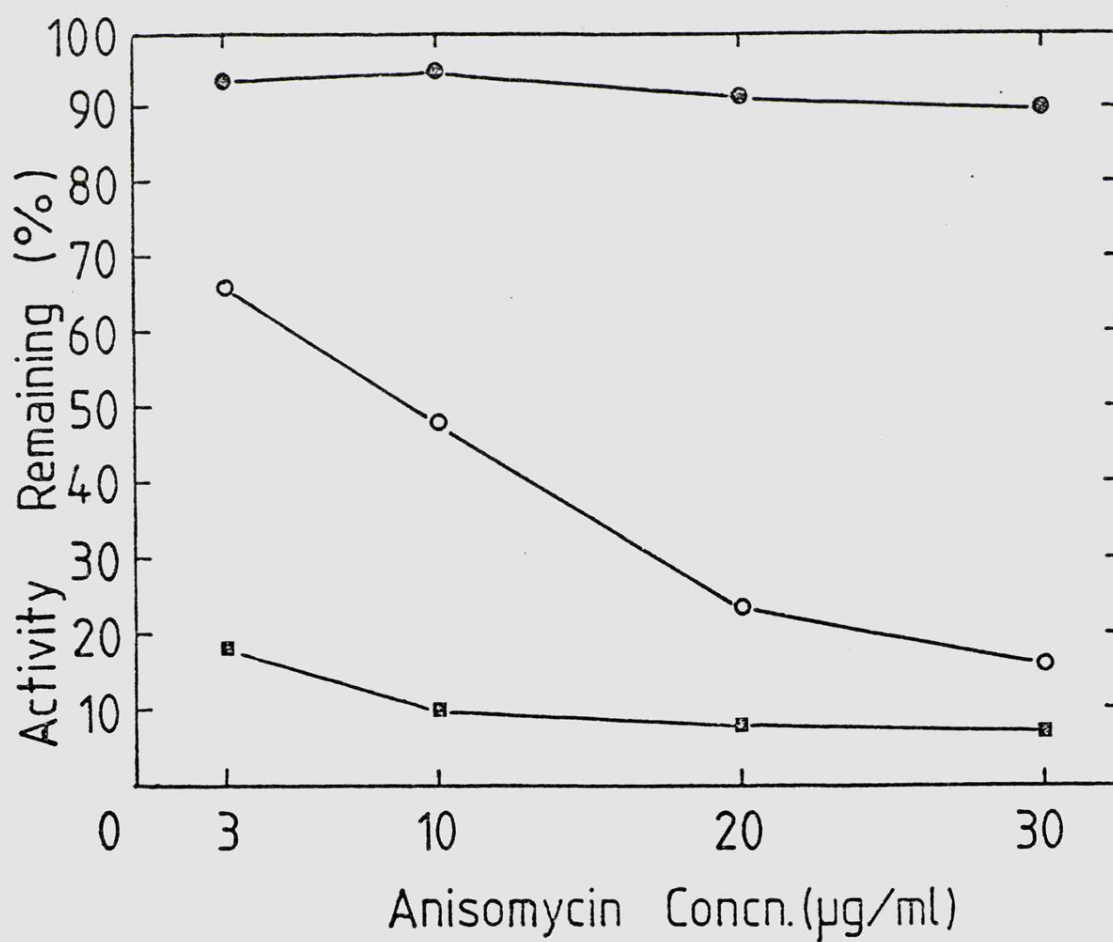
Legend to Table 6.7

Inhibition of Incubation Mixtures containing  
either Yeast or M. verrucaria S-30 extracts

Mixtures for poly U-directed protein synthesis  
(Section 3.14) containing either yeast or  
M. verrucaria S-30 extracts (A<sub>260</sub> = 36, final concn.)  
were incubated in the presence of various  
concentrations of antibiotics at 25°C. Samples  
(5 µl) were removed at 10, 20, 30, 40 and 60 minutes,  
precipitated with TCA and radioactivity in the  
precipitates estimated by liquid-scintillation  
counting (Section 3.16).

The percentage inhibition of protein synthesis  
given here is an average value for the 60 min.  
period when compared to an uninhibited mixture.

FIGURE 6.8 Effect of Anisomycin on PolyU-Directed Protein Synthesis



Legend to Figure 6.8

Effect of Anisomycin on Poly U-directed Protein  
Synthesis

Mixtures for Poly U-directed protein synthesis (Section 3.14) containing yeast strains Y<sub>166</sub>, TR<sub>1</sub> or M. verrucaria S-30 extracts (A260 = 36, final concn.) were incubated in the presence of various concentrations of anisomycin at 25°C. Samples (5 µl) were removed at 10, 20, 30, 40 and 60 minutes, precipitated with TCA and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16). The percentage activity in protein synthesis given here is an average value for the 60 min. period compared to an uninhibited mixture.

- M. verrucaria ribosomes
- TR<sub>1</sub> "
- Y<sub>166</sub> "

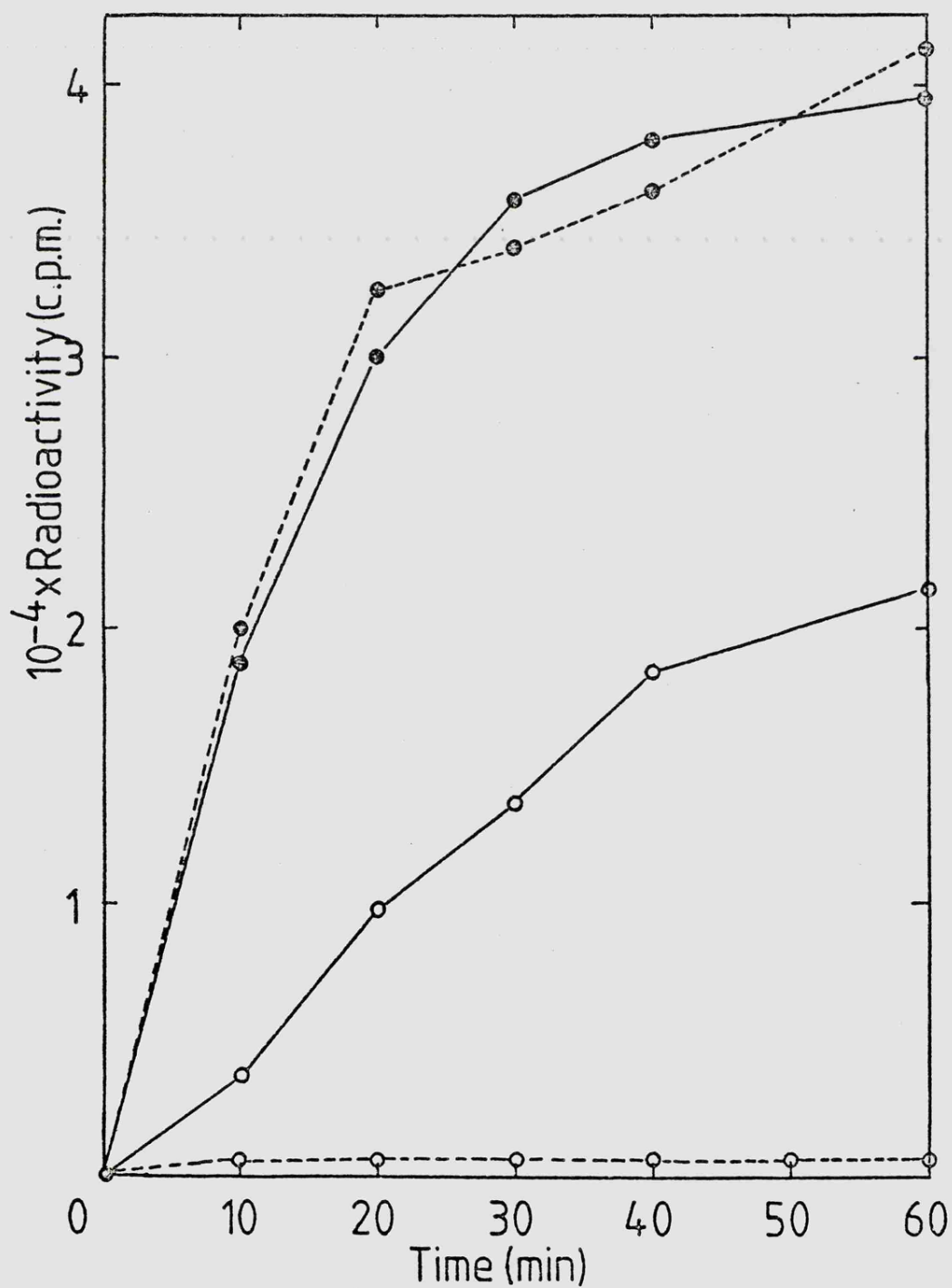
M. verrucaria cell-free extracts were investigated.

No data for inhibition of incubation mixtures containing strain TR<sub>1</sub> S-30 extracts are given (except for the effect of anisomycin, Fig.6.8) because such data has already been published (Jimenez et al., 1975).

Incubation mixtures containing strain Y<sub>166</sub> or M. verrucaria S-30 extracts were equally sensitive to the following antibiotics: alpha sarcin, cycloheximide and sparsomycin (Table 6.7). However, unlike strain Y<sub>166</sub>, M. verrucaria S-30 extracts were resistant both to antibiotics of the 12,13 epoxytrichothecene family and the chemically-unrelated antibiotics anisomycin and homoharringtonine (Table 6.7). Although M. verrucaria S-30 extracts were resistant to high concentrations of anisomycin, extracts of strain TR<sub>1</sub> showed resistance only when low levels of anisomycin were used (Fig.6.8) indicating that resistance in these two organisms was not effected in the same way.

The resistance of M. verrucaria S-30 extracts to inhibition was investigated further by examining the effect of the antibiotics on yeast and M. verrucaria ribosomes when combined with yeast S100. Ribosomes were confronted with antibiotic prior to the addition of the remainder of the incubation mixture and, in this case, inhibition of poly U-directed protein synthesis by yeast ribosomes was increased in comparison to inhibition of

FIGURE 6.9 Inhibition of PolyU- Directed Protein Synthesis by T-2 Toxin



Legend to Figure 6.9

Inhibition of Poly U-Directed Protein Synthesis  
by T-2 Toxin

Mixtures for poly U-directed protein synthesis (Section 3.14) containing either yeast strain Y<sub>166</sub> (A260 = 19 final concn.) or M. verrucaria (A260 = 22 final concn.) ribosomes and yeast S100 were incubated at 25°C. T-2 toxin (10 µg/ml final concn.) was mixed with the ribosomes prior to the addition of S100.

Samples (5 µl) were precipitated with TCA and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).

- , control with yeast strain Y<sub>166</sub> ribosomes;
- , yeast strain Y<sub>166</sub> ribosomes plus T-2 toxin;
- , control with M. verrucaria ribosomes;
- , M. verrucaria ribosomes plus T-2 toxin.

TABLE 6.10

INHIBITION OF INCUBATION MIXTURES CONTAINING EITHER  
YEAST OR M. VERRUCARIA RIBOSOMES AND YEAST S100

Antibiotic	Final concentration μg/ml	% Inhibition of Synthesis		
		Y <sub>166</sub> R	TR <sub>1</sub> R	<u>M. verrucaria</u> R
Anisomycin	3	79%	39%	18%
Anisomycin	10	84%	64%	18%
Homoharringtonine	10	46%	-	10%
Homoharringtonine	30	62%	-	14.5%
T-2 toxin	2	97%	65%	0.5%
T-2 toxin	10	99%	78%	0.5%
Verrucaric acid	30	81%	-	9%

R = Ribosomes



Legend to Table 6.10

Inhibition of Incubation Mixtures Containing  
Either Yeast or M. verrucaria Ribosomes and Yeast  
S100.

Mixtures for poly U-directed protein synthesis  
(Section 3.14) containing either yeast or M. verrucaria  
ribosomes (A260 = 26 final concn.) and yeast  
strain Y<sub>166</sub> S100 were incubated at 25°C. Antibiotics  
at various final concentrations were added to the ribo-  
somes prior to the addition of the remainder of  
the incubation mixture. Samples (5 µl) were removed  
at 10, 20, 30, 40 and 60 minutes, precipitated with  
TCA and radioactivity in the precipitates estimated  
by liquid-scintillation counting (Section 3.16).  
The percentage inhibition of protein synthesis  
was averaged over the whole time course.

yeast S-30 extracts. However, M. verrucaria ribosomes remained unaffected by the 12,13 epoxytrichothecenes, anisomycin or homoharringtonine (Fig.6.9 and Table 6.10). Clearly, resistance to these antibiotics was a function of the M. verrucaria ribosomes rather than the S100 as indeed was the case for TR<sub>1</sub> (Jimenez et al., 1975).

The 12,13 epoxytrichothecenes, anisomycin and homoharringtonine are all believed to share common or over-lapping binding sites within the peptidyltransferase centre of the yeast ribosome (Barbacid and Vazquez, 1974a, 1974b; Jimenez et al., 1975; Fresno et al., 1977). It would seem, therefore, that resistance to all these antibiotics is linked and may be a consequence of the need for M. verrucaria to protect itself from its own toxic metabolites.

In agreement with others (Schindler, 1974; Carrasco et al., 1973), trichodermin, a 12,13 epoxytrichothecene which inhibits elongation of polypeptides (Cundliffe et al., 1974; Carter et al., 1976), was a very poor inhibitor of poly U-directed protein synthesis by yeast extracts and, therefore, no data is presented here for inhibition by trichodermin. This phenomenon was investigated and the results are reported later (Chapter 9).

#### 6.8. EFFECT OF 12,13 EPOXYTRICHTHOCENES ON THE PEPTIDYLTRANSFERASE ACTIVITY OF YEAST AND M. VERRUCARIA RIBOSOMES

Since the 12,13 epoxytrichothecenes, anisomycin

and homoharringtonine have all been shown to be inhibitors of the yeast peptidyltransferase (Carrasco et al., 1973; Jimenez et al., 1975; Fresno et al., 1977), the effect of these antibiotics upon the 'fragment reaction' catalysed by M. verrucaria ribosomes was investigated. The 'fragment reaction' is an assay reputed to be specific for the peptidyltransferase (Monro and Marcker, 1967; Monro, 1969; Monro et al., 1969) and requires the presence of a tRNA fragment, CACCA-(acetyl-Leu) or UACCA-(acetyl-Leu), puromycin, methanol or ethanol and ribosomes (or large ribosomal subunits). The reaction may be represented by:

$$\text{CACCA-(acetyl-}\text{L}\text{-}^3\text{H}\text{-Leu)} + \text{Puromycin} \longrightarrow \text{acetyl-}\text{L}\text{-}^3\text{H}\text{-Leu-puromycin} + \text{CACCA}$$

(a) Preparation of Fragment

(i) Preparation of E.coli S100

High speed supernatant (S100) was prepared from E.coli (strain MRE 600) cells as follows: cells were grown in liquid medium (containing 13g. nutrient broth and 5g. glucose per litre of water) at 37°C until the medium had an absorbance at 600 n.m. of approximately 1.0. They were then harvested by centrifugation at 3000 r.p.m. for 20 minutes and washed in buffer (10mM-Tris. acetic acid (pH7.6), 10mM-magnesium acetate, 50mM-ammonium acetate and 3mM-2-mercaptoethanol). After resuspension in one volume of ice-cold buffer (as above), the cells were disrupted in a French pressure cell at 10,000 psi and an S-30 extract was prepared by centrifuging the mixture at

20,000 r.p.m. for 15 minutes and dialysing the resultant supernatant against two changes of buffer (10 mM-Tris.acetic acid (pH7.6) 10mM-magnesium acetate, 50mM-ammonium acetate and 3mM-2-mercaptoethanol). Finally, an S100 was obtained by centrifugation of the S-30 extract at 50,000 r.p.m. for three hours.

(ii) Charging of tRNA with  $\gamma$ -<sup>3</sup>H leucine.

The tRNA was charged at 37°C in an incubation mixture of the following composition:

'Salts'	10 µl	
Energy mix	10 µl	
L- $\gamma$ -4-5- <sup>3</sup> H leucine	10 µl	(10.2 Ci/mMol)
<u>E.coli</u> tRNA	10 µl	(initial concentration 50 mg/ml)
200mM-magnesium acetate	5 µl	
Water	<u>5 µl</u>	
	50 µl	
<u>E.coli</u> S100	<u>50 µl</u>	
	100 µl	
<u>'Salts'</u>	200 mM-Hepes/KOH pH7.5	
	500 mM-potassium acetate	
	35 mM-magnesium acetate	
<u>Energy mix</u>	3.7mM - GTP (Li <sub>3</sub> )	
	2.5mM - ATP (Na <sub>2</sub> )	
	50 mM - PEP	

After incubating for 20 minutes, the mixture was made 60% (v/v) in ethanol, left overnight at -20°C to precipitate RNA, centrifuged and the resultant pellet was washed in absolute ethanol before being

dried in vacuo.

(iii) Acetylation and Digestion of Leucyl-tRNA

The  $\text{[}^{-3}\text{H]}$  Leu-tRNA obtained as above was acetylated and then digested with ribonuclease- $\text{T}_1$  (RNase- $\text{T}_1$ ) as described by Monro (1971). The Leucyl-tRNA was acetylated by incubating 20mg of charged tRNA in 1 ml 0.2M-sodium acetate (pH5.0) with 20  $\mu\text{l}$  of acetic anhydride at  $0^\circ\text{C}$  for 40 minutes. Two further portions of 20  $\mu\text{l}$  of acetic anhydride were added with 40 minutes incubation after each addition. After isolation of tRNA using ethanol precipitation (as described above), the N-acetylleucyl-tRNA was digested for 30 minutes at  $37^\circ\text{C}$  with RNase- $\text{T}_1$  in a reaction mixture containing 20 mg of charged, acetylated tRNA and 0.1 mg of RNase- $\text{T}_1$  per ml of 0.25M-sodium acetate (pH5.4) and 2mM-disodium diaminoethanetetra-acetate ( $\text{Na}_2$  EDTA).

The two fragments produced by this procedure, namely CACCA - acetyl  $\text{[}^{-3}\text{H]}$  Leu and UACCA - acetyl  $\text{[}^{-3}\text{H]}$  Leu, were separated by electrophoresis on Whatman No.52 paper at 2500 V for 2 hours (pH3.5). Following electrophoresis, fragments were first detected by scintillation spectrometry of thin strips of the electrophoregram and were then eluted in 0.1 mM-sodium acetate (pH5.0) at  $4^\circ\text{C}$ . Eluates were lyophilised, redissolved in small quantities of water and were stored as aliquots at  $-70^\circ\text{C}$ .

The fragments CACCA- acetyl  $\text{[}^{-3}\text{H]}$ Leu and UACCA -

acetyl [ $^3\text{H}$ ] Leu moved 4 cm and 9 cm, respectively, in the direction of the anode when electrophoresed as described above.

(b) Conditions for Fragment Reaction

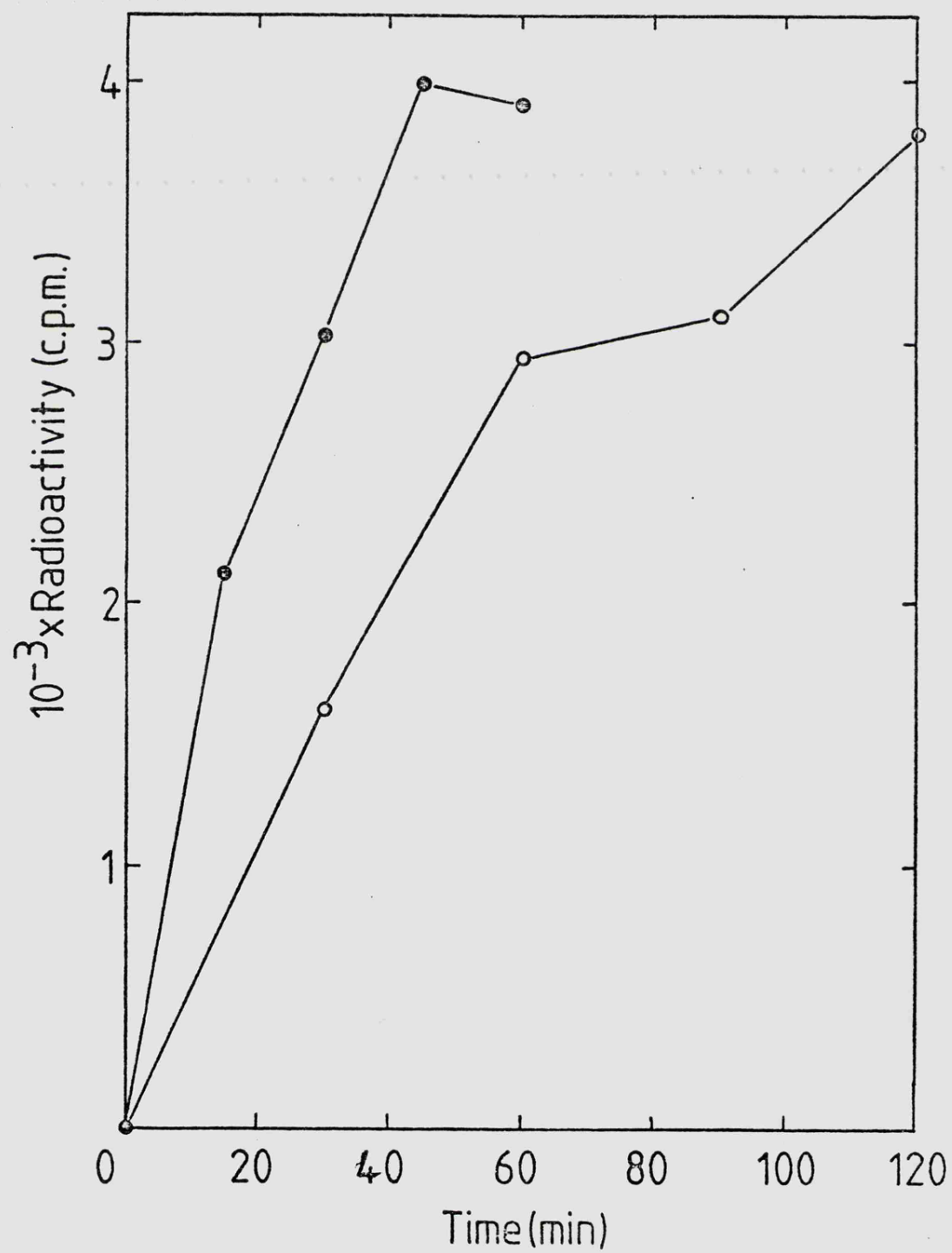
Incubations were carried out either at 0°C or 20°C (see figure legends) in ionic conditions derived as below:

1M-Tris.HCl (pH7.6)	5 $\mu\text{l}$
2M-KCl	20 $\mu\text{l}$
1M - $\text{MgCl}_2$	2 $\mu\text{l}$
Puromycin (5 mg/ml)	5 $\mu\text{l}$
Water	68 $\mu\text{l}$
Methanol	50 $\mu\text{l}$
	<hr/>
	150 $\mu\text{l}$

Ribosomes (20 - 25 p moles) were added to 150  $\mu\text{l}$  of buffer (as above) followed by 2,000 - 4,000 cpm of fragment. After 15 - 120 minutes the reaction was stopped by addition of 0.1 ml of 0.3 M-sodium acetate. acetic acid (pH5.5), saturated with  $\text{Mg SO}_4$  at room temperature. The acetyl [ $^3\text{H}$ ] Leu-puromycin formed during the reaction was extracted into 1.5 ml ethyl acetate and 1 ml of this solvent was taken for liquid-scintillation spectrometry. Samples of ethyl acetate were mixed with 3 ml water-miscible scintillant (Section 3.16).

Counting efficiency for tritium in scintillant/

FIGURE 6.11 Effect of Temperature on the Fragment Reaction



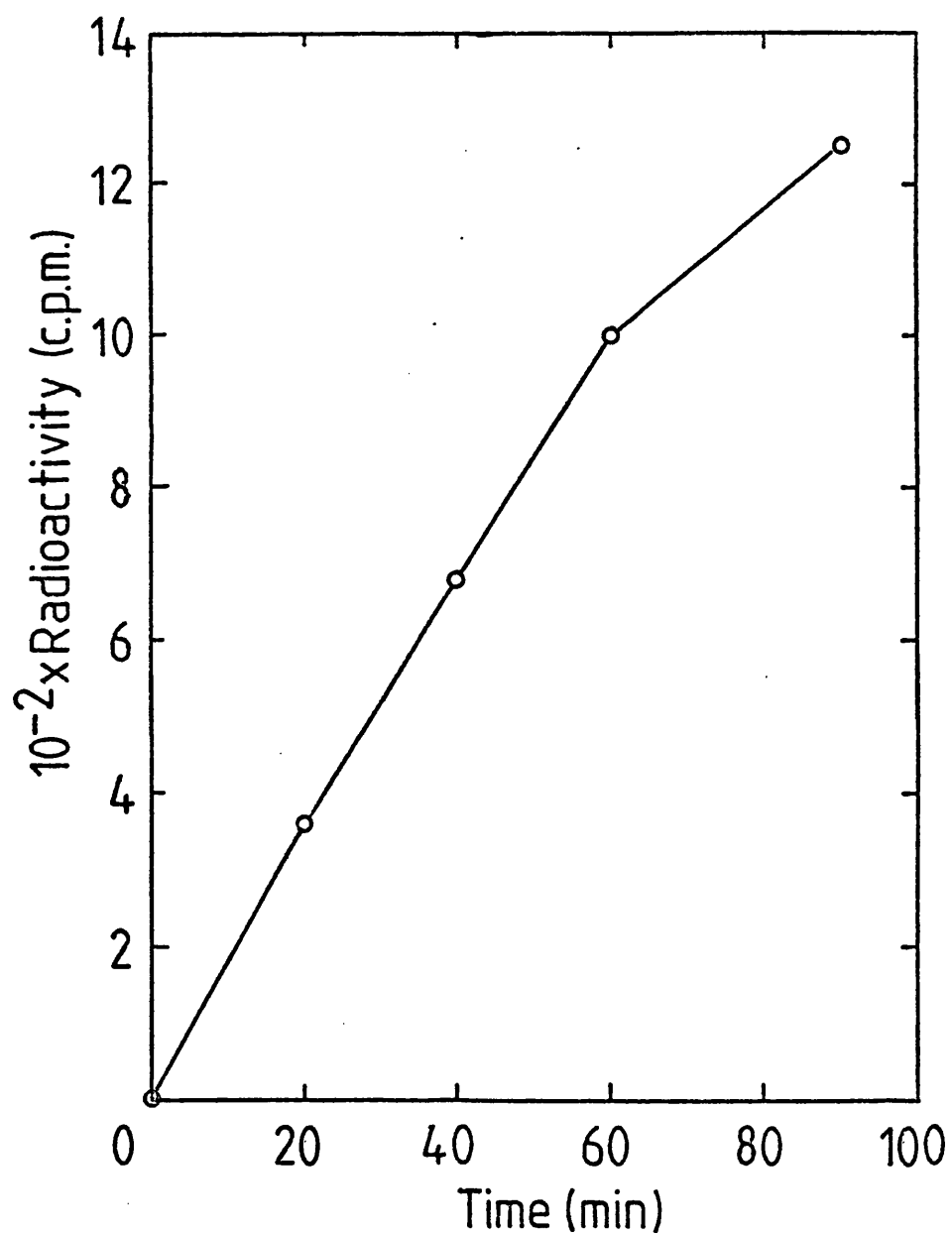
## Legend to Figure 6.11

### Effect of Temperature on the Fragment Reaction

Mixtures for fragment reactions (Section 6.8b) containing yeast ribosomes (A260 = 10 final concn.) and 7700 c.p.m. of Ac[ $^3\text{H}$ ] leu-ACCAC were incubated at either (●) 20°C or (○) 0°C. At various times the reaction was stopped by addition of 0.1ml 0.3M - sodium acetate, acetic acid (pH5.5), saturated with  $\text{MgSO}_4$ . The amount of Ac[ $^3\text{H}$ ] leu-puromycin formed was estimated by liquid-scintillation counting of an ethyl acetate extract (Section 6.8b). Parallel experiments were performed in the absence of ribosomes and these 'backgrounds' were subtracted from each time point.



