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PROPERTIES OF 70 S AND 80 S RIBOSOMES FROM TOBACCO LEAVES

**Eigenschappen van 70 S en 80 S ribosomen
uit tabaksbladeren**

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I. INTRODUCTION

The process of plant virus multiplication is still a subject of intensive research, although much is known about the effect of a virus infection. Viral RNA is responsible for its own multiplication as well as for the synthesis of virus specific proteins. Virus multiplication has to take place as a sequence of specific steps. The RNA polymerizing enzymes have to be synthesized before the RNA can be multiplied, coat protein and other proteins will be produced and finally the virus particles will be assembled.

Since viral RNA itself is able to act directly as a messenger for protein synthesis, the following questions arise. What interaction takes place between the viral RNA and the plant ribosomes during the sequence of steps in virus multiplication. In which way is the structure of the viral RNA responsible for the specific order of these steps.

At least two types of ribosomes occur in green plants, viz., 70 S and 80 S ribosomes. Little is known about the interaction of messenger RNA with 80 S ribosomes. Because of the many differences between 70 S and 80 S ribosomes it is interesting to study such an interaction with both 70 S and 80 S ribosomes. For such studies viral RNA can be used as a natural messenger. Stimulation of amino acid incorporation in a system for protein synthesis *in vitro* can be used as a means to measure messenger activity of RNA.

In 1961 Nirenberg and Matthaei prepared a system from *E. coli* which was highly active in synthesizing protein *in vitro*. When RNA from bacterial viruses was used as a messenger in such a system proteins could be synthesized one of which corresponded to the viral coat protein (NATHANS et al., 1962; CAPECCHI, 1966). When plant viral RNA was used as a messenger in the *E. coli* system various difficulties arose. Only when mono and dicistronic plant viral RNAs were used the results indicated that synthesis of specific viral proteins had taken place (CLARK et al., 1965; VAN RAVENSWAAY CLAASEN, 1967). Larger plant viral RNAs, such as TMV-RNA, however, did not lead to conclusive results (AACH et al., 1967; SCHWARTZ, 1967). This might be due to a difference in the interaction of plant viral RNA with 70 S bacterial ribosomes *in vitro* and with 80 S ribosomes from the plant cytoplasm *in vivo*. The secondary structure of the longer RNA molecules may also be involved in this difference. The negative results obtained with plant viral RNAs in a heterologous bacterial cell-free system made it of interest to study the interaction between plant viral RNA and ribosomes in a homologous cell-free system. Plant viral RNA has been found to occur in the ribosomal fraction of TMV-infected tobacco leaves (VAN KAMMEN, 1963). The presence of 80 S as well as 70 S ribosomes in green leaves made it possible to compare the interaction of both types of ribosomes with messenger RNA in a homologous system.

In the present article we describe our efforts to prepare from tobacco leaves a system for the *in vitro* synthesis of protein. First the relevant literature will be reviewed. The characteristics of this cell-free system will be described in

chapter 3. The separation of 70 S and 80 S ribosomes is described in chapter 4 and some of the physical properties of these ribosomes are compared. The ability of these ribosomes to incorporate amino acids into polypeptides is discussed in chapter 5. As a result of these studies methods were developed for studying the interaction of exogenous messengers with both types of ribosomes. In these experiments poly U was used as an exogenous messenger. As yet, no satisfactory results have been obtained with viral RNAs (chapter 6).

These results and the difficulties encountered in studying protein synthesis in vitro will be discussed in chapter 7.

1.1. DIFFERENCES BETWEEN THE VARIOUS SIZE CLASSES OF RIBOSOMES

Ribosomes are ribonucleoprotein particles and have an important function in protein synthesis. In fact, protein synthesis occurs on the surface of the ribosomes. Ribosomes are composed of 40–60% of RNA and of 60–40% of protein, depending on the organism. They have been isolated from a vast number of organisms (see for a review PETERMANN, 1964). They have often been found to occur in clusters when observed in vivo. Such clusters consist of several ribosomes attached to a messenger RNA molecule. However, it is difficult to isolate these so-called polyribosomes because of their rapid breakdown by RNases. WETTSTEIN, STAEHELIN and NOLL (1963) have been able to isolate a considerable part of the ribosomes from rat liver in the form of polyribosomes. They introduced the name *ergosome* for the functionally active polyribosome. It has been more difficult to isolate polyribosomes from plant material and only a very small part of the total ribosomal material was obtained as polyribosomes. Using a nuclease inhibitor, CLARK, MATTHEWS and RALPH (1963) were able to isolate polyribosomes from Chinese cabbage, with an average sedimentation constant of 200 S. Light stimulated the formation of polyribosomes. At the end of the dark period 50–80% of the ribosomes was present in the monoribosome form. The polyribosomes increased from the beginning of the light period until 90% was present in forms greater than 80 S. *Chenopodium album* proved to be a good source for the isolation of polyribosomes in the absence of nuclease inhibitors (LYTTLETON, 1967).

Chloroplasts were found to have their own class of ribosomes, which differs from the cytoplasmic class of ribosomes (LYTTLETON, 1962). Polyribosomes have also been isolated from chloroplasts and their formation was even more rapid after exposure to light than that of cytoplasmic polyribosomes (CLARK, 1964). The chloroplast ribosomes in Chinese cabbage represent only about 25% of the total leaf ribosomes and the two classes of ribosomes could not be separated from each other (CLARK, MATTHEWS and RALPH, 1964). Under our experimental circumstances (2.1) we usually found even a lower percentage of chloroplast ribosomes, but this was highly dependent on the conditions of growth (4.1).

BARKER and RIEBER (1967) have isolated polyribosomes from pea seed and LEAVER and KEY (1967) from carrot roots. CHEN and WILDMAN (1967) showed

their presence in tobacco leaves. They have also been found in the latter material in the present study (chapter 4). These findings support the generally accepted view that ribosomes active in protein synthesis occur in the form of polyribosomes.

1.1.1. *Functional differences*

Although most of the ribosomes are present in vivo as polyribosomes, yet the monoribosome should be considered in comparing the different classes of ribosomes. Ribosomes have been found to sediment in the analytical ultracentrifuge with a sedimentation coefficient in the range of 70 to 80 S.

With the introduction of new separation techniques, like zonal centrifugation and polyacrylamide gel electrophoresis, a more satisfactory classification of the different classes of ribosomes and their RNAs became possible. It has been generally accepted that ribosomes from prokaryotes are of the 70 S type, whereas those from eukaryotes were thought to be also of one kind: the 80 S type. The discovery by LYTTLETON (1962) that chloroplasts have their own, characteristic ribosomes has led to a detailed search resulting in the discovery of different protein synthesizing systems each using their own specific ribosomes (see for a review also SVETAILO, PHILIPPOVICH and SISSAKIAN, 1967). BRAWERMAN (1963) found characteristic differences in the base composition of ribosomal RNA isolated from *Euglena gracilis* chloroplasts as compared to that obtained from the cytoplasm. TEWARI and WILDMAN (1966) have isolated a specific DNA from tobacco chloroplasts, different from nuclear DNA. This DNA was shown to be responsible for the synthesis of chloroplast proteins (GOFFEAU and BRACHET, 1965). Similar results were obtained by SCHWEIGER and BERGER (1964), also with tobacco leaves, by SHAH and LYMAN (1966) with *Euglena gracilis* and by CHAPMAN, NUGENT and SCHREIBER (1966). The latter authors demonstrated that in chloroplasts of *Acetabularia mediterranea* RNA synthesis occurs beside protein synthesis. Recent hybridization experiments by TEWARI and WILDMAN (1968) have confirmed that chloroplast DNA contains cistrons coding for chloroplast ribosomal RNA but not for cytoplasmic ribosomal RNA. Nuclear DNA, however, was said to contain cistrons coding for both cytoplasmic and chloroplast ribosomal RNA. RICHARDS (1967) found that considerable hybridization is possible between chloroplast DNA and nuclear DNA. Assuming that chloroplasts are autonomous organelles, these findings are not easily understood.

Concerning mitochondria a similar development has taken place (see for literature review DURE, EPLER and BARNETT, 1967 and POLLARD, STEMLER and BLAYDES, 1966). Also, *Neurospora crassa* mitochondria have been found to contain some specific tRNAs and aminoacyl-tRNA synthetases different from the cytoplasmic ones (BARNETT and EPLER, 1966). These findings have led DURE et al. (1967) to conclude that within mitochondria and chloroplasts a complete sequence of transcriptional and translational events takes place and that the macromolecules involved are unique for the organelle and distinct from those found in and used by the cytoplasm.

Species specificity in protein synthesis has been demonstrated by PARISI, MILANESI, VAN ETEN, PERANI and CIFERRI (1967). They prepared an amino acid incorporating system from *E. coli*, *Bacillus subtilis* (70 S class), *S. cerevisiae*, castor bean seedlings and rat liver (80 S class). Within each class it was possible to mix ribosomes and supernatant enzymes from different organisms. Mixing of ribosomes and enzymes prepared from organisms having ribosomes of the two different classes, however, gave very little if any synthesis. It was concluded that this incompatibility was due to the lack of interaction between ribosomes of one size and polymerizing enzymes extracted from organisms containing ribosomes of the other size.

1.1.2. Chemical and physical differences

Beside on the basis of functional properties, ribosomes can also be classified on their chemical and physical properties.

It is now generally accepted that ribosomes consist of two subunits, a smaller and a larger one, each containing one major RNA molecule¹ (CLICK and TINT, 1967). In many cases it has been difficult or impossible to isolate the intact RNA from ribosomes, probably because of degradation during the isolation (SPENCER and WHITFIELD, 1966; LOENING and INGLE, 1967). NOLL (1967) developed a method to demonstrate differences in sedimentation coefficient of 5% by using isokinetic gradients and used this method to characterize ribosomes and their RNAs from different organisms (KÜNTZEL and NOLL, 1967). Their results are summarized in table 1.1. The smaller RNA component isolated from bean cytoplasmic ribosomes sedimented at the same rate as the analogous component from mitochondrial, *E. coli* and chloroplast ribosomes. LOENING and INGLE (1967), using polyacrylamide gel electrophoresis, were able to distinguish between the smaller plant cytoplasmic ribosomal RNA and the smaller RNA of the other ribosomal class. They calculated a sedimentation coefficient of 18 S for the former. This difference could not be detected by gradient centrifugation (STUTZ and NOLL, 1967).²

TABLE 1.1. Sedimentation coefficients of ribosomes and their RNAs (according to KÜNTZEL and NOLL, 1967).

Source of ribosomes	Relative S value	S values of RNA components	
Rat liver (cytoplasm)	80.0	30.4	18.9
Bean (cytoplasm)	78.6	26.5	16.0
<i>Neurospora</i> (cytoplasm)	76.9	25.8	16.5
<i>Neurospora</i> (mitochondria)	73.2	23.0	16.2
<i>E. coli</i>	70.0	22.6	16.0
Bean (chloroplasts)	66.8	22.6	15.9

¹ The larger one contains an additional small RNA component: the 5 S RNA.

² Very recently RAWSON and STUTZ (1969) have found that 87 S ribosomes, containing 24 S and 20 S RNA components, occur in the cytoplasm of *Euglena gracilis*. They were able to separate on sucrose gradients these RNAs from each other and from the 22 S and 17 S RNAs occurring in the 68 S chloroplast ribosomes.

On the basis of the above data ribosomes have been divided into three classes, i.e., ribosomes from bacteria and organelles, ribosomes from plant cytoplasm and ribosomes from animal cytoplasm. This division into three classes can also be seen with several properties of the ribosomes. In table 1.2 the base composition of ribosomal RNAs from several organisms is summarized. There are clear differences in base composition of the RNAs between each of the groups, although within each group there is a certain degree of variation (see for a survey PETERMANN, 1964). Also, there is a difference in the composition of the larger and the smaller component. Thus, each component must have its own cistrons on the DNA (CLICK and HACKETT, 1966).

TABLE 1.2. Base composition of various ribosomal RNAs

Source of the ribosomes	S value of the RNA	A	G	U	C
<i>E. coli</i> (a)	23	25.4	33.5	19.6	21.5
	16	24.8	31.0	21.5	22.7
<i>Neurospora</i> (b)					
cytoplasm	25 + 17	24.1	28.1	24.3	21.1
mitochondria	21 + 16	27.2	22.9	29.8	14.8
Spinach (c)					
cytoplasm		24.6	33.0	18.8	23.6
chloroplasts		25.8	33.4	17.3	23.5
Potato tuber (d)	25	25.1	31.7	21.2	22.0
	16	25.4	27.2	25.2	22.2
<i>Chlorella</i> (e)					
cytoplasm		22.7	29.5	19.6	27.1
chloroplasts		29.3	27.4	23.9	19.4
Rat liver (f)	30 + 19	20.9	27.0	23.0	27.1

(a) MIDGLEY (1962)

(b) KÜNTZEL and NOLL (1967)

(c) SPENCER and WHITFIELD (1966)

(d) CLICK and HACKETT (1966)

(e) BRAWERMAN (1963)

(f) GOSWAMI, BARR and MUNRO (1962)

Another property which bacterial, chloroplast and mitochondrial ribosomes have in common is the inhibition of protein synthesis by chloramphenicol but not by cycloheximide, whereas 80 S ribosomes are inhibited by cycloheximide but not by chloramphenicol (WINTERSBERGER, 1965; MARGULIES, 1964; WHEELDON and LEHNINGER, 1966; PARTHIER, 1965).

Finally, the three classes of ribosomes differ remarkably in their dissociation behaviour in low Mg^{2+} concentrations. As *E. coli* ribosomes chloroplast ribosomes very easily dissociate into their subunits at Mg^{2+} concentrations of 10^{-3} M or lower. This dissociation is reversible (BOARDMAN, FRANCKI and WILDMAN, 1966; the present article, 4.3).

Plant cytoplasmic ribosomes do not dissociate as easily as chloroplast ribosomes, and this dissociation is only partially reversible (see chapter 4 of the present article). Animal ribosomes dissociate reversibly into their subunits at low Mg^{2+} concentrations, but apparently, the smaller subunit is very unstable,

since both subunits are not present in equal amounts (Ts'o and VINOGRAD, 1961).¹ When comparing the different classes of ribosomes KÜNTZEL and NOLL (1967) and REISNER, ROWE and MACINDOE (1968) concluded that the ribosomes and their RNAs have become larger during the evolution of life. The latter authors suggested that photosynthesis must have its origin at the very threshold of life. They made this suggestion because chloroplast ribosomes have the lowest sedimentation coefficient and photosynthetic bacteria have similar ribosomes (see table 1.1). Respiration might have developed later. Both functions have been preserved in separate cellular compartments in higher plants.

With the enlargement of ribosomes during evolution the structure of their RNAs became more complicated, as revealed by the pattern of a nuclease digest of ribosomes from the various classes on polyacrylamide gels. With increasing size of the ribosomes there is an increase in the resistance of the RNA to enzymatic hydrolysis (GOULD, BONANOU and KANAGALINGHAM, 1966). These experiments, of course, do not provide sufficient evidence as yet for the conclusion that there is a complete parallelism between the functional and the chemical specificity of the different types of ribosomes.

1.2. PROTEIN SYNTHESIS IN VITRO

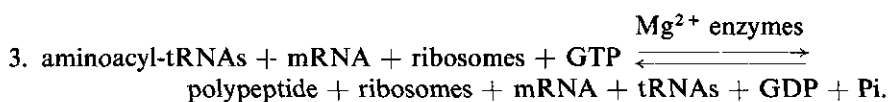
In this section first the mechanism of protein synthesis will be briefly summarized. Then protein synthesizing systems extracted from various plants will be discussed.