FIGURE 6.12 Fragment Reaction in the  
Absence of Ribosomes



## Legend to Figure 6.12

### Fragment Reaction in the Absence of Ribosomes

Mixtures for fragment reactions (Section 6.8b) containing 3850 c.p.m. of acetyl  $\angle^{-3}\text{H}_7\text{leu-ACCAC}$  but no ribosomes were incubated at 20°C. At various times the reaction was stopped by addition of 0.1ml 0.3M-sodium acetate, acetic acid (pH5.5), saturated with  $\text{MgSO}_4$ . Formation of acetyl  $\angle^{-3}\text{H}_7\text{leu-puromycin}$  was estimated by liquid-scintillation counting of an ethyl acetate extract (Section 6.8b).

ethyl acetate was 13%.

(c) Fragment Reaction with Yeast and *M. verrucaria* Ribosomes.

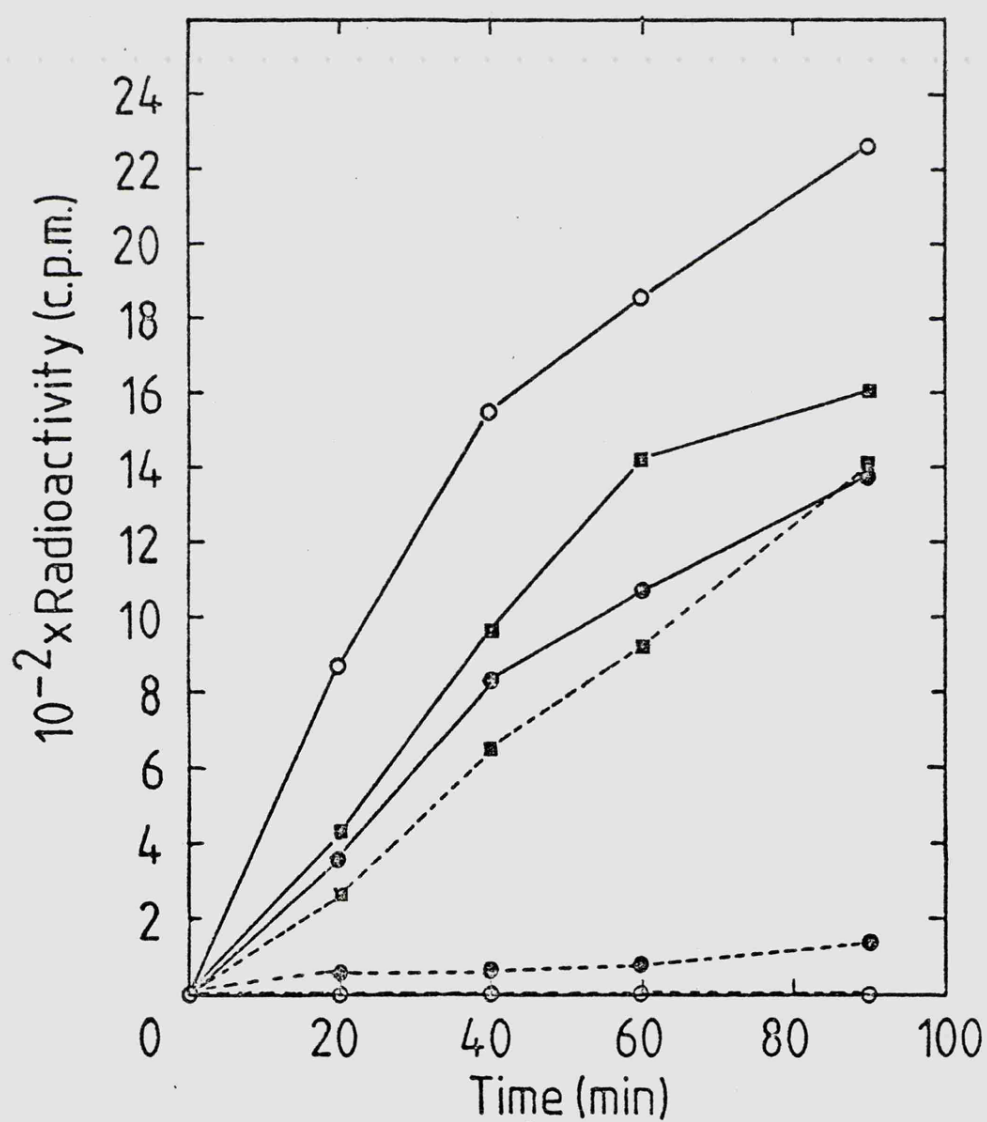
All 'fragment reactions' were performed at 20°C since, at this temperature, the reaction proceeds at more than twice the rate at 0°C (Fig.6.11). It was necessary to have controls for every time point of an incubation because 'fragment' reacted with puromycin even in the absence of ribosomes and gave rise to an increase in background with time (Fig.6.12).

The 12,13 epoxytrichothecene, T-2 toxin, totally inhibited the 'fragment reaction' catalysed by yeast, strain Y<sub>166</sub>, ribosomes but only inhibited by 90% when ribosomes from the mutant TR<sub>1</sub> were used (Fig.6.13).

'Fragment reactions' catalysed by *M. verrucaria* ribosomes were virtually unaffected by T-2 toxin - being inhibited by about 10% (Fig.6.13). Although trichodermin was unable to inhibit poly U-directed protein synthesis by yeast extracts, the 'fragment reaction' was inhibited by 78% at 30 µg/ml trichodermin (final concentration). The ribosomes of *M. verrucaria* were unaffected by trichodermin when assayed both for poly U-directed protein synthesis and the 'fragment reaction' (data not given).

Therefore, resistance to the 12,13 epoxytrichothecenes appears, in *M. verrucaria*, to be a

FIGURE 6.13 Inhibition of the Fragment Reaction  
by T-2 Toxin



Legend to Figure 6.13

Inhibition of the Fragment Reaction by T-2 Toxin

Mixtures for fragment reactions (Section 6.8b) containing 3850 c.p.m. of acetyl[ $^{-3}\text{H}$ ] leu-ACCAC and either yeast strain Y<sub>166</sub> or TR<sub>1</sub>, or M.verrucaria ribosomes (A260 = 6.5 final concen.) were incubated at 20°C. At various times the reaction was stopped by addition of 0.1ml 0.3M-sodium acetate, acetic acid (pH5.5), saturated with MgSO<sub>4</sub>. Formation of acetyl[ $^{-3}\text{H}$ ] leu-puromycin was estimated by liquid-scintillation counting of an ethyl acetate extract (Section 6.8b). All data are presented with the appropriate 'no ribosome background' subtracted.

○, yeast strain Y<sub>166</sub> ribosomes; ●, yeast strain TR<sub>1</sub> ribosomes; ■, M.verrucaria ribosomes.

——, plus ribosomes; -----, ribosomes plus T-2 toxin (2µg/ml final concen.).

property of the ribosome and specifically the peptidyltransferase. Since the peptidyltransferase is reported always to be an integral part of the large ribosomal subunit (Monro, 1967; Maden et al., 1968; Traut and Monro, 1964; Vazquez et al., 1969) resistance to the antibiotics was likely to be a property of the large ribosomal subunit of M.verrucaria.

This hypothesis was tested and the results are shown in Chapter 7.

It is interesting that sparsomycin, whilst being an inhibitor of the peptidyltransferase (Section 4.2) inhibited poly U-directed protein synthesis by M.verrucaria ribosomes. This evidence supports other reports that the binding sites of the 12,13 epoxytrichothecenes and sparsomycin are different (Barbacid and Vazquez, 1974a,b; Jimenez and Vazquez, 1975) and helps to explain why sparsomycin inhibits both eukaryotic and prokaryotic ribosomes whilst the 12,13 epoxytrichothecenes only inhibit eukaryotic ribosomes (for a review, see Vazquez, 1974).

## 7. PREPARATION OF RIBOSOMAL SUBUNITS AND RIBOSOMAL PROTEINS

### 7.1. MATERIALS

Commercial sources of materials were as follows: [<sup>14</sup>C]-U protein hydrolysate from The Radiochemical Centre, Amersham, Bucks.; Coomassie brilliant blue -G and Bromophenol blue from Raymond A. Lamb, London; TEMED (N,N,N<sup>1</sup>,N<sup>1</sup>-tetramethyl-ethylenediamine), MES (2-[N-morpholino]ethanesulphonic acid), BIS-TRIS (bis [2-hydroxyethyl]imino-tris [hydroxy-methyl]methane) and Basic fuchsin were from Sigma Chemical Co., Kingston-upon-Thames, Surrey. All other chemicals were obtained as described in Section 3.2.

### 7.2. INTRODUCTION

Eukaryotic ribosomes may be split into 40S and 60S subunits from the 80S parent (Chao, 1957). In contrast to bacterial ribosomes which readily dissociate into subunits when the magnesium ion concentration is lowered (Tissières et al., 1960), eukaryotic ribosomes do not. When mammalian ribosomes are suspended in low concentrations of magnesium ion many partially-unfolded ribosomes are formed (Lamfrom and Glowacki, 1962). This was also found to be true of yeast and fungal ribosomes (Section 7.3). High concentrations of potassium cations, however, do cause ribosomes from many different eukaryotic sources to dissociate into

their component subunits (Martin and Wool, 1968, 1969; Martin et al., 1969). Here a method is described for the preparation of 40S and 60S ribosomal subunits from both yeast and fungi. The subunits may be recombined to give ribosomes which are active in poly U-directed protein synthesis.

Experiments involving heterologous recombinations of ribosomal subunits from yeast and M. verrucaria were performed in order to identify the ribosomal subunit of M. verrucaria responsible for antibiotic resistance. Additionally heterologous recombinations between the ribosomal subunits of yeast strains Y166 and TR<sub>1</sub> were performed to confirm that resistance to the 12,13 epoxytrichothecenes in strain TR<sub>1</sub> is a function of the 60S ribosomal subunit (Jimenez et al., 1975).

The gene-product which is altered in strain TR<sub>1</sub> and causes resistance to the 12,13 epoxytrichothecenes is unknown. Proteins were prepared from the ribosomal subunits of strains Y<sub>166</sub> and TR<sub>1</sub> and subjected to 2-D gel electrophoresis (Section 7.5) in an attempt to identify any altered proteins. Also, the 2-D gel profile of the proteins from the ribosomes of strain Y<sub>166</sub> was compared to that of the ribosomal proteins from M. verrucaria.

### 7.3. PREPARATION OF RIBOSOMAL SUBUNITS

Ribosomal subunits have been prepared from many



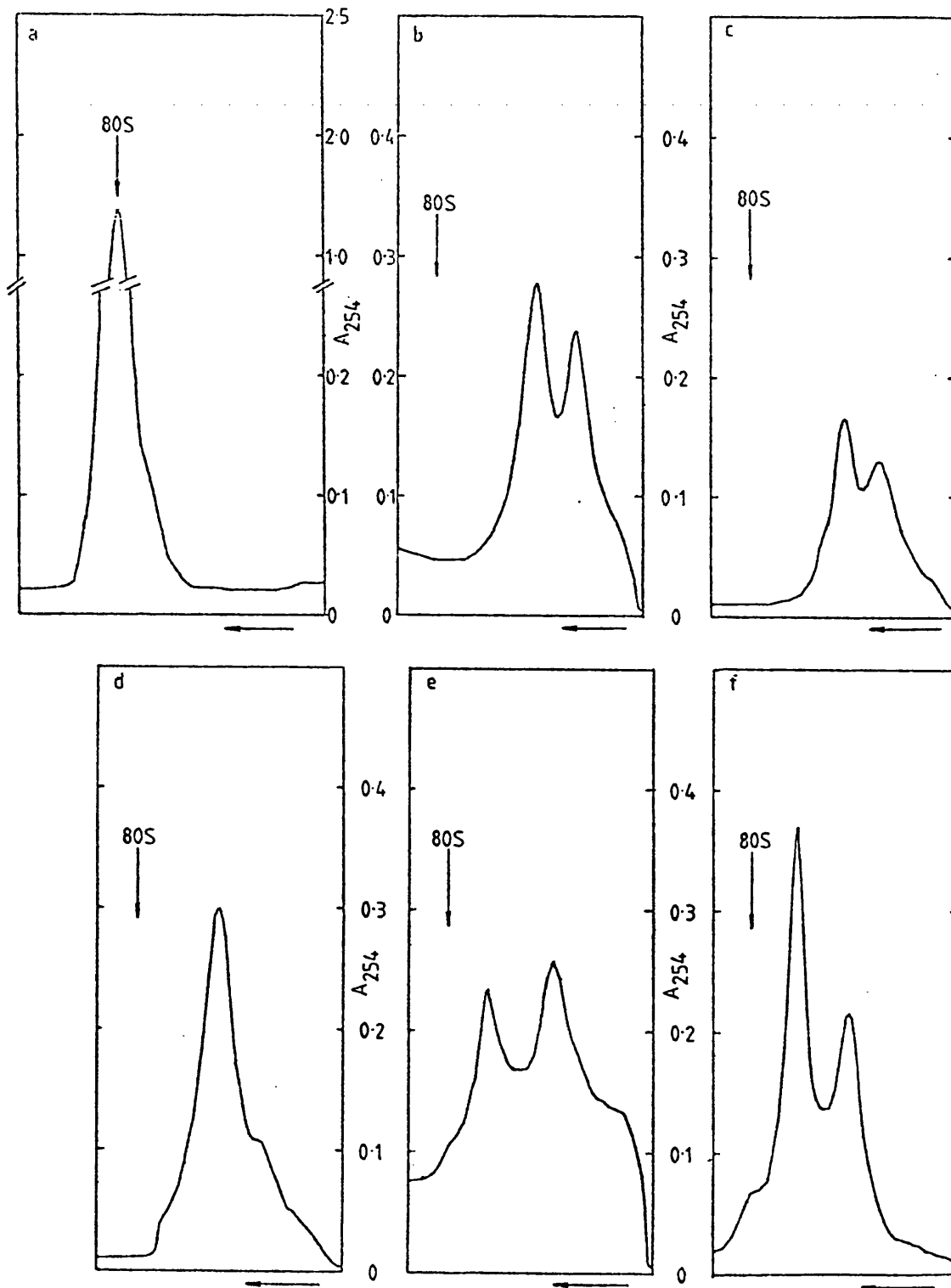
types of eukaryotes (Martin et al., 1971; Staehelin and Falvey, 1971; Battaner and Vazquez, 1971; Schindler et al., 1974; Brown et al., 1974). All these techniques involve suspending ribosomes in high potassium ion and low magnesium ion concentrations and some involve pre-treatment with puromycin to remove nascent peptides. The ionic conditions for ribosomal subunit preparation described by these authors, namely:  $K^+$ -880 mM,  $Mg^{2+}$ - 12.5mM (Martin et al., 1971);  $K^+$ -500mM,  $NH_4^+$  - 100 mM,  $Mg^{2+}$ - 5mM (Staehelin and Falvey, 1971);  $NH_4^+$ - 50mM,  $Mg^{2+}$  - 0.2mM (Battaner and Vazquez, 1971);  $K^+$ -500 mM,  $Mg^{2+}$  - 5mM (Schindler et al., 1974); preincubation with 0.1mM-puromycin then  $K^+$ -150mM,  $Mg^{2+}$  - 1mM (Brown et al., 1974) were used in this work in an attempt to produce 40S and 60S subunits from yeast and M. verrucaria ribosomes. However, without exception these conditions caused the ribosomes to dissociate into partially-unfolded structures which did not sediment at the same rate as ribosomal subunits (data not given). Accordingly, ionic conditions were varied in order to determine optimum conditions for the preparation of ribosomal subunits from yeast and M. verrucaria.

The ribosomes, from which the subunits were to be prepared, were isolated from cells which had been grown to

the phase of logarithmic growth and then rapidly cooled on ice prior to harvesting. If the cells were harvested without prior cooling the ribosomes would not dissociate readily into subunits upon suspension in high potassium (data not given). It is well documented that ribosomes carrying peptidyl-tRNA dissociate less readily into subunits than do free subunits (Martin *et al.*, 1969; Lawford, 1969; Faust and Matthaei, 1972). Presumably cooling the medium (and cells) causes some 'run off' of ribosomes from polyribosomes. Preincubation of ribosomes with puromycin was found to have no effect upon subunit formation (data not given).

Ribosomes which consisted only of 80S monomers (Fig. 7.1) in 'eukaryotic dialysis buffer' (10mM-Tris. acetic acid (pH7.6); 90mM-potassium acetate, 1mM-magnesium acetate, 3mM-2-mercaptoethanol) were dialysed at 0°C into buffers of varying ionic conditions in the range:  $K^+$  or  $Na^+$ , 90 - 500 mM;  $Mg^{2+}$ , 0.1 - 20 mM (see figure legends) and were then centrifuged through 5 ml 10-30% (<sup>w</sup>/v) sucrose density-gradients (in appropriate ionic conditions) in an SW50.1 rotor for two hours at 40,000 r.p.m. and 2°C. After centrifugation, ultra-violet absorbance of materials in the gradients was monitored continuously as the gradient solution was pumped through an ISCO absorbance monitor set at 254 nm.

FIGURE 7.1 Effect of  $Mg^{2+}$  and  $K^{+}$  Concn. on the Dissociation of Yeast Ribosomes



Legend to Figure 7.1

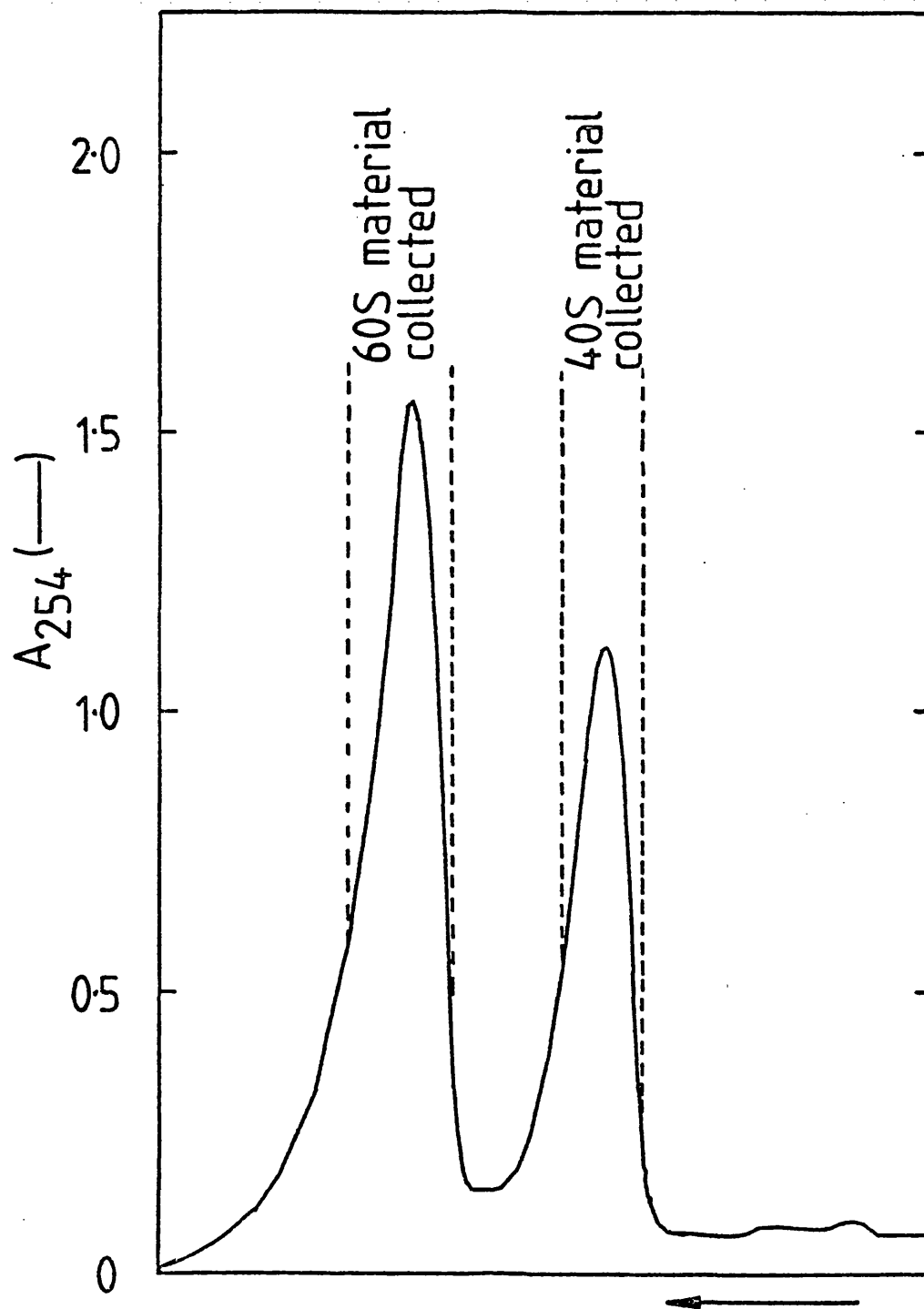
Effect of  $Mg^{2+}$  and  $K^{+}$  Concentrations on the  
Dissociation of Yeast Ribosomes

A crude preparation of yeast ribosomes (1.2 A260 units; Section 3.10) was diluted in 0.25 ml of 10mM-Tris. acetic acid (pH7.6), 90mM-potassium acetate, 1mM-magnesium acetate, 3mM-2-mercaptoethanol and either (a) centrifuged through a 10-30% (<sup>w</sup>/v) sucrose density-gradient containing this buffer or dialysed into 10mM-Tris. HCl (pH7.6), 500mM-KCl, 3mM-2-mercaptoethanol and (b) 0.1mM-MgCl<sub>2</sub>; (c) 0.5mM-MgCl<sub>2</sub>; (d) 1mM-MgCl<sub>2</sub>; (e) 5mM-MgCl<sub>2</sub>; (f) 12.5mM-MgCl<sub>2</sub> and then centrifuged through a sucrose density-gradient containing the appropriate buffer. Gradients were centrifuged at 40,000 r.p.m. for 2 hours at 2°C in a Spinco SW50.1 rotor. Horizontal arrows represent the direction of sedimentation of ribosomes.

Very low magnesium ion concentrations caused the ribosomes to form structures which did not correspond to either 40S or 60S ribosomal subunits (Fig.7.1). The best ionic conditions for production of subunits was found to be 10mM - Tris.HCl (pH7.6), 500 mM-KCl, 12.5 mM-MgCl<sub>2</sub>, 3mM-2-mercaptoethanol. In these ionic conditions, ribosomes were dissociated almost completely to subunits and no other products were apparent (Fig.7.1). Subunits were still produced at 15-20 mM-MgCl<sub>2</sub> but less 80S monomers were dissociated. The correct positions within sucrose density-gradients of 40S and 60S ribosomal subunits and 80S monomers were determined by substituting rabbit reticulocyte lysates for yeast ribosomes and centrifuging the lysates under conditions in which their ribosomes dissociate into subunits (see Section 5.1).

For the large scale preparation of ribosomal subunits, ribosomes were dialysed into 10mM-Tris.HCl (pH7.6), 500mM-KCl, 12.5mM-MgCl<sub>2</sub>, 3mM-2-mercaptoethanol at 0°C and approximately 5 mg ribosomes were centrifuged through 34 ml 10-30% (<sup>w</sup>/v) sucrose in an SW27 rotor for six hours at 26,000 r.p.m. and 2°C. Ribosomal subunits were detected by pumping gradient material through an ISCO absorbance monitor. Portions of the gradients containing 40S or 60S ribosomal subunits were collected (Fig.7.2) and dialysed against 'eukaryotic dialysis buffer' at 0°C. The subunits were then centrifuged at 50,000 r.p.m. for

FIGURE 7.2 Preparation of Ribosomal Subunits

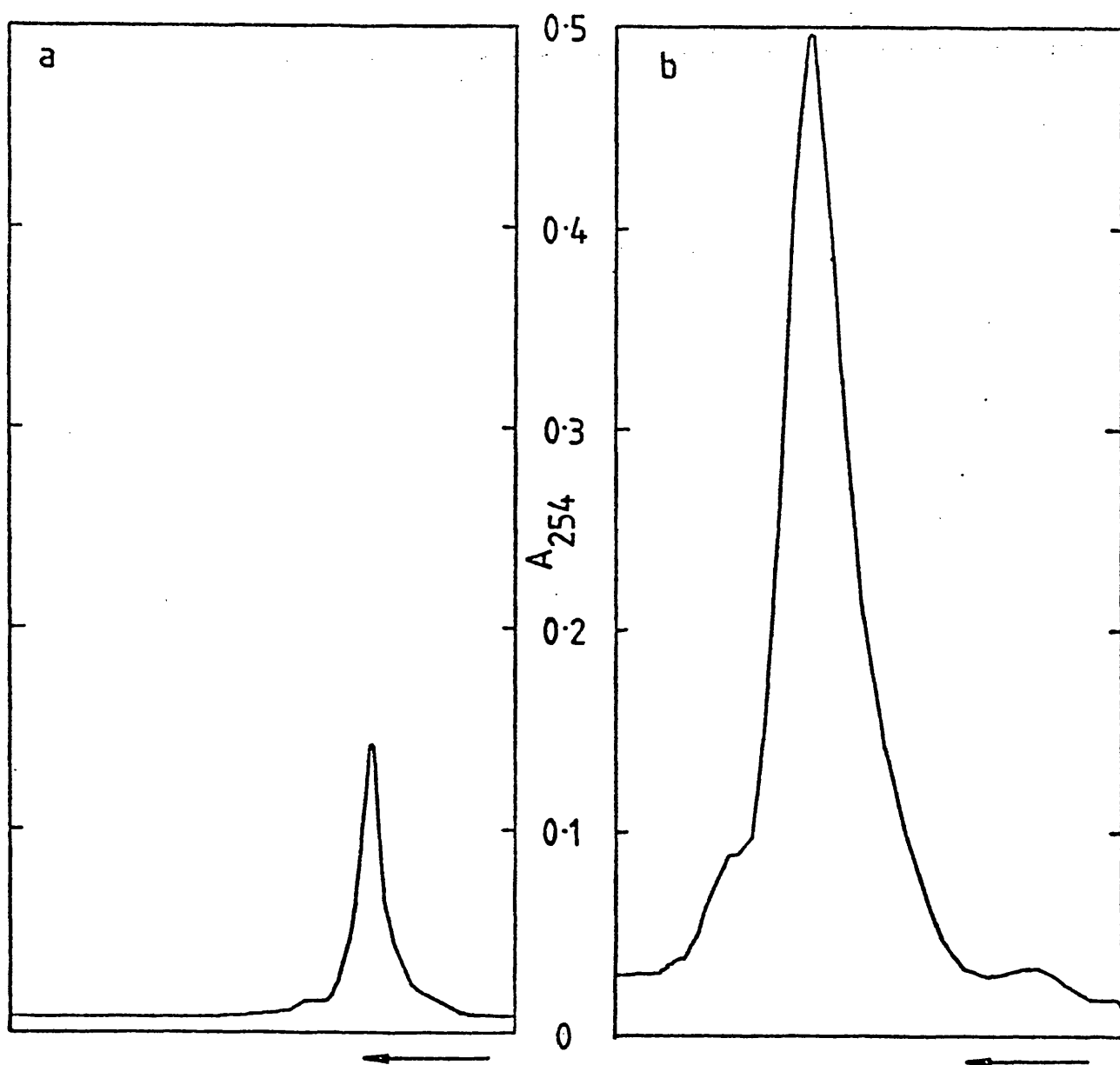


## Legend to Figure 7.2

### Preparation of Ribosomal Subunits

A crude preparation of ribosomes (36 A260 units) in 1 ml of 10mM-Tris.acetic acid (pH7.6), 90mM-potassium acetate, 1mM-magnesium acetate, 3mM-2-mercaptoethanol was dialysed against 10mM-Tris.HCl (pH7.6), 500mM-KCl, 12.5mM-MgCl<sub>2</sub>, 3mM-2-mercaptoethanol. The suspension was then centrifuged through a 34ml 10-30% (<sup>w</sup>/v) sucrose density-gradient containing the appropriate buffer for six hours at 26,000 r.p.m. and 2°C. Material collected from each gradient is indicated in the figure. The horizontal arrow represents the direction of sedimentation of ribosomes.

FIGURE 7.3 Analysis of rRNA from Ribosomal Subunits





### Legend to Figure 7.3

#### Analysis of rRNA from Ribosomal Subunits

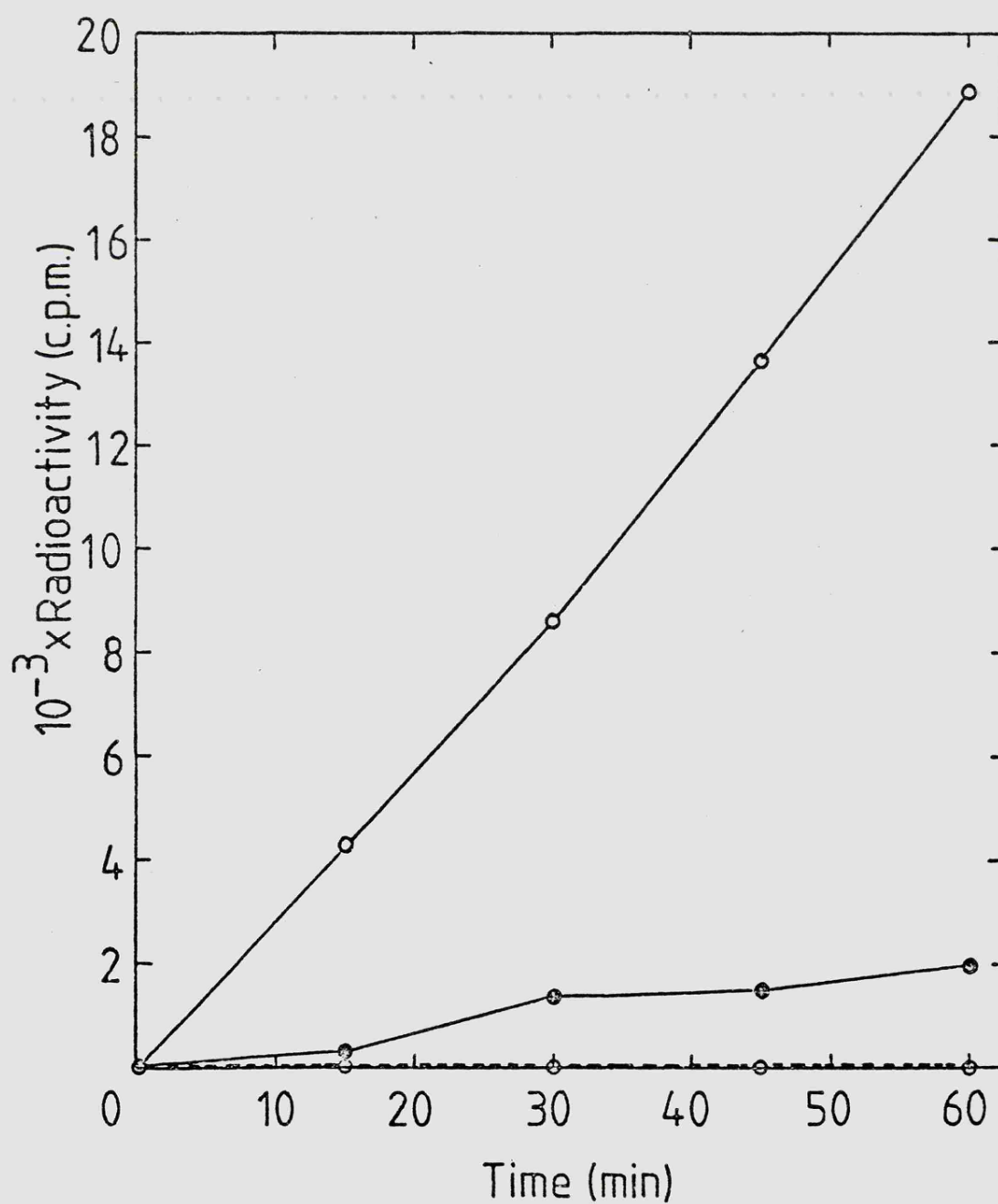
Ribosomal RNA prepared from (a) 40S ribosomal subunits or (b) 60S ribosomal subunits (Section 7.3) was layered onto 5ml 10-40% (<sup>W</sup>/v) sucrose density-gradients containing 10mM-Tris.HCl (pH7.6), 100mM-NaCl, 1mM-Na<sub>2</sub> EDTA and 0.1% (<sup>W</sup>/v) SDS and centrifuged at 45,000 r.p.m. and 15°C for 3½ hours in a Spinco SW50.1 rotor. The gradients contained (a) 0.2 A260 units and (b) 2.5 A260 units of rRNA. The horizontal arrows represent the direction of sedimentation of ribosomes.

six to twelve hours and resuspended in a small quantity of 'eukaryotic dialysis buffer'.

It is always possible that 60S ribosomal subunit preparations are contaminated either with small quantities of undissociated ribosomes or dimers of 40S subunits (which sediment at 55S, Martin et al., 1971). In order to check for contamination, rRNA was prepared from both 60S and 40S subunits and analysed on sucrose density-gradients. The rRNA was extracted and analysed as follows: ribosomal subunits were made 100 mM in magnesium acetate and 66% (<sup>V</sup>/v) in acetic acid and the mixture centrifuged to precipitate RNA. After washing in more 66% (<sup>V</sup>/v) acetic acid, the precipitate was dried in vacuo and dissolved in 10mM-Tris. HCl (pH7.6), 100 mM-NaCl, 1mM-Na<sub>2</sub> EDTA by warming at 37°C for 20 - 30 minutes. The RNA was then centrifuged through sucrose density-gradients containing the buffer described above plus 0.1% (<sup>W</sup>/v) sodium dodecyl sulphate (SDS) and detected by pumping the gradient material through an ISCO absorbance monitor.

Ribosomal RNA extracted from the 40S subunit consisted only of one species (Fig.7.3). However, RNA prepared from the 60S subunit despite being predominantly one species (26S) did have a small quantity of RNA with a lower sedimentation coefficient

FIGURE 7.4 Activity of Yeast Ribosomal Subunits in Protein Synthesis

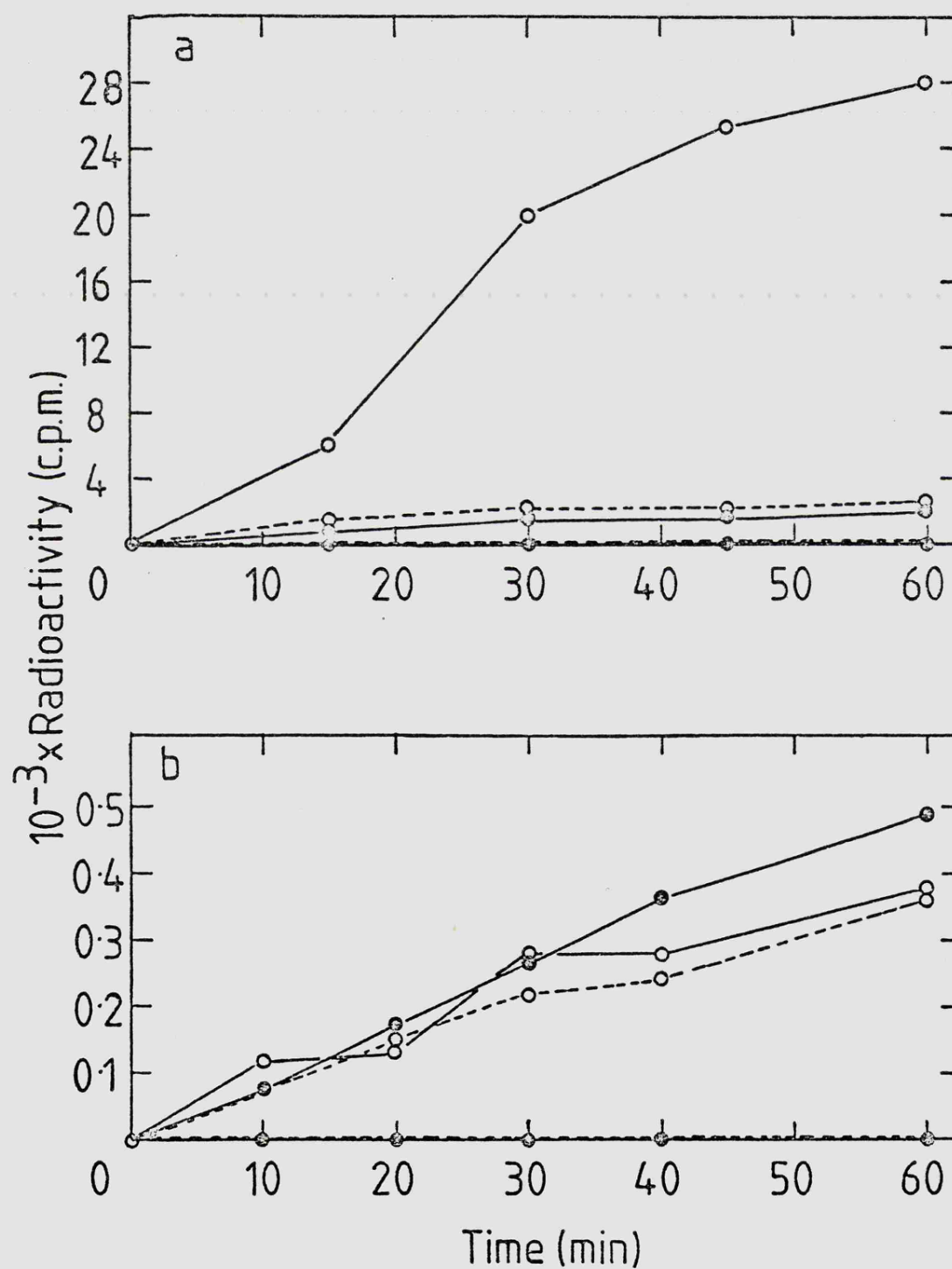


#### Legend to Figure 7.4

##### Activity of Yeast Ribosomal Subunits in Protein Synthesis

Mixtures for poly U-directed protein synthesis (Section 3.14) containing o — o, yeast strain Y<sub>166</sub> 40S and 60S ribosomal subunits; ●, yeast strain Y<sub>166</sub> 60S ribosomal subunits; o --- o, yeast strain Y<sub>166</sub> 40S ribosomal subunits and yeast S100 were incubated at 25°C. Each incubation (50  $\mu$ l) contained 16 pmoles of ribosomal subunits. Samples (10  $\mu$ l) were precipitated with TCA and radioactivity in the precipitates was estimated by liquid-scintillation counting (Section 3.16).

FIGURE 7.5 Heterologous Combination of Ribosomal Subunits of Yeast and *M. verrucaria*



Legend to Figure 7.5

Heterologous Combination of Ribosomal Subunits of  
Yeast and M. verrucaria

Mixtures for poly U-directed protein synthesis (Section 3.14) containing (a) 60S ribosomal subunits from yeast strain Y<sub>166</sub> and/or 40S ribosomal subunits from M. verrucaria or (b) 60S ribosomal subunits from M. verrucaria and/or 40S ribosomal subunits from yeast strain Y<sub>166</sub> and yeast S100 were incubated at 25°C. Each incubation (50 µl) contained 16 pmoles of ribosomal subunits. Samples (10 µl) were precipitated with TCA and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).

o — o, plus 40S and 60S ribosomal subunits;

o --- o, 40S and 60S ribosomal subunits plus T-2 toxin (10 µg/ml final concn.); ● — ●, plus 60S ribosomal

subunits; ● --- ●, plus 40S ribosomal subunits.

(Fig. 7.3; 5.8S and 5S rRNA were not detected by this technique).

It was assumed that the ratio of absorbance at 260 n.m. (A<sub>260</sub>) of 60S to 40S ribosomal subunits from yeast or M. verrucaria was the same as the ratio for reticulocyte ribosomal subunits (= 2.5, Loening, 1968). Therefore, 250 pmoles of 40S or 60S subunits had an A<sub>260</sub> of 3.7 or 9.3 respectively (see Section 3.10). Incubation mixtures (50 µl) for poly U-directed protein synthesis contained 18.5 pmoles of ribosomal subunits (≅ 1.5 mg ribosomes /ml).

Ribosomal subunits were stored as aliquots at -70°C.

#### 7.4. RECOMBINATION OF RIBOSOMAL SUBUNITS

The small (40S) ribosomal subunits of yeast and M. verrucaria were inactive in poly U-directed protein synthesis in the absence of 60S subunits. In contrast, the 60S ribosomal subunit preparations had some synthetic activity although this was greatly stimulated by the addition of 40S subunits (Fig. 7.4).

Heterologous recombination experiments with ribosomal subunits from strain Y<sub>166</sub> and strain TR<sub>1</sub> or strain Y<sub>166</sub> and M. verrucaria were performed (Fig. 7.5 and table 7.6) in order to determine which subunit of strain TR<sub>1</sub> and M. verrucaria ribosomes were responsible for resistance to T-2 toxin. It was found that an

TABLE 7.6

LOCALISATION OF RESISTANCE TO T-2 TOXIN ON  
S.CEREVISIAE AND M. VERRUCARIA RIBOSOMES

Source of Subunits		pmoles [ $^{14}\text{C}$ ] Phe incorporated (5 $\mu\text{l}$ )		% Activity + T-2 toxin
		Control	+T-2 toxin 10 $\mu\text{g/ml}$	
40S	60S			
Y <sub>166</sub>	Y <sub>166</sub>	11.5	0.5	4
Y <sub>166</sub>	TR <sub>1</sub>	9.0	2.0	22
TR <sub>1</sub>	TR <sub>1</sub>	8.8	2.0	23
TR <sub>1</sub>	Y <sub>166</sub>	10.5	0.6	6
Y <sub>166</sub>	<u>M. verrucaria</u>	0	0	-
<u>M. verrucaria</u>	Y <sub>166</sub>	28	2.0	7
<u>M. verrucaria</u>	<u>M. verrucaria</u>	25	23	92

Mixtures for poly U-directed protein synthesis (Section 3.14) containing 40S and 60S ribosomal subunits and yeast S100 were incubated in the presence or absence of T-2 toxin (10  $\mu\text{g/ml}$  final concn.) at 25°C. Each incubation (25  $\mu\text{l}$ ) contained 8 pmoles of ribosomal subunits. A sample (5  $\mu\text{l}$ ) was removed from each incubation after 60 minutes, precipitated with TCA and radioactivity in the precipitate estimated by liquid-scintillation counting (Section 3.16).



active ribosome could be produced from M. verrucaria 40S and Y<sub>166</sub> 60S ribosomal subunits but not from strain Y<sub>166</sub> 40S and M. verrucaria 60S ribosomal subunits. No explanation is offered for this phenomenon since all the subunits were active when tested in homologous recombination experiments (data not given).

The 60S ribosomal subunits of both yeast strain TR<sub>1</sub> and M. verrucaria were found to be responsible for resistance to T-2 toxin (Table 7.6) as predicted from evidence presented elsewhere (Chapter 6).

#### 7.5. ANALYSIS OF RIBOSOMAL PROTEINS BY TWO-DIMENSION GEL-ELECTROPHORESIS

Whilst it is clear that strain TR<sub>1</sub> is resistant to trichodermin by virtue of altered 60S ribosomal subunits as compared to wild-type (Table 7.6), no altered gene-product has ever been implicated. Other workers (Grant et al., 1976) have subjected another trichodermin-resistant yeast (CLR<sub>1</sub>) to genetic analysis and have mapped the mutation to a site on chromosome XV. However, this site has not been demonstrated to code for any component of the ribosome and, therefore, the mutation might affect a ribosomal protein, rRNA or an enzyme capable of modifying the ribosome. In order to define the mutation, techniques for studying the proteins and RNA of the ribosome are necessary.

Ribosomal proteins may be separated easily from

rRNA (Section 7.5a) so that they can be studied in isolation.

Separation of RNA species from one another has been achieved by electrophoresis using a variety of gel-electrophoresis techniques (Richards and Gratzer, 1964; Bachvaroff and McMaster, 1964; Beney and Szekely, 1966). However, these techniques are of little use for the study of rRNA species since they are all large molecules and small modifications of their structure are unlikely to be noticed. Despite this problem, mutations in rRNA species have been detected by digestion of the RNA and analysis of oligonucleotide fragments produced by this treatment (Helser et al., 1972; Lai and Weisblum, 1971).

Alterations in ribosomal proteins caused by replacement, modification or deletion of amino acids are far easier to detect than those in rRNA. The proteins are smaller than rRNA and may be separated from one another by virtue of size or charge differences. An altered ribosomal protein may be differentiated from its wild-type counterpart by a change in mobility when subjected to gel-electrophoresis. However, since the 60S ribosomal subunits of yeast contain 43-45 proteins (Cannon et al., 1977; Otaka and Kobata, 1978) which are of similar sizes and mostly very basic, the technique of gel-electrophoresis has to be used at the limit of resolution. One-dimensional gel-

electrophoresis is inadequate for resolving all the ribosomal proteins and, therefore, two-dimensional systems were used in this work. This latter technique allows the pH and composition of gels to be varied independently between the first and second dimension (see 7.5b).

Ribosomal RNA species have not been studied in this work but two techniques of gel-electrophoresis have been used here to analyse ribosomal proteins. These are a modification (Howard and Traut, 1973) of the 2-D method of Kaldschmidt and Wittmann (1970 a,b) and the 2-D method of Mets and Bogorad (1974).

In the system of Howard and Traut, proteins are electrophoresed at pH8.7 in 6M-urea and 4% (<sup>w</sup>/v) polyacrylamide in the first dimension and at pH4.5 in 6M-urea and 18% (<sup>w</sup>/v) polyacrylamide in the second. The procedure of Mets and Bogorad separates proteins by electrophoresis in the first dimension at pH5.0 in 8M-urea and 4% (<sup>w</sup>/v) polyacrylamide and in a sodium dodecyl sulphate (SDS) 10% (<sup>w</sup>/v) polyacrylamide gel in the second dimension. Electrophoresis of protein molecules in the presence of anionic detergent SDS separates them on the basis of size (and to a lesser extent, shape) and is independent of net charge on the proteins (Shapiro et al., 1967).

#### (a) Preparation of Ribosomal Proteins

Proteins were extracted from ribosomal subunits

TABLE 7.7

SOLUTIONS FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

IN TWO DIMENSIONS (HOWARD AND TRAUT, 1973)

GEL SOLUTIONS per litre

<u>1-D</u>	Urea	360g	)	
	Acrylamide	40g	)	
	MBA	1.33g	)	Polymerised with 0.2
	Na <sub>2</sub> EDTA	80g	)	ml 10% ( <sup>W</sup> /v) ammonium
	Boric Acid	32g	)	persulphate/100 ml
	Tris base	48.6g	)	gel solution
	TEMED	1 ml	)	(pH8.7 unadjusted)

<u>2-D</u>	Urea	360g	)	
	Acrylamide	180g	)	
	MBA	2.5g	)	Polymerised with 3.0
	Acetic Acid	53 ml	)	ml 10% ( <sup>W</sup> /v) ammonium
	KOH	2.7g	)	persulphate/100 ml gel
	TEMED	3 ml	)	solution
			)	(pH4.5 unadjusted)

RUNNING-BUFFERS per litre

<u>1-D</u>	Na <sub>2</sub> EDTA	2.4g	)	
	Boric Acid	4.8g	)	
	Tris base	7.25g	)	(pH8.2 unadjusted)
<u>2-D</u>	Glycine	14g	)	
	Acetic acid	1.5 ml	)	(pH4.0 unadjusted)

TABLE 7.8

SOLUTIONS FOR POLYACRYLAMIDE GEL ELECTROPHORESIS IN

TWO DIMENSIONS ( METS AND BOGORAD, 1974 ).

GEL SOLUTIONS per litre

<u>1-D</u>	Urea	40 g	)	
	Acrylamide	1 g	)	
	MBA	480 g	)	Polymerised with 5 ml
	Bis-Tris	11.92g	)	10% ( <sup>w</sup> /v) ammonium
	TEMED	5 ml	)	persulphate/litre of
	Acetic acid to pH5.0		)	gel solution.
<u>2-D</u>	Acrylamide	100 g	)	
	MBA	2.5 g	)	Polymerised with 2 ml
	Bis-Tris	30 g	)	10% ( <sup>w</sup> /v) ammonium
	TEMED	1 ml	)	persulphate/litre of
	HCl to pH 6.75		)	gel solution.

RUNNING BUFFERS per litre

<u>1-D</u>	Upper buffer	Bis-Tris	2.00 g
		Acetic acid to pH4.0	
	Lower buffer	Potassium acetate	17.5 g
		Acetic acid to pH4.5	
<u>2-D</u>	Upper buffer	MES	13.7 g
		Bis-Tris	14.6 g
		SDS	1 g
		2-mercaptoethanol	3mM
	Lower buffer	Bis-Tris	5.85 g
		HCl to pH6.75	

according to the method of Dohme and Nierhaus (1976). The subunits were made 100 mM in magnesium acetate and 66% (<sup>v</sup>/v) in acetic acid and the mixture centrifuged to precipitate RNA. Five volumes of acetone were added to the supernatant and, following centrifugation, the precipitate of proteins was washed twice in acetone, dried in vacuo and redissolved in 8M-urea containing 30mM-2-mercaptoethanol.

(b) Two-dimensional polyacrylamide gel-electrophoresis

The composition of the various gel solutions and buffers are given in Tables 7.7 and 7.8. The apparatus for 2-D gel-electrophoresis was essentially as described by Howard and Traut (1974).

(i) First dimension:

Descending disc-gel electrophoresis was performed in glass tubes (14 cm x 3 mm i.d. for Mets and Bogorad gel systems and, when only the basic proteins were to be separated, in Kaldschmidt and Wittmann-type 2-D gel systems; 9 cm x 3 mm i.d. for Kaldschmidt and Wittmann-type gels containing both acidic and basic proteins). The tubes were washed in chromic acid and the bottom of each tube sealed before being filled with gel solution which had been degassed under vacuum and polymerised with freshly-prepared ammonium persulphate solution (see Tables 7.7 and 7.8). Gels were poured in the tubes to a length of 12 cm or 7 cm (see above) and overlaid with butanol which had been saturated with water. After the

gel had polymerised (10 to 15 minutes) the butanol was removed and the upper gel surface washed with running-buffer (Tables 7.7 and 7.8). The tubes were then placed in the gel-electrophoresis apparatus (Howard and Traut, 1974) and filled with running buffer before loading protein samples which were in 8M-urea, 30mM-2-mercaptoethanol, 20% (<sup>v</sup>/v)-glycerol and 0.05% (<sup>w</sup>/v)-marker dye onto the top of the gels. Basic fuchsin was used as marker for runs from anode to cathode and bromophenol blue in runs from cathode to anode. Times and conditions for electrophoresis were as described in figure legends.

Following electrophoresis in the first dimension, gels were removed from their tubes by injecting first-dimension buffer between the gels and the glass with a hyperdermic syringe. If the gels did not slide out of the tubes they could be expelled by applying gentle air pressure to one end of the tube.

(ii) Second dimension:

For Kaldschmidt and Wittmann-type gels containing both acidic and basic proteins, the two gels (one containing acidic and one basic proteins) were trimmed to about 6 cm each and placed origin to origin on a glass plate. Gels of 12 cm length were placed directly onto a glass plate without trimming. The arrangement of glass plates for the second dimension was as described by Howard and Traut (1974). The gel was placed at the top of the plate between perspex spacers but so that there was a gap of 3 mm between the

spacer and the end of the gel. Another glass plate was then clamped on top of the first plate so as to trap the gel in place. After the space between the plates had been blocked at the sides and bottom by plastic tubing, the second-dimension gel solution was poured through the gap between gel and spacer so that, after polymerisation, the first-dimension gel formed the top edge of the slab gel. The second dimension gel solution was degassed and mixed with ammonium persulphate solution before pouring. Polymerisation was complete within 30 minutes.

After the gel had polymerised, the plastic tubing was removed and the glass plates clamped in position in the apparatus for slab-gel electrophoresis (Studier, 1972).

The two buffer chambers were then filled and the proteins electrophoresed from anode to cathode (Kaldschmidt and Wittmann) or cathode to anode (Mets and Bogorad) using the conditions described in figure legends. Finally, the slab-gels were prised from between the glass plates and stained for two hours in a solution of 0.04% (<sup>W</sup>/v) Coomassie Brilliant Blue - G in 3.5% (<sup>V</sup>/v) perchloric acid. The gels were then placed in several changes of destaining solution (15% (<sup>V</sup>/v) methanol, 7.5% (<sup>V</sup>/v) acetic acid) to remove stain that was not bound to protein.

Proteins prepared from 60S ribosomal subunits of yeast strains Y<sub>166</sub> and TR<sub>1</sub> were electrophoresed using identical conditions by the method of Howard and Traut (1973). The acidic proteins were not studied, however,



**FIG 7.9**



**b**



Legend to Figure 7.9

Analysis of Basic Proteins from the 60S  
Ribosomal Subunits of Yeast Strains Y<sub>166</sub>  
and TR<sub>1</sub> by 2-D Gel Electrophoresis (Howard  
and Traut, 1973)

Proteins (0.5 mg) from the 60S ribosomal  
subunits of (a) yeast strain Y<sub>166</sub> or (b) yeast  
strain TR<sub>1</sub> (Section 7.5a) were electrophoresed  
in two dimensions by the method of Howard and Traut  
(1973; see Section 7.5b and Table 7.7).

Electrophoresis was performed at 4°C for  
13 hours at 200 V in the first dimension and  
24 hours at 120 V in the second dimension. All  
electrophoresis steps were from anode to  
cathode and, therefore, acidic proteins were not  
detected.

Following electrophoresis, gels were stained in  
0.04% (w/v) Coomassie Brilliant Blue-G in 3.5% (v/v)  
perchloric acid and destained with 15% (v/v)  
methanol, 7.5% (v/v) acetic acid.