1.2.1. Mechanism of protein synthesis

The biosynthesis of proteins is a complicated series of reactions. In this process the amino acids are linked to each other by means of peptide links between the carboxyl-group of one and the α -amino-group of a second amino acid. The amino acid sequence is determined genetically. These reactions can be summarized as follows:

1. amino acid + aminoacyl-tRNA synthetase + ATP $\xrightleftharpoons{\text{Mg}^{2+}}$ aminoacyl-adenylate-enzyme + PP.
 Mg^{2+}
2. aminoacyl-adenylate-enzyme + tRNA $\xrightleftharpoons{\text{Mg}^{2+}}$ aminoacyl-tRNA + aminoacyl-tRNA synthetase + AMP

¹ After the experiments described in this article had been concluded, it was found by Wool and coworkers that skeletal muscle ribosomes, when exposed at 28°C to 880 mM potassium chloride in the presence of 12.5 mM Mg^{2+} , dissociate into 40 S and 60 S subunits. Removal of the potassium chloride led to reassociation into 80 S ribosomes able to catalyse protein synthesis in the presence of added template RNA. It was also possible to reassociate subunits derived from rat, rabbit and *Tetrahymena pyriformis* ribosomes into active hybrid ribosomes. It would be of interest to see whether plant ribosomes will behave similarly (MARTIN, ROLLESTON, Low and WOOL, 1969; MARTIN and WOOL, 1969).



In the first step, the amino acid activation, each amino acid is raised to a higher energy level by means of a specific enzyme and ATP as an energy source in order to make possible the formation of an energy requiring peptide link. The activated amino acid is transferred to a tRNA, specific for this amino acid, to which it becomes attached by means of an ester bond between the carboxyl group of the amino acid and the 3'OH-group of the ribose moiety of the terminal adenosine residue of the tRNA (2).

The third step is the most complicated one. Amino acids are arranged in the right order by means of their tRNAs as directed by the messenger RNA. Each time 3 successive nucleotides in a tRNA (anticodon) are bound to 3 nucleotides on the mRNA (codon) according to the WATSON and CRICK base pairing principle. The α -amino-group of an amino acid will form a bond with the carboxyl-group of the previous one. In order to make these reactions possible, aminoacyl-tRNAs, messenger RNA and enzymes are maintained in the proper conformation by the ribosomes. It is generally accepted now that on each ribosome there are two sites to which tRNA is bound, i.e., a peptidyl site, which is occupied by the last tRNA carrying the growing polypeptide chain and an aminoacyl site, occupied by the next aminoacyl-tRNA to which the peptide chain is transferred. Then the discharged tRNA will be released from the messenger-ribosome-complex. The ribosome and the mRNA will shift one triplet with respect to each other and the process will be repeated with another incoming aminoacyl-tRNA. In this way peptides will grow step by step from the amino end to the carboxyl end. They remain continuously attached to tRNA molecules with their C-terminal amino acid. For chain initiation and termination special mechanisms exist involving specific tRNAs and factors.

Recently, the process of protein synthesis has been reviewed by SCHWEET and HEINTZ (1966), NOVELLI (1967), VAN RAVENSWAAY CLAASEN (1967), REINECKE (1968) and AB (1968).

1.2.2. *Protein synthesis by systems extracted from plants*

Some of the first experiments on the amino acid incorporation in vitro were carried out with plant material. RAACKE (1959) and WEBSTER (1959) obtained a very active system from peas. This, however, could not be confirmed (CAMPAGNE and GRUBER, 1962; LETT and TAKAHASHI, 1962; LETT, TAKAHASHI and BIRNSTIEL, 1963).

The isolation of a very active cell-free system from *E. coli* (MATTHAEI and NIRENBERG, 1961; NIRENBERG and MATTHAEI, 1961) attracted the attention largely to the *E. coli* system. However, the interest in ribosomes from higher organisms remained, because they differ from those in the bacteria. Furthermore, the isolation of a special class of ribosomes from spinach chloroplasts, different from the known plant ribosomes (LYTTLETON, 1962), and the demon-

stration of amino acid activating enzymes in spinach chloroplasts (BOVÉ and RAACKE, 1959), may have given a new impetus to the investigation of the synthesis of proteins in vitro by means of cell-free plant systems. First three of the most important and extensive investigations will be described.

MANS and NOVELLI (1964) reported a very active system for the in vitro incorporation of amino acids prepared from maize seedlings which possessed all the characteristics of true polypeptide synthesis. The authors were able also to isolate tRNA and soluble enzymes from maize.

These were demonstrated to stimulate the amino acid incorporation (MANS, PURCELL and NOVELLI, 1964). Over 1000 $\mu\mu$ moles of labeled leucine were incorporated in this maize system, but the authors indicated that other amino acids were much less incorporated. Moreover, GRAEBE and NOVELLI (1966) obtained a leucine incorporating system from tissue cultures of maize endosperm which proved to be characteristic in its requirements with respect to ions, high-energy compounds etc. However, not even 100 $\mu\mu$ moles per mg protein were incorporated, which is comparable to the low activity found in the other plant systems. Therefore, it is questionable, whether the previous system was really as active as was described. WILLIAMS and NOVELLI (1968) have found that maize seedlings grown in the dark are less active in the in vitro incorporation of amino acids than plants grown in the light, and they suggested that light might control protein synthesis in plants, possibly only indirectly by stimulating messenger synthesis. In this connection it is noteworthy that CLARK (1964) and CLARK, MATTHEWS and RALPH (1964) were able to demonstrate that light induces the formation of polyribosomes in Chinese cabbage leaves. These authors demonstrated that this phenomenon was associated with the synthesis of new RNA.

BRAWERMAN (1963) isolated chloroplast ribosomes from *Euglena gracilis*. This organism is colourless when grown in the dark. When exposed to light, the formation of chloroplasts and of chloroplast ribosomes was induced and there appeared large amounts of proteins associated with the chloroplast structure. Thus, the chloroplast ribosomes may be responsible for the synthesis of these specific proteins. These ribosomes (EISENSTADT and BRAWERMAN, 1963) as well as the cytoplasmic ribosomes (EISENSTADT and BRAWERMAN, 1964a), were able to incorporate amino acids in vitro, but only the chloroplast ribosomes could be stimulated by added mRNA. The specific activities of both classes of ribosomes appeared to be equal, but their sensitivity to chloramphenicol was different (EISENSTADT and BRAWERMAN, 1964b). The chloroplast ribosomes proved to possess a low affinity for messenger RNA and it was difficult to isolate these ribosomes without loss of the endogenous messenger. BRAWERMAN and EISENSTADT (1964) suggested that the two classes of ribosomes might differ in their interaction with messenger RNA and this might have a specific regulatory function in the process of protein synthesis. Physiological alterations in this process might operate in part through modifications at the ribosome level.

SPENCER and WILDMAN (1964) studied the amino acid incorporating activity in cell-free extracts from tobacco leaves and concluded that the major activity

is found in the chloroplasts. This activity proved to be localized in the mobile phase of the chloroplasts, which surrounds the stationary, chlorophyll containing component (FRANCKI, BOARDMAN and WILDMAN, 1965). When released from the chloroplasts, most of the activity was associated with monoribosomes (BOARDMAN, FRANCKI and WILDMAN, 1965). These ribosomes, the 70 S chloroplast ribosomes, occurred in the same quantity in tobacco leaves as the 80 S cytoplasmic ribosomes, but were 10 to 20 times more active in protein synthesis. However, most of the activity was lost when the chloroplasts were disrupted together with the whole leaves, and not purified first as whole chloroplasts. When chloroplast ribosomes were pelleted, their activity decreased also and it was demonstrated that no nuclease action or deficiencies in tRNA or enzymes were responsible for this loss in activity (BOARDMAN, FRANCKI and WILDMAN, 1966). On the other hand, cytoplasmic ribosomes were more active after purification. The two classes of ribosomes differed in their magnesium requirements for maximum incorporating activity.

Several other organisms have also been used for preparing cell-free systems. From *Chlorella pyrenoidosa* a system was prepared, incorporating amino acids into acid-insoluble material (GALLING, 1966). HALL and COCKING (1966) found amino acid incorporation by an aseptically isolated chloroplast fraction from tomato seedlings, which also showed photosynthetic activity. ALLENDE and BRAVO (1966) isolated an amino acid incorporating system from wheat embryos, which had a low endogenous activity. A great stimulation, however, was obtained when poly U was added. The maize seedling system was also stimulated by poly U (WILLIAMS and NOVELLI, 1968), whereas the other plant systems described were not. This may indicate a special property of seedlings and embryos.

BARKER and RIEBER (1967), found that dormant pea seeds do not contain polyribosomes, whereas imbibition led to rapid polyribosome formation, active in amino acid incorporation. ELLIS and MACDONALD (1967) showed that microsomal fractions from sterile disks of red beet root are active in amino acid incorporation. Ageing the disks stimulated the activity. This may be caused by messenger RNA synthesis during ageing. LEAVER and KEY (1967) came to similar conclusions. When they were ageing carrot root tissue, a rapid increase of polyribosomes was one of the earliest biochemical changes observed, which also suggests messenger RNA synthesis. From discs of Jerusalem artichoke tubers CHAPMAN and EDELMAN (1967) isolated a cell-free protein synthesizing system. The activity increased with ageing of the discs. It was shown to be associated largely with the mitochondria.

1.3. MESSENGER ACTIVITY OF VIRAL RNA

Shortly after MATTHAEI and NIRENBERG (1961) had discovered that synthetic messenger RNAs can be translated by a system synthesizing proteins in vitro, successful attempts were made to synthesize in vitro specific proteins, using viral RNAs as naturally occurring messengers. NATHANS, NOTANI, SCHWARTZ

and ZINDER (1962) were the first investigators to report the synthesis of a specific protein. They used *E. coli* extracts and RNA from the coli phage f2. Later on it was demonstrated that this RNA is able also to direct the synthesis of its coat protein in a cell-free system derived from *Euglena gracilis* (SCHWARTZ, EISENSTADT, BRAWERMAN, ZINDER, 1965). This means that at least with bacteriophage f2-RNA there does not exist a barrier for the translation in a heterologous system. CAPECCHI (1966), using bacteriophage R17-RNA in the *E. coli* system, was able to demonstrate the synthesis of two different functional proteins.

Also plant viral RNAs have been used for such studies. When larger RNAs such as TMV-RNA and TYMV-RNA, were used, it was found that *E. coli* ribosomes do accept them as a messenger, but no specific products were found (AACH et al., 1964; VOORMA, 1965; SCHWARTZ, 1967).

So the earlier statement that TMV-RNA is able to direct the synthesis of its coat protein in the *E. coli* system proved to be erroneous (NIRENBERG and MATTHAEI, 1961; TSUGITA, FRAENKEL-CONRAT, NIRENBERG and MATTHAEI, 1962). Small viral RNAs consisting of 1 or 2 cistrons, as sTNV-RNA and AMV – top a component – RNA, however, have been said to be able to direct the synthesis of virus specific proteins when used in the *E. coli* cell-free system (CLARK, CHANG, SPIEGELMAN and REICHMANN, 1965; VAN RAVENSWAAY CLAASEN, 1967; REINECKE, 1968). Also, a stimulation of the amino acid incorporation in the *E. coli* system has been observed with brome grass mosaic virus RNA, but this did not constitute sufficient evidence for the synthesis of specific proteins (STUBBS and KAESBERG, 1967).

2. MATERIALS AND METHODS

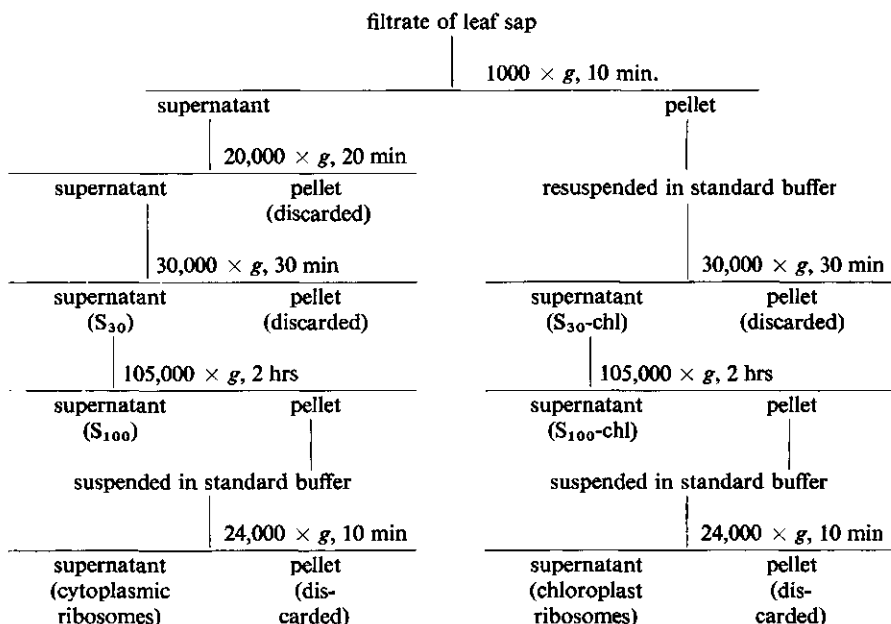
2.1. PLANT MATERIAL

In all experiments plants of *Nicotiana tabacum* var. Samsun (Turkish tobacco) were used. Plants were grown in soil consisting of a mixture of sand, compost, leaf mold and farm yard manure. The soil was sterilized by steaming for 2 hours at 100°C and sieved afterwards. The seeds were sown in sterile baskets. After about 3 weeks the seedlings were transplanted into boxes and after another 2 weeks the plants were placed in pots. During the whole period of growth the plants were kept in a growing chamber under controlled lighting conditions (16 hours light of about 19,000 erg/sec. cm², produced by 40 W Philips 'TL' fluorescent tubes, white light, and 8 hours darkness per 24 hours), temperature (18–20°C), and humidity (70–80% relative humidity).

2.2. PREPARATION OF THE RIBOSOME FRACTIONS

Ribosomes were prepared from the cytoplasm as well as from the chloroplasts, as will be described in 2.2.1 and 2.2.2, respectively. A survey of the fractionation procedure is given in scheme 2.1. Both ribosome preparations consisted of several components. The purification of these components will be described in 2.3.

SCHEME 2.1. Flow sheet of the fractionation procedure



2.2.1. *Preparation of ribosomes from the cytoplasm*

Ribosomes were isolated from leaves of two months old plants, which had about 10 leaves. The upper 4 to 5 leaves were picked, washed in distilled water, dried between sheets of filterpaper after removing the midribs and chopped into a fine mince with a razor blade in a 0.05 M Tris buffer pH 7.2, containing 0.5 M sucrose, 0.01 M magnesium acetate and 0.006 M MCE (1 ml of buffer per g of leaf material). The whole procedure was performed in the cold room at 0–4°C. The homogenate was filtered through cheese cloth. The filtrate was centrifuged at $1000 \times g$ for 10 minutes. The pellet was used for preparing the ribosomes from the chloroplasts (2.2.2). The supernatant was centrifuged first at $20,000 \times g$ for 20 minutes and then at $30,000 \times g$ for 30 minutes. From the supernatant (S_{30}) the ribosomes were sedimented by centrifugation at $105,000 \times g$ for 2 hours in a preparative ultracentrifuge (Spinco, model L.) The supernatant (S_{100}) was used for preparing the soluble enzyme fraction (see chapter 2.7). The pellet was suspended in a standard buffer (0.01 M Tris, pH 7.8, 0.06 M KCL (or 0.075 M NH_4Cl), 0.01 M MgCl_2 and 0.006 M MCE) and the suspension was cleared by centrifugation at $24,000 \times g$ for 10 minutes. The supernatant contained the cytoplasmic ribosomes. The method used for preparing the cytoplasmic ribosomes was the same method as that used by VAN KAMMEN (1967a).