**FIG 7.10**



Legend to Figure 7.10

Analysis of the Proteins of the 60S Ribosomal Subunits of M. verrucaria by 2-D Gel Electrophoresis (Howard and Traut, 1973)

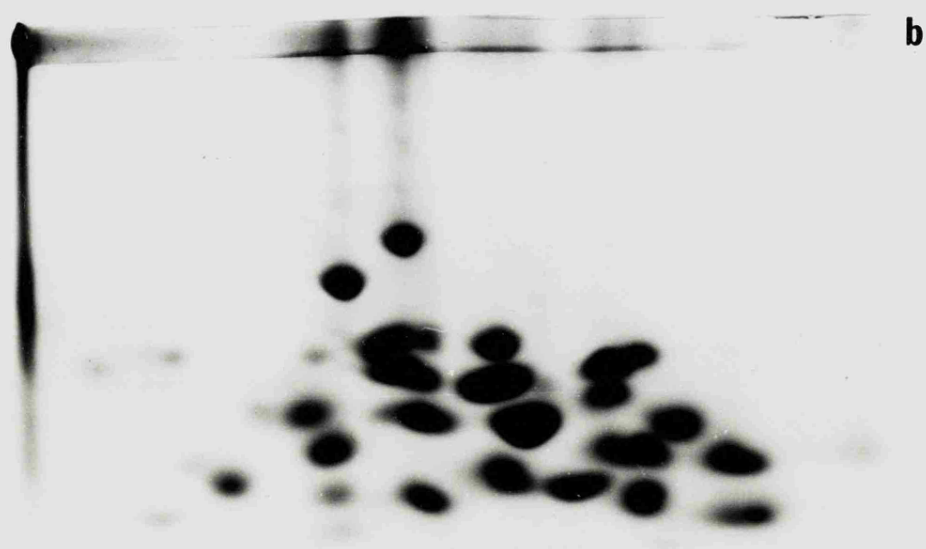
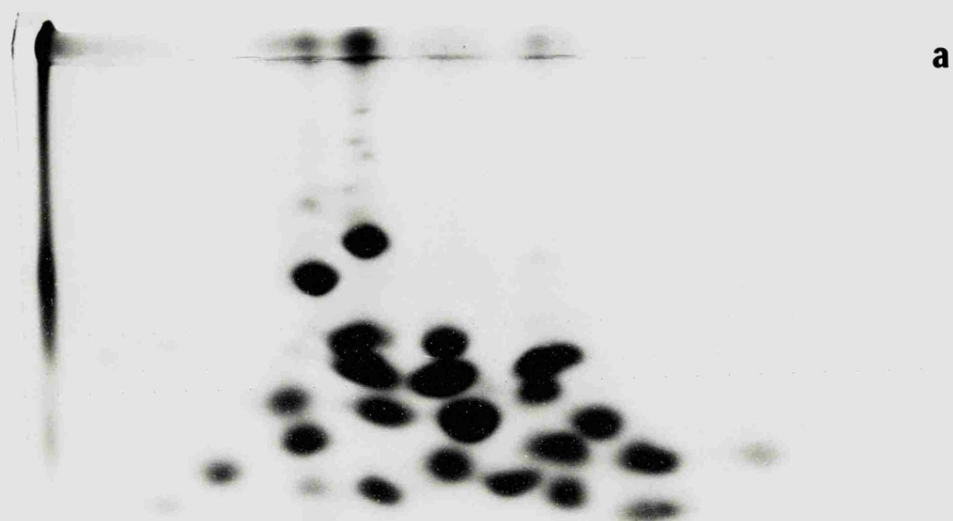
Proteins (0.5 mg) from the 60S ribosomal subunits of M. verrucaria (Section 7.5a) were electrophoresed in two dimensions by the method of Howard and Traut (1973; see Section 7.5b and Table 7.7). Electrophoresis was performed at 4°C for 13 hours at 200 V (anode to cathode) or 2½ hours at 200 V (cathode to anode) in the first dimension. The 2 gels were then placed origin to origin in a slab-gel and electrophoresed for 24 hours at 120 V and 4°C in the second dimension (anode to cathode). The gel was stained in 0.04% (<sup>w</sup>/v) Coomasie Brilliant Blue-G in 3.5% (<sup>v</sup>/v) perchloric acid and destained with 15% (<sup>v</sup>/v) methanol, 7.5% (<sup>v</sup>/v) acetic acid.

since they did not form clearly stained spots within the gel (data not given). There were no obvious differences between the electrophoretic mobilities of the basic proteins from wild-type and mutant yeast (Fig.7.9). The ribosomal proteins from the large subunit of M. verrucaria were electrophoresed also according to Howard and Traut (1973). However, there were few proteins with electrophoretic mobilities similar to those found in yeast (Fig.7.10). Clearly, comparison of proteins from M. verrucaria and S. cerevisiae is unable to provide any clues to the method by which resistance to trichothecenes is achieved.

The proteins of the 60S ribosomal subunits of strains Y<sub>166</sub> and TR<sub>1</sub> were compared also in the gel-electrophoresis system of Mets and Bogorad (1974). This system does not distinguish between acidic and basic proteins thus making analysis of ribosomal proteins less complicated. However, the proteins are less well separated in this system when compared to that of Howard and Traut (1974). Again, there were no obvious differences between the electrophoretic mobilities of the proteins from wild-type and mutant ribosomes (Fig.7.11). The proteins of the 40S ribosomal subunit were also analysed by the method of Mets and Bogorad (Fig.7.12).

It is extremely difficult to compare protein profiles

**FIG 7.11**

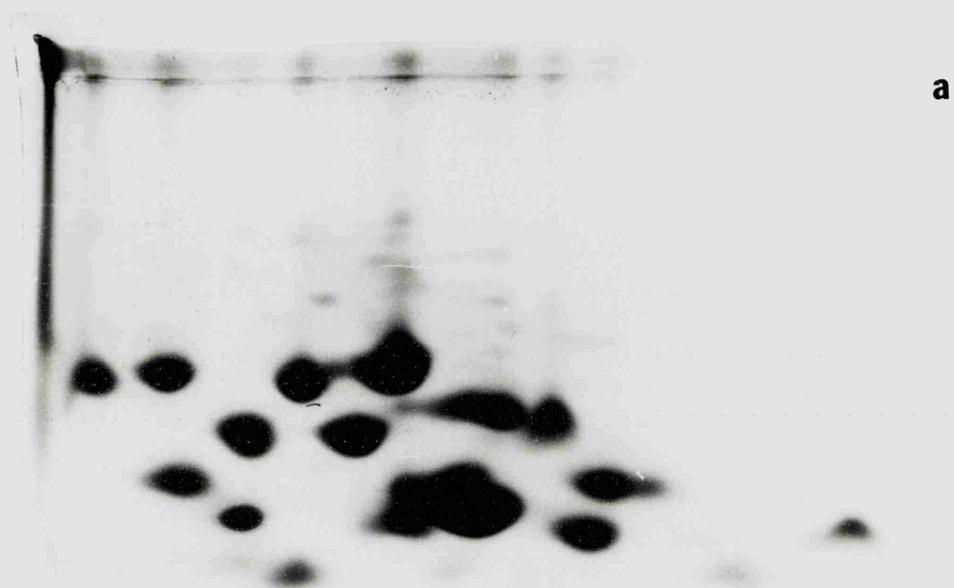


#### LEGEND TO FIGURE 7.11

Analysis of the Proteins of the 60S Ribosomal  
Subunits of Yeast Strains Y<sub>166</sub> and TR<sub>1</sub> by 2-D Gel  
Electrophoresis (Mets and Bogorad, 1974)

Proteins (0.5 mg) from the 60S ribosomal subunits  
of (a) yeast strain Y<sub>166</sub> or (b) yeast strain TR<sub>1</sub> (Section  
7.5a) were electrophoresed in two dimensions by the  
method of Mets and Bogorad (1974); (see Section 7.5b  
and Table 7.8). Electrophoresis was performed at  
4°C for 12 hours at 80 V in the first dimension  
(anode to cathode) and at room temperature for 5 hours  
at 50 V in the second dimension (cathode to anode).  
The gels were stained in 0.04% (<sup>W</sup>/v) Coomasie Brilliant  
Blue-G in 3.5% (<sup>V</sup>/v) perchloric acid and destained  
with 15% (<sup>V</sup>/v) methanol, 7.5% (<sup>V</sup>/v) acetic acid.

**FIG 7.12**



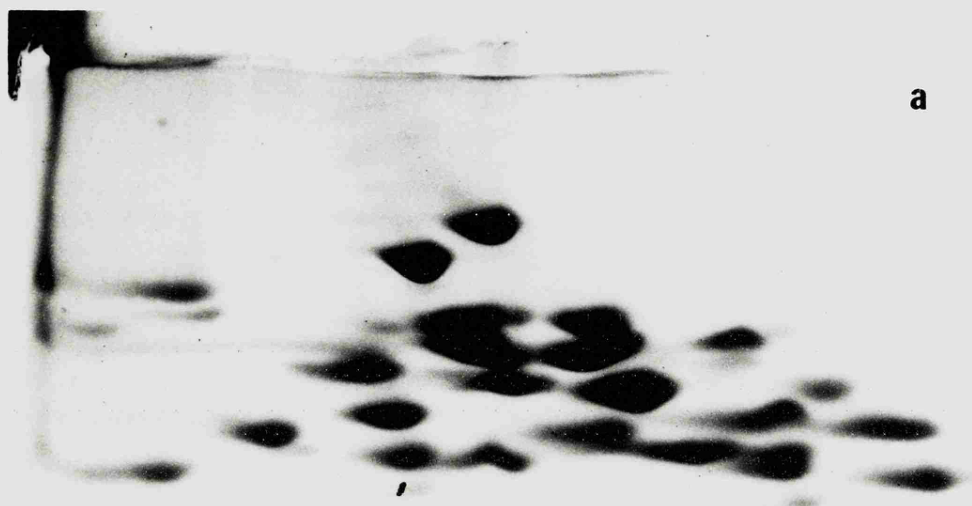


Legend to Figure 7.12

Analysis of the Proteins of the 40S Ribosomal  
Subunits of Yeast Strains Y<sub>166</sub> and TR<sub>1</sub> by 2-D  
Gel Electrophoresis (Mets and Bogorad, 1974)

Proteins (0.5 mg) from the 40S ribosomal  
subunits of (a) yeast strain Y<sub>166</sub> or (b) yeast  
strain TR<sub>1</sub> (Section 7.5a) were electrophoresed in  
two dimensions by the method of Mets and Bogorad  
(1974); (see Section 7.5b and Table 7.8).  
Electrophoresis was performed at 4°C for 12 hours  
at 80 V in the first dimension (anode to cathode)  
and at room temperature for 5 hours at 50V in the  
second dimension (cathode to anode). The gels  
were stained in 0.04% (<sup>W</sup>/v) Coomasie Brilliant Blue-G  
in 3.5% (<sup>V</sup>/v) perchloric acid and destained with 15%  
(<sup>V</sup>/v) methanol, 7.5% (<sup>V</sup>/v) acetic acid.

**FIG 7.13**



**a**



**b**

from two different sources since no two gels ever run exactly alike. There is always some variation in the pattern even when comparing two gels which contain the same proteins. Therefore, a small difference in mobility between wild-type and mutant polypeptide is unlikely to be noticed by such comparisons. In order to avoid this problem, it is necessary to run both sets of proteins on the same gel and, thus, ensure that conditions are identical. The ribosomal proteins of strain Y<sub>166</sub> were differentiated from those of strain TR<sub>1</sub> by growing Y<sub>166</sub> cells in medium containing [<sup>14</sup>C]-amino acids and preparing ribosomal proteins from this source.

#### (c) Growth of Radio-Labelled Yeast Cells

A small quantity (200 ml) of 'minimal amino acid medium' (Section 2.2) was inoculated with a single colony of yeast strain Y<sub>166</sub> and incubated with shaking at 30°C until the medium had an absorbance at 650 n.m. (A<sub>650</sub>) of 0.1. Ten microcuries of [<sup>14</sup>C]-protein hydrolysate (59 m Ci/mAtom) were then added and the incubation continued until the medium had an A<sub>650</sub> of 0.8. The medium was rapidly cooled to 0°C and the cells harvested as described previously (Section 2.4).

#### (d) Preparation and Analysis of Radio-labelled Ribosomal Proteins

Ribosomes, ribosomal subunits and the proteins of

### Legend to Figure 7.13

Analysis of the Proteins of the 60S Ribosomal Subunits of Yeast Strain TR<sub>1</sub> and  $\angle^{-14}\text{C}_-$  Proteins of the 60S Ribosomal Subunits of Yeast Strain Y<sub>166</sub> by 2-D Gel Electrophoresis. (Mets and Bogorad, 1974)

Proteins (0.4mg) from the 60S ribosomal subunits of yeast strain TR<sub>1</sub> (Section 7.5a) and  $\angle^{-14}\text{C}_-$  proteins (0.2mg) prepared from radiolabelled 60S ribosomal subunits of yeast strain Y<sub>166</sub> (Section 7.5d) were mixed and electrophoresed in the two dimensional gel-system of Mets and Bogorad (1974); (see Section 7.5b and Table 7.8). Electrophoresis was at 4°C and 80V for 12 hours in the first dimension (anode to cathode) and at room temperature and 50V for 5 hours in the second dimension (cathode to anode). The gel was stained in 0.04% (<sup>W</sup>/v) Coomassie Brilliant Blue-G in 3.5% (<sup>V</sup>/v) perchloric acid and destained in 15% (<sup>V</sup>/v) methanol, 7.5% (<sup>V</sup>/v) acetic acid.

Finally the gel was dried onto Whatmann No.1 paper and autoradiographed for 5 weeks using KODIREX X-ray film.

(a) autoradiogram; (b) Stained gel.

the 60S ribosomal subunit were prepared as described previously (Sections 3.9, 3.10, 7.3 and 7.5a).

Radio-labelled proteins from the 60S ribosomal subunit of strain Y<sub>166</sub> were mixed with the equivalent unlabelled proteins from strain TR<sub>1</sub> and then electrophoresed in the two-dimensional gel-electrophoresis system of Mets and Bogorad (1974). After electrophoresis, proteins within the gel were stained with Coomassie Brilliant Blue - G and the gel then dried onto a paper support. Finally, an autoradiogram was prepared by exposing X-Ray film to the dried gel. Regions of radioactivity within the gel were compared to regions containing protein (as judged by stain) to determine whether maximum radioactivity corresponded to maximum density of stain. There were no obvious differences between the radioactivity and stain profiles of the gels (Fig.7.13).

It is not possible to rule out mutation of a ribosomal protein as the means by which yeast become resistant to the 12,13 epoxytrichothecenes. However, such a mutation must be fairly conservative in nature. An amber mutation giving rise to a smaller ribosomal protein or a radical change of one amino acid within the polypeptide should cause a change in the electrophoretic mobility of the protein which would be detected by this procedure.

Chemical modification of the ribosomal proteins of eukaryotes by phosphorylation or methylation is well-documented and may provide mechanisms for antibiotic-resistance. Several proteins within the large and small ribosomal subunits of yeast are known to be methylated (Cannon et al., 1977; Hernandez et al., 1978). However, since such methylations in eukaryotes are normally of lysine and arginine residues within the polypeptides (Reporter 1973, 1974; Chang et al., 1976) one would normally expect an over- or under-methylated mutant protein to have a different overall charge and, therefore, different electrophoretic mobility when compared to its wild-type counterpart. Methylated derivatives of histidine (Reporter, 1973), alanine and methionine (Chen et al., 1977) have also been reported in ribosomal proteins and, in the case of the latter two amino acids, methylation of these residues is less likely to alter electrophoretic mobility.

Ribosomal proteins in a wide variety of eukaryotes have been shown to be phosphorylated in vivo (Kabat, 1970, 1972; Loeb and Blat, 1970; Bitte and Kabat, 1972; Rankine et al., 1977). However, it is unlikely that the resistance of strain TR<sub>1</sub> to trichothecenes is due to over- or under-phosphorylation of a ribosomal protein since the electrophoretic mobility of the altered protein would be different from its wild-type counterpart.

Presumably, the mutation within the large ribosomal subunit of strain TR<sub>1</sub> which confers resistance to the 12,13 epoxytrichothecenes is either a conservative substitution of an amino acid (e.g. neutral amino acid replaced by a different neutral amino acid) or an altered rRNA. Further experiments are needed to sort out these possibilities.

## 8. PARTIAL-RECONSTITUTION OF THE RIBOSOMES OF YEAST AND M. VERRUCARIA

### 8.1. MATERIALS

Commercial sources of materials were as follows:-  
[ $1-^{14}\text{C}$ ] acetic anhydride (29.7 mCi/mmol), S-adenosyl-L-[ $\text{methyl-}^3\text{H}$ ] methionine (1 Ci/mmol) and [ $^3\text{H}$ ] sodium borohydride (661 mCi/mmol) were from the Radiochemical Centre, Amersham, Bucks.; guanylyl ( $\beta$ ,  $\gamma$  -methylene) - diphosphate (GMPPCP) was from Sigma Chemical Co., Kingston-upon-Thames, Surrey; Carbowax 6000 was from Union Carbide U.K. Ltd. Hythe, Southampton, Hants. All other materials were obtained as mentioned previously.

### 8.2. INTRODUCTION

Antibiotics have been used as tools in ribosome research for several years (Chapter 1) and have provided valuable insights into ribosome structure and function. Their use in determining which ribosomal proteins are required for particular steps in protein biosynthesis has been exploited extensively in prokaryotes. Experiments with the antibiotic thiostrepton provide a good example of the techniques employed.

Thiostrepton is a low-molecular weight peptide produced by Streptomyces azureus (Pagano et al., 1956). Binding studies using [ $^{35}\text{S}$ ] thiostrepton and ribosomes from B. megaterium and other prokaryotes have shown that a very stable complex is formed (Dixon, 1976; Highland



et al., 1975). If, however, some of the ribosomal proteins ('split-proteins') are removed by washing the ribosomes of B.megaterium in 2M-Li Cl, the antibiotic is no longer able to bind to the resultant core-particle. The core-particle may be reconstituted so that it binds thiostrepton merely by the recombination of core-particle and a single split-protein (Dixon, 1976). This split-protein has been shown to be altered in a thiostrepton-resistant mutant of B.megaterium (M.Stark and E.Cundliffe, personal communication).

The organism which produces thiostrepton, S.azureus, was found to be intrinsically-resistant to the antibiotic both in vivo and in vitro (Dixon et al., 1975; Dixon, 1976). In an experiment involving total-reconstitution of a 50S ribosomal subunit from ribosomal proteins of B.stearothermophilus and rRNA of S.azureus or the related species S.coelicolor, Cundliffe (1978) was able to demonstrate that S.azureus protected itself against thiostrepton by specifically methylating the RNA of its ribosome. Thus, a ribosomal protein and rRNA have been implicated<sup>in</sup> the action of thiostrepton.

Experiments of this nature have never been reported with eukaryotic ribosomes because of two major obstacles: (a) No antibiotics have been reported which bind to the eukaryotic ribosome as strongly as thiostrepton does to

the prokaryotic ribosome; (b) Total-reconstitution of the eukaryotic ribosome has not been achieved and only very limited success has been reported with partial-reconstitution experiments.

Many attempts have been made to mimic with eukaryotic ribosomes the conditions for total- and partial-reconstitution of prokaryotic ribosomes. Most success has been obtained with partial-reconstitution experiments on 40S ribosomal subunits (Lerman, 1968; Terao and Ogata, 1971; Reboud et al., 1972) although there are reports of partial reconstitution of the large ribosomal subunit (Richter and Möller, 1974; Cox and Greenwell, 1976; Reyes et al., 1977). However, only one of these latter papers claims any reconstitution of the peptidyltransferase (Cox and Greenwell, 1976).

Clearly, since the antibiotics, to which yeast strain TR<sub>1</sub> and M. verrucaria are resistant, inhibit the peptidyltransferases of sensitive organisms, partial-reconstitution experiments involving ribosomal proteins which make up the peptidyltransferase are likely to be very informative. Such experiments were attempted and the results are given below.

As mentioned above, another approach to the problem of detecting reconstitution is to use radio-labelled antibiotics as probes. Accordingly [<sup>14</sup>C] trichodermin, [<sup>3</sup>H] anisomycin and [<sup>3</sup>H]methyl-alpha

sarcin were prepared and tested for binding to ribosomes, ribosomal core-particles and 'reconstituted' ribosomes.

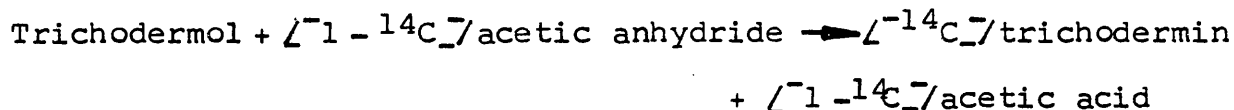
### 8.3. PREPARATION OF RADIO-LABELLED ANTIBIOTICS

#### (a) $\angle^{-14}\text{C}\angle$ trichodermin

Radioactive trichodermin was prepared by acetylation of trichodermol with  $\angle^{-1-^{14}\text{C}}\angle$  acetic anhydride using the method of Barbacid and Vazquez (1974).

One millicurie of  $\angle^{-1-^{14}\text{C}}\angle$  acetic anhydride (29.7 m Ci/m mol) was dissolved in 0.3 ml dry pyridine containing 12.5 mg trichodermol. The mixture was incubated at 40°C for 20 hours, dried in vacuo and redissolved in a small volume of pyridine before being chromatogrammed on Chrom. A.R. 500 sheet (Mallinckrodt) using benzene:ethyl acetate: n-pentane (4 : 1 : 1) as solvent. Radioactivity on the sheet was detected by autoradiography and the band at  $R_F$  0.81-0.84 was eluted with ethanol. The preparation of  $\angle^{-14}\text{C}\angle$ trichodermin was dried in vacuo, redissolved in dimethylsulphoxide (DMSO) and assayed for purity by chromatography using the above solvent system and comparing its  $R_F$  value with those of unlabelled trichodermin and trichodermol. Material on the chromatogram was detected by spraying the sheet with concentrated sulphuric acid and heating at 100°C for a few minutes. Additionally, the  $\angle^{-14}\text{C}\angle$  trichodermin inhibited the growth of yeast strain Y<sub>166</sub> cells on agar plates but had no effect on the growth of yeast strain TR<sub>1</sub> cells.

The specific activity of the  $\angle^{-14}\text{C}\angle$  trichodermin was calculated to be 14.85 m Ci/m mol by consideration of the reaction:



Four techniques were employed in order to detect binding of  $\angle^{-14}\text{C}\angle$  trichodermin to yeast ribosomes:

(i) Gel-filtration of the reaction mixture through a sepharose 6B column (c.f. Dixon, 1976).

This method failed to detect significant binding of  $\angle^{-14}\text{C}\angle$  trichodermin to yeast ribosomes despite a large input of ribosomes. Presumably,  $\angle^{-14}\text{C}\angle$  trichodermin does not bind to yeast ribosomes sufficiently strongly for it to be detected by this technique.

(ii) Ultracentrifugation of reaction mixtures in 0.8 ml cellulose nitrate tubes in order to precipitate ribosomes.

Although this method has been used previously to detect  $\angle^{-14}\text{C}\angle$  trichodermin-binding (Barbacid and Vazquez, 1974; Jimenez et al., 1975; Cannon et al., 1976), it gave widely variable results in my hands. The tubes could absorb different amounts of  $\angle^{-14}\text{C}\angle$  trichodermin from the reaction mixture and, therefore, loss of counts from the supernatant upon centrifugation could not be attributed to ribosome binding alone.

(iii) Ethanol precipitation of ribosomes.

The reaction mix (in small plastic tubes) was made

33% (v/v) in ethanol and centrifuged at 10,000 r.p.m. for two minutes and 0°C to precipitate ribosomes. The amount of antibiotic bound to the ribosome was calculated by comparing radioactivity in the supernatant [using water-miscible scintillation fluid (Section 3.16)] before and after centrifugation. Again, no binding of [<sup>14</sup>C] trichodermin to ribosomes could be detected.

(iv) Equilibrium Dialysis.

Ribosomes were placed in a well within a perspex block and separated from another well containing [<sup>14</sup>C] trichodermin by a dialysis membrane which permitted the passage of trichodermin but not ribosomes. The solutions were left to equilibrate at 4°C for 24 hours and the extent of antibiotic-binding was determined from the difference in radioactivity concentrations between the two sides of the membrane. Considerable amounts of [<sup>14</sup>C] trichodermin were lost apparently during the course of dialysis and no significant binding of [<sup>14</sup>C] trichodermin to ribosomes was detectable.

These experiments were performed using high salt-washed, crude (Section 3.10) or 'run-off' ribosomes prepared from yeast cells which had been inhibited by 1 mM-azide prior to harvesting. No explanation is offered for the inability of [<sup>14</sup>C] trichodermin to bind to yeast ribosomes although this may be a function of the high

dissociation constant for trichodermin binding to yeast ribosomes ( $0.72 \mu\text{M}$  -  $2.1 \mu\text{M}$ ; Barbacid and Vazquez, 1974a; Jimenez and Vazquez, 1975; Cannon et al., 1976) coupled with its apparent affinity for plastics and other organic material.

(b)  $\text{[}^{-3}\text{H}\text{]}$  Anisomycin

A crude preparation of  $\text{[}^{-3}\text{H}\text{]}$  anisomycin, produced by tritium exchange with unlabelled anisomycin, was the kind gift of Dr. D. Vazquez. The  $\text{[}^{-3}\text{H}\text{]}$  anisomycin was purified as described by Barbacid and Vazquez (1974c) except that the high-voltage paper-electrophoresis step was omitted since this degraded the anisomycin (R. Skinner, personal communication).

The specific activity of the  $\text{[}^{-3}\text{H}\text{]}$  anisomycin was estimated, on the basis of the value given in Barbacid and Vazquez (1974c) and the half-life of tritium, to be 208 m Ci/ m mol.

Binding to yeast ribosomes was detected by the use of the ethanol-precipitation technique described above (Section 8.3 (a) (iii) ).

(c)  $\text{[}^{-3}\text{H}\text{]}$  Methyl-AlphaSarcin

The lysine residues of alpha sarcin were reductively-methylated by the method of Means and Feeney (1968).

Ten milligrams ( $0.625 \mu\text{mol}$ ) of alpha sarcin in 1 ml 0.2M -borate buffer (pH9.0) were mixed with 12.5 m Ci of

[ $^3\text{H}$ ] sodium borohydride (661 m Ci/m mol). Two portions of 5  $\mu\text{l}$  of 37% (v/v) formaldehyde were then added to the mixture at  $0^\circ\text{C}$  with an interval of one minute between additions. After ten minutes at  $0^\circ\text{C}$  the mixture was dialysed exhaustively against water to remove unreacted  $\text{Na BH}_4$  and formaldehyde.

A sample of the [ $^3\text{H}$ ] methyl-alpha sarcin was estimated for radioactivity in water-miscible scintillation fluid (Section 3.16). Assuming a molecular weight for alpha sarcin of 16,000 daltons (Schuurmans et al., 1964), the extent of methylation was on average 6.56 methyl groups per molecule of alpha sarcin. Therefore, the specific activity of [ $^3\text{H}$ ] methyl-alpha sarcin was 1.08 Ci/m mol.

In order to show that radioactivity was incorporated into peptide, [ $^3\text{H}$ ] methyl-alpha sarcin and unlabelled alpha sarcin were electrophoresed into a sodium dodecyl sulphate (SDS) 15% (w/v) polyacrylamide gel using the method of Laemmli (1970). The peptides were detected either by staining the gel in a solution of 0.04% (w/v) Coomassie Brilliant Blue - G in 3.5% (v/v) perchloric acid and then destaining in 15% (v/v) methanol, 7.5% (v/v) acetic acid or by fluography: the gel was soaked in two changes of dimethylsulphoxide (DMSO) before dialysing it against 22% (w/v) 2,5-diphenyloxazole (PPO) in DMSO for three hours. After washing the gel in distilled water overnight followed by a solution of 1% (v/v) glycerol and 10% (v/v) acetic

acid for thirty minutes, it was dried onto Whatman No.1 paper and placed in contact with X-ray film. Methylated alpha sarcin had the same mobility in an SDS polyacrylamide-gel as had authentic alpha sarcin and was also radioactive (date not given).