2.2.2. *Preparation of ribosomes from chloroplasts*

The $1000 \times g$ pellet, obtained during the preparation of the ribosomes as described in 2.2.1, was washed carefully and then suspended, both in standard buffer, in order to disrupt the chloroplasts. This suspension was centrifuged during 30 minutes at $30,000 \times g$. The ribosomes were sedimented from the supernatant ($S_{30}\text{-chl}$) by centrifugation at $105,000 \times g$ for 2 hours. The pellet was suspended in standard buffer and the suspension was cleared by centrifugation at $24,000 \times g$ for 10 minutes. The supernatant contained the chloroplast ribosomes. This method was very similar to that used by SPENCER and WILDMAN (1964).

2.3. PURIFICATION OF RIBOSOMES BY SUCROSE GRADIENT CENTRIFUGATION

- a. The ribosomal fraction, as obtained in 2.2.1, consisted of several components which initially were separated according to WETTSTEIN, STAEHELIN and NOLL (1963) in the following way:

In a tube of the Spinco SW 25 rotor two layers of 5 ml (2 M and 0.5 M sucrose in standard buffer, respectively) were pipetted and on top of these about 16 ml S_{30} (see 2.2.1), was layered. The tubes were run at 25,000 rpm for 4–5 hours. After the run polyribosomes, if present, were found as a pellet and the monoribosomes in the interphase between the two sucrose layers. The monoribosomes were removed by means of a hypodermic syringe, diluted two to threefold with standard buffer and the ribosomes were sedimented from the suspension by centrifugation at $105,000 \times g$ for 2 hours. Monoribosomal and

polyribosomal pellets were each suspended in standard buffer and the suspensions were cleared by centrifugation at $24,000 \times g$ for 10 minutes.

b. The method of 2.3.a resulted in the elimination of low molecular weight substances together with small chloroplast fragments. When no fractionation of the ribosomes was desired and only washed ribosomes were needed, this method was modified. Ribosomes were then layered on 3 ml 0.5 M sucrose in standard buffer and centrifuged in the Spinco R 40 rotor at $105,000 \times g$ for 2–3 hours. This procedure made unnecessary a second run and increased the ribosome yield, as ribosomes could never be isolated quantitatively from the interphase (2.3.a). The pellets were suspended again in standard buffer and the suspension was clarified. The supernatant will be referred to as purified ribosomes.

c. Ribosomes as prepared in 2.2 and 2.3 contained 70 S and 80 S ribosomes as well as polyribosomes. For separating these components ribosomes were layered on a gradient consisting of layers of 40, 30, 20 and 10% sucrose in standard buffer, 6 ml each. Runs were at 25,000 rpm in the SW 25 rotor for 3 hours. The gradients were prepared about 1 hour before use as a discontinuous gradient. During centrifugation the components became distributed in specific layers. These layers were removed as described in 2.4.

2.4. FRACTIONATION OF SUCROSE GRADIENTS

a. In most cases the components, as obtained in 2.3.a, were removed from the tubes with a hypodermic syringe fitted with a bent needle. After dilution with standard buffer they were sedimented by centrifugation at $105,000 \times g$ for 2–3 hours. The pellets were suspended in standard buffer and the suspensions were cleared by centrifugation.

b. In some experiments the bottom of the tube was pierced with a hollow needle, through which the whole tube was emptied dropwise. Thus, fractions of any desirable volume could be obtained.

c. Another method used for isolating various components from sucrose gradients was described by VAN KAMMEN (1967b). This method consists of pumping CCl_4 into the tube at a constant rate through a hole pierced into the wall, close to the bottom. The tube was first closed with a cap ground conically at the inner side. A needle was inserted into a hole in the middle of the cap and ultraviolet absorption at 254 m μ was recorded continuously with an LKB Uvi-cord. The zones containing the various components were collected and the ribosomes were concentrated by centrifugation at $105,000 \times g$.

2.5. PURIFICATION OF 70 S AND 80 S RIBOSOMES BY ZONAL CENTRIFUGATION

At the end of this investigation 70 S and 80 S ribosomes were purified by zonal centrifugation, using the B₁₄ zonal rotor of the MSE superspeed 50TC ultracentrifuge. The rotor was filled with a gradient of 10–40% sucrose in standard buffer linear by volume, (350 ml 10% and 350 ml 40% sucrose); 10

ml of a ribosome suspension in 2% sucrose in standard buffer were pumped on top of the gradient with 100 ml standard buffer as an overlayer. The rotor was run at 30,000 (35,000) rpm for 5 (3½) hours and the gradient was pumped out of the rotor by 45% sucrose in water and collected in fractions of about 10 ml. These fractions were scanned in a Zeiss or Beckman DU spectrophotometer by measuring the extinction at 260 mμ. The desired fractions were collected and the ribosomes were sedimented at 105,000 × g. This method resulted in very pure 70 S and 80 S ribosomes.

2.6. DETERMINATION OF THE RIBOSOME CONCENTRATION

The concentration of the ribosomes was determined according to VAN KAMMEN (1963). The absorbancy of a water-diluted sample was read at 260 mμ against an appropriate blank, using $A_{260}^{1\text{ cm}} = 1.316$ for a concentration of 55 μg ribosomal RNA per ml.

2.7. PREPARATION OF THE SOLUBLE FRACTION FROM TOBACCO LEAVES

The soluble fraction added to the incubation mixtures was prepared by fractionating the supernatant of the 105,000 × g centrifugation (S_{100}) mentioned in 2.2.1 on a Sephadex G75 column. Quantities of 3 ml of the S_{100} were placed on the Sephadex column (height 20 cm and diameter 0.8 cm), eluted with standard buffer and collected in fractions of 1–1.5 ml. A standard elution pattern is shown in figure 2.1, where the absorbancies at 260 and 280 mμ (A_{260} and A_{280}) of the various fractions are given. The first peak contained most of the protein. Usually this peak was colourless, while, as indicated in the figure, the second peak was yellowish-green with the darkest colour in the fractions 12 and 13.

The activities of the various fractions were tested by incubating complete incubation mixtures (see 2.9) without ribosomes. During the subsequent wash-

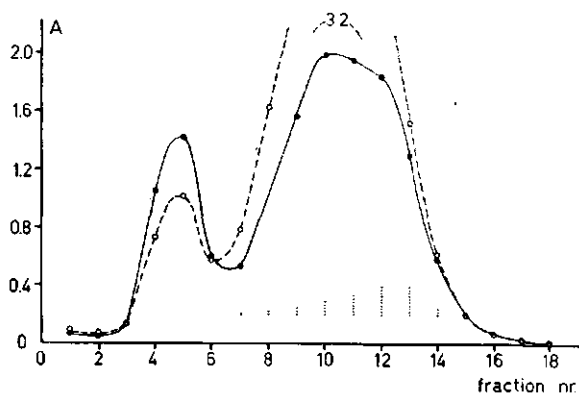
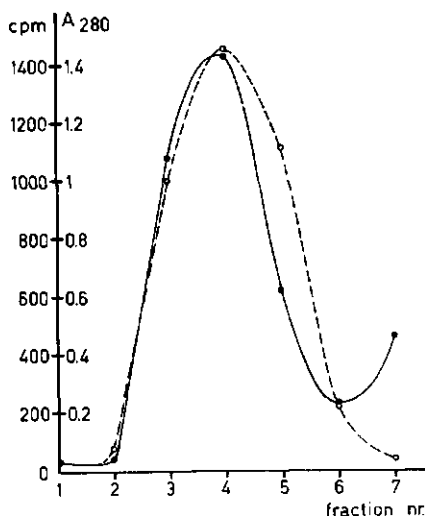


FIG. 2.1. Elution pattern of the 105,000 × g supernatant on a Sephadex G75 column. 3 ml were placed on the column (20 cm in height and 0.8 cm in diameter) and eluted with standard buffer. Fractions of 1.5 ml were collected. o — — — o A_{260} , ● — — — ● A_{280} indicates the yellowish-green colour of the eluate. Fraction nr. 5 was used for the incorporation experiments.

FIG. 2.2. Elution pattern of UV absorbing material and enzyme activity. The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 7.8, 75 μ moles NH_4Cl , 11 μ moles MgCl_2 , 6 μ moles MCE, 100 μ g tRNA, 100 μ l enzymes and 0.002 μ mole ^{14}C -leucine (200 mC/mmole). Incubation was for 50 min. To prevent hydrolysis of aminoacyl-tRNA further processing was done at $0^\circ\text{--}4^\circ\text{C}$. After precipitation of the proteins ribosomes were added and the activities (cpm) were corrected for identical amounts of protein (see text). ● — ● extinction at 280 m μ . ○ — ○ incorporation in cpm.



ing procedure the samples were not heated in order to prevent hydrolysis of aminoacyl-tRNAs. The activities of the fractions obtained from the Sephadex column are shown in figure 2.2. The incorporation curve and the optical density curve almost coincided. Although the fractions with the highest optical density had the highest absolute activity, the specific activity of the next fraction was higher. So the latter fraction was used as a source of soluble enzymes. It was mixed with the previous one only when a larger amount of soluble enzymes was required. Figure 2.4.b shows the dependence of the ^{14}C -leucyl-tRNA synthesis on the amount of soluble enzymes added. This will be discussed in greater detail in section 2.8.

2.8. PREPARATION OF tRNA FROM TOBACCO LEAVES

Transfer RNA was prepared mainly according to VAN KAMMEN (1967a), whose procedure was based on methods of KIRBY (1956) and HOLLEY (1963). Leaves were freed of the midribs, washed and dried. To 150 g of leaf tissue was added 150 ml of a 1% suspension of bentonite in 0.06 M phosphate buffer at pH 7.95 and 150 ml of water-saturated redistilled phenol and a fine mince was prepared in a Waring Blendor run for a few minutes at maximum speed. The mixture was then centrifuged at $1000 \times g$ for 10 minutes. The debris of the leaves was recovered in the phenol layer, which was extracted with 150 ml of fresh phosphate buffer. The combined buffer layers were extracted with 75 ml of phenol by stirring at $0\text{--}4^\circ\text{C}$ for 30 minutes. After separating the water and phenol phases by centrifugation at low speed, the RNA was precipitated from the water phase by adding 2.5 volumes of ethanol. The precipitated RNA was dissolved in a small volume of water, centrifuged again, the insoluble parts were extracted once more with water and the solution was clarified by centrifugation.

After dialysis against water for 2 hours, the RNA was reprecipitated with 2.5 volumes of ethanol and dissolved again in distilled water. This precipitate usually dissolved completely in water. Insoluble material was discarded each time.

To fractionate the RNA it was adsorbed on a DEAE-cellulose column (height 8 cm and diameter 3 cm), equilibrated with 0.012 M phosphate buffer at pH 7.95. The column was washed with 0.012 M phosphate buffer pH 7.95 and thereafter with a solution of 0.2 M NaCl in the same buffer. This resulted in the elution of first low molecular weight material. The tRNA was eluted with 1 M NaCl in the same buffer, while the ribosomal RNA could be eluted with 1 M NaOH.

The elution pattern usually was as shown in figure 2.3. Four peaks were always present. Their relative heights varied and depended a.o. on the age of the plant material. The eluted tRNA was collected and precipitated by the addition of 2.5 volumes of ethanol. The precipitate was dissolved in water, dialyzed against water for two hours and insoluble material, if present, was centrifuged off. The tRNA was precipitated again with ethanol and finally dissolved in water. In each case its ability to accept amino acids was tested under standard conditions as specified in the legend of figure 2.4.a. Figures 2.4.a and 2.4.b show that the synthesis of aminoacyl-tRNA was completely dependent on the addition of both tRNA and soluble enzymes. The enzyme fraction was free of tRNA.

The active tRNA was diluted to the desired concentration and stored frozen. It was assumed that the $A_{260}^{1\text{ cm}}$ of 1 mg tRNA/ml was 24. The yield usually was in the range of 15–50% of the total column-adsorbed absorption units at an average of 20%, or 8–16 mg tRNA from 150 g of leaves. The $A_{\text{max}}/A_{\text{min}}$ was 2.2–2.3.

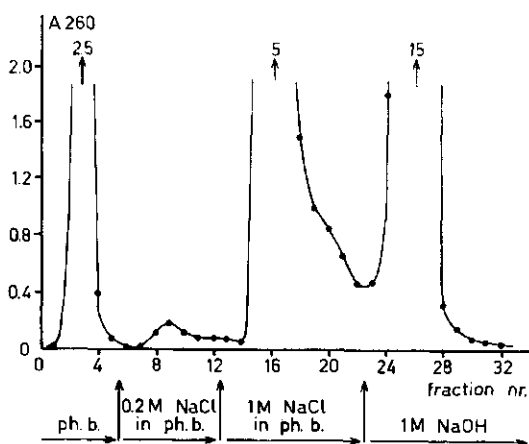


FIG. 2.3. Elution pattern of an RNA mixture on DEAE-cellulose. The column (8 cm in height and 3 cm in diameter) was first washed with 0.012 M phosphate buffer (ph. b.) pH 7.95 and then eluted with 0.2 M NaCl in the same buffer. The tRNA was subsequently eluted with 1 M NaCl in the same buffer and the ribosomal RNA could be eluted by 1 M NaOH. The tRNA fractions (in this case 15–20) were collected and combined.

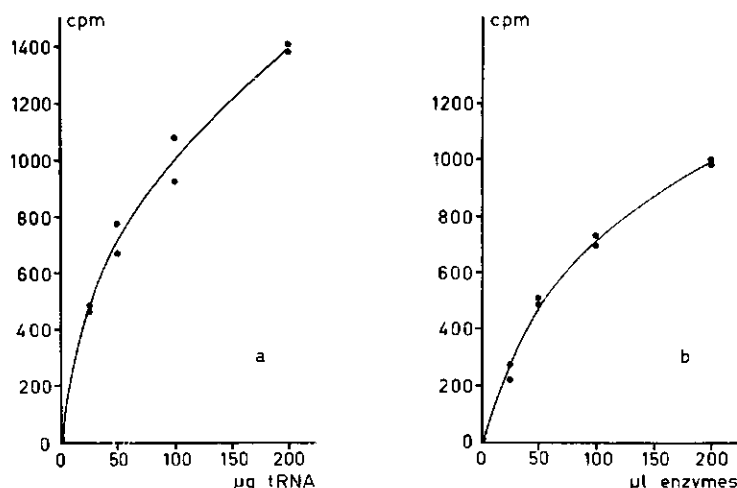


FIG. 2.4. The effect of tRNA (a) and soluble enzymes (b) on the synthesis of ^{14}C -leucyl-tRNA. The incubation mixture contained 10 μmoles PEP, 20 μg PK, 0.03 μmole GTP, 1 μmole ATP, 100 μmoles Tris, pH 7.8, 11 μmoles MgCl_2 , 75 μmoles NH_4Cl , 6 μmoles MCE, 0.003 μmole ^{14}C -leucine (150 mC/mmmole), tRNA (in a as indicated, in b 100 μg) and enzymes (in a 100 μl and in b as indicated). Incubation was for 40 minutes. To prevent hydrolysis of aminoacyl-tRNA further processing was done at $0^\circ\text{--}4^\circ\text{C}$. After precipitation of the proteins ribosomes were added and the activities (cpm) were calculated on the basis of the weighed protein (see text).

2.9. PROCEDURE USED IN THE AMINO ACID INCORPORATION EXPERIMENTS

The incubation mixture contained PEP, PK, GTP, ATP, Tris buffer, MgCl_2 , KCL or NH_4Cl , MCE, tRNA, soluble enzymes, ribosomes, poly U or other messengers and ^{14}C -amino acids. The concentrations varied and will be indicated in each case. The total volume of the incubation mixture was always 1 ml. Incubation was at 30°C , unless otherwise indicated.

Incorporation was terminated by adding an equal volume of 10% trichloroacetic acid and the precipitated protein washed with 5% trichloroacetic acid, heated at 90°C for 15 minutes, and washed subsequently with 5% trichloroacetic acid, ethanol, with a mixture of ethanol-chloroform-ether (2:1:1 by volume) and ether (according to SIEKEVITZ, 1952) and then suspended in 90% acetone. The suspensions were placed on planchettes, dried and counted in a Philips anticoincidence counter with a counting efficiency of about 5%. The samples were counted twice each time for 10 minutes. The radioactivity was corrected for background (10–12 cpm), the incorporating activity was expressed as cpm per mg protein (cpm/mg). Protein weights were determined by weighing the samples. Duplicate counts measured in one sample differed less than 10%. The washing procedure was checked by adding 1 ml 10% trichloroacetic acid at zero time. The radioactivity of these controls should be at about the same level as the background.

The results shown in the figures and tables constitute either representative examples of more than one experiment or are averages for two or more experiments.

2.10. ASSAY OF PROTEIN AND RNA

Protein was estimated with Folin-Ciocalteu reagent according to the method of LOWRY et al. (1951). A standard curve was prepared from ovalbumin. The blue colour was read at 750 m μ in a Unicam SP 600 spectrophotometer about 2 hours after the reagent was added.

RNA was estimated by the orcinol reaction (SCHNEIDER, 1957). The green colour was read at 660 m μ . A standard curve was prepared from purified yeast RNA.

2.11. PREPARATION OF RNA FROM RIBOSOMES

Ribosomal RNA was prepared according to a procedure based on the method used by GIERER and SCHRAMM (1956) for TMV-RNA. Samples of 6 ml of a ribosome suspension containing 1–3 mg ribosomal RNA, 0.1% sodium lauryl sulphate and 1% bentonite, in 0.01 M phosphate buffer pH 7.0 were stirred magnetically with an equal volume of redistilled water saturated phenol at 0–4°C for 10 minutes. The emulsion was broken by centrifugation at low speed. The phenol layer was extracted once more with 3 ml phosphate buffer for 10 minutes. The combined buffer layers were extracted twice with phenol. Each time the phenol layer was discarded. The buffer layer was washed three times with an equal volume of a mixture of chloroform-octanol (24:1) to remove the phenol. The RNA was precipitated with 2.5 volumes of ethanol. The precipitate was collected by centrifugation, dissolved in water and stored in the frozen state. The RNA concentration was estimated by determining the absorbancy at 260 m μ . An $A_{260}^{1\text{ cm}}$ in water of 24 was assumed for 1 mg/ml. The yield usually was 60–70%.

2.12. PREPARATION OF BENTONITE

Bentonite was prepared according to the method of KASSANIS and WELKIE (1963). The fraction used was that which sedimented between 3000 and 10,000 rpm.

2.13. DETERMINATION OF THE SEDIMENTATION COEFFICIENTS

Sedimentation coefficients were determined in the analytical ultracentrifuge Spinco Model E, using Schlieren optics. They were calculated by means of the graphical method of MARKHAM (1960). The sedimentation coefficients at infinite dilution S_0 were determined by running various concentrations and extrapolating the values to zero concentration. All determinations were done at 20°C. The solvent used in each case will be specified.

2.14. PREPARATION OF VIRAL RNAs

TMV was prepared according to the standard procedure used in our laboratory (KNIGHT, 1962). For preparing the TMV-RNA the procedure of 2.11 was used, but 80–100 mg TMV per 6 ml were taken and no bentonite was added. The final RNA suspension was dialyzed against water to remove phosphate. One mg of RNA per ml was considered to have an extinction $A_{260}^{1\text{ cm}}$ of 24. The RNA was stored in the frozen state.

TYMV-RNA was prepared in the same way as TMV-RNA.

Purified TYMV was a gift from Prof. Dr. L. Bosch (Biochemistry Department, State University of Leiden).

CPMV-RNA was also prepared as TMV-RNA. The CPMV middle and bottom components were gifts from Dr. A. van Kammen.

AMV top a – RNA was a gift from Dr. J. C. van Ravenswaay Claasen (Biochemistry Department, State University of Leiden).

2.15. MATERIALS AND ABBREVIATIONS

AMP	Adenosine monophosphate
AMV	Alfalfa mosaic virus
ATP	Adenosine 5'-triphosphate, disodium salt (P-L Biochemicals Inc.)
¹² C-amino acids	(The British Drug House LTD)
¹⁴ C-amino acids	(New England Nuclear Corp.)
Chloramphenicol	B grade (Calbiochem)
CPMV	Cowpea mosaic virus
Cycloheximide	(3 [2 (3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl] glutarimide) (Sigma)
DNA	Deoxyribonucleic acid
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetra-acetic acid (2Na-salt)
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
GTP	Guanosine-5'-triphosphate sodium salt, grade 11-s (Sigma)
Leucovorin	Formyltetrahydropteroylglutamic acid (Lederle Laboratories Division)
MCE	2-Mercaptoethanol
mRNA	Messenger ribonucleic acid
PEP	Phospho (enol) pyruvic acid trisodium salt hydrate (Sigma)
PK	Pyruvate kinase (Boehringer und Soehne)
Poly U	Polyribouridylic acid Potassium salt, A grade (Calbiochem)
PP	Pyrophosphate
RNA	Ribonucleic acid
RNAse	Ribonuclease, bovine pancreas (Sigma)
rRNA	Ribosomal ribonucleic acid
sTNV	Satellite of tobacco necrosis virus
TCA	Trichloroacetic acid
TMV	Tobacco mosaic virus
Tris	Tris-(hydroxy-methyl)-amino-methane
tRNA	Transfer ribonucleic acid
TYMV	Turnip yellow mosaic virus

3. CHARACTERIZATION OF THE SYSTEM FOR AMINO ACID INCORPORATION

This chapter contains a description of the properties of the system for amino acid incorporation as isolated initially from healthy plant material and after purification by various methods. This system contained heterogeneous ribosome material, since the 70 S and 80 S ribosomes were not separated, although it consisted mainly of 80 S ribosomes because most of the 70 S ribosomes were lost during purification.

We shall describe first the influence of each of the components of the incubation mixture on the incorporation as studied in the preliminary experiments. The system appeared to be dependent on the addition of nucleoside triphosphates, ribosomes, magnesium and potassium ions and on the incubation time. Thereafter purification methods will be described. With purified ribosomes the incorporation was dependent on the addition of tRNA and soluble enzymes. Finally, the observed rapid decrease in the rate of incorporation will be discussed.

3.1. PROPERTIES OF THE NON-PURIFIED SYSTEM FOR AMINO ACID INCORPORATION

In preliminary experiments the requirements were studied to obtain optimal amino acid incorporation by the system derived from tobacco leaves. Incubation mixtures were as described in 2.9. Incorporation proved to be highly dependent on the presence of ATP, GTP, PEP and PK. In their absence the incorporation was reduced by 85–97%. This indicated that the incorporation was not due to contaminating micro-organisms but to incorporation of amino acids into polypeptides by the cell-free system. The concentrations used were 1 μ mole ATP, 0.03 μ mole GTP, 10 μ moles PEP and 20 μ g PK in most of the following experiments, although higher concentrations slightly increased the incorporation.

Magnesium ions were indispensable, although the concentration could be varied over a wide range without much influencing the incorporation. As the influence of magnesium ions on 70 S and 80 S ribosomes was different, this point will be discussed in detail in the chapters 4 and 5.

Varying the potassium ion concentration between 25–100 μ moles/ml did not influence greatly the level of incorporation. For this reason and because they were substituted by ammonium ions (see 3.3.2) this was not investigated further.

The addition of ^{12}C -amino acids did not have any effect on the incorporation. It was found that an endogenous amino acid pool of about 0.0012–0.0025 μ mole/ml was present in the ribosome preparations and was responsible for this feature. These endogenous amino acids could not be washed out.

The effect of the concentration of ribosomes on the amino acid incorpora-

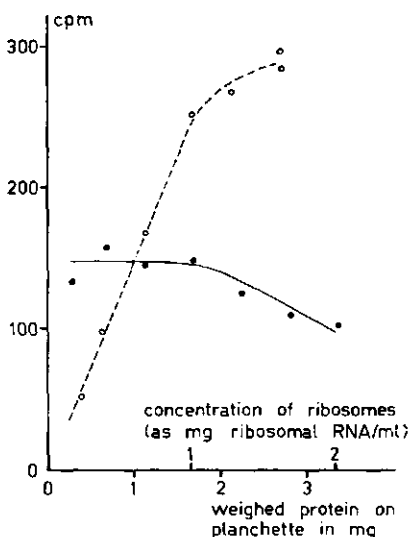


FIG. 3.1. The effect of the ribosome concentration on the amino acid incorporation. The incubation mixture contained 5 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 7.8, 100 μ moles KCl, 20 μ moles MgCl_2 , 12 μ moles MCE, ribosomes (ribosomal RNA from 0.17–2.04 mg) and 0.003 μ mole ^{14}C -leucine (150 mC/mmole). The incubation time was 40 minutes. ●—● amino acid incorporation as specific activity, dependent on the concentration of ribosomes in mg ribosomal RNA/ml (cpm/mg protein). ○—○ total amino acid incorporation dependent on the total amount of protein weighed on the planchettes (cpm).

tion can be seen in figure 3.1. Up to one mg RNA per ml the incorporation increased linearly with the ribosome concentration, i.e., the specific activity remained constant for ribosome concentrations not above one mg RNA per ml in the incubation mixture used. To avoid serious weighing errors approximately constant, ponderable amounts of protein, were added as ribosomes, corresponding to 0.4–0.8 mg of ribosomal RNA. When less than 0.4 mg ribosomal RNA per sample was present, carrier ribosomes were added to obtain the desired amount of protein on the planchette. The specific activity was then corrected for the added amount of carrier.

Soluble enzymes and tRNA had no effect on the amino acid incorporation by the non-purified system. Since both enzymes and tRNA, as isolated, were active (see 2.7 and 2.8) these factors were probably present in the ribosome preparations. In 3.2 we shall return to this subject.

The time course of the amino acid incorporation is given in figure 3.2. The rate of incorporation decreased very rapidly to almost zero within 60 minutes

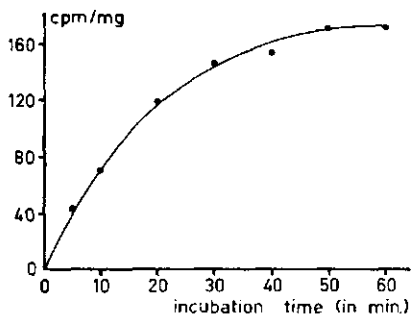


FIG. 3.2. The effect of the incubation time on the amino acid incorporation. The incubation mixture contained 5 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 7.8, 100 μ moles KCl, 20 μ moles MgCl_2 , 12 μ moles MCE, ribosomes (0.95 mg ribosomal RNA) and 0.003 μ mole ^{14}C -leucine (150 mC/mmole).

and although such curves have been found for the ribosomes of most organisms, it was striking that even during the first minutes the rate of incorporation was not constant. Addition of fresh ribosomes to an incubation mixture after 60 minutes resulted in additional incorporation for at least 25 minutes. This means that the decrease in incorporation rate was not due to depletion of factors in the incubation mixture but was inherent to the ribosomes themselves. Possible causes of this phenomenon were studied and will be described in the following sections.

3.2. AMINO ACID ACTIVATION

In order to obtain some information about the amino acid activating reaction, the acid-precipitable counts were measured in a duplicate incubation mixture. Only one of the tubes was heated with TCA to hydrolyze aminoacyl-tRNA. The non-heated sample gave the sum of amino acid incorporation and activation, the heated sample incorporation only. Figure 3.3 gives a comparison of the time course of the amino acid activation reaction and the amino acid incorporation. There was a rapid synthesis of an excess of aminoacyl-tRNA. Omitting from the incubation mixture tRNA and soluble enzymes gave similar results, but for a smaller excess of synthesized aminoacyl-tRNA. Since the amount of aminoacyl-tRNA in excess did not diminish during peptide synthesis, it may be concluded that the ribosome preparations contained sufficient tRNA and soluble enzymes and that the addition of purified tRNA and enzy-

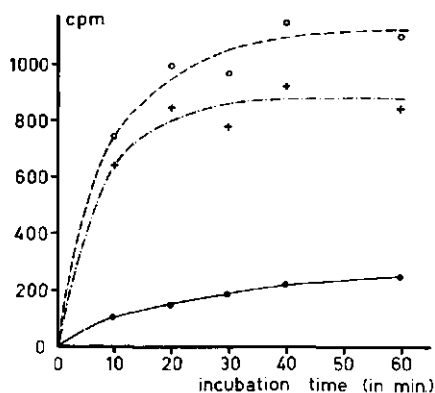


FIG. 3.3. The effect of the incubation time on the amino acid activation and amino acid incorporation. The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 7.8, 75 μ moles NH_4Cl , 11 μ moles MgCl_2 , 6 μ moles MCE, 100 μ g tRNA, 100 μ l enzymes, ribosomes (0.72 mg ribosomal RNA) and 0.003 μ mole ^{14}C -leucine (150 mC/mmmole). The incubation time was as indicated. \bullet — \bullet amino acid incorporation (heated) \circ — \circ sum of amino acid incorporation and aminoacyl-tRNA formation (unheated) $+$ — $+$ difference between the heated and unheated counts, indicating the amount of aminoacyl-tRNA in excess.

mes caused an increase in aminoacyl-tRNA formation, but did not affect polypeptide synthesis. Thus, amino acid activation was not a limiting step in the system for amino acid incorporation.

3.3. PURIFICATION OF THE RIBOSOME PREPARATIONS

3.3.1. Sucrose gradient centrifugation

A simple method to fractionate a ribosome preparation has been described by WETTSTEIN, STAEHELIN and NOLL (1963). Ribosome preparations were layered on 0.5 M sucrose placed on a 2.0 M sucrose cushion and were centrifuged in the Spinco SW 25 rotor for 4 to 5 hours. The ribosomes were collected in the interphase between the two sucrose layers, whereas the polyribosomes formed a pellet. VAN KAMMEN (1967a) demonstrated that such ribosomes are more dependent on added tRNA and soluble enzymes than are ribosomes purified by differential centrifugation only. Apparently centrifugation in sucrose layers washes out fractions of lower molecular weight.

Ribosomes were purified according to this method (see chapter 2.3.a) and the effect of tRNA and enzymes on the amino acid incorporation was investigated. As summarized in table 3.1.a, the amino acid incorporation appeared to be

TABLE 3.1. The effect of tRNA and soluble enzymes on the amino acid incorporation as dependent on the method of purification of the ribosomes

Purification method	Amino acid incorporation in cpm/mg					
	a		b		c	
	Sucrose gradient centrifugation		Washing in 0.5 M NH ₄ Cl		Sephadex G200 treatment	
Conditions	1	2	1	2	1	2
Complete – ATP, GTP, PEP and PK	12	16	12	11	12	10
Complete	203	66	115	112	128	119
Complete + tRNA	213	79	–	–	174	119
Complete + enzymes	235	193	–	–	215	199
Complete + tRNA + enzymes	240	220	143	196	268	252

The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 7.8, KCl or NH₄Cl (see below), 11 μ moles MgCl₂, 6 μ moles MCE, 50 μ g tRNA and 100 μ l enzymes (when indicated), 0.003 μ mole ¹⁴C-leucine (150 mC/mmmole) and ribosomes. The incubation time was 40 minutes.