Binding of  $\text{[}^{-3}\text{H]}$  methyl-alpha sarcin to yeast ribosomes was detected by gel-filtration using a Sepharose 6 B column (Dixon, 1976). By this criterion, approximately one molecule of  $\text{[}^{-3}\text{H]}$  methyl-alpha sarcin bound per yeast ribosome but radioactivity already bound to the ribosomes could not be competed off by addition of alpha sarcin. Equally, the amount of radioactivity bound to the ribosomes was unchanged even if the ribosomes were incubated with alpha sarcin prior to the addition of  $\text{[}^{-3}\text{H]}$  methyl-alpha sarcin (data not given). It is not clear, therefore, whether  $\text{[}^{-3}\text{H]}$  methyl-alpha sarcin is a good analogue for authentic alpha sarcin.

#### 8.4. PREPARATION OF RIBOSOMAL CORE-PARTICLES AND SPLIT-PROTEIN FRACTIONS

Core-particles and split-proteins were produced from yeast, strains Y<sub>166</sub> and TR<sub>1</sub>, and M. verrucaria ribosomes by the method of Cox and Greenwell (1976). Unfortunately, these two fractions could not be recombined to give a reconstituted ribosome active in either poly U-directed protein synthesis or the 'fragment reaction' (data not given). Also, the core-particles and



'reconstituted-ribosomes' would not bind  $\angle^{-3}\text{H}_7$  anisomycin. Therefore, another technique was employed for the production of ribosomal core-particles and split-proteins.

High salt-washed ribosomes (1.1-1.2 n mol) in 'eukaryotic dialysis buffer' (10 mM-Tris-acetic acid (pH7.6), 90mM-potassium acetate, 1mM-magnesium acetate, 3mM-2-mercaptoethanol) were centrifuged at 40,000 r.p.m. and 2°C through 5 ml 20% (<sup>w</sup>/v) sucrose containing 10mM-Tris.HCl (pH7.6), 1.5 to 3.5 M-NH<sub>4</sub>Cl (see figure legends), 100mM-MgCl<sub>2</sub>, 3mM-2-mercaptoethanol which was itself layered onto 2ml 40% (<sup>w</sup>/v) sucrose containing 'eukaryotic dialysis buffer'. The core-particles produced by this procedure were suspended in a small quantity of 'eukaryotic dialysis buffer' and stored as aliquots at -70°C. Split-proteins were prepared from the supernatant as follows:

The supernatant was dialysed against 'eukaryotic dialysis buffer' at 0°C and then concentrated to approximately 0.1 ml by further dialysis against 20% (<sup>w</sup>/v) carbowax 6000 containing 'eukaryotic dialysis buffer'. During this procedure, some split-proteins precipitated out of solution. However, the mixtures of split-proteins were vortexed and samples removed as if all proteins were dissolved.

It was assumed that only ribosomal proteins were lost from the ribosome by this procedure and, therefore,

TABLE 8.1

BINDING OF  $\angle^{-3}\text{H}\angle$  ANISOMYCIN TO RIBOSOMES AND  
RIBOSOMAL CORE-PARTICLES

Particle (98 p mol)		$\angle^{-3}\text{H}\angle$ anisomycin in reaction mixture (p mol)	$\angle^{-3}\text{H}\angle$ anisomycin bound to particles (p mol)
<u>M.verr</u>	80S	170	2.0
Y <sub>166</sub>	80S	85	20.3
TR <sub>1</sub>	80S	85	15.5
Y <sub>166</sub>	80S	170	27.6
TR <sub>1</sub>	80S	170	23.8
Y <sub>166</sub>	80S	235	29.4
TR <sub>1</sub>	80S	255	28.6
Y <sub>166</sub>	1.5M-core	170	23.3
Y <sub>166</sub>	2.0M-core	170	7.1
Y <sub>166</sub>	2.5M-core	170	4.6
Y <sub>166</sub>	3.0M-core	170	2.6
Y <sub>166</sub>	3.5M-core	170	0.2

## Legend to Table 8.1

### Binding of $\text{[}^3\text{H]}$ Anisomycin to Ribosomes and Ribosomal Core-Particles

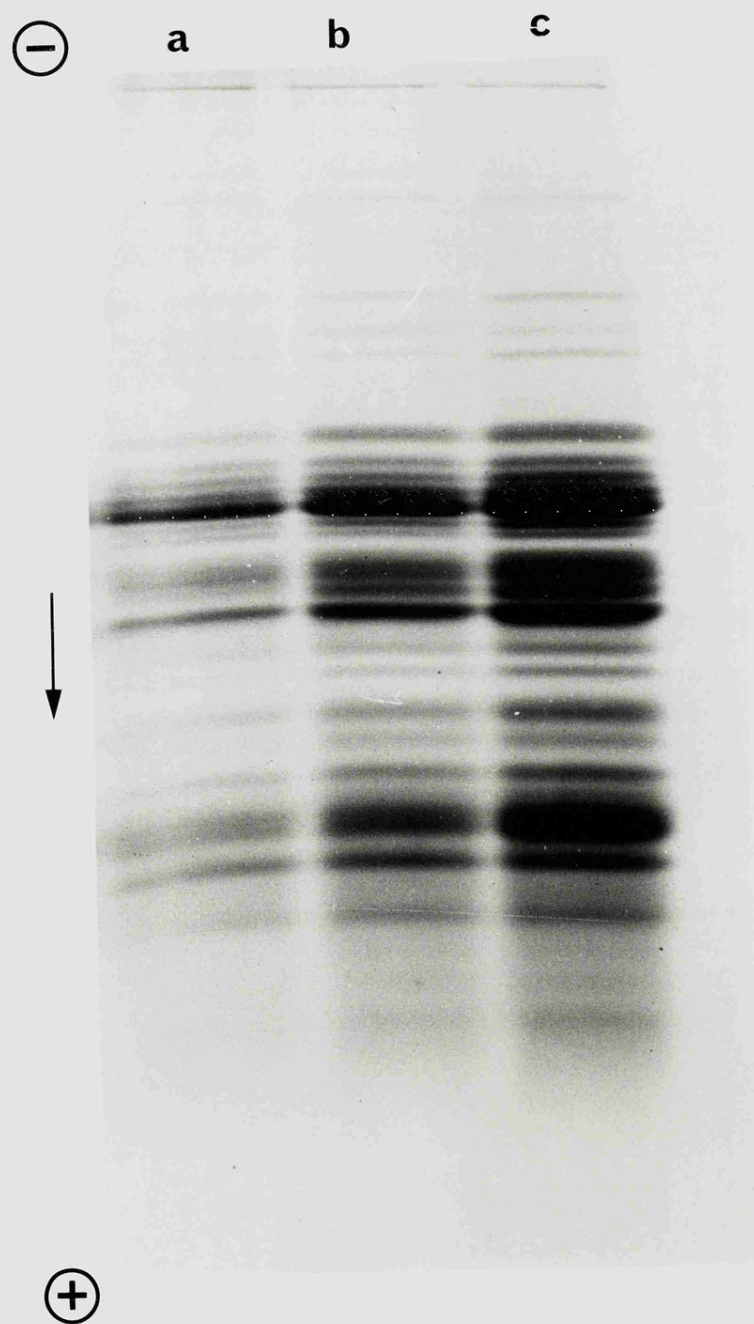
Ribosomes or ribosomal core-particles (98 pmol; Section 8.3) were mixed with various amounts of  $\text{[}^3\text{H]}$  anisomycin and the volume was adjusted to 50  $\mu\text{l}$  with 10mM-Tris.acetic acid (pH7.6), 90mM-potassium acetate, 1mM-magnesium acetate, 3mM-2-mercaptoethanol. After incubating at  $0^{\circ}\text{C}$  for 10 minutes, two samples (10  $\mu\text{l}$ ) were removed and radioactivity in the samples estimated by liquid-scintillation counting (Section 3.16). The remaining mix was made 33% ( $\text{V/v}$ ) in ethanol and centrifuged to precipitate ribosomes. A further two samples (10  $\mu\text{l}$ ) were removed and their radioactivity estimated as above. The quantity of bound  $\text{[}^3\text{H]}$  anisomycin was estimated from these values.

that an extinction coefficient of 13A<sub>260</sub> units was equivalent to 250 pmol. of core-particles (see Section 3.10). A 'pmol. equivalent' of split-proteins was defined as that quantity of ribosomal split-proteins which had been washed off 1 pmol. of ribosomes.

Core-particles made from ribosomes of yeast strain Y<sub>166</sub> lost some of their ability to bind  $\angle^{-3}\text{H}_\angle$  anisomycin. Thus, core-particles made by washing through 1.5M-NH<sub>4</sub>Cl (1.5M-cores) bound almost as much  $\angle^{-3}\text{H}_\angle$  anisomycin as did high salt-washed ribosomes (Table 8.1) whereas 3.5M-cores bound little or no  $\angle^{-3}\text{H}_\angle$  anisomycin (Table 8.1). High salt-washed ribosomes from yeast strain TR<sub>1</sub> and M.verrucaria were also tested for their ability to bind  $\angle^{-3}\text{H}_\angle$  anisomycin. In contrast to the results reported by Jimenez and Vazquez (1975), the ribosomes of strain TR<sub>1</sub> bound less  $\angle^{-3}\text{H}_\angle$  anisomycin than did wild-type ribosomes when tested at low drug concentrations (Table 8.1). At higher concentrations of  $\angle^{-3}\text{H}_\angle$  anisomycin, Y<sub>166</sub> and TR<sub>1</sub> ribosomes bound approximately the same amount of antibiotic (data not given). The ribosomes of M.verrucaria bound  $\angle^{-3}\text{H}_\angle$  anisomycin only very poorly (Table 8.1).

As may be seen from Table 8.1, the ability of

**FIG 8.2**



## Legend to Figure 8.2

### Analysis of Ribosomal Split-Proteins by SDS-Gel Electrophoresis

Ribosomal split-proteins (88 pmol equivalents) made by centrifuging yeast strain Y<sub>166</sub> ribosomes through (a) 1.5M-NH<sub>4</sub>Cl (b) 2.0M-NH<sub>4</sub>Cl or (c) 3.5M-NH<sub>4</sub>Cl (Section 8.3) were electrophoresed through an SDS 15% (<sup>W</sup>/v) polyacrylamide-slab-gel according to the method of Lämmlí (1970). Electrophoresis was at room temperature and 20 mA for 4 hours (cathode to anode). The gel was stained in 0.04% (<sup>W</sup>/v) Coomasie Brilliant Blue-G in 3.5% (<sup>V</sup>/v) perchloric acid and destained with 15% (<sup>V</sup>/v) methanol, 7.5% (<sup>V</sup>/v) acetic acid.

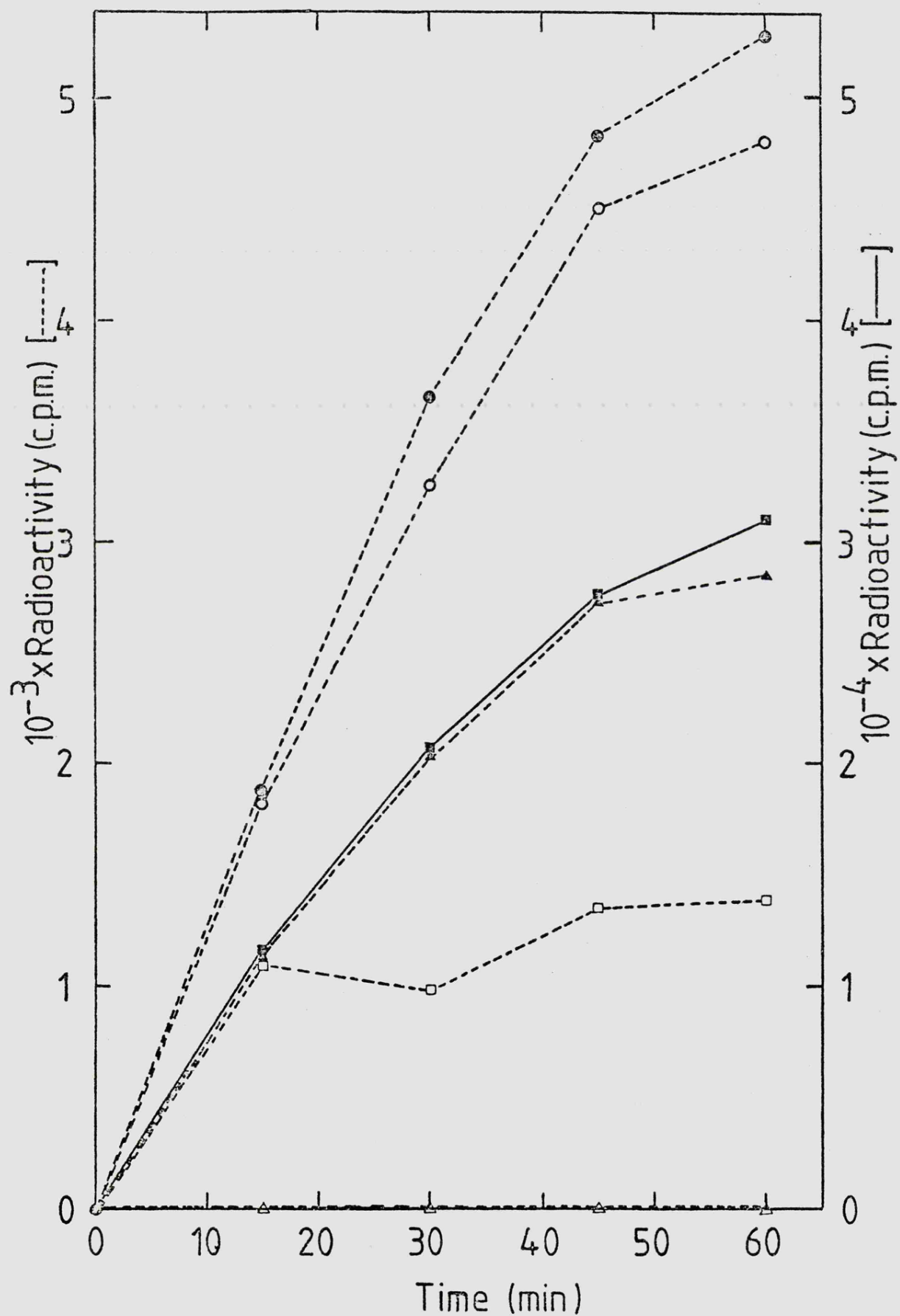
ribosomal core-particles from strain Y<sub>166</sub> to bind [<sup>3</sup>H] anisomycin was lost mainly at the stage of production of a 2.0M-core. Therefore, portions of the split-proteins were electrophoresed through an SDS 15% (w/v) polyacrylamide-gel according to the method of Lammler (1970). There were no obvious differences between the proteins split off by treatment with 3.5M-NH<sub>4</sub>Cl and those split off by treatment with 1.5M-NH<sub>4</sub>Cl or 2.0M-NH<sub>4</sub>Cl (Fig.8.2) except that more protein was removed at the higher salt concentrations. It would seem, therefore, that the high ammonium salt-wash procedure does not allow sequential removal of ribosomal proteins and may exert the effects observed here by virtue of unfolding or altering the conformation of ribosomal particles.

Ribosomal core-particles were also assayed for their activity in poly U-directed protein synthesis. Even 3.5M-core-particles retained some activity although this was considerably less than that of 1.5M-cores and about 3% of the activity of the ribosome (data not given). Split-proteins had no activity in poly U-directed protein synthesis (Fig.8.3).

#### 8.5. RECONSTITUTION OF RIBOSOMES FROM YEAST STRAIN Y<sub>166</sub>

Ribosomal core-particles and split-proteins from yeast strain Y<sub>166</sub> were recombined and assayed for

FIGURE 8.3 Activity of Reconstituted Yeast Ribosomes in Protein Synthesis





### Legend to Figure 8.3

#### Activity of Reconstituted Yeast Ribosomes in Protein Synthesis

Mixtures (50  $\mu$ l) for poly U-directed protein synthesis (Section 3.14) containing yeast strain Y<sub>166</sub> ribosomes (17.9 pmoles), yeast strain Y<sub>166</sub> 3.0M-ribosomal core-particles (17.9 pmoles) and various amounts of yeast ribosomal split-proteins, or ribosomal split-proteins alone (Section 8.3) and yeast S100 were incubated at 25°C. Samples (10  $\mu$ l) were precipitated with TCA and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).

■ , plus yeast ribosomes; □ , yeast core-particles, ▲ , core-particles plus 2 equivalents of split-proteins; o, core-particles plus 4 equivalents of split-proteins; ●, core-particles plus 6 equivalents of split-proteins; Δ , 6 equivalents of split-proteins alone.

reconstitution of [ $^3\text{H}$ ] anisomycin binding and activity in poly U-directed protein synthesis.

No significant increase in the ability of core-particles to bind [ $^3\text{H}$ ] anisomycin could be detected (data not given). However, core-particles could be reconstituted for poly U-directed protein synthesis and the extent of reconstitution was found to depend upon the number of equivalents of split-proteins added back (Fig.8.3). For 3.0M-cores, their activity could be stimulated from 5.4% to 17.3% of the activity of the intact ribosome by the addition of 6 equivalents of split-proteins (Fig.8.3). Split-proteins produced by a 2.5M-NH<sub>4</sub>Cl washing procedure were also capable of stimulating poly U-directed protein synthesis by 3.0M-cores although more equivalents were required in order to cause the same degree of stimulation (data not given).

In order to ensure that the stimulation in poly U-directed protein synthesis by split-proteins represented true reconstitution and was not an artefact, the following precautions were taken:

(a) All incubations for poly U-directed protein synthesis were performed at 20mM-Mg<sup>2+</sup> i.e. the optimum for yeast ribosomes (Fig.6.2b). Therefore, ionic conditions were optimum for protein synthesis and any alteration of ionic conditions by addition of split-proteins would cause a

decrease in incorporation.

(b) Split-proteins and core-particles were tested in isolation for their activity in poly U-directed protein synthesis in all experiments. They were treated exactly as for assays involving reconstitution and, therefore, any conformational changes in the core-particles (i.e. reconstitution not involving addition of split-proteins) during reconstitution were not responsible for increases in synthetic activity.

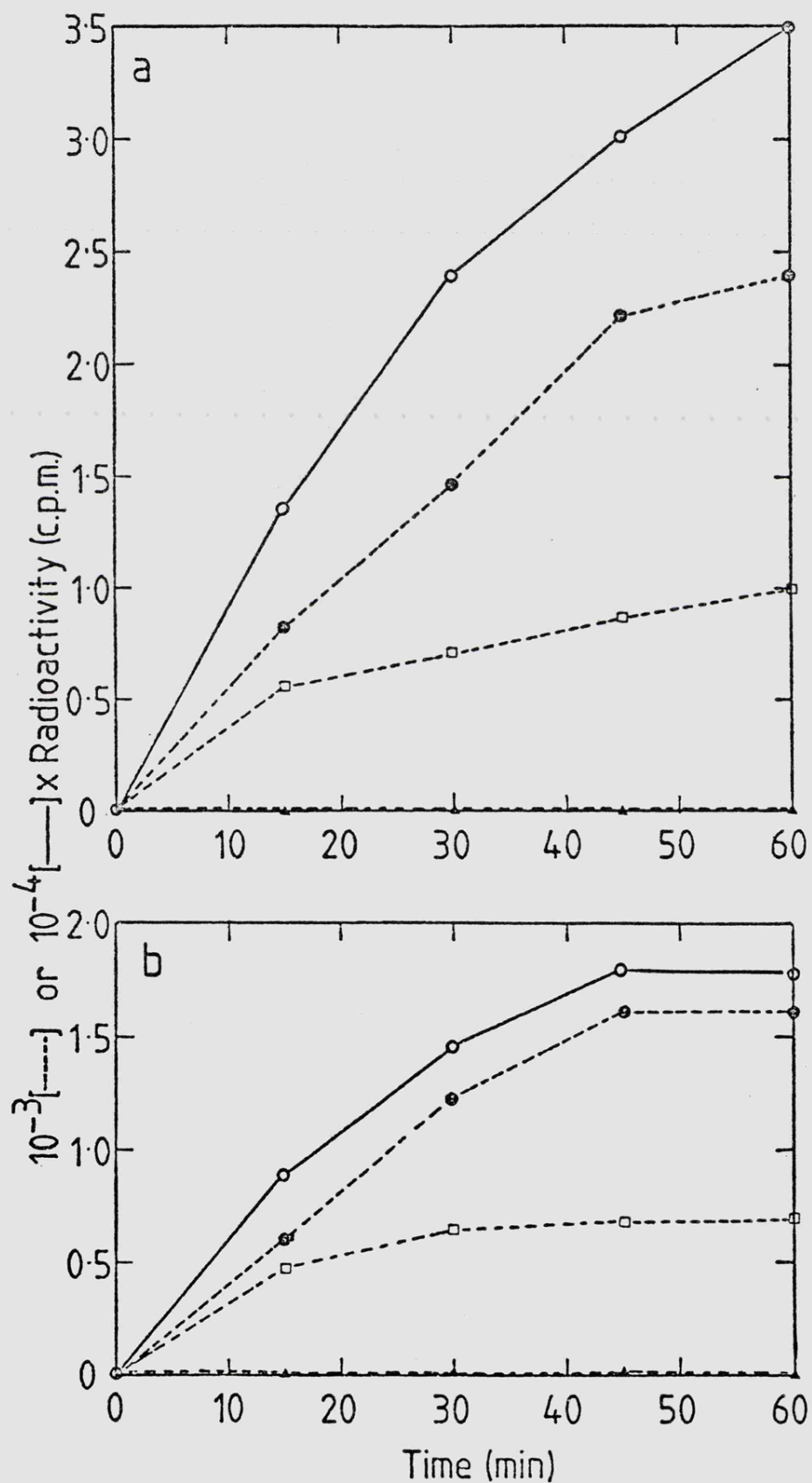
(c) No effect of split-proteins on the activity of ribosomes in poly U-directed protein synthesis was observed (data not given) which demonstrated that the split-proteins did not contain elongation factors etc. which might have caused a stimulation of protein synthesis.

All reconstitutions were performed by mixing core-particles and split-proteins for 15 minutes at 25°C before addition of yeast S100 and the remainder of the incubation mixture. Pre-incubation for longer periods or at higher temperatures did not stimulate reconstitution above the levels already observed and, in the case of temperatures of 37°C and above, pre-incubation reduced the activity of the core-particles (data not given).

#### 8.6. HETEROLOGOUS RECOMBINATIONS OF RIBOSOMAL CORE-PARTICLES AND SPLIT-PROTEINS FROM YEAST, STRAINS Y<sub>166</sub> and TR<sub>1</sub> and M. VERRUCARIA

Ribosomal core-particles and split-proteins were produced from yeast strain TR<sub>1</sub> and M. verrucaria as

FIGURE 8.4 Activity of Reconstituted Ribosomes from Yeast Strain TR<sub>1</sub> or M. verrucaria in Protein Synthesis



Legend to Figure 8.4

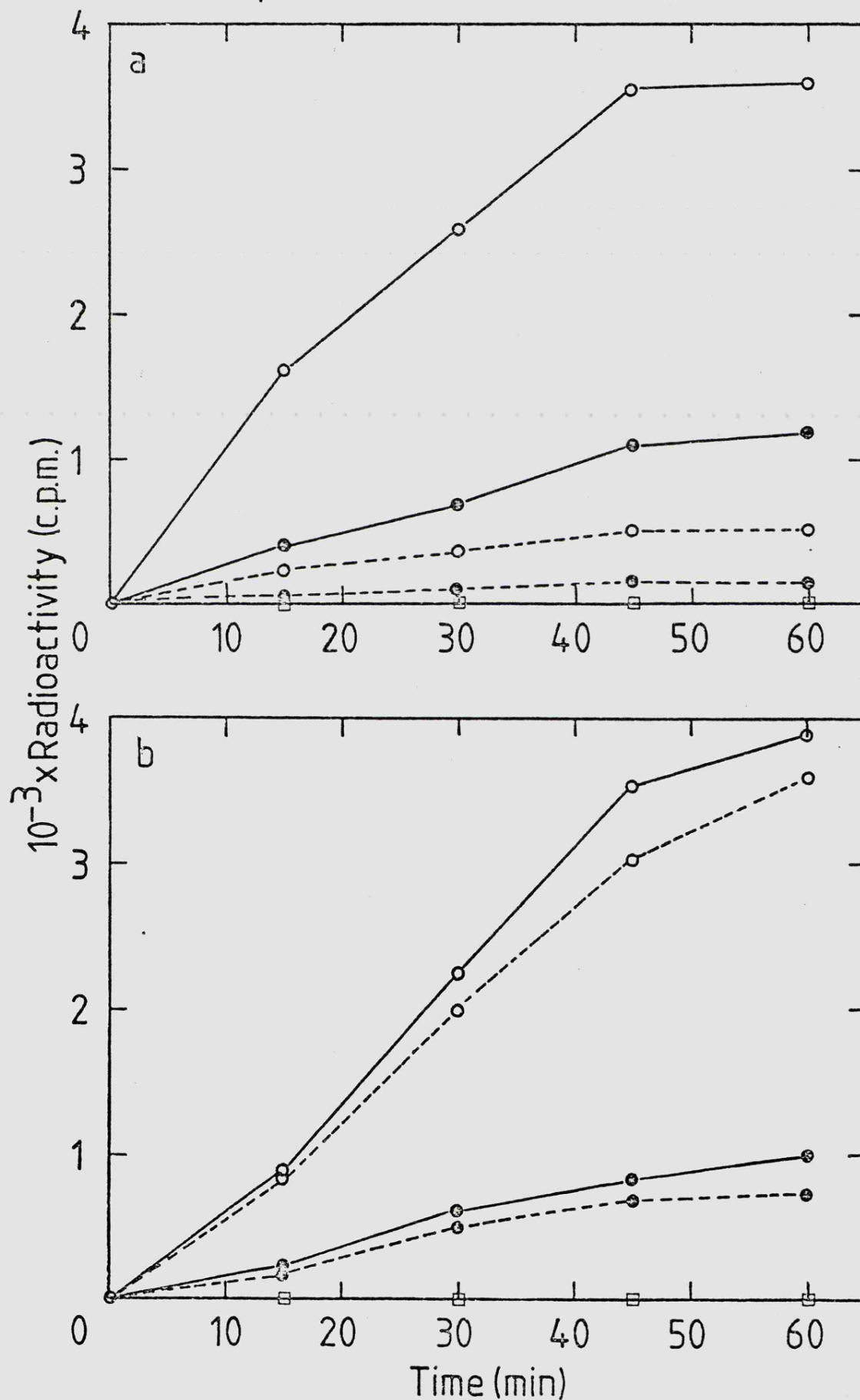
Activity of Reconstituted Ribosomes from Yeast Strain  
TR<sub>1</sub> or M. verrucaria in Protein Synthesis

Mixtures (50  $\mu$ l) for poly U-directed protein synthesis (Section 3.14) containing (a) yeast strain TR<sub>1</sub> ribosomes (17.9 pmol), ribosomal 3.0M-core-particles (17.9 pmol) and/or ribosomal split-proteins; (b) M. verrucaria ribosomes (17.9 pmol) and/or ribosomal split-proteins and yeast S100 was incubated at 25°C. Samples (10  $\mu$ l) were precipitated with TCA and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).

○, plus ribosomes; □, plus core-particles;

●, core-particles plus 2 equivalents of split-proteins (35.8 pmol); ▲, plus 2 equivalents of split-proteins.