- Ribosomes purified by sucrose gradient centrifugation as described in 3.3.1. Ribosomes were added at a concentration of 0.6 mg RNA and KCl at a concentration of 60 μ moles. The results obtained with purified ribosomes (2) are compared to those with unpurified ones (1).
- Ribosomes washed in 0.5 M NH₄Cl (2) compared with unwashed ribosomes (1); 0.65 mg ribosomal RNA and 60 μ moles KCl and 75 μ moles NH₄Cl respectively in (1) and (2) (See 3.3.2.).
- NH₄⁺-ribosomes, purified on a Sephadex G200 column (2) and non-treated NH₄⁺-ribosomes (1); 0.35 mg ribosomal RNA was added and 75 μ moles NH₄Cl (see 3.3.3.).

dependent on the addition of enzymes, which clearly were removed from the ribosomes to a large extent. However, the addition of tRNA still did not seem to have much effect on the incorporation. This could not be due to the presence of tRNA in the enzyme fraction, (figure 2.4.a), since an incubation of the enzymes in the incubation medium in the absence of ribosomes and tRNA hardly gave any synthesis of aminoacyl-tRNA.

The method of WETTSTEIN et al. (1963) was developed to separate ribosomes from polyribosomes. Since the ribosomes are difficult to collect from the interphase, another method was followed to prepare these for subsequent experiments. The ribosome preparation was placed on one sucrose layer of 0.5 M and centrifuged in the Spinco R 40 rotor as described in chapter 2.3.b. Ribosomes were then obtained as pellet together with polyribosomes and there was no further need to concentrate them.

3.3.2. Purification by centrifugation in 0.5 M NH_4Cl

LUBIN and ENNIS (1964) and VOORMA (1965) have discussed the influence of K^+ and NH_4^+ ions on the amino acid incorporation. They demonstrated that protein synthesis by the *E. coli* system increased when the K^+ ions were replaced by NH_4^+ ions, since K^+ ions activate an RNA splitting phosphodiesterase. The enzyme was not activated by NH_4^+ ions. Since the rate of amino acid incorporation showed a very rapid decrease, (figure 3.2), it seemed worthwhile to investigate whether the replacement of the K^+ ions by NH_4^+ ions also had a favourable effect on the amino acid incorporation by tobacco ribosomes. For this purpose different procedures were compared, namely ribosomes prepared according to the modified sucrose gradient centrifugation method as described in chapter 2.3.b (K^+ -ribosomes) and ribosomes prepared according to VOORMA (1965) by centrifugation on 0.5 M sucrose containing 0.5 M NH_4Cl in stead of KCl (NH_4^+ -ribosomes). In addition, in the latter case KCl was replaced by 75 μmoles NH_4Cl in the incubation mixture. Figure 3.4 gives a comparison between the time course of the amino acid incorporation by K^+ and NH_4^+ -ribosomes. The incorporation rate did not decrease as

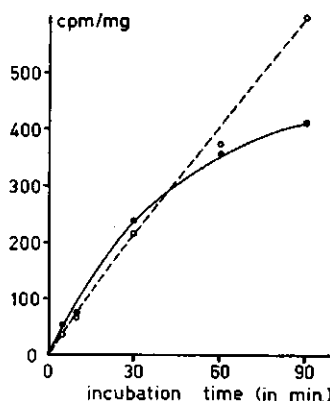


FIG. 3.4. The effect of K^+ and NH_4^+ ions on the amino acid incorporation by ribosomes. The complete incubation mixture contained 10 μmoles PEP, 20 μg PK, 0.03 μmole GTP, 1 μmole ATP, 100 μmoles Tris, pH 7.8, 11 μmoles MgCl_2 , 60 μmoles KCl or 75 μmoles NH_4Cl , respectively, 6 μmoles MCE, ribosomes (0.56 mg ribosomal RNA), 50 μg tRNA, 100 μl enzymes and 0.003 μmole ^{14}C -leucine (150 mc/mmmole). The incubation time was as indicated. ● — ● K^+ -ribosomes
○ — — ○ NH_4^+ -ribosomes.

rapidly with NH_4^+ -ribosomes as with K^+ -ribosomes. The effect of tRNA and soluble enzymes on the amino acid incorporation is shown in table 3.1.b. This effect was greater for NH_4^+ -ribosomes than for K^+ -ribosomes. Thus, the soluble fraction caused a smaller inactivation in the NH_4^+ -system than in the K^+ -system. There may also be present a phosphodiesterase activated by K^+ ions in plant ribosome systems.

Taking the above conclusion into account, in all the following experiments K^+ was replaced by NH_4^+ , whereas 0.5 M NH_4Cl was added to the sucrose buffer used for centrifugation in the following experiments described in this chapter.

3.3.3. *Purification by means of Sephadex G200*

Sephadex columns can be used successfully for purification on the basis of molecular size. Thus, it was thought that Sephadex G200 might be suitable for purifying the ribosomes. Ribosomes prepared as described in 3.3.2 were placed on a G200 Sephadex column (20×1 cm), equilibrated with standard buffer containing NH_4^+ in stead of K^+ ions (standard- NH_4^+ -buffer) and eluted with the same buffer. The fractions containing the ribosomes were collected and the yield was about 80%. The amino acid incorporation by these ribosomes was compared to that by untreated ribosomes. The results are shown in table 3.1.c. The incorporating activity was increased nor decreased by Sephadex filtration, but became considerably more dependent on the addition of tRNA and enzymes. Thus, it can be concluded that the combination of sucrose gradient centrifugation and Sephadex G200 chromatography had eliminated most of the tRNA and soluble enzymes from the ribosomal fraction. Therefore, the apparent independence of the amino acid incorporation on the addition of tRNA and enzymes mentioned in 3.1 was only due to the presence of tRNA and soluble enzymes in the ribosome fraction.

3.3.4. *Summary of the effects of purification*

The purification as described above led to a more pronounced dependence of the incorporation on added tRNA and soluble enzymes and also to a change in optical properties. Ribonucleoprotein particles such as ribosomes, have characteristic values for the ratio of the extinctions at 260 and 238 $\text{m}\mu$ (A_{260}/A_{238}). For this reason, in a number of experiments these average ratios were determined and compared (see table 3.2). Purification of the ribosomes increased the A_{260}/A_{238} ratio from 1.46 to 1.58 and the A_{260}/A_{280} ratio from 1.88 to 1.92. The ratios for ribosomes purified by only one cycle of differential centrifugation (table 3.2 line 1) were the same as those found by VAN KAMMEN (1963) for ribosomes given three of such cycles. This again underlines the advantage of the present method for fractionating leaf extracts (chapter 2.2). A more extensive purification increased those ratios even more, which indicates that material different from ribonucleoproteins was removed.

E. coli ribosomes can be stored frozen (VOORMA, 1965). So far, plant ribosomes always proved to be inactivated when they were kept frozen (VAN KAM-

TABLE 3.2. Optical properties of ribosomes, purified in different ways.

Method of ribosome purification	A_{260}/A_{238}	A_{260}/A_{280}
1	1.46 ± 0.01	1.88 ± 0.02
2	1.52 ± 0.02	1.90 ± 0.02
3	1.52 ± 0.01	1.90 ± 0.01
4	1.54 ± 0.02	1.89 ± 0.03
5	1.58 ± 0.02	1.92 ± 0.03

1. Ribosomes purified by one cycle of differential centrifugation as described in 2.2.1.
2. Ribosomes purified according to WETTSTEIN et al. (1963) on two layers of sucrose, as described in 2.3.a and 3.3.1.
3. Ribosomes purified on one layer of sucrose, as described in 2.3.b and 3.3.1.
4. Ribosomes purified with the aid of 0.5 M NH_4Cl , as described in 3.3.2.
5. Ribosomes purified on Sephadex G200, as described in 3.3.3.

MEN, 1963) We became interested to see whether the purified ribosomes would remain active upon storage in the frozen state. When comparing various ribosome preparations, each time fresh enzyme preparations had to be used since soluble enzymes lost already activity when stored overnight at 0°C . To avoid the effect of possible differences in the activity of different enzyme preparations, fresh and stored enzymes were used in paired experiments.

Sephadex G200 treatment of the ribosomes appeared to have a favourable effect on the activity remaining after storage overnight at 0°C . Freezing at -15°C , though, decreased the activity to about 40%. In view of the almost complete loss of the activity upon storage overnight at 0°C or frozen of ribosomes given only one cycle of differential centrifugation, it could be concluded that a further purification led to an improvement in stability upon storage. Even the most purified ribosomes could not be stored, however, without any loss of activity. It was attempted to store ribosomes at 0°C for a period up to one week. As bacteria started to grow after a few days, this method could not be used. The addition of penicillin and streptomycin did not suppress bacterial growth.

3.4. THE DEPENDENCE OF THE AMINO ACID INCORPORATION ON THE ADDITION OF tRNA, SOLUBLE ENZYMES AND AMINO ACIDS

Since tRNA and soluble enzymes were largely removed from the purified ribosomes (see 3.3), the dependence of the amino acid incorporation on the concentration of these factors was studied to find optimal conditions. The results are shown in figure 3.5. The dependence on soluble enzymes (curve a) was practically absolute, whereas apparently sufficient tRNA (curve b) remained in the ribosome preparations to reach 60% of maximum incorporation. Only catalytically small amounts of both enzymes and tRNA had to be added to reach an optimal effect.

From curve 3.5.b it could be estimated that there was 25 μg or less tRNA

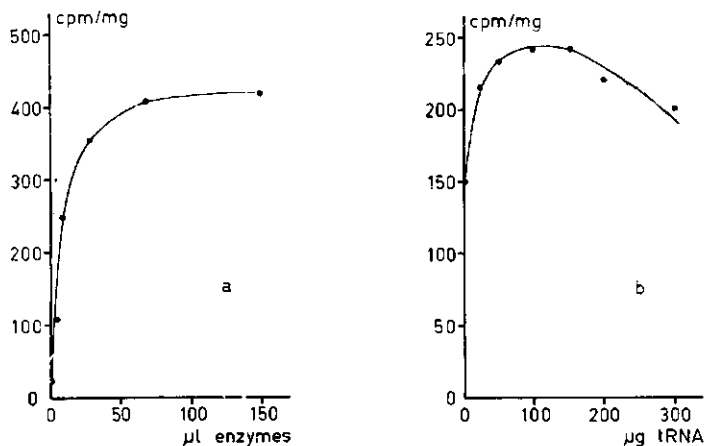


FIG. 3.5. Amino acid incorporation as dependent on soluble enzymes (a) and tRNA (b). The incubation mixture contained 10 μmoles PEP, 20 μg PK, 0.03 μmole GTP, 1 μmole ATP, 100 μmole Tris, pH 7.8, 11 μmoles MgCl_2 , 75 μmoles NH_4Cl , 6 μmoles MCE, 0.003 μmole ^{14}C -leucine (150 mC/mmmole); for curve a) 0.63 mg ribosomal RNA and 25 μg tRNA; for curve b) 0.51 mg ribosomal RNA and 100 μl enzymes. The incubation time was 50 min.

left in the ribosome preparations per 0.5–0.6 mg ribosomal RNA. Assuming a molecular weight of about 26,000 for tRNA (average molecular weight per nucleotide of 330 and 75–80 nucleotides per tRNA molecule) and of about 4.2×10^6 for the ribosomes (Ts'o, BONNER and VINOGRAD, 1958), this would amount to an average of 2–3 tRNA molecules per ribosome. It is generally accepted now that two tRNA molecules can be bound functionally to one ribosome (WARNER and RICH, 1964), although WETTSTEIN and NOLL (1965) have suggested that three tRNA molecules can be bound, i.e. one charged with an amino acid, one attached to the end of the nascent polypeptide chain and one without an amino acid. Since the binding of the latter is not specific, an average binding of 2–3 bound tRNA molecules per ribosome seems to be quite acceptable.

The effect of added, unlabeled amino acids on the amino acid incorporation was determined once more. The endogenous pool of leucine was estimated to be 0.0012 $\mu\text{mole/ml}$ when ribosomes were used which were purified in 0.5 M NH_4Cl . Taking into account that there were 900 $\mu\mu\text{moles}$ or less tRNA left in the ribosome preparation, all of these 1200 $\mu\mu\text{moles}$ leucine could not possibly be bound to tRNA molecules. Therefore, they must have remained rather tightly bound to the ribosomes during the washing procedure. Keeping this in mind, no further attention was paid to the effect of added, unlabeled amino acids.

3.5. INACTIVATION OF THE ACTIVE COMPLEX DURING INCUBATION

As shown in this chapter, *in vitro* amino acid incorporation proceeded longer with more extensively purified ribosomes. However, the reaction rate decreased ultimately with ribosomes purified by the Sephadex method (3.3.3). In order to obtain some information on this decrease the following experiment was made. Ribosomes were preincubated for different lengths of time both in a complete incubation mixture without the labeled amino acid, or in standard buffer only. Then the omitted components of the incubation mixture were added and the whole mixture was incubated for an additional period. Such an experiment is shown in figure 3.6. Curve a represents a normal time course, curves b and c the amino acid incorporation after 35 minutes by ribosomes which were preincubated in a medium as indicated in the legend of the figure. Ribosomes which were preincubated in a medium suitable for amino acid incorporation (curve c) retained hardly any activity after a preincubation of 120 minutes (ca. 15%), whereas ribosomes which were preincubated in standard buffer only (curve b) lost not more than 25% of their activity during preincubation. This means that inactivation occurred only during the process of amino acid incorporation and not when ribosomes were kept at 30°C for 2 hours, i.e., there was no endogenous inactivation. The inactivation could be due to several causes, viz. completion of the translation of messenger RNA or its degradation.

To decide among these possibilities the following experiment was carried out. Ribosomes were preincubated as in the previous experiment, but now

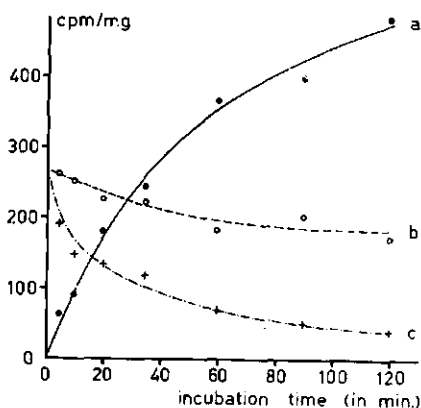


FIG. 3.6. Inactivation of the active complex during incubation. The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 7.8, 11 μ moles $MgCl_2$, 75 μ moles NH_4Cl , 6 μ moles MCE, 0.002 μ mole ^{14}C -leucine (250 mc/mmole), 25 μ g tRNA, 100 μ l enzymes and ribosomes, purified by means of 0.5 M NH_4Cl (0.7 mg ribosomal RNA). a. non-preincubated ribosomes, b. activity (amino acid incorporation in cpm/mg in 35 min.) of ribosomes, preincubated in standard buffer for the given lengths of time, c. activity of ribosomes, preincubated for the given lengths of time in the incubation mixture without ^{14}C -leucine.