FIGURE 8.5 Heterologous Combination of Ribosomal Core-Particles and Split-Proteins



Legend to Figure 8.5

Heterologous Combination of Ribosomal Core-Particles  
and Split-Proteins

Mixtures (50  $\mu$ l) for poly U-directed protein synthesis (Section 3.14) containing (a) yeast strain Y<sub>166</sub> ribosomal 3.0M-core-particles (17.9 pmol) and/or M.verrucaria ribosomal split-proteins; (b) M.verrucaria ribosomal 3.0M-core-particles (17.9 pmol) and/or yeast strain Y<sub>166</sub> ribosomal split-proteins and yeast S100 were incubated in the presence or absence of T-2 toxin (10  $\mu$ g/ml final concn.) at 25°C. Samples (10  $\mu$ l) were precipitated with TCA and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).

□ , 2 equivalents (35.8 pmol) split-proteins;  
● — ●, core-particles; ●---●, core-particles plus  
T-2 toxin; ○ — ○, core-particles plus 2 equivalents  
split-proteins; ○ --- ○, core-particles plus 2 equivalents  
split-proteins and T-2 toxin.

described for yeast strain Y<sub>166</sub> (Section 8.3). They could be recombined to give a stimulation of poly U-directed protein synthesis when treated as for strain Y<sub>166</sub> ribosomal cores and splits (Fig.8.4).

Heterologous reconstitutions involving the ribosomal core-particles and split-proteins from yeast strains Y<sub>166</sub> and TR<sub>1</sub> were performed and the resultant particles tested for their activity in poly U-directed protein synthesis and their sensitivity to T-2 toxin. The extent to which the reconstituted particles were inhibited by T-2 toxin was dependent only upon the source of the core-particles (Table 8.6).

Similar experiments were performed for ribosomal core-particles and split-proteins from yeast strain Y<sub>166</sub> and M. verrucaria. The split-proteins from M. verrucaria ribosomes were able to stimulate the activity of ribosomal core-particles from strain Y<sub>166</sub> in poly U-directed protein synthesis (Fig.8.5a) and vice versa (Fig.8.5b). Sensitivity of the reaction mixtures to T-2 toxin was dependent only upon the source of the core-particles (Fig.8.5a, b and Table 8.6).

Clearly, the ribosomal proteins which were involved in the reconstitution process described above were not responsible for antibiotic sensitivity or resistance in yeast or M. verrucaria. Further experiments were performed in an attempt to determine which step(s) in protein synthesis was/were affected by these proteins.



TABLE 8.6.

INHIBITION OF PROTEIN SYNTHESIS ON RECONSTITUTED  
RIBOSOMES BY T-2 TOXIN

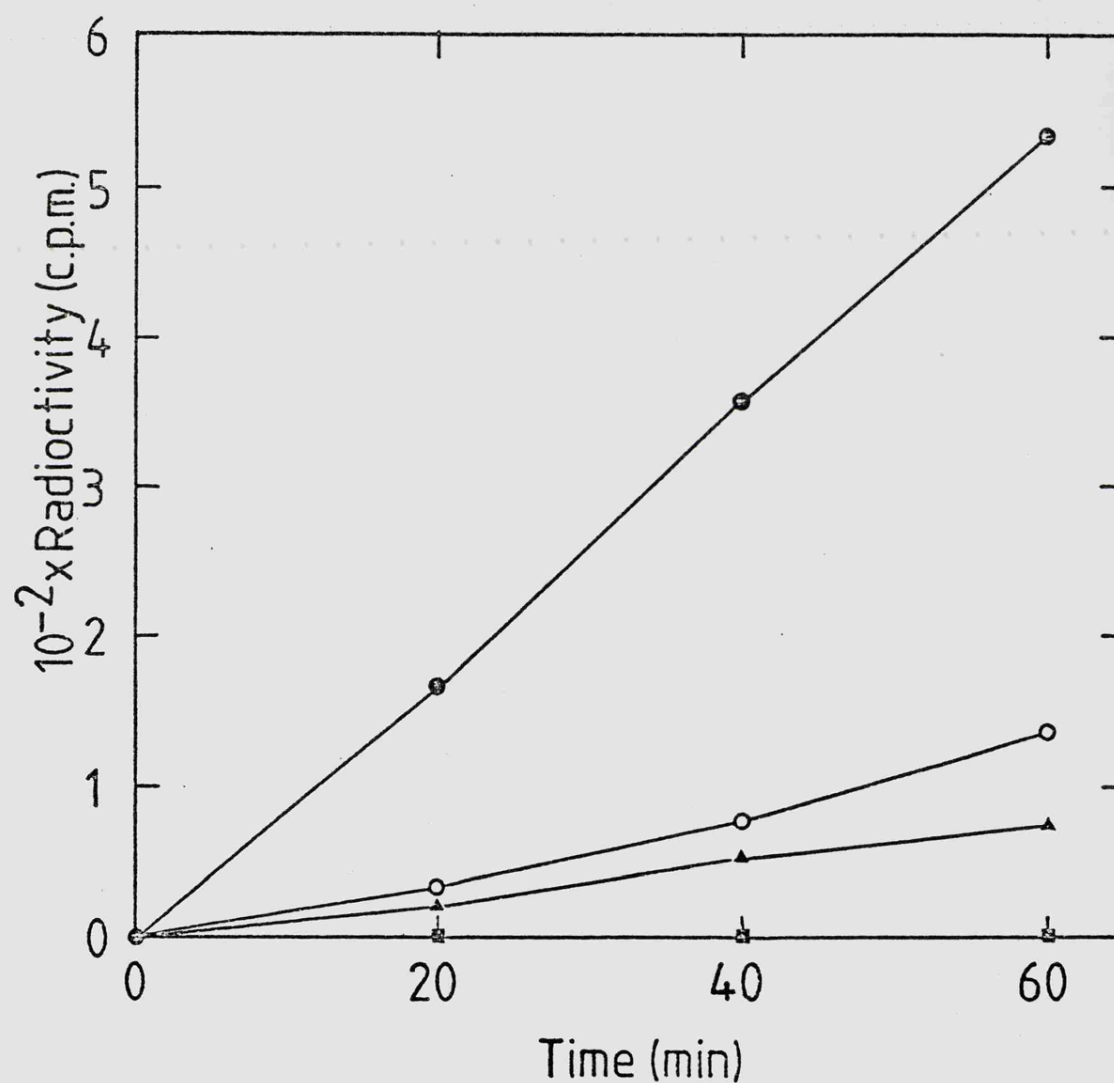
Source of core-particles	Source of split-proteins	% Inhibition by 10 $\mu$ g/ml T-2 toxin
Y <sub>166</sub>	Y <sub>166</sub>	86
Y <sub>166</sub>	TR <sub>1</sub>	83
TR <sub>1</sub>	TR <sub>1</sub>	70
TR <sub>1</sub>	Y <sub>166</sub>	69
<u>M.verr</u>	<u>M.verr</u>	8
<u>M.verr</u>	Y <sub>166</sub>	10.5
Y <sub>166</sub>	<u>M.verr</u>	80.5

Legend to Table 8.6

Inhibition of Protein Synthesis on Reconstituted  
Ribosomes by T-2 toxin

Mixtures (50  $\mu$ l) for poly U-directed protein synthesis (Section 3.14) containing 3.0M-ribosomal core-particles (17.9 pmol) and 2 equivalents (35.8 pmol) of ribosomal split-proteins from various sources were incubated at 25°C in the presence or absence of T-2 toxin (10  $\mu$ g/ml). Samples (10  $\mu$ l) were precipitated with TCA and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16). The percentage inhibition by T-2 toxin was calculated as an average of the whole time-course (0-60 min.).

FIGURE 8.7 Activity of Ribosomal Core-  
Particles and 'Reconstituted'  
Ribosomes in the Fragment Reaction



Legend to Figure 8.7

Activity of Ribosomal Core-Particles and  
'Reconstituted' Ribosomes in the Fragment Reaction

Mixtures for the fragment reaction (Section 6.8b) containing (●) yeast strain Y<sub>166</sub> ribosomes (17.9 pmol); (○) yeast strain Y<sub>166</sub> 3.0M-ribosomal core-particles (17.9 pmol); ▲ , ribosomal 3.0M-core-particles (17.9 pmol) and ribosomal split-proteins (35.8 pmol): ■ , ribosomal split-proteins (35.8 pmol) were incubated at 20°C with 3850 c.p.m. of acetyl  $\text{L-}^3\text{H-}$  leu-ACCAC. At various times the reaction was stopped by addition of 0.1 ml 0.3M-sodium acetate. acetic acid (pH5.5), saturated with MgSO<sub>4</sub>. Radioactivity present as acetyl  $\text{L-}^3\text{H-}$  leu-puromycin was estimated by liquid-scintillation counting of an ethyl acetate extract (Sections 8.5b and 3.16). Parallel reactions containing no ribosomes were performed and the 'background' values obtained were subtracted.

8.7. ACTIVITY OF THE RIBOSOMES AND RIBOSOMAL CORE-PARTICLES  
of YEAST STRAIN Y<sub>166</sub> IN THE STEPS OF PROTEIN BIOSYNTHESIS

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The core-particles and split-proteins produced from yeast ribosomes were tested for their activity in two partial reactions of the ribosome.

(a) The Fragment Reaction

Yeast ribosomes and 3.OM-cores were tested for their activity in the 'fragment reaction' (Section 6.8) and for the ability of split-proteins to stimulate that activity. The 3.OM-cores retained 23% of the activity of their parent ribosomes (Fig.8.7) but were slightly inhibited in the fragment reaction by the addition of split-proteins (Fig.8.7).

Comparison of the 3.OM-cores particles' activity in the 'fragment reaction' with that in poly U-directed protein synthesis (Fig.8.3) suggests that wherever the reconstitution was taking place, it was not at the site of the peptidyltransferase. Thus, the activity of the reconstituted particle in poly U-directed protein synthesis (as a percentage of the activity of the parent ribosome) was similar to the activity of the 3.OM-core in the 'fragment reaction' (Figs. 8.3 and 8.7). It may be that, using the above reconstitution technique, only a small percentage of the 3.OM-cores can be reconstituted because the remainder of the cores have lost ribosomal components of the peptidyltransferase.

(b)  $\text{[}^{14}\text{C] Phe-tRNA Binding to the Ribosome}$

Ribosomes or core-particles were incubated with a reaction mixture similar to that for poly U-directed protein synthesis (Section 3.14) but containing guanylyl ( $\beta, \gamma$ -methylene)-diphosponate (GMPPCP) instead of GTP. The reaction mixtures were incubated for 20 minutes at 25°C and then filtered through Sartorius cellulose nitrate-filters (pore size 0.45  $\mu$ ) and the filters were washed 5 times with buffer containing 10mM-Tris.acetic acid (pH7.6), 90mM-potassium acetate, 14mM-magnesium acetate and 3mM-2-mercaptoethanol. Finally, the filters were dried and their radioactivity estimated by scintillation-counting (Section 3.16). Additionally, mixtures were precipitated with hot TCA and the precipitates dried and scintillation-counted to determine whether any poly U-directed protein synthesis had occurred. No protein synthesis was observed (data not given).

By these criteria, 50% of 3.0M-core-particles retained their ability to bind  $\text{[}^{14}\text{C] Phe-tRNA}$  (data not given) although this value was reduced to 17% upon recombination of core-particles with split-proteins (data not given).

The reason for the inhibition by split-proteins is unclear although the results do correlate with those

of an experiment involving the binding of [ $^3\text{H}$ ] methyl-alpha sarcin to ribosomal core-particles. In this latter experiment, 3.0M-cores bound more [ $^3\text{H}$ ] methyl-alpha sarcin than did the parent ribosomes but addition of split-proteins to the cores inhibited the extra binding (data not given). Since alpha sarcin is reported to cut rRNA at a specific point (Schindler and Davies, 1977), it is possible that the extent of binding of [ $^3\text{H}$ ] methyl-alpha sarcin to the ribosome reflects the degree to which rRNA is exposed on the ribosomal surface.

It has not proved possible either to detect which proteins were responsible for the small amount of reconstitution that has been observed in these experiments or to determine which step in protein synthesis was affected. However, comparison of my data with that of others (Richter and Möller, 1974; Reyes et al., 1977) suggests that the reconstitution reported here might have involved merely the addition of the acidic proteins. These proteins, L40 and L41 (Stöffler et al., 1974), may be removed from rat liver ribosomes without affecting the activity of the resulting core-particles in the 'fragment reaction' (Reyes et al., 1977). Additionally, the acidic proteins of yeast proteins may be substituted by the acidic proteins L7 and L12 of the E.coli ribosome without loss of activity (Richter and

Møller, 1974). Clearly, if these proteins have been conserved through nature so as to be interchangeable between prokaryotes and eukaryotes, it would not be surprising if the split-proteins of M. verrucaria ribosomes could complement yeast core-particles.

#### 8.8 ENZYMIC-METHYLATION OF RIBOSOMES AND RIBOSOMAL CORE-PARTICLES

In prokaryotes, drug resistance is determined sometimes by the rRNA (Helser, et al., 1971; Lai and Weisblum, 1971; Cundliffe, 1978) and, in all these cases, the state of methylation of the rRNA has been the determining factor. Therefore, extracts of M. verrucaria and yeast were examined for methylase activities capable of altering the antibiotic-sensitivities of each other's ribosomes.

Crude ribosomes from M. verrucaria, prepared without the high salt-washing procedure, were centrifuged through 20% (<sup>w</sup>/v) sucrose containing 10mM-Tris.HCl, (pH7.6) 1M-NH<sub>4</sub>Cl, 10mM-MgCl<sub>2</sub> and 3mM-2-mercaptoethanol. The supernatant was dialysed against 'eukaryotic dialysis buffer' (10mM-Tris.acetic acid (pH7.6), 90mM-potassium acetate, 1mM-magnesium acetate, 3mM-2-mercaptoethanol) and assayed for its ability to methylate yeast ribosomes or ribosomal core-particles using S-adenosyl-L- $\gamma$ -methyl-<sup>3</sup>H-methionine (  $\gamma$ -<sup>3</sup>H-SAM) as substrate.

After incubation for four hours, yeast ribosomes could be methylated to the extent of approximately one



TABLE 8.8

ENZYMIC-METHYLATION OF RIBOSOMES AND RIBOSOMAL  
CORE-PARTICLES

Particle (7.6 pMol)	Source of crude Methylase	TCA-Precipitable material (c.p.m.)	Methyl groups/ particle
No ribosomes	<u>M. verr.</u>	583	-
Yeast 80S	<u>M. verr.</u>	4653	0.94
Yeast 3.5M-core	<u>M. verr.</u>	596	0
<u>M. verr.</u> 80S	<u>M. verr.</u>	832	0.05
No Ribosomes	Yeast	652	-
Yeast 80S	Yeast	721	0.02
<u>M. verr.</u> 80S	Yeast	4491	0.88

Legend to Table 8.8

Enzymic-Methylation of Ribosomes and  
Ribosomal Core-Particles

Ribosomes or ribosomal core-particles (38 pmol) were incubated at 25°C with 4  $\mu$  Ci of s-adenosyl-L-[methyl-<sup>3</sup>H] methionine (1 Ci/mmol) and 10  $\mu$ l ribosomal washate (Section 8.7). The volume was adjusted to 50  $\mu$ l with 10mM-Tris.acetic acid (pH7.6), 90mM-potassium acetate, 1mM-magnesium acetate and 3mM-2-mercaptoethanol initially. Samples (10  $\mu$ l) were precipitated after four hours with hot TCA (Section 3.15) and radioactivity in the precipitates was estimated by liquid-scintillation counting (Section 3.16).

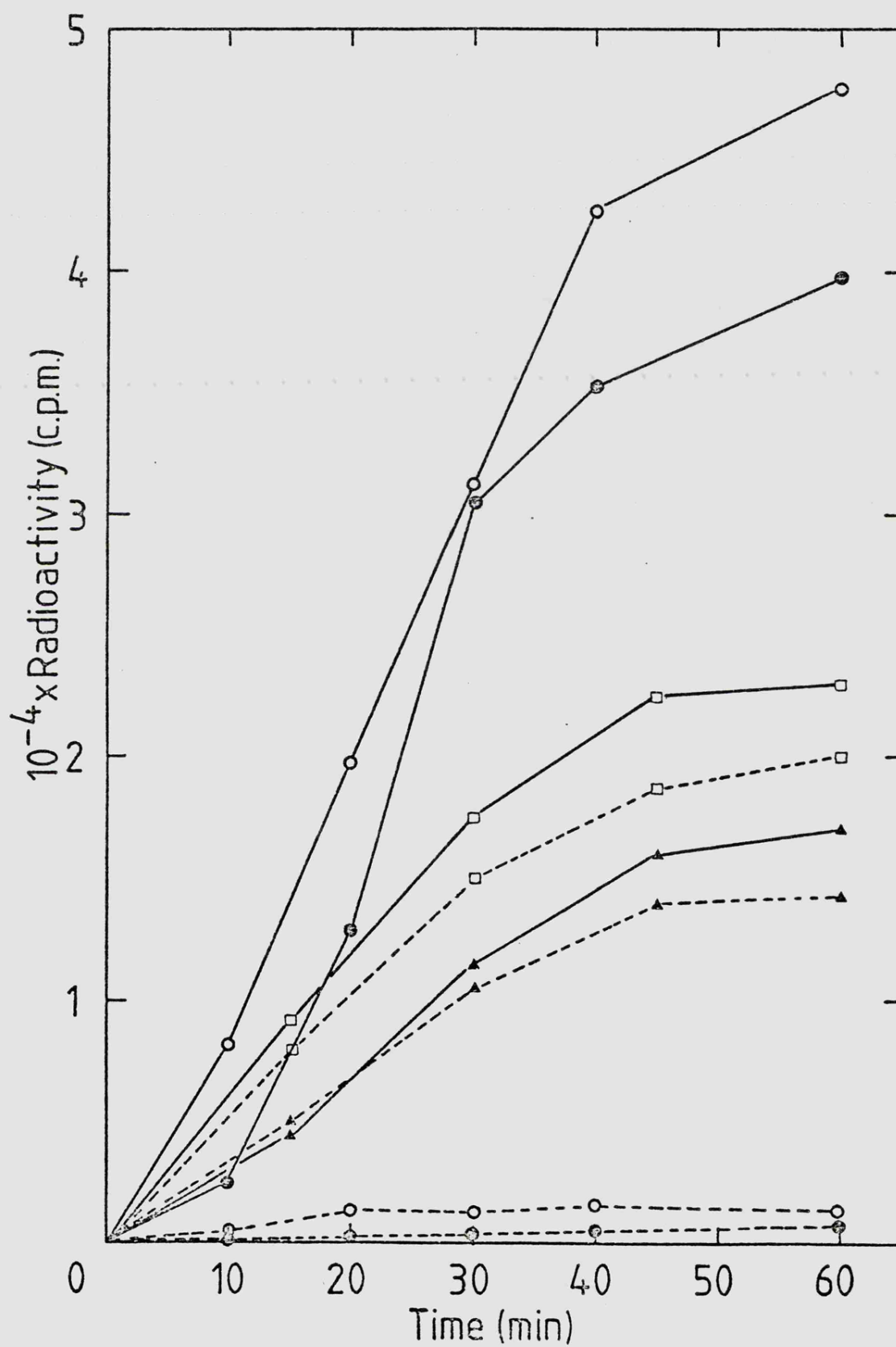
methyl group per ribosome (Table 8.8) whereas yeast 3.5M-cores and ribosomes from M. verrucaria were unaffected (Table 8.8). The methyl groups were assumed to be attached to protein since the radioactivity was precipitated by hot TCA.

The ribosomes of M. verrucaria could, however, be methylated when incubated with a high salt-wash from yeast strain Y<sub>166</sub> (prepared as described above for M. verrucaria) and [<sup>3</sup>H]-SAM (Table 8.8). Again, methylation was on a ribosomal protein.

Ribosomes of M. verrucaria or yeast strain Y<sub>166</sub> were methylated (as described in the legend to Table 8.8 except that unlabelled SAM was used) and tested for their sensitivity to T-2 toxin in poly U-directed protein synthesis. There was no alteration in the sensitivity of the ribosomes (Fig. 8.9).

These results do not deny the possibility that over or under-methylation of ribosomal proteins or RNA causes the phenomena of resistance to 12,13 epoxytrichothecenes in yeast and M. verrucaria. These organisms are not necessarily capable of methylating each other's ribosomes and, thus, conferring resistance or sensitivity to antibiotics. Equally, methylation of the ribosome may occur at an early stage in assembly. It is reported, for example, that many methylations occur on the 26S rRNA of the yeast ribosome whilst it is still part of the 37S precursor RNA (Brand et al., 1977). Further work on the mechanism of resistance

FIGURE 8.9 Effect of T-2 Toxin upon Methylated Ribosomes



Legend to Figure 8.9

Effect of T-2 Toxin upon Methylated Ribosomes

Mixtures (50  $\mu$ l) for poly U-directed protein synthesis (Section 3.14) containing ribosomes (21 pmol) or ribosomes which had been methylated previously (as described in legend to Table 8.8 except that unlabelled SAM was used; 21 pmol) and yeast S100 were incubated in the presence or absence of T-2 toxin (10  $\mu$ g/ml) at 25°C. Samples (10  $\mu$ l) were precipitated with TCA (Section 3.15) and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).

———, ribosomes only; -----, ribosomes plus T-2 toxin; o, yeast strain Y<sub>166</sub> ribosomes; ●, yeast strain Y<sub>166</sub> methylated ribosomes; □, M. verrucaria ribosomes; ▲, M. verrucaria methylated ribosomes.

to the 12,13 epoxytrichothecenes in M. verrucaria must await the development of a technique for the reversible dissociation of the eukaryotic ribosome.

9. INHIBITION OF POLY U-DIRECTED PROTEIN SYNTHESIS BY THE 12,13 EPOXYTRICHOTHECENES

9.1. MATERIALS

Commercial sources of materials were as follows:  
L-phenyl [ $^2$ , 3 -  $^3\text{H}$ ] alanine was from The Radiochemical Centre, Amersham; anhydrous hydrazine from Pierce and Warriner (U.K.) Limited, Chester; dansyl chloride and hydrazine sulphate from Sigma Chemical Co. and pyruvate kinase was from Boehringer and Soehne. Other chemicals were obtained from the sources mentioned previously.

9.2. INTRODUCTION

The 12,13 epoxytrichothecene, trichodermin, whilst being an inhibitor of polypeptide elongation (Section 4.4) was unable to inhibit poly U-directed protein synthesis in yeast extracts when assayed in the ionic conditions described in Section 3.14 ( $95\text{mM-K}^+$ ,  $14\text{mM-Mg}^{2+}$ ). In contrast, however, T-2 toxin, an inhibitor of polypeptide chain initiation (Section 5.4), was a potent inhibitor of poly U-directed protein synthesis (Fig.6.9). These results, in agreement with the work of others (Carrasco et al., 1973; Schindler et al., 1974; Schindler, 1974; Jimenez et al., 1975), support the suggestion (Schindler, 1974) that the 12,13 epoxytrichothecenes can be divided into

two groups on the basis of their ability to inhibit poly U-directed protein synthesis in yeast extracts.

Other workers report strong inhibition of poly U-directed protein synthesis by trichodermin with residual activity ranging from 20 - 50% of the control incubation (Tate and Caskey, 1973; Wei et al., 1974; Wei and McLaughlin, 1974; Cannon et al., 1976a). Incubation mixtures which were inhibited strongly by trichodermin, however, were relatively insensitive to T-2 toxin (Tate and Caskey, 1973; Cannon et al., 1976a). Therefore, there appears to be two types of poly U-directed protein synthesis, one which is inhibited by T-2 toxin and not by trichodermin and one which has the opposite characteristics. Consideration of the data leads one to implicate magnesium ion concentration in these responses. Thus, at 18mM-Mg<sup>2+</sup>, trichodermin inhibited by 5% (Schindler et al., 1974) or 13% (Schindler, 1974) and, at 8mM - Mg<sup>2+</sup>, inhibition was 80% (Tate and Caskey, 1973). Additionally, Carter (1978) reported that the ability of T-2 toxin to inhibit poly U-directed protein synthesis in yeast was increased as the magnesium ion concentration was raised. There remain, however, several discrepancies in the data: Wei and McLaughlin (1974) found only 72% inhibition of poly U-directed protein synthesis by



trichodermin at  $7\text{mM-Mg}^{2+}$  and Cannon et al., (1976a) could inhibit synthesis by 75% at  $12.5\text{mM-Mg}^{2+}$  under which conditions Wei et al., (1974) only observed 50% inhibition.

The ionic conditions for poly U-directed protein synthesis by yeast extracts were varied to see whether trichodermin gained the ability to inhibit and the results were compared to those obtained for a yeast incubation mixture prepared by the method of Sissons (1974).

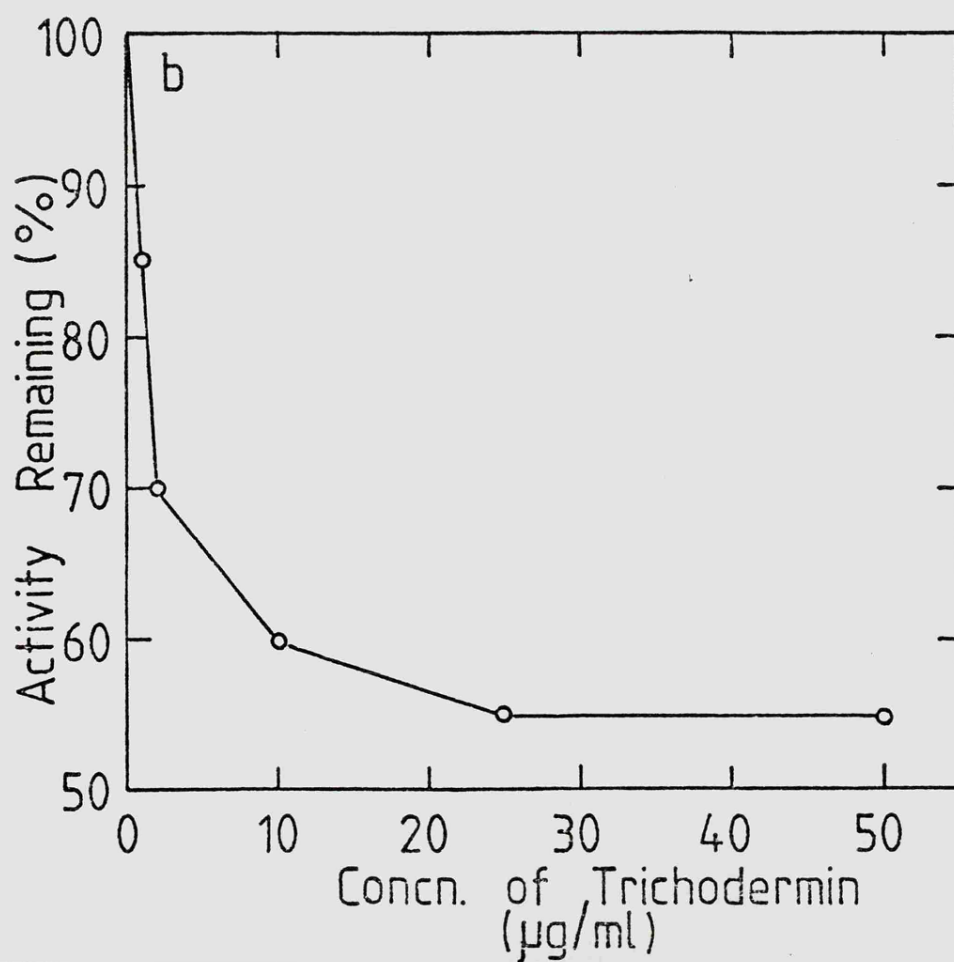
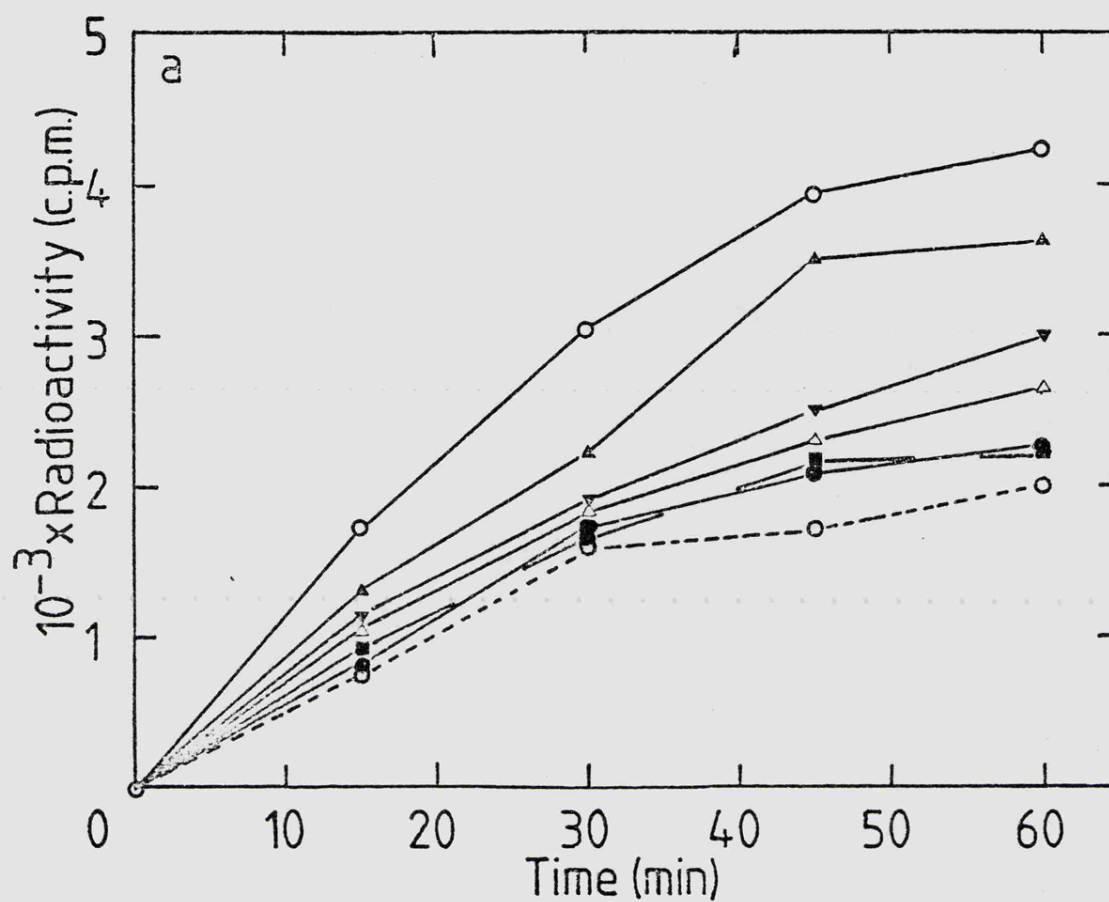
### 9.3. PREPARATION AND ASSAY OF YEAST S-30 EXTRACTS BY THE METHOD OF SISSONS (1974).