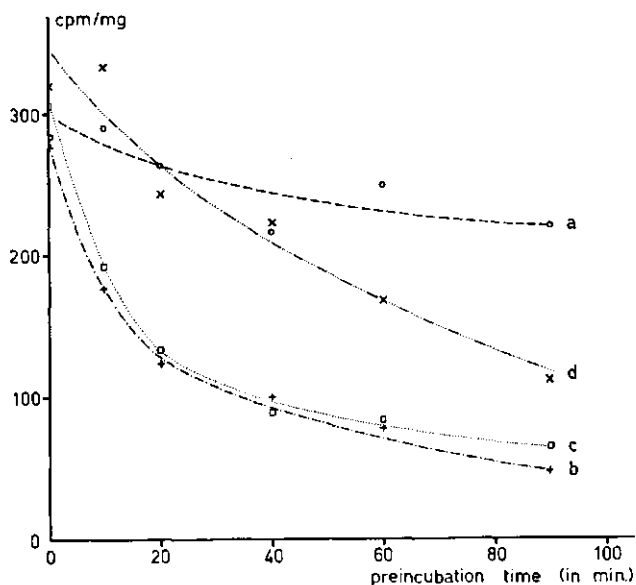


FIG. 3.7. The effect of preincubation under various conditions. The composition of the incubation mixture was as mentioned in figure 3.6. Ribosomes were added in the concentration of 0.6 mg ribosomal RNA. a. activity (amino acid incorporation in cpm/mg in 35 min.) of ribosomes, preincubated in standard buffer for the given lengths of time, b. activity of ribosomes, preincubated in the complete incubation mixture without ^{14}C -leucine for the given lengths of time, c. activity of ribosomes, preincubated in the complete incubation mixture without ^{14}C -leucine and GTP for the given lengths of time, d. activity of ribosomes, preincubated in the complete incubation mixture without ^{14}C -leucine, ATP, GTP, PEP and PK for the given lengths of time.

under various conditions, viz., a) in standard buffer, b) in the incubation mixture without the labeled amino acid, c) in the incubation mixture without the labeled amino acid and GTP, and d) in the incubation mixture without the labeled amino acid, GTP and ATP, PEP and PK. Then the omitted components were added and the entire mixture was incubated for an additional 35 minutes. The results of this experiment are given in figure 3.7. Omission of GTP appeared to have no effect, for curves b and c are nearly identical. Preincubation without ATP, GTP, PEP and PK gave some indication of what was happening during incubation. When a complete incubation mixture, minus ATP, GTP, PEP and PK was incubated, (see table 3.1), only very little (3-10%) radioactivity was incorporated. As shown in curve d, preincubating such a mixture for 90 minutes, diminished the incorporation to about 50%. Thus, it may be suggested that, in spite of the slight incorporation, degrading enzymes had broken down the messenger RNA. This is not in agreement with the conclusion of Voorma (1965), according to which, even when NH_4^+ -ribosomes are used, the

messenger (TYMV-RNA) is degraded in the *E. coli* system only when the ribosomes move along that messenger, i.e., when incorporation takes place.

The results did not allow a conclusion to which extent the completion of messenger translation was also involved. It is likely that both degradation and completion of translation caused a decrease in the incorporation rate to zero.

3.6. DISCUSSION AND CONCLUSIONS

This chapter contains a description of the properties of the system for amino acid incorporation as isolated from healthy plant material. It was concluded that the present system was really a cell-free system because of its absolute dependence on nucleoside triphosphates. However, it was independent on the addition of tRNA and soluble enzymes and the incorporation rate decreased very rapidly.

For this reason the amino acid activation process was investigated. Highly active tRNA and soluble enzymes could be isolated from leaf material. The addition of ribosomes did not inhibit amino acid activation. The purified ribosomes appeared to contain a considerable amount of tRNA and soluble enzymes, which could be largely removed by various purification methods, in particular by washing with 0.5 M NH_4Cl and elution from a Sephadex G200 column. After the latter treatment, the amino acid incorporation became almost completely dependent on the addition of soluble enzymes. It was not possible to remove completely the tRNA from the ribosomes. However, the amino acid incorporation became partially dependent on added tRNA after Sephadex G200 treatment, but it was estimated that tRNA was firmly bound by the ribosomes to a maximum of 2–3 molecules of tRNA per ribosome. This figure is in agreement with the number of tRNA molecules which can be functionally bound to ribosomes.

The amino acid activating process was shown to occur at a high rate, since after less than 10 minutes of incubation, half of the maximal aminoacyl-tRNA had been synthesized already (figure 3.3). This process was completed after about 30 minutes of incubation. The initial rate of the amino acid activation was estimated to be about 9 $\mu\text{moles } ^{14}\text{C-leucine/mg/minute}$, whereas the initial rate of peptide synthesis was only about 0.72 $\mu\text{mole } ^{14}\text{C-leucine/mg/minute}$. From these results it can be concluded that the amino acid activating process was not the rate limiting step in the process of peptide synthesis.

Purified ribosomes were still not influenced by the addition of ^{12}C -amino acids, although 50% of the endogenous amino acids had been removed by the washing procedures. It may be remarked that in most of the plant systems described in the literature the effect of ^{12}C -amino acids was absent or at least doubtful (MANS and NOVELLI, 1964; SPENCER and WILDMAN, 1964; BOARDMAN et al., 1966; VAN KAMMEN, 1967a).

Further purification also resulted in a less rapidly decreasing incorporation rate (cf. figures 3.2 and 3.4). Presumably, this is caused by the removal of factors inhibiting the amino acid incorporation. On average 10 to 20 μmoles of

leucine per mg protein were incorporated by the purified system in 40 minutes. This amount is similar to the incorporation by the cytoplasmic fraction from tobacco leaves described by BOARDMAN et al. (1966). Maize seedlings seemed to yield a more active system, for MANS and NOVELLI (1964) reported an incorporation of 200 to 500 μmoles per mg protein by their system. However, these values were obtained only when leucine was used as a label. When other amino acids were used these results could not be reproduced. Bacterial ribosomes are also more active. In their *E. coli* system, MATTHAEI and NIRENBERG (1961) found an endogenous incorporation of 500 to 1000 μmoles leucine per mg protein. Still higher values were obtained with rat liver polysomes. These polysomes were able to incorporate about 4500 μmoles leucine per mg protein (BLOEMENDAL, BONT and BENEDETTI, 1964). Thus, in general, it can be said that ribosomal systems from plants are less active than those from bacteria and animals.

As will be discussed further in the chapters 4 and 5, sucrose gradient centrifugation revealed (3.3.1) that only a very small amount of polyribosomes was present in the ribosomal preparations. These polyribosomes which are now generally accepted to be the active complexes in vivo, were apparently broken down during isolation. This would mean that the messengers were also fragmented, so that no chain termination (CAPECCHI, 1967), and therefore probably no release of messenger and no reinitiation were possible. This is a suggestive explanation for the decrease of the incorporation rate. Figure 3.7 suggests that degrading enzymes were involved in this process. TAKANAMI and ZUBAY (1964) found that an RNase treatment of an *E. coli* - ribosome - poly U - complex left about 27 uridylic residues on a ribosome. Probably in each case a messenger fragment of at least this size remains bound, meaning that each active ribosome actually will incorporate a certain number of amino acids dependent on the size of the fragment. Since the messengers will break down at random, the incorporation rate will decrease gradually, as is shown in figure 3.6.

One may conclude that all components, mentioned in this chapter, were necessary for the process of amino acid incorporation and that the ribosomes could be purified in order for the process of incorporation to become dependent on the addition of these components except of ^{12}C -amino acids. The process of incorporation continued at a decreasing rate for more than two hours and stopped finally because of read out and, probably in part, breakdown of the messenger fragments. It is likely that the pieces of messenger were translated only once. The incorporation by a tobacco ribosome system proved to be a rather slow process. Highly purified ribosomes could be stored at 0°C overnight with only about 10% loss of activity.

4. PHYSICAL PROPERTIES OF 70 S AND 80 S RIBOSOMES

The ribosome preparations proved to be inhomogeneous in the analytical ultracentrifuge. LYTTLETON (1962) demonstrated the presence of at least two classes of ribosomes in spinach plants, i.e., cytoplasmic and chloroplast ribosomes. These classes differed in their sedimentation coefficients. Similarly, BRAWERMAN (1963) and EISENSTADT and BRAWERMAN (1963, 1964) isolated two types of ribosomes from *Euglena gracilis* and investigated their properties, while CLARK (1964) and CLARK, MATTHEWS and RALPH (1964) have demonstrated two classes of ribosomes in *Brassica pekinensis*. Regarding their significance in protein synthesis, SPENCER and WILDMAN (1964) concluded that in tobacco plants the chloroplasts made the greatest contribution to protein synthesis in vitro.

The studies presented in this chapter show that also in our system both chloroplast and cytoplasmic ribosomes occurred. The ribosome mixtures were analyzed and the various components were purified. Finally, some of the physical properties were determined, in particular those of chloroplast and cytoplasmic ribosomes.

4.1. COMPONENTS OF THE RIBOSOME PREPARATIONS

When plants were grown in the greenhouse, the particle composition of the ribosome preparations varied and depended on the weather. Various samples were run in the analytical ultracentrifuge and as can be seen in figure 4.1.a, it was chiefly the ratio between the 70 S and 80 S peaks which was varying. For this reason plants used in our experiments were grown under controlled conditions as described in 2.1. Although some variation was still present even under these conditions, usually the various components were obtained in amounts sufficient for further study. The ratio between 70 S and 80 S ribosomes was on the average as indicated in figure 4.1.a.3.

Several methods for fractionating the ribosome preparations were investigated. Various sucrose gradients were tried. Finally it appeared that a discontinuous gradient from 10–40% sucrose in standard buffer, run for 2.5–3 hours at 25,000 rpm as described in chapter 2.3.c gave the best separation. In addition to a protein layer (18 S) near to the top of the gradient, always four bands were present. These bands were removed from the gradient with a hypodermic syringe as described in 2.4.a and subsequently the material was sedimented down by centrifugation at $105,000 \times g$ and resuspended in standard buffer. Samples of these suspensions were run in the analytical ultracentrifuge. In figure 4.1.b a picture of each band is shown. It is clear that the upper two bands contained mainly 70 S and 80 S material, whereas the two lower ones contained polyribosomal material in addition to an 80 S peak (80–150 S and 80–200 S). Since the two upper bands were very close to each other, the 70 S and 80 S material could not always be separated as well as shown in figure 4.1.b. Usually

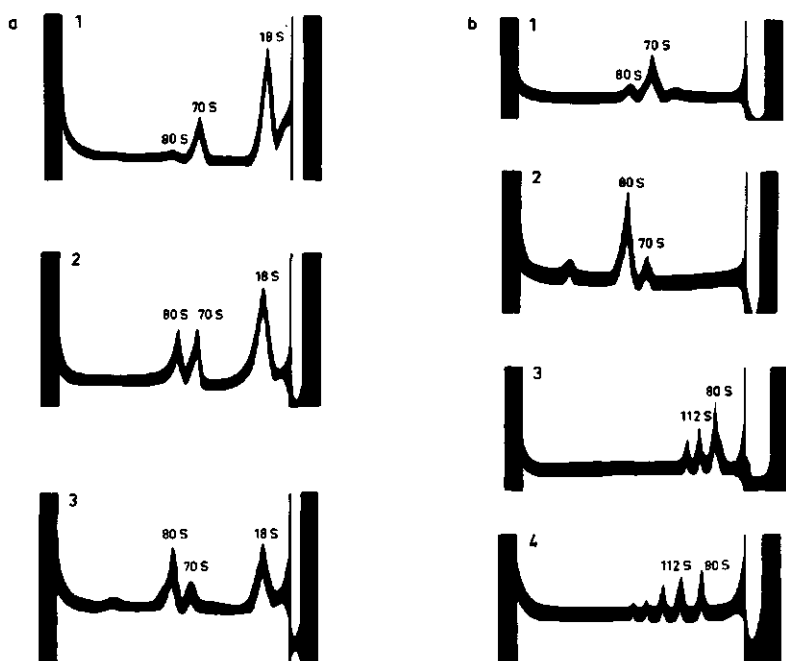


FIG. 4.1. Sedimentation patterns of ribosome preparations isolated, (a) from tobacco plants grown under constant controlled conditions in different sucrose gradient layers obtained with an extract of one batch of tobacco leaves. The solvent was standard buffer. Sedimentation was from right to left. Speed 37,020 rpm. Temperature 20 °C.

- a. 1. Plants sown in juli; experiment on september 21, 1964.
2. Plants sown in august; experiment on october 14, 1964.
3. Plants sown in december 1964; experiment on february 24, 1965.
- b. 1. 70 S ribosomes.
2. 80 S ribosomes.
3. Smaller polyribosomes.
4. Larger polyribosomes.

the 80 S band contained over 70% of all the material placed on the gradient, hence the yield of 70 S material and polyribosomes, especially of the larger polyribosomes, was very poor. The polyribosome samples were never free of 80 S particles, probably due to breakdown of the messenger RNA during the various treatments after isolation.

Some years ago, it has been proposed that the 70 S material originates from the chloroplasts and the 80 S material from the cytoplasm (LYTTLETON, 1962; CLARK, 1964; MATTHEWS and RALPH, 1964). This was confirmed by BOARDMAN et al. (1965), in studying the structure of chloroplasts. FRANCKI et al. (1965) distinguished two so-called phases in these structures, i.e., a stationary phase with the chlorophyll-containing grana, and a mobile phase surrounding the other one, containing ribosomes, enzymes, tRNA and other factors, necessary

for protein synthesis. The mobile phase is surrounded by a semipermeable membrane, which can burst easily when the chloroplasts are suspended in a buffer with a low osmotic value. Apparently the chloroplasts will remain intact during the sucrose extraction, whereas their outer membranes will burst when they are resuspended in standard buffer. The ribosomes will then be released and the stationary phase can be centrifuged off. We have adopted this method with a minor modification (see 2.2.2).

In this way, it was confirmed that the 70 *S* particles originate from the chloroplasts and the 80 *S* particles from the cytoplasm. The 70 *S* ribosomes were always contaminated with cytoplasmic ribosomes and the 80 *S* with chloroplast ribosomes. Further purification of each type could be obtained by sucrose gradient centrifugation as described previously. It was advantageous to isolate the chloroplasts prior to the purification of the ribosomes in that higher yields of 70 *S* ribosomes were obtained.

It was tested whether the polyribosomes were real polyribosomes or merely aggregates by treating a ribosome preparation with RNase at 0°C. After such treatment most material larger than 80 *S* had disappeared and could be recovered from an 80 *S* zone as shown in figure 4.2. Since the 70 *S* peak did not increase after RNase treatment, it was presumed that no polyribosomes consisting of 70 *S* ribosomes were isolated.

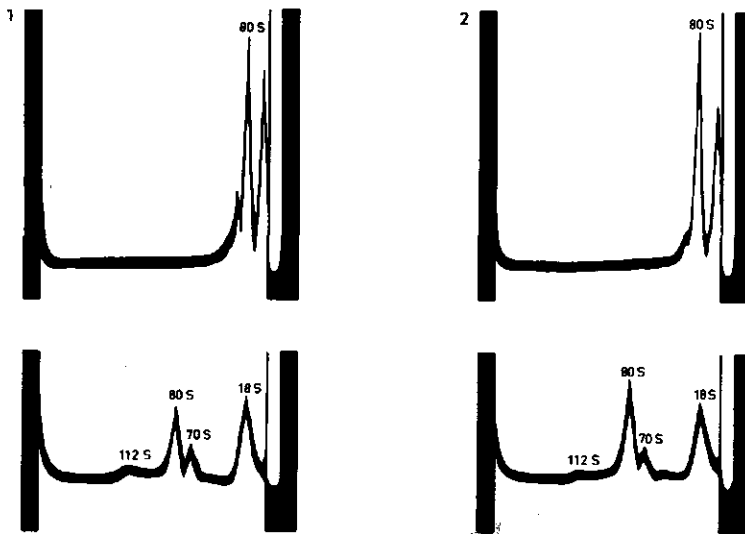


FIG. 4.2. Breakdown of polyribosomes by treatment with RNase. The solvent was standard buffer. Sedimentation conditions as in figure 4.1.

1. Ribosome mixture, untreated.

2. Ribosome mixture, incubated for 1 hour at 0°C with pancreas RNase (1 µg/ml).

4.2. THE INFLUENCE OF HIGH SALT CONCENTRATIONS ON 70 S AND 80 S RIBOSOMES

As pointed out in the previous chapter, washing the ribosomes through 0.5 M NH_4Cl had a favourable effect on their activity for amino acid incorporation. Peculiarly enough, it was found that during purification most of the 70 S ribosomes were lost, so that the results obtained with highly purified ribosomes, described in 3.3.2, pertain to 80 S ribosomes. It seemed of interest to investigate which treatment was leading to the elimination of the 70 S ribosomes. As shown in the analytical ultracentrifuge 70 S ribosomes were unstable in 0.5 M NH_4Cl . Both 70 S and 80 S ribosomes were first centrifuged in duplicate on a sucrose layer as described in 2.3.b; in one case 0.5 M NH_4Cl was added to the sucrose, in the other not. Samples of those ribosomes were then run in the analytical ultracentrifuge. The results, shown in figure 4.3, demonstrate clearly that 70 S ribosomes are partly broken down to low molecular weight material by the 0.5 M NH_4Cl treatment, whereas 80 S ribosomes are not. We cannot explain why some of the 70 S ribosomes always remained unaffected by this treatment.

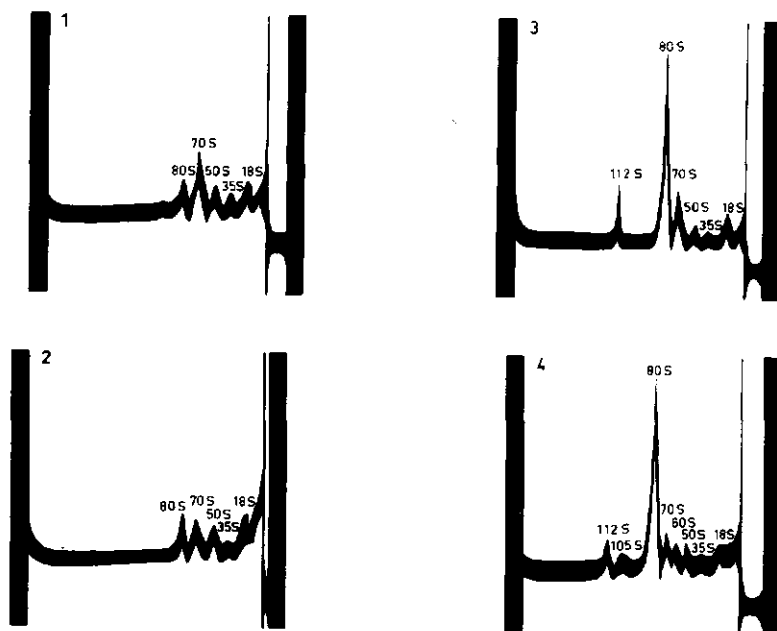


FIG. 4.3. Effect of 0.5 M NH_4Cl on 70 S and 80 S ribosomes. The solvent was standard buffer. Sedimentation conditions as in figure 4.1.

1. 70 S preparation, pelleted in 0.5 M sucrose.
2. 70 S preparation, pelleted in 0.5 M sucrose, containing 0.5 M NH_4Cl .
3. 80 S preparation, pelleted in 0.5 M sucrose.
4. 80 S preparation, pelleted in 0.5 M sucrose, containing 0.5 M NH_4Cl .

4.3. THE EFFECT OF LOW MAGNESIUM ION CONCENTRATIONS ON 70 S AND 80 S RIBOSOMES

Since ribosomes are stabilized by magnesium ions (see chapter 1), the removal of these ions might show differences between 70 S and 80 S ribosomes. In our experiments the magnesium ion concentration of ribosome preparations was reduced by dialysing them against a standard buffer containing 10^{-4} M MgCl_2 (standard- 10^{-4} M MgCl_2 buffer). Two hours of dialysis against this buffer appeared to be sufficient to dissociate the 70 S ribosomes almost completely into their subunits, i.e., 50 S and 35 S particles. This dissociation process was reversible since dialysing against standard buffer (containing 10^{-2} M MgCl_2) caused the 50 S and 35 S particles to reassociate into 70 S ribosomes. Reassociation was nearly complete after one hour of dialysis. These results can be seen in figure 4.4.

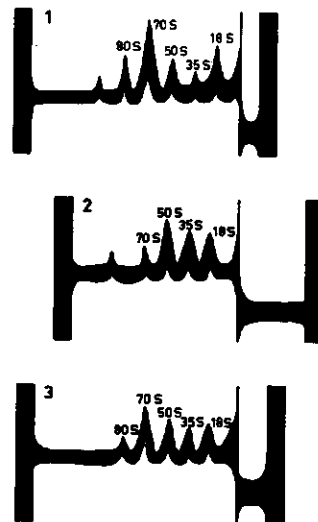


FIG. 4.4. Dependence of 70 S ribosome dissociation and reassociation on the Mg^{2+} concentration. Sedimentation conditions as in figure 4.1.

1. Prepared as described in 4.1, untreated. Solvent: standard buffer.
2. After dialysis for 2 hours against standard 10^{-4} M Mg^{2+} buffer. Solvent: the same buffer.
3. First dialysed as in (2), and dialysed subsequently for $1\frac{1}{2}$ hours against standard buffer. Solvent: standard buffer.

The 80 S ribosomes behaved quite differently during dialysis against 10^{-4} M MgCl_2 . Firstly, the 80 S ribosomes appeared much more stable in low Mg^{2+} concentrations than the 70 S ribosomes, and after 24 hours of dialysis some of the 80 S ribosomes were still intact. Secondly, no dissociation into 2 distinct subunits could be observed. In order to demonstrate what was happening during the removal of Mg^{2+} ions, samples of 80 S ribosomes which had been dialysed for different periods, were run in the analytical ultracentrifuge. Such a series is shown in figure 4.5 (1-4). It is clear that during dialysis the 80 S peak decreased gradually. At the same time a 60 S peak appeared, which decreased again later on. In conjunction with the decrease of the 60 S peak, there appeared 50 S and 40 S peaks in increasing amounts. Especially the first stages of 80 S ribosome 'dissociation' were indistinct because of the presence of 50 S and 35 S subunits originating from the 70 S ribosomes. The

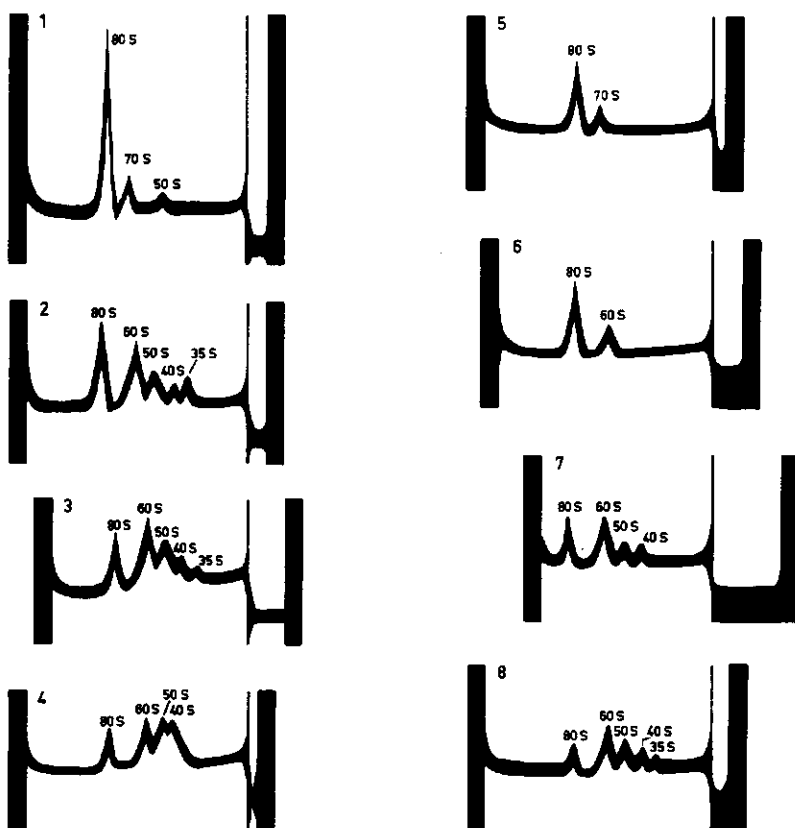


FIG. 4.5. Effect of low Mg^{2+} concentrations on the 80 S ribosomes. Ribosomes were prepared as described in 4.1. Sedimentation conditions as in figure 4.1.

1. Untreated. Solvent: standard buffer.
2. After dialysis for 8 hours against standard 10^{-4} M $MgCl_2$ buffer. Solvent: the same buffer.
3. As (2), but dialysed for 16 hours.
4. As (2), but dialysed for 24 hours.
5. Untreated. Solvent: standard buffer.
6. After dialysis for 3 hours against standard 10^{-4} M $MgCl_2$ buffer. Solvent: the same buffer.
7. As (6), but dialysed for 5.5 hours.
8. As (6), but dialysed for 8 hours.

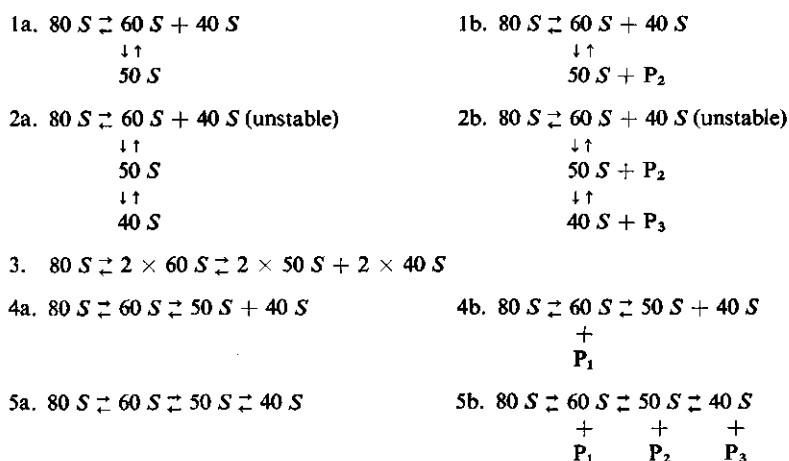
peaks of these subunits interfered with those of the 80 S 'subparticles'. Upon prolonged dialysis the 35 S subunit disappeared since in picture 4.5.4 no such particles were observed anymore.

To a certain extent, the presence of 70 S and 35 S particles was useful because they could serve as good markers for comparing the 80 S ribosomal 'subparticles' with the 70 S ribosomal subunits. It seemed that removal of

Mg^{2+} ions transformed the 80 S ribosomes into 60 S particles, whereas later on 50 S and 40 S particles appeared. To illustrate the first stages in this process, another series was run, in which dialysis was for shorter periods. This series is shown in figure 4.5 (5–8). It is clear that already after 3 hours of dialysis 60 S particles appeared, whereas hardly any smaller particles could be seen at that time. This illustrates that initially there is a transformation of 80 S to 60 S particles. It becomes not clear from the pictures what happened with the 70 S ribosomes present in this experiment.

There are several possibilities to explain these observations, as is summarized schematically in scheme 4.1. In 4 of the 5 possibilities unfolding of the particles (a) and removal of proteins (b) are pictured as distinct steps. The following experiments were designed to decide among these possibilities.

SCHEME 4.1. Possible relationships between 80 S, 60 S, 50 S and 40 S particles.



P_1 , P_2 , and P_3 are proteins, split from the 80 S, 60 S and 50 S particles, respectively.

Stable subunits are characterized by their capacity to recombine into the original particles. Thus, after dialysis of the samples of the 80 S ribosomes against 10^{-4} M $MgCl_2$ for different periods, the Mg^{2+} concentration was raised again by dialysis against standard buffer for 4 hours. The results of such an experiment can be seen in figure 4.6. After 8 hours of dialysis against standard- 10^{-4} M $MgCl_2$ buffer, the 60 S peak was relatively highest, and these particles could be transformed back into 80 S ribosomes almost quantitatively (pictures 3 and 4). After 16 hours more 50 S and 40 S particles were already present which could not be transformed back quantitatively into 80 S ribosomes (pictures 5 and 6). After 24 hours no 60 S particles were remaining and only very few 80 S ribosomes could be recovered after the Mg^{2+} concentration was raised again (pictures 7 and 8). Moreover, when the Mg^{2+} concentration was raised, most of the 40 S and 50 S particles disappeared without being transformed back into 80 S ribosomes. There appeared to be an inverse relationship

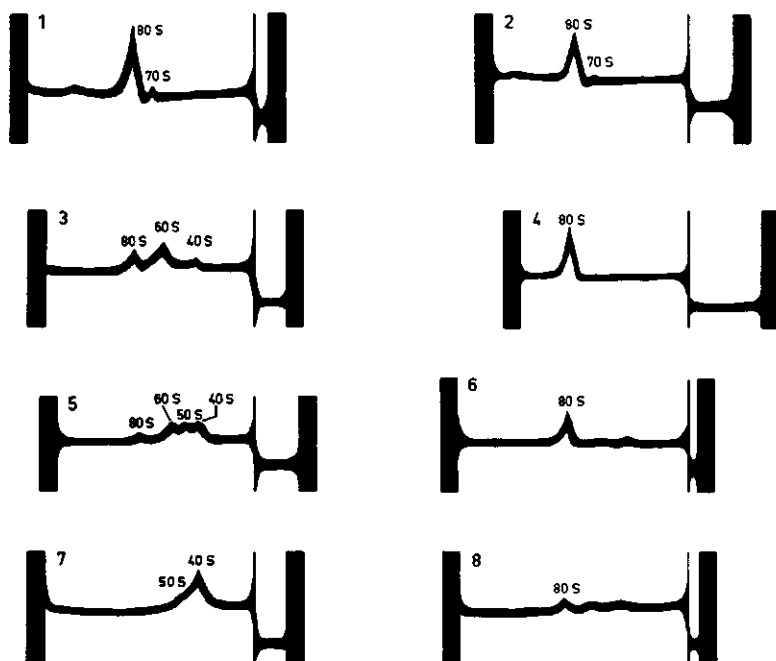


FIG. 4.6. Reversion of the effect of low Mg^{2+} concentration on the 80 S ribosomes. Ribosomes were prepared as described in 4.1. Sedimentation conditions as in figure 4.1.

1. Untreated. Solvent: standard buffer.
2. Dialysed for 24 hours against standard buffer.
3. Dialysed for 8 hours against standard 10^{-4} M $MgCl_2$ buffer. Solvent: the same buffer.
4. As in (3), but dialysed subsequently for 4 hours against standard buffer. Solvent: the same buffer.
5. As (3), but dialysed for 16 hours.
6. As in (5), but dialysed subsequently for 4 hours against standard buffer. Solvent: the same buffer.
7. As (3), but dialysed for 24 hours.
8. As in (7), but dialysed subsequently for 4 hours against standard buffer. Solvent: the same buffer.

between the percentage of recovery of 80 S ribosomes and the degree of change into 40 S and 50 S particles. Furthermore, a direct correlation exists between the amount of 60 S particles present and the extent to which 80 S ribosomes could be recovered. It was felt that these observations excluded the possibilities 1 and 2 (scheme 4.1), as 80 S ribosomes would require intact 40 S particles for the reassociation.