Yeast cells (strain Y166) were grown and harvested as described in Section 2.4 except that they were washed in 'isolation buffer' containing  $50\text{mM-Tris.HCl}$  (pH7.6),  $8\text{mM-magnesium acetate}$ ,  $60\text{mM-NH}_4\text{Cl}$ ,  $0.5\text{mM-spermidine}$ ,  $3\text{mM-2-mercaptoethanol}$  and 10% (V/v) glycerol. An S-30 extract was prepared as described in Section 3.9 except that 'isolation buffer' was used throughout.

An incubation mixture for poly U-directed protein synthesis was derived as follows:-

Energy mix	100 $\mu\text{l}$	
Poly U	16 $\mu\text{l}$	(Initial concentration, 10 mg/ml)
Yeast tRNA (phe specific)	20 $\mu\text{l}$	(Initial concentration, 2 mg/ml)
50mM-spermidine	7 $\mu\text{l}$	
pyruvate kinase	5 $\mu\text{l}$	(Initial concentration, 10mg/ml)
Water	<u>152 <math>\mu\text{l}</math></u>	
	300 $\mu\text{l}$	
Yeast S-30	100 $\mu\text{l}$	
	<u>400 <math>\mu\text{l}</math></u>	

FIGURE 9.1 Effect of Trichodermin on Protein Synthesis



## Legend to Figure 9.1

### Effect of Trichodermin on Protein Synthesis

Mixtures for poly U-directed protein synthesis as described by Sissons, 1974 (Section 9.3) containing yeast strain Y<sub>166</sub> S-30 extract (A<sub>260</sub> = 19.4 final concn.) was incubated with various final concentrations of trichodermin or T-2 toxin (10 µg/ml) at 25°C. Samples (10 µl) were precipitated with TCA (Section 3.15) and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16). Antibiotics were in 50% DMSO and, therefore, the control was adjusted accordingly.

(a) incorporation into poly Phe; o — o, control;  
o --- o, plus T-2 toxin; plus trichodermin at final concentrations of ▲ , 1 µg/ml; ▼ , 2 µg/ml;  
△ , 10 µg/ml; ● , 25 µg/ml; ■ , 50 µg/ml.

(b) Activity against concentration of trichodermin.

Energy mix: 150 mM-Tris.HCl, 24mM - magnesium acetate, 180 mM-NH<sub>4</sub>Cl, 20 mM-phosphoenolpyruvate, 5.32 mM-ATP, 0.668 mM-GTP, 32mM-glutathione, 0.16 mM-L-amino acids (except phenylalanine), 0.04 mM-L-phenyl [2, 3-<sup>3</sup>H] alanine (660 mCi/mmol).

Final conditions for poly U-directed protein synthesis were 60mM-NH<sub>4</sub><sup>+</sup>, 8mM-Mg<sup>2+</sup>, 1mM-spermidine, 8mM-glutathione.

#### 9.4. INHIBITION OF YEAST EXTRACTS BY TRICHODERMIN AND T-2 TOXIN

Polyuridylic acid-directed protein synthesis in a yeast incubation mixture made by the method of Sissons (1974) was inhibited by a maximum of 45% at concentrations of trichodermin of 25 µg/ml and above (Fig.9.1). In contrast, yeast S-30 extracts prepared and assayed by the method described in Section 3.9 and 3.14 were inhibited by approximately 3% even at high concentrations of trichodermin (data not given).

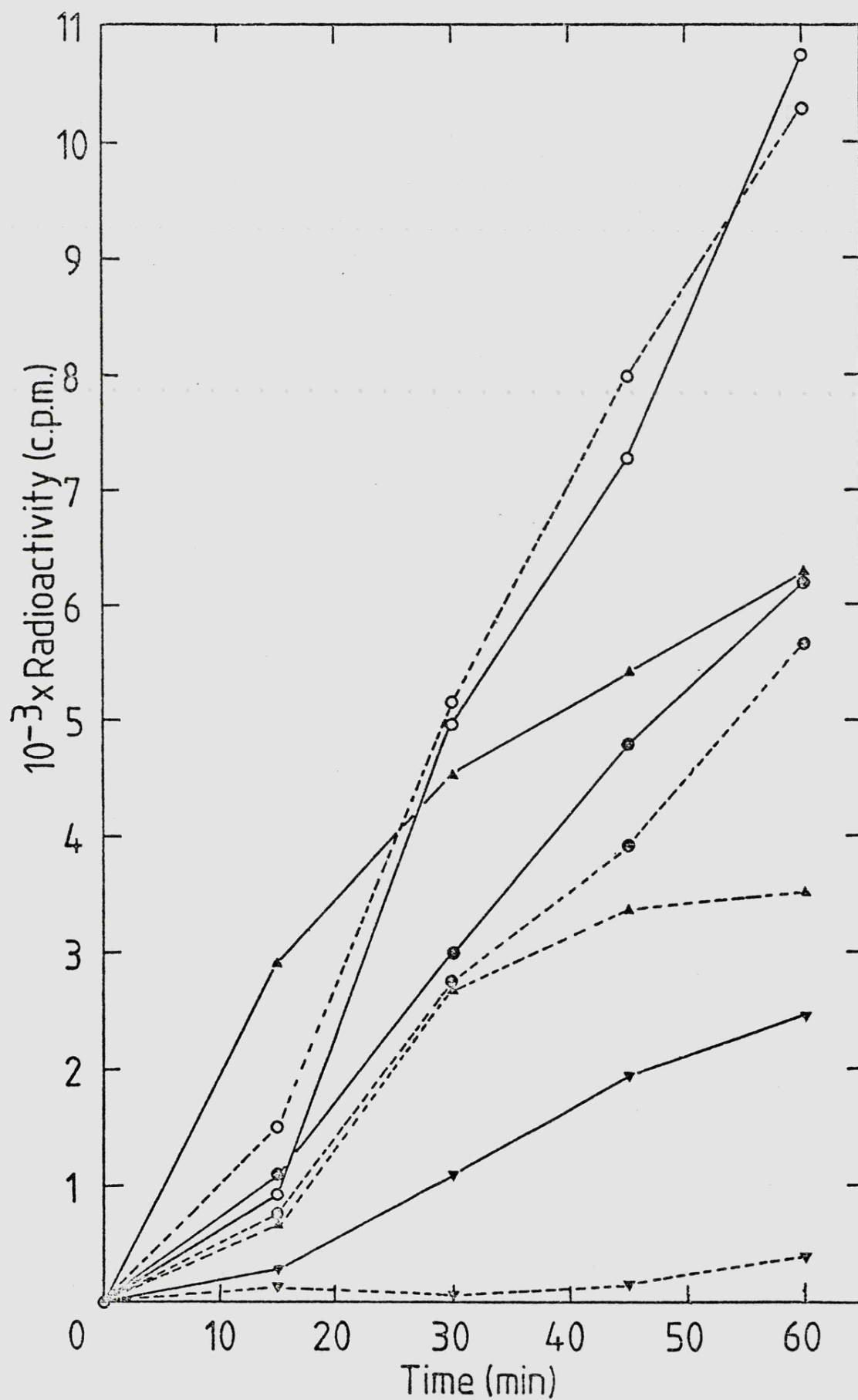
Inhibition of poly U-directed protein synthesis by T-2 toxin (10 µg/ml, final concentration) was, however, only 50% (Fig.9.1) for the incubation mixture of Sissons (1974) whereas the standard yeast incubation mixtures were inhibited by 74% at 10 µg T-2 toxin/ml (Table 6.7).

Although the ionic conditions of the yeast incubation mixtures were dissimilar, the differential effects of trichodermin on protein synthesis could have been

attributable to the state of the ribosomes. Therefore, high salt-washed ribosomes (Section 3.10) were used in all other experiments here. These ribosomes were assayed either using S100 prepared from standard yeast S-30 extracts (Section 3.10) or with S100 made from yeast S-30 extract of the Sissons' type. The latter type of S100 was prepared simply by centrifuging yeast S-30 at 50,000 r.p.m. for 4 hours.

Incubation mixtures containing high salt-washed ribosomes and yeast S100 in the standard assay for poly U-directed protein synthesis (Section 3.14) were uninhibited by trichodermin (Fig.9.2) but when the ribosomes were combined with yeast S100 and incubation mixture for the assay described by Sissons (1974), inhibition by trichodermin was 46% (Fig.9.2). Additionally, yeast S100 prepared as described in Section 3.10 was dialysed against 'isolation buffer' and assayed with high salt-washed ribosomes using the incubation conditions of Sissons (1974). In this case, inhibition by the same concentration of trichodermin was 88% (Fig.9.2). When S100 made in 'isolation buffer' was dialysed against 'eukaryotic dialysis buffer' and used in the standard assay (Section 3.14), inhibition by trichodermin was 13% (Fig.9.2). Therefore, the manner of preparation of

FIGURE 9.2 Differential Effects of Trichodermin on Protein Synthesis



## Legend to Figure 9.2

### Differential Effects of Trichodermin on Protein Synthesis

Mixtures (50  $\mu$ l) for poly U-directed protein synthesis of my type (O and ●, Section 3.14) or Sisson's type (▲ and ▼, Section 9.3) containing high salt-washed yeast strain Y<sub>166</sub> ribosomes (21 pmol), (Section 3.10) and yeast S100 (prepared in different ways) were incubated in the presence or absence of trichodermin (25  $\mu$ g/ml final concn.) at 25°C. Samples (10  $\mu$ l) were precipitated with TCA (Section 3.15) and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).

——, control; ----, plus trichodermin.

○, yeast S100 (Section 3.10); ●, yeast S100 dialysed from 'isolation buffer' (Section 9.3) into 'eukaryotic dialysis buffer' (Chapter 3);

▲, yeast S100 in isolation buffer (Section 9.4);

▼, yeast S100 (Section 3.10) dialysed into 'isolation buffer' (Section 9.3).

of the S100 as well as the ionic conditions appears to be important in determining the sensitivity of poly U-directed protein synthesis to trichodermin.

As mentioned previously, Carter (1978), using the assay conditions of Sissons (1974), found that inhibition of protein synthesis by T-2 toxin increased as the magnesium concentration was raised. Since an increase in sensitivity to T-2 toxin is linked to a loss of sensitivity to trichodermin (Tate and Caskey, 1973; Cannon et al., 1976a), experiments were performed to determine whether lowering the magnesium ion concentration in the standard incubation mixture (Section 3.14) increased its sensitivity to trichodermin.

Very little inhibition of protein synthesis was noticed even at 8mM-magnesium ions (the same  $Mg^{2+}$  concentration as the yeast incubation mixture described by Sissons, 1974). Equally, addition of 1mM-spermidine or substitution of ammonium for potassium ions or both did not increase the inhibition by trichodermin (data not given). However, addition of 8mM-glutathione did make the incubation mixtures sensitive to trichodermin in some experiments. This latter result was, however, variable and addition of glutathione also resulted in no alteration in sensitivity to trichodermin in at least half of the assays. There was no apparent correlation



between the differential effects of glutathione and the other ionic conditions. Therefore, no explanation is offered here for these apparently anomalous results.

#### 9.5. ANALYSIS OF PRODUCTS OF POLY U-DIRECTED PROTEIN SYNTHESIS

It has been reported that cell-free extracts from yeast (Van der Zeijst et al., 1973), rat muscle (Castles et al., 1971) and Neurospora crassa (Sturani et al., 1971) are capable of two types of poly U-directed protein synthesis. At high magnesium ion concentrations, polyphenylalanine is formed, whereas at low magnesium ion concentrations, phenylalanine molecules are added to the carboxy-termini of peptides which are present on the ribosomes as peptidyl-tRNA. It was, therefore, proposed (Carter, 1978) that the differential effects of trichodermin could be explained in terms of these two types of poly U-directed protein synthesis. Thus, trichodermin could inhibit the addition of phenylalanine to the carboxy-termini of nascent peptidyl-tRNA but not if the peptide moiety was (Phe)<sub>n</sub> or, presumably peptidyl-(Phe)<sub>n</sub>. It follows also that T-2 toxin would be unable to inhibit ribosomes engaged in the second type of protein synthesis (at low Mg<sup>2+</sup>) since they would carry peptidyl-tRNA even at the start of cell-free

synthesis (see Cannon et al., 1976a).

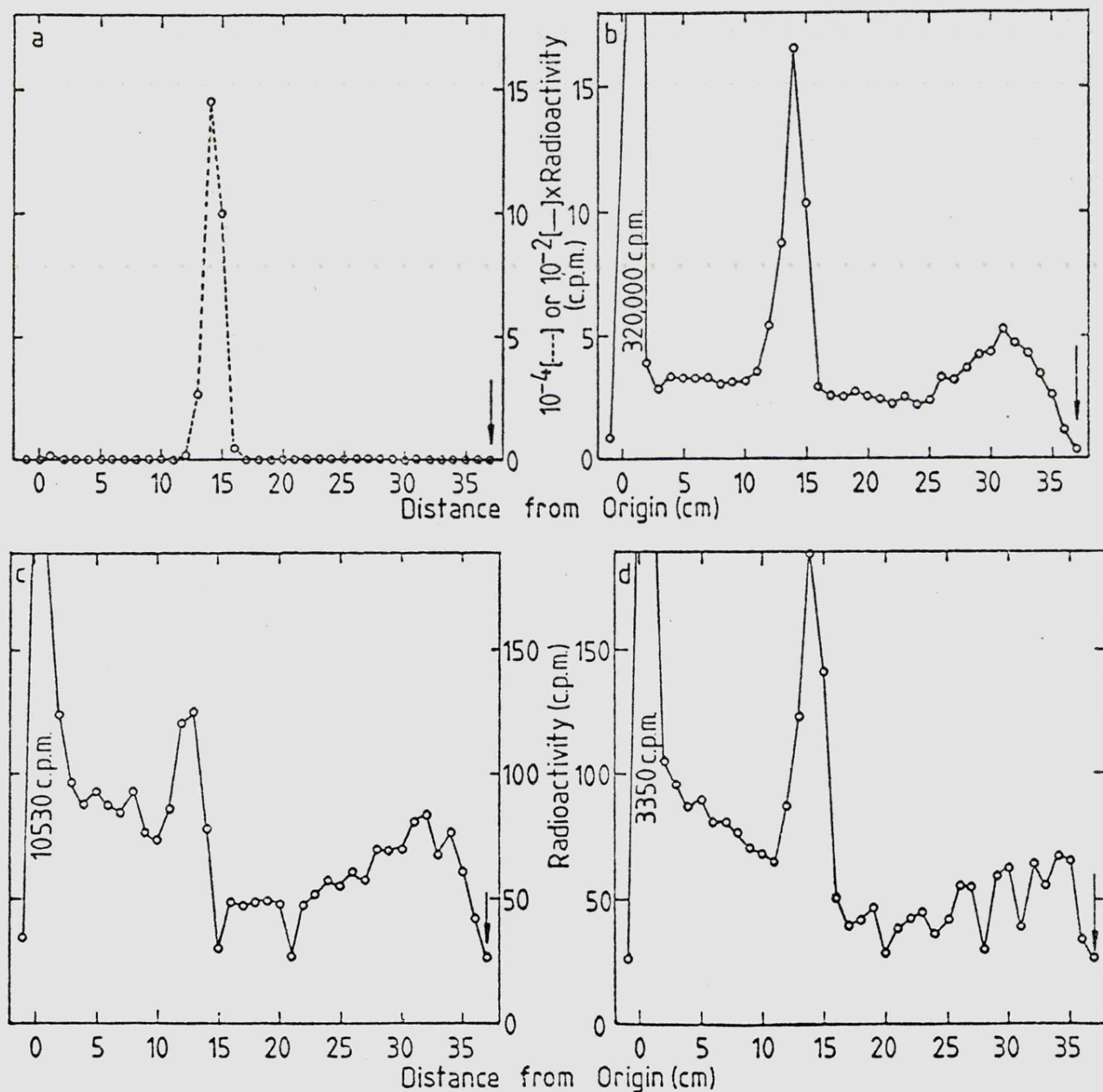
Evidence for the hypothesis of Carter (1978) is apparently contradictory, however. If only ionic conditions were important, why did yeast S100 extracts prepared in different ionic conditions cause the ribosomes to have different sensitivities to trichodermin? Also, if trichodermin is unable to inhibit the synthesis of polyphenylalanine it presumably is unable to inhibit synthesis of the polypeptide, peptidyl-(Phe)<sub>n</sub>. The results of Cannon et al. (1976a) show, however, that trichodermin inhibited poly U-directed protein synthesis by reticulocyte lysates equally well whatever the stage at which it was added, i.e. inhibition was 70% whether trichodermin was added at the start or after 25 minutes of incubation. Clearly, either this reticulocyte incubation mixture was only capable of producing peptidyl-Phe-tRNA or the synthesis of peptidyl(Phe)<sub>n</sub>-tRNA was also inhibited by trichodermin.

The products of poly U-directed protein synthesis in the standard incubation mixture (Section 3.14) or in the assay mixture of Sissons (with or without inhibition by trichodermin) were analysed to determine whether they consisted solely of polyphenylalanine.

#### (a) Analysis of Products by Chromatography

Mixtures for poly U-directed protein synthesis

FIGURE 9.3 Analysis of the Products of PolyU-Directed Protein Synthesis by Chromatography



### Legend to Figure 9.3

#### Analysis of the Products of Poly U-directed Protein Synthesis by Chromatography

Mixtures (100  $\mu$ l) for poly U-directed protein synthesis of (b) my type (Section 3.14); (c) Sissons' type (Section 9.3); (d) Sissons' type plus trichodermin (25  $\mu$ g/ml final concn.) containing high salt-washed yeast strain Y<sub>166</sub> ribosomes (42 pmol; Section 3.10) and S100 were incubated at 25°C. After sixty minutes the mixtures were centrifuged at 50,000 r.p.m. through a 20% (<sup>w</sup>/v) sucrose shelf containing 'eukaryotic dialysis buffer'. The ribosomes were resuspended in 1M-NH<sub>4</sub>OH and warmed at 37°C for 30 min. before the suspension was spotted onto Whatman<sup>®</sup> No.1 paper. The chromatogram was developed in n-butanol : 0.88 s.g. ammonia solution : water (100 : 3 : 18) and divided into 1 cm portions. Radioactivity in the portions was estimated by liquid-scintillation counting (Section 3.16). A pure preparation of  $\text{L-}^{14}\text{C}$ -phenylalanine was also chromatogrammed (a). Vertical arrows represent the solvent front.

(Section 3.14 or Sissons, 1974) were incubated for sixty minutes at 25°C. A sample (0.1 ml) of each mixture was then removed and centrifuged at 50,000 r.p.m. through 20% (<sup>w</sup>/v) sucrose containing 'eukaryotic dialysis buffer' (10mM-Tris.acetic acid (pH7.6), 90mM-potassium acetate, 1mM-magnesium acetate, 3mM-2-mercaptoethanol). The ribosomes were resuspended in 1M-NH<sub>4</sub>OH and warmed at 37°C for 30 minutes. Finally, the suspensions were chromatogrammed on Whatman No.1 paper using n-butanol : 0.88 s.g. ammonia solution : water (100 : 3 : 18) as solvent and radioactivity on the chromatograms was detected by scintillation spectrometry (Section 3.16) of 1 cm sections. In these conditions, homopolymers of phenylalanine with a chain length of five or greater remain at the origin as do peptides of mixed composition (Castles et al., 1971).

In all the incubations, the [<sup>14</sup>C] phenylalanine which was recovered from the ribosomes was found (Fig.9.3) to be present as material which ran on chromatograms at R<sub>F</sub> 0.37 ( [<sup>14</sup>C] phenylalanine), R<sub>F</sub> 0.67 - 0.97 ( [<sup>14</sup>Phe]<sub>2</sub> to [<sup>14</sup>Phe]<sub>4</sub> ; as judged by comparison with the data of Castles et al., 1971) and material which remained at the origin (polyphenylalanine consisting of five or more amino acids and/or other large peptides).

There were, however, differences in the quantity of each constituent. Thus, an uninhibited incubation mixture of Sissons' type contained less  $\text{L}^{-14}\text{C}_7$ -Phe-tRNA but more  $\text{L}^{-14}\text{Phe}_2$  - to  $\text{L}^{-14}\text{Phe}_4$  - tRNA bound to the ribosomes than did a mixture inhibited by trichodermin (Fig.9.3).

(b) Analysis of Amino - and Carboxy-Terminal Amino Acids

The large peptides which remained at the origin of the chromatograms (Fig.9.3) were isolated and the proportion of radioactive phenylalanine at their N- and carboxy-terminal were determined as below.

Samples (0.1ml) of the incubation mixtures (see above) were precipitated with hot TCA and the precipitates which contained peptides and polyphenylalanine of four residues and greater (Pestka et al., 1969) were dried in vacuo.

The carboxy-terminal amino acids were analysed by the method of Akabori et al., (1952). Anhydrous hydrazine (0.4ml) containing 1M-hydrazine sulphate was added to precipitates obtained from the three incubations, i.e. my type (Section 3.14), Sissons' type and Sissons' type containing trichodermin, and the mixtures were heated in vacuo. A control mixture containing  $\text{L}^{-14}\text{C}_7$ -phenylalanine was also incubated with hydrazine. After fifty hours at  $60^\circ\text{C}$  the mixtures were dried in vacuo and redissolved in 0.6ml water. A sample of the water (0.1ml)

was removed and its radioactivity estimated by scintillation-counting (Section 3.16), another was removed after extracting the remainder of the water with 0.25 ml benzaldehyde which removes hydrazides. The percentage of carboxy-terminal radioactivity in the precipitated peptides was calculated by dividing the radioactivity remaining in water after benzaldehyde treatment by the total radioactivity and making allowance for the efficiency of extraction (value obtained from  $[^{14}\text{C}]$  phenylalanine control). By this criterion, only 2% of phenylalanine molecules (in peptide) were present at the carboxy-termini of hot TCA-precipitable peptides produced during the standard assay (Table 9.4) but 17 - 21% of the phenylalanine molecules incorporated into hot TCA-precipitable material were at the carboxy-termini of peptides produced during the Sissons' type assays (Table 9.4). However, it is important to remember when considering these figures that phenylalanine is insoluble in most solvents and all may not, as a result, have reacted with hydrazine.

The phenylalanine in the TCA-precipitable material could be present either as  $(\text{Phe})_n$  or  $\text{peptidyl}(\text{Phe})_n$  or a mixture of both. In order to distinguish between these possibilities, the percentage of

TABLE 9.4

ANALYSIS OF N- AND C- TERMINI OF HOT TCA-PRECIPITABLE  
PEPTIDES PRODUCED BY POLY U-DIRECTED PROTEIN SYNTHESIS

C- TERMINUS ANALYSIS

Experiment	c.p.m. in water		% of cpm in C- terminus	% of cpm in C- terminus (corrected figure)
	Before benzaldehyde	After benzaldehyde		
My system	98,833	1,628	1.65	2.1
Sissons' system	3,455	454	13.15	16.9
Sissons' system + trichodermin	1,667	265	15.9	20.5
Control ( $\gamma$ - $^{14}\text{C}$ - Phe only)	746,890	580,020	77.7	100.0

N-Terminus Analysis

Experiment	cpm in 0.1ml TCA-precipitable material	cpm in spot on chromatogram	% of cpm at N-terminus
My system	252,000	7,570	3.0
Sissons' system	13,300	2,124	16.0
Sissons' system + trichodermin	4,800	892	18.6



#### Legend to Table 9.4

##### Analysis of N- and C- Termini of Hot TCA- Precipitable Peptides Produced by Poly U- Directed Protein Synthesis

Mixtures (100  $\mu$ l) for poly U-directed protein synthesis of my type (Section 3.14), Sissons' type (Section 9.3) or Sissons' type plus trichodermin (25  $\mu$ g/ml final concn.) containing yeast strain Y<sub>166</sub> ribosomes (Section 3.10) and S100 were incubated at 25°C. After 60 minutes the mixtures were precipitated with hot TCA and the precipitates collected. The percentages of radioactivity at the N- and C- termini of the precipitated peptides were determined by dansylation and hydrazinolysis respectively (Section 9.5b). Preparations of  $\text{L-}^{14}\text{C-}$  phenylalanine were used as controls for the assays.

radioactive phenylalanine at the N-termini of TCA-precipitable material was also determined. The dansylation method described by Hartley (1970) was used.

Dried TCA-precipitates (see above) were mixed with 10  $\mu$ l 0.2M-NaHCO<sub>3</sub> and the mixture evaporated to remove ammonia. Then 20  $\mu$ l of 5mM-dansyl chloride, 50%(<sup>V</sup>/v) acetone were added and, after sealing the tube with parafilm, the mix was heated at 45°C. After 30 minutes, the solvent was evaporated, 20  $\mu$ l 6M-HCl were added and the tubes were heated in vacuo at 105°C for 18 hours. Finally, the HCl was removed by evaporating over NaOH and 20  $\mu$ l ethanol were added. The products (in ethanol) were chromatogrammed on polyamide sheet using 1.5% (<sup>V</sup>/v) formic acid in the first dimension and benzene : acetic acid (9:1) in the second. An authentic sample of dansyl-phe was added to the mixtures before chromatography and the appropriate spot (after detection in U.V.light) was excised from the sheet and its radioactivity was estimated by scintillation-counting (Section 3.16).

The percentage of radioactive phenylalanine present at the N-termini of the TCA-precipitable peptides was found to be similar to that at the carboxy-termini (Table 9.4). Therefore, the great majority of the

products of poly U-directed protein synthesis in either my assay system (Section 3.14) or Sissons' system appeared to be authentic polyphenylalanine and not peptidyl-(Phe)<sub>n</sub>. Although it is possible that [<sup>14</sup>C] phenylalanine at the N-terminal of peptides was produced by enzymic addition this is unlikely since no 'synthesis' was observed in the absence of ribosomes.

The results presented here suggest that the differential effects of trichodermin reported by other authors were genuine and were not caused by contamination of the antibiotic with other toxins. Therefore, sensitivity or resistance to trichodermin appears to be independent of the products of protein synthesis and must depend upon other variables. It remains to be shown how ionic and other conditions affect the ratio of the two types of synthesis, i.e. one sensitive and one insensitive to trichodermin. Presumably, however, the conformation of the ribosome is important in the binding of antibiotic and this might be affected by ionic conditions, presence of aminoacyl- and peptidyl-tRNA and by peptides and other molecules in the supernatant. Thus, a combination of these variables may allow trichodermin to bind to the ribosomes.

## 10. PROTEIN SYNTHESIS IN EXTRACTS OF ANTIBIOTIC-PRODUCING FUNGI

### 10.1. INTRODUCTION

Antibiotics are very potent toxins made by living organisms and yet many producing-organisms can remain metabolically active in their own soups e.g. Streptomyces griseus which produces streptomycin (Woodruff, 1966) and S. venezuelae which produces chloramphenicol (Malik, 1972). It has been proposed (Demain, 1974) that all antibiotics have a functional role in the survival of the producing organism and there are several theories which seek to rationalize antibiotic-production. However, no one theory fits all the available evidence, suggesting either that the antibiotics' true purpose has not yet been determined or that it is impossible to produce a unified theory for all producing-organisms. The theories are outlined below.

#### (a) Competition

Initially, perhaps the most attractive idea, this hypothesis proposes that producing-organisms kill competing species by the production of antibiotic. In fact, antibiotic-production has only been demonstrated to occur in a few species under 'natural' conditions. For instance, the fungus Penicillium

chrysogenum produces penicillin when placed in unsupplemented soils (Hill,1972).

(b) Enzyme Maintenance

Antibiotics are produced in the stationary stage (idiophase) following the phase of logarithmic growth (trophophase). During idiophase the cell contains many enzymes which were required for growth but no longer have a use. It is proposed (Bu'lock, 1961) that these enzymes are protected from degradation (and are, thus, available immediately for the next period of rapid growth) by keeping them in operation and putting their products to different uses e.g. as precursors for antibiotics.

(c) Detoxification

An extension of the theory of Bu'lock (1961) was offered by Woodruff (1966). Following the period of rapid growth, control mechanisms are insufficient in certain organisms to prevent over-synthesis of primary metabolites. Unless these metabolites are converted and the products (including antibiotics) excreted from the cell, death would result due to imbalanced growth.

As a variation, Dhar and Khan (1971) proposed that producing-organisms convert toxic molecules which have accumulated in the cell into (nontoxic) antibiotics. Given the toxicity of antibiotics it is hard to

rationalise why any organism should wish to do this - especially as few production processes for antibiotics involve toxic precursors (Demain,1973). However, this proposal is supported by the fact that the penicillin precursor, phenylacetic acid, is more toxic to P.chrysogenum than is benz<sup>y</sup>penicillin, the antibiotic it produces (Polya and Nyiri,1966).

(d) Sporulation

More evidence is available to support this hypothesis than any other although there are several exceptions amongst producing organisms. It is proposed (Demain,1974) that antibiotic production is linked to differentiation and sporulation. Thus, inhibitors of sporulation such as ethyl malonate and fluoroacetate also inhibit antibiotic production in bacilli (Sarkar and Paulus,1972).

Producing-organisms have been studied extensively since, in many cases, their products have been used clinically. Data are, therefore, available concerning the antibiotic-sensitivities of prokaryotes which produce anti-bacterial agents. For instance, Streptomyces griseus and Streptomyces venezuelae are sensitive to their products (streptomycin and chloramphenicol, respectively) whilst in trophophase but develop resistance during idiophase (Woodruff,1966;

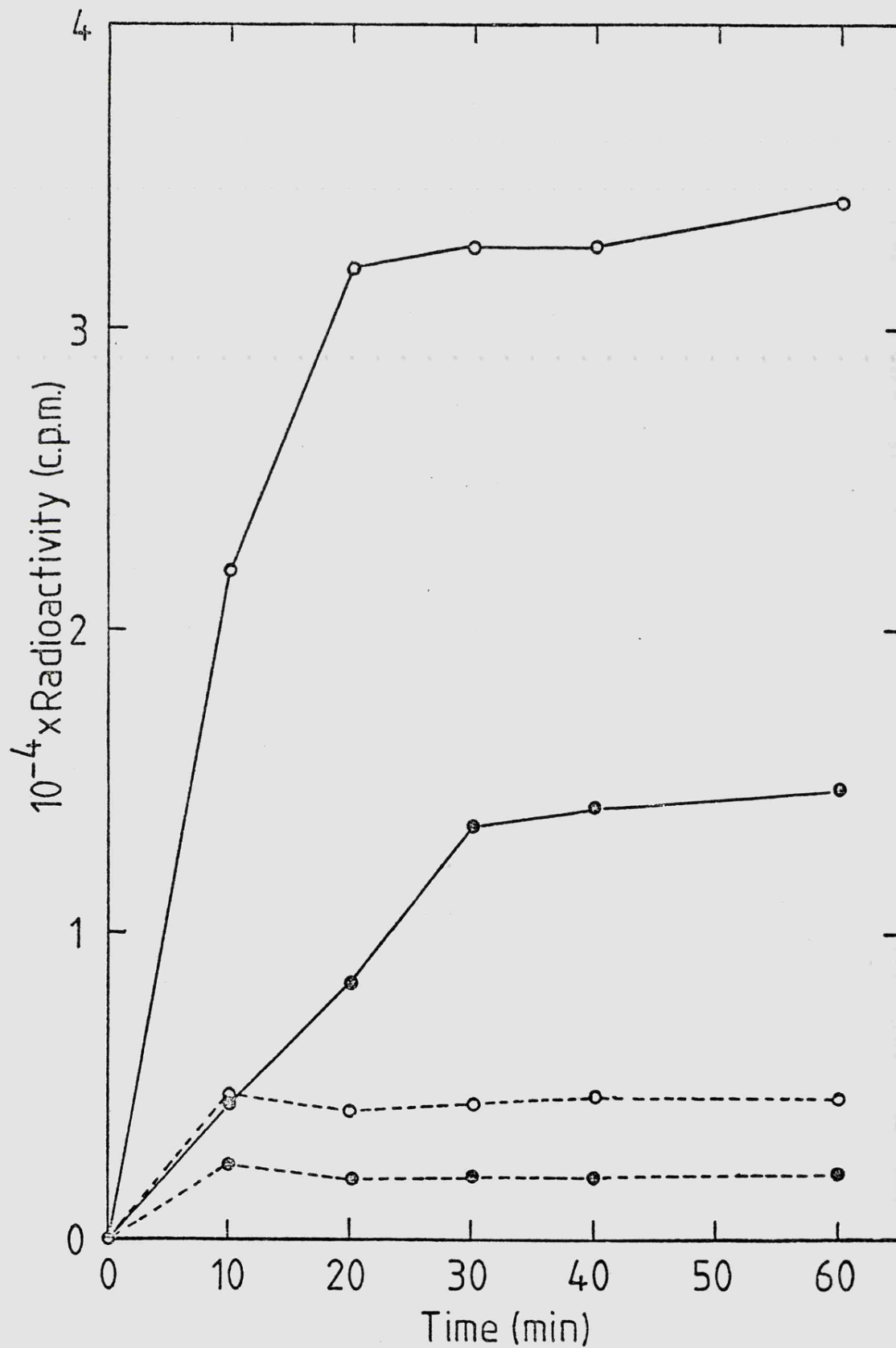
Malik and Vining, 1970; Malik, 1972). On the other hand, Streptomyces azureus is resistant to its product, thiostrepton, even during trophophase (Cundliffe, 1978).

Several fungi which are reputed to be capable of producing eukaryote-specific toxins were investigated in this work. In all cases, the organisms were resistant to their products in vivo (Table 2.2) and, therefore, cell-free extracts were prepared and the nature of the resistance investigated. The results for M. verrucaria have been presented elsewhere in this dissertation (Chapters 6 and 7) and show that extracts of the organism are resistant in vitro due to a modified target organelle even when the cells are in trophophase. The implications of this result are discussed in relation to other results presented in this chapter.

#### 10.2. CELL-FREE EXTRACTS FROM FUSARIUM POAE AND FUSARIUM SPOROTRICHIODES

The fusarium species, F. poae and F. sporotrichiodes, have been positively identified as the cause of alimentary toxic aleukia, a serious and in most cases lethal disease caused by consumption of mouldy corn (Joffe, 1974). Strains of these two fungi were all reported to produce the 12,13 epoxytrichothecenes, T-2 toxin, HT-2 toxin, neosolaniol and T-2 tetraol although

FIGURE 10.1 Effect of T-2 Toxin on Protein Synthesis by Extracts of F.poae and F.sporotrichiodes





Legend to Figure 10.1

Effect of T-2 Toxin on Protein Synthesis  
by Extracts of F.poae and F.sporotrichiodes

Mixtures for poly U-directed protein synthesis (Section 3.14) containing F.poae S-30 extract (●, A260 = 13 final concn.) or F.sporotrichiodes (○, A260 = 60 final concn.) were incubated in the presence or absence of T-2 toxin (10 µg/ml) at 25°C. Samples (5 µl) were precipitated with TCA (Section 3.15) and radioactivity in the precipitates estimated by liquid-scintillation counting, (Section 3.16).

————— , control;      - - - - - , plus T-2 toxin.

the amounts of each toxin varied between strains (Szathmary et al., 1976).

The strains used in these experiments were not shown to produce toxins (Section 2.5) although this possibility cannot be ruled out.

The organisms were grown as described in Section 2.4 and cell-free extracts were prepared and assayed for poly U-directed protein synthesis (Sections 3.13 and 3.14).

Extracts of both F.poae and F.sporotrichiodes were very active in protein synthesis but they were sensitive to T-2 toxin (Fig.10.1).

These results were not unexpected since the organisms had been shown to be sensitive to trichodermin and anisomycin in vivo (Table 2.2) and these antibiotics share a common or over-lapping binding site with T-2 toxin on the ribosome (see Chapter 6 for a discussion of this point). No experiments were performed on cells grown to stationary phase since extracts of cells at this stage of growth were generally inactive in poly U-directed protein synthesis (data not given). Therefore, it is unclear whether these organisms become resistant to trichodermin during idiophase. Since, however, resistance to their product (T-2 toxin) has already been achieved by cell-

permeability phenomena <sup>they</sup> ~~though~~ may have no need for further modifications. This hypothesis demands that the toxic metabolites are exported from the cell and do not exist within the cell either in high concentrations or as active molecules.

### 10.3. CELL-FREE EXTRACTS OF FUSARIUM EQUISETI

The Fusarium species, F.equiseti has not been implicated in any toxicoses although various strains have been shown to produce diacetoxyscirpenol, a 12,13 epoxytrichothecene (Brian et al., 1961; Ishii et al., 1971). A colony of F.equiseti growing on an agar plate failed to inhibit yeast strain Y<sub>166</sub> cells from growing around it (Section 2.5). However, this result does not provide evidence to determine whether the strain of F.equiseti used in these experiments produced diacetoxyscirpenol since yeast strain Y<sub>166</sub> was resistant to this toxin in vivo (Table 2.2).

Although F.equiseti was resistant in vivo to both trichodermin and anisomycin, a cell-free extract of the organism which was capable of poly U-directed protein synthesis was inhibited by T-2 toxin (data not given).

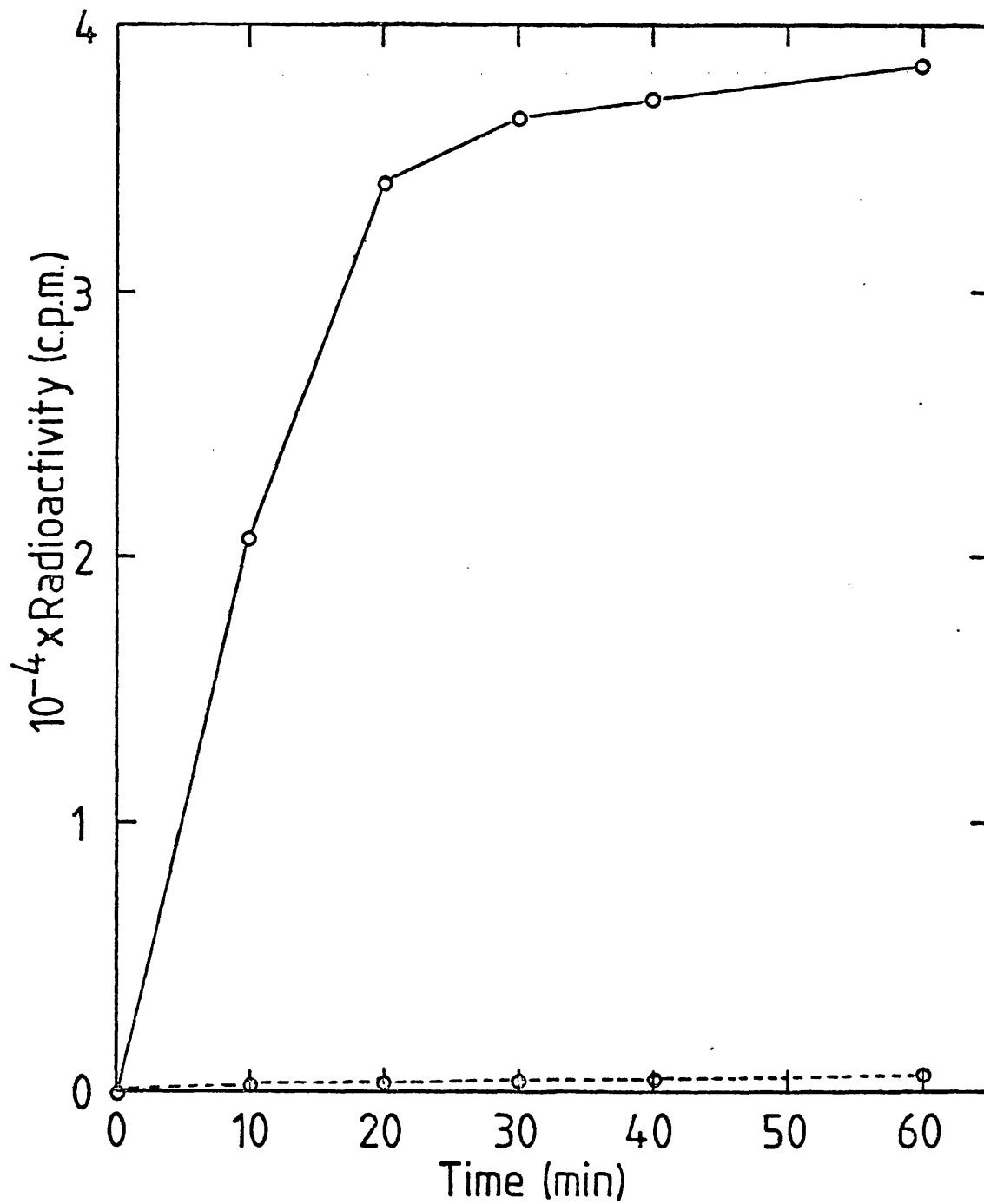
Resistance to the organism's toxic products is probably achieved by the cell being impermeable to these toxins. However, further experiments are needed to determine whether all Fusarium species achieve resistance by this means or whether they gain resistance

during idiophase. It is possible that some species are always resistant to the trichothecenes by virtue of a modified ribosome (c.f. M. verrucaria) or that a mechanism of resistance e.g. modification of the ribosome or production of an enzyme capable of detoxifying the antibiotic is induced as a result of antibiotic-production during idiophase. Clearly, any reaction involving modification of the ribosome during idiophase would involve the intact ribosome rather than a precursor. Otherwise the cell would have all or most of its ribosomes inhibited by the toxin. Such modifications should be far easier to identify than those in M. verrucaria where the ribosomes appear always to be resistant to 12,13 epoxytrichothecenes and, presumably, are modified during assembly. Detoxification of the antibiotics may be brought about by the enzymes which are responsible for its production i.e. they may be reversible and capable of converting the trichothecenes to non-toxic precursors when the toxin reaches a critical level.

#### 10.4. CELL-FREE EXTRACTS OF ASPERGILLUS GIGANTEUS

The strain of Aspergillus giganteus used in these experiments has been shown to produce the polypeptide, alpha sarcin (Olson, 1963; Schuurmans

FIGURE 10.2 Effect of Alpha Sarcin on Protein Synthesis by an Extract of A.giganteus



Legend to Figure 10.2

Effect of Alpha Sarcin on Protein Synthesis by  
an Extract of A.giganteus

Mixtures for poly U-directed protein synthesis  
(Section 3.14) containing A.giganteus S-30  
extract (A260 = 53 final concn.) were incubated  
in the presence or absence of alpha sarcin (100 µg/ml  
final concn.) at 25°C. Samples (5 µl) were  
precipitated with TCA (Section 3.15) and radio-  
activity in the precipitates estimated by liquid-  
scintillation counting (Section 3.16).

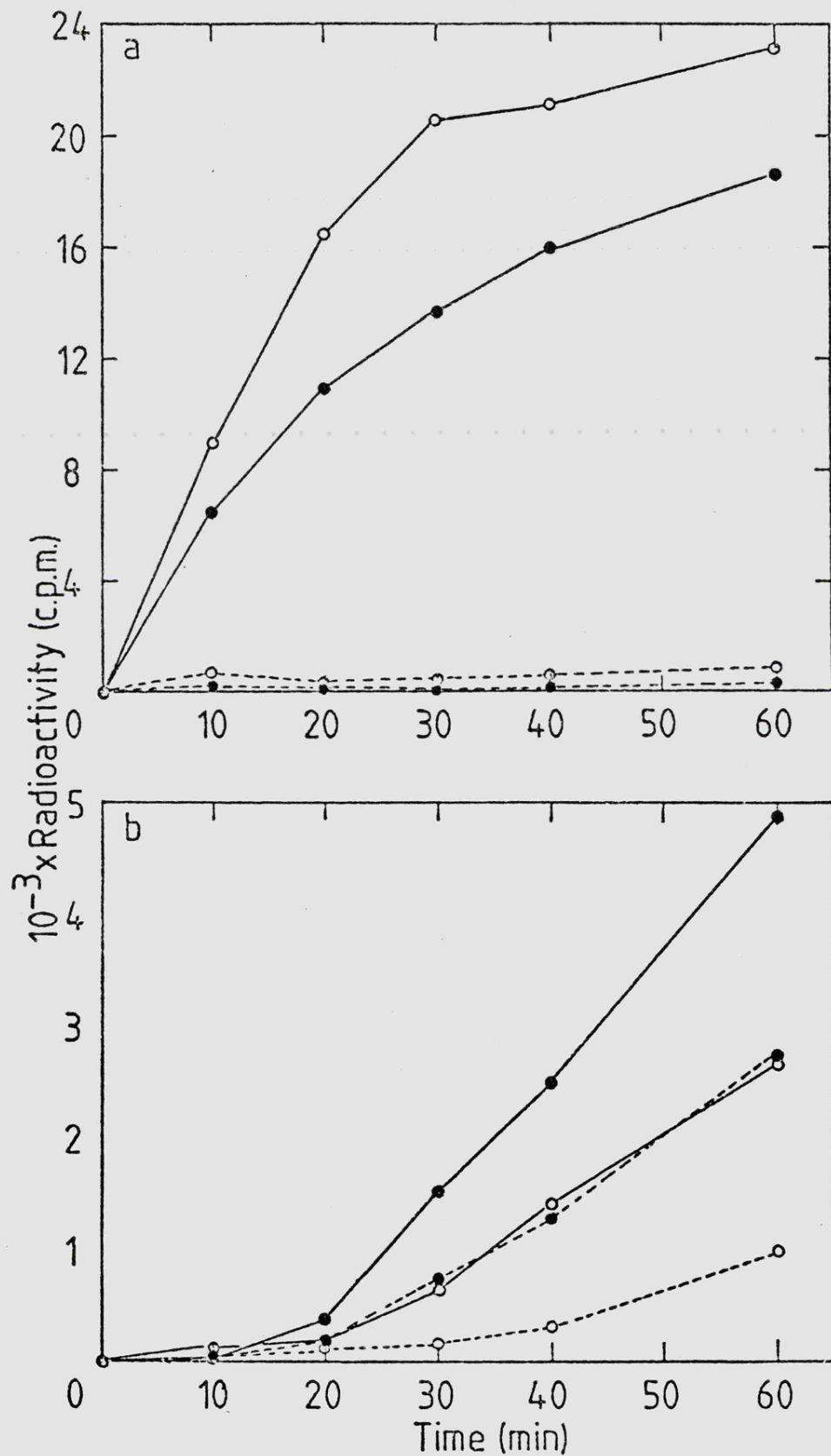
——— , control; ----- , plus alpha sarcin.

et al., 1964). Production of the toxin was not observed, however, until the cells were in idiophase (Olson et al., 1965).

For these experiments, cells were grown until they were in the phase of logarithmic growth and were then harvested and disrupted as described previously (Sections 3.13 and 3.14). Extracts of the cells were very active in poly U-directed protein synthesis but were inhibited by the addition of alpha sarcin (Fig.10.2).

Since alpha sarcin cleaves rRNA specifically (Schindler and Davies, 1977), as does the bacterial toxin colicin E<sub>3</sub> (Bowman et al., 1971a), it was thought possible that the cells contained an immunity protein similar to that reported for colicinogenic cells (Bowman et al., 1971b; Jakes and Zinder, 1974). Therefore, the S-30 extract of A.giganteus cells was confronted with lower concentrations of alpha sarcin. The S-30 was found to be just as sensitive as a yeast S-30 extract (data not given). Additionally, ribosomes and S100 were prepared from the S-30 extract of A.giganteus cells and yeast or A.giganteus ribosomes were confronted with alpha sarcin using S100 from A.giganteus and that from yeast. Again both types of ribosome were inhibited equally and there

FIGURE 10.3 Effect of Alpha Sarcin on the Ribosomes of Yeast or A.giganteus





Legend to Figure 10.3

Effect of Alpha Sarcin on the Ribosomes of  
Yeast or A.giganteus

Mixtures (50  $\mu$ l) for poly U-directed protein synthesis (Section 3.14) containing yeast strain Y<sub>166</sub> ribosomes (12 pmol) or A.giganteus ribosomes (12 pmol) and (a) yeast S100; (b) A.giganteus S100 were incubated with alpha sarcin ( (a) 10  $\mu$ g/ml; (b) 0.1  $\mu$ g/ml final concn.) at 25°C. Samples (5  $\mu$ l) were precipitated with TCA (Section 3.15) and radioactivity in the precipitates estimated by liquid-scintillation counting (Section 3.16).

——— , controls; -----, plus alpha sarcin.

●, yeast ribosomes; o, A.giganteus ribosomes.

was no difference in inhibition using yeast or A.giganteus S100 extract (Fig.10.3). Finally, alpha sarcin was preincubated with S100 from A.giganteus prior to the addition of yeast ribosomes in order to determine whether the S100 could detoxify alpha sarcin. This procedure, however, had no effect upon inhibition by alpha sarcin (data not given).

It would appear, therefore, that A.giganteus is resistant to alpha sarcin during trophophase by virtue of a cell-permeability phenomenon. Further experiments are needed to determine whether the ribosomes of A.giganteus remain sensitive whilst the antibiotic is being produced. If the ribosomes do remain sensitive, then the mechanism of resistance to alpha sarcin will be of great interest.

Presumably, alpha sarcin, a polypeptide of molecular weight 16,000 daltons (Schuurmans, et al., 1964) is made on the ribosome rather than by a series of specific enzymes. Therefore, in the absence of resistant ribosomes, alpha sarcin would have to be produced either as an inactive precursor or in the presence of immunity proteins in order to protect those ribosomes. It is known that alpha sarcin contains the unusual amino acid, sarcinine (Olson and Goerner, 1965) and, since this amino acid may be required for

antibiotic-activity, the cell may avoid suicide by converting the precursor amino acid to sarcosine immediately before alpha sarcosine is exported.

Alternatively, the precursor may be produced on rough endoplasmic reticulum where the growing chain is drawn through the membrane into the lumen, thus, preventing contact of the completed molecule with the ribosome.

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THE INTERACTIONS OF ANTIBIOTICS WITH EUKARYOTIC  
RIBOSOMES

- by -

Adrian N.Hobden

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ABSTRACT

Precipitation of peptidyl-tRNA by cetyltrimethylammonium bromide affords an extremely convenient and accurate means of following the reaction of nascent polypeptides with puromycin. Using this assay, I have investigated the modes of action of several antibiotics which inhibit polypeptide chain elongation. The polypeptide, alpha sarcin, has been shown to inhibit the binding of aminoacyl-tRNA to the ribosomal 'A' site as have the antibiotics, chartreusin and emetine. Additionally, it has been confirmed that sparsomycin and trichodermin inhibit the peptidyltransferase whereas cycloheximide probably inhibits translocation.

The technique for measuring peptidyl-tRNA concentrations has also been used to detect inhibitors of initiation of protein synthesis. By this and other techniques, homoharringtonine has been shown to inhibit a step in initiation - possibly the same step as that inhibited by the chemically-unrelated antibiotic T-2 toxin.

Cell-free extracts of S.cerevisiae and M.verrucaria have been prepared which are very active in poly U-directed protein synthesis. These extracts have been used to study resistance to the 12,13 epoxytrichothecene group of toxins either in a yeast mutant, strain TR<sub>1</sub>, or in the organism M.verrucaria which produces several 12,13 epoxytrichothecenes. Both strain TR<sub>1</sub> and M.verrucaria are resistant to T-2 toxin in vitro because of features of their 60S ribosomal subunits.

The effect of the 12,13 epoxytrichothecene, trichodermin, on poly U-directed protein synthesis by yeast cell-free extracts was also investigated. It was found that there were apparently two types of synthesis - one sensitive and one insensitive to trichodermin. The ratio of the two types of synthesis appeared independent of the manner in which the ribosomes were prepared but was influenced by ion concentrations and the mode of preparation of the high-speed supernatant fraction (S100). Both types of poly U-directed protein synthesis involved production of polyphenylalanine rather than peptidyl-polyphenylalanine.

Cell-free extracts capable of poly U-directed protein synthesis were also produced from strains of the 12,13 epoxytrichothecene-producing organisms F.poae, F.sporotrichiodes and F.equiseti and the fungi, A.giganteus, which produces alpha sarcin. All the organisms were sensitive to their products in vitro but resistant in vivo.