To obtain more definite information on the 60 S particles we tried to purify them on sucrose gradients similar to those used to separate the 80 S and the 70 S ribosomes. However, no pure 60 S particles could be obtained in this way for the following reasons:

- a. The 60 S particles were too unstable under the conditions of such an isolation, as will be shown below.
- b. The amount of material available was too small for gradient studies.

Ad a) The problem of instability could only partly be solved, as will be shown hereafter. Several methods were tried in order to obtain stable 60 S particles. First a ribosome pellet was resuspended in phosphate buffer (0.05 M, pH 7.0) and the suspension was run in the analytical ultracentrifuge. As can be seen in figure 4.7 (1 and 2) the results were similar to those obtained when the ribosomes were dialysed for 16 hours against 10^{-4} M Mg^{2+} (figure 4.6.5).

To other samples, various concentrations of EDTA were added. These samples were also run in the analytical ultracentrifuge and the pictures are shown in figure 4.7 (3–6). At 0.017 M EDTA some 60 S particles could be observed (figure 4.7.5), but both with phosphate and EDTA this appeared to be insufficient for our purpose. In addition, these results obtained with both methods were similar to those obtained by dialysis against 10^{-4} M Mg^{2+} . Thus, neither of these methods did answer the expectations.

After this, it was tried to obtain better results by varying the Mg^{2+} concentration. For this purpose samples of 80 S ribosomes were dialysed for 24 hours against standard buffer, containing various Mg^{2+} concentrations, and then run in the analytical ultracentrifuge. The pictures of this experiment are shown in figure 4.8 (1–6). In fact, a series was obtained similar to those of the figures 4.5 and 4.6, i.e., when a constant Mg^{2+} concentration (10^{-4} M) was used for various periods of dialysis. Since a fair amount of 60 S particles was present when $5 \cdot 10^{-4}$ M Mg^{2+} was used (4.8.4), Mg^{2+} concentrations around this

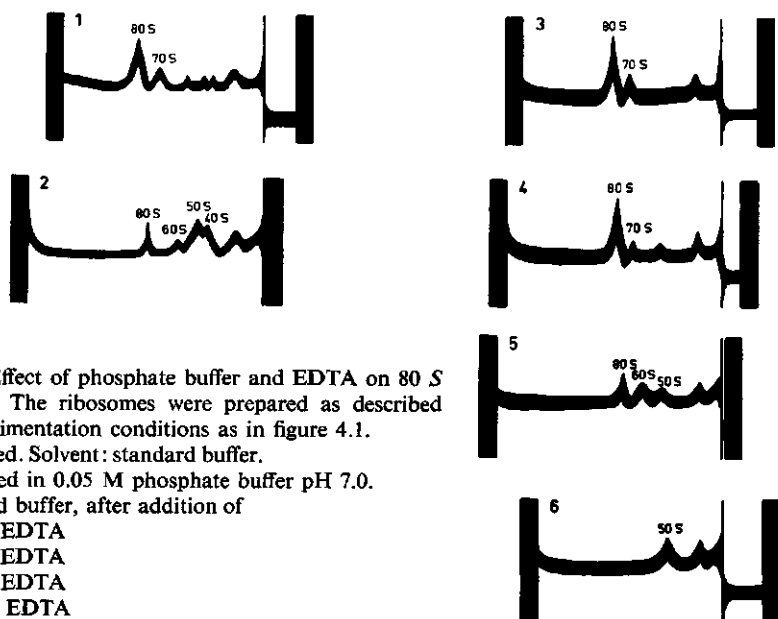


FIG. 4.7. Effect of phosphate buffer and EDTA on 80 S ribosomes. The ribosomes were prepared as described in 2.2. Sedimentation conditions as in figure 4.1.

1. Untreated. Solvent: standard buffer.
2. Suspended in 0.05 M phosphate buffer pH 7.0. In standard buffer, after addition of
3. 0,005 M EDTA
4. 0,010 M EDTA
5. 0,017 M EDTA
6. 0,025 M EDTA

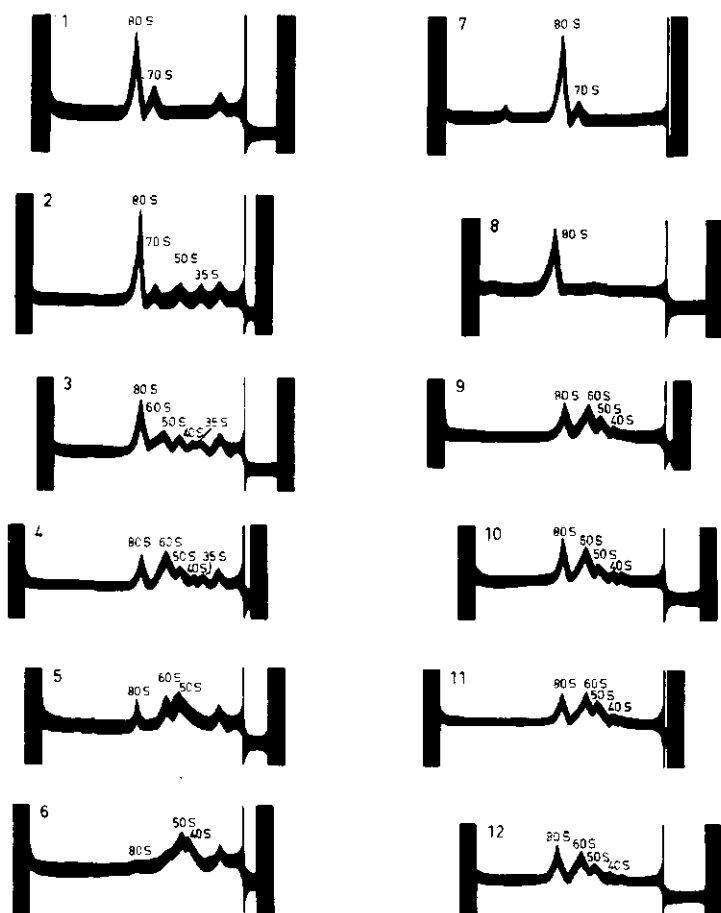


FIG. 4.8. Stability of the 60 S particles. The 80 S ribosomes were prepared as described in 2.2. Sedimentation conditions as in figure 4.1.

1. 80 S ribosomes, untreated. Solvent: standard buffer.

2-6. 80 S ribosomes, dialysed for 24 hours against 2.10^{-3} , 10^{-3} , 5.10^{-4} , 2.10^{-4} and 10^{-4} M $MgCl_2$ in standard buffer, respectively. Each time the solvent was the dialysis buffer.

7. 80 S ribosomes, untreated. Solvent: standard buffer.

8. 80 S ribosomes, dialysed against standard 4.10^{-4} M $MgCl_2$ buffer for 24 hours, then dialysed against standard buffer for 4 hours. Solvent: standard buffer.

9 and 10. 80 S ribosomes, dialysed for 16 hours against 4.10^{-4} and 6.10^{-4} M $MgCl_2$ in standard buffer, respectively. Solvent: the dialysis buffer.

11 and 12. 80 S ribosomes, dialysed for 24 hours against 4.10^{-4} and 6.10^{-4} M $MgCl_2$ in standard buffer, respectively. Solvent: the dialysis buffer.

value were further examined. In order to get some information about the stability of the 60 S particles, samples dialysed for 16 hours and 24 hours were compared. In addition, the effect of small differences in the Mg^{2+} concentration was examined by varying the Mg^{2+} concentration. The results can be seen in figure 4.8 (7–12). It is clear that the patterns at $4 \cdot 10^{-4}$ M or $6 \cdot 10^{-4}$ M Mg^{2+} were very similar, neither did they change much when the material was dialysed for 24 hours instead of 16 hours. This meant to us that at a Mg^{2+} concentration of 4–6 times 10^{-4} M the 60 S particles were rather stable. We confirmed this by dialysing sample 11 back to 10^{-2} M Mg^{2+} , which enabled the 80 S ribosomes to be recovered almost completely (picture 8).

Ad b) The availability of a B_{14} zonal rotor (see chapter 2.5) in the final stages of this investigation made it possible to solve the second difficulty, as in the zonal rotor more than 10 times as much material (30–50 mg of ribosomal RNA) could be treated. Other advantages of this rotor were a good separation of 70 S and 80 S ribosomes – i.e., very pure ribosomes of each type could be obtained – and a convenient collection of the various fractions.

In figure 4.9 the results of a zonal centrifugation are shown. Ribosomes (24 mg ribosomal RNA) were layered on the gradient and run for 5 hours at 30,000 rpm as described in chapter 2.5. Fractions of about 10 ml were collected and the A_{260} was measured. When compared to the same ribosomal mixture, run in the analytical ultracentrifuge (figure 4.10.1), both pictures were very similar. The fractions 16–21 and 23–26, presumed to contain the 80 S and 70 S ribosomes (figure 4.9), were collected and the ribosomes were sedimented by centrifugation for 3 hours at $105,000 \times g$. After resuspending in standard buffer samples were run again in the analytical ultracentrifuge (figure 4.10, pictures 2 and 3). In this way it was shown that the alleged 70 S and 80 S peaks indeed consisted of such ribosomes and further that the 70 S preparation was essentially free from 80 S particles and, likewise, the 80 S preparation free from 70 S particles. Particle recovery was about 50%. More than 80%, ex-

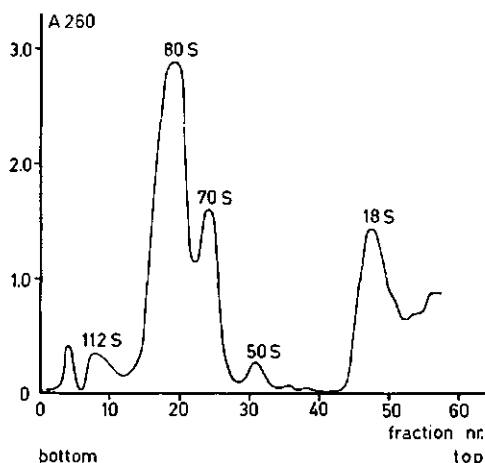


FIG. 4.9. Separation of 70 S and 80 S ribosomes by zonal centrifugation. Ribosomes were prepared as described in 2.2 and centrifuged in the zonal rotor as described in 2.5. They were layered on the gradient in a concentration of 24 mg ribosomal RNA. Centrifugation was at 30,000 rpm for 5 hours. Fractions of about 10 ml were collected and the A_{260} was measured. Fractions 16–21 and 23–26 were collected and the ribosomes were concentrated by centrifugation at $105,000 \times g$ for 3 hours.

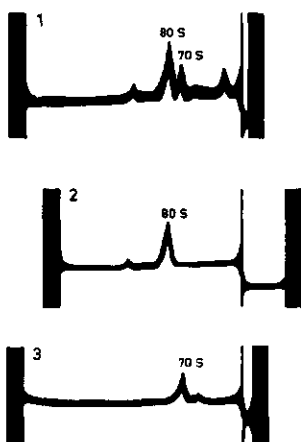


FIG. 4.10. Composition of the ribosome preparation before and after zonal centrifugation. The solvent was standard buffer. Sedimentation conditions as in figure 4.1.

1. Ribosomes, untreated.
 2. 80 S peak, as collected from the zonal centrifugation.
 3. 70 S peak, as collected from the zonal centrifugation.
- For further explanations see text.

pressed in OD units, was recovered from the zonal rotor. The loss of material during the concentration of the ribosomes may be due to the fact that the suspension from which they were sedimented was very dilute.

The 80 S ribosomes, purified as described above, were used for the following experiments. Ribosomes were dialysed against 5.10^{-4} M MgCl_2 for 14 hours in order to obtain a fair amount of 60 S particles (compare figure 4.8.4). Then the whole sample was centrifuged in the zonal rotor, using a gradient containing 5.10^{-4} M MgCl_2 . At the end of the run, the fluid was pumped

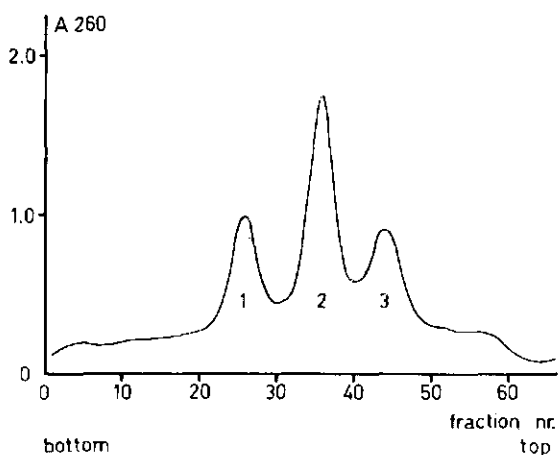


FIG. 4.11. Isolation of 80 S, 60 S and 50 S particles by means of zonal centrifugation. The 80 S ribosomes were prepared as shown in figure 4.9. These ribosomes were dialysed against standard 5.10^{-4} M MgCl_2 buffer for 16 hours and centrifuged again in the zonal rotor at 35,000 rpm for 3 hours. Fractions of 10 ml were collected and the A_{260} was measured. The fractions 24-28, 33-38 and 42-74, representing the peaks 1, 2 and 3, respectively, were combined and the material was pelleted at $105,000 \times g$.

out of the rotor as usual and fractions of 10 ml were collected. The A_{260} was measured and in figure 4.11 the OD pattern is shown. When comparing this figure to figure 4.8.4, it becomes clear that the separation profile again is in fair agreement with the pattern obtained in the analytical ultracentrifuge. Therefore, it was assumed that the three peaks contained 50 S, 60 S and 80 S particles from top to bottom. The fractions belonging to these peaks were collected and the material was concentrated as described above and resuspended in standard- 5.10^{-4} M $MgCl_2$ buffer. A sample of each peak was run in the analytical ultracentrifuge. The patterns are shown in figure 4.12 (1,3 and 5). Indeed, in each case most of the material was present in 50 S and 40 S peaks (5), a 60 S peak (3) and a 80 S peak (1). Other samples of the same material were dialysed against 10^{-2} M $MgCl_2$ for 3 hours in order to increase the Mg^{2+} concentra-

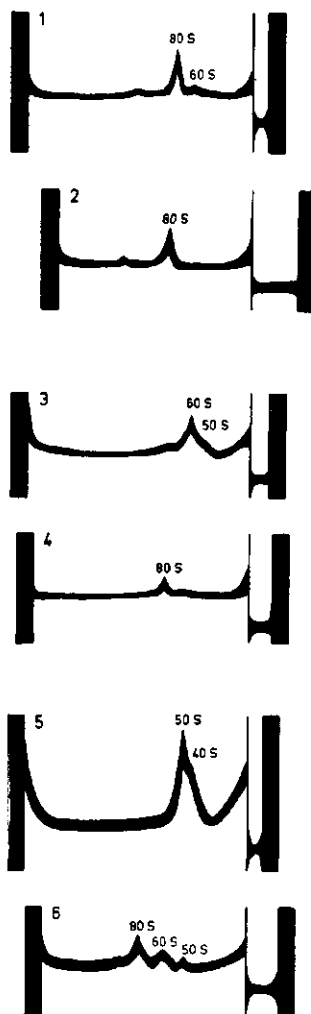


FIG. 4.12. Analysis of 80 S, 60 S and 50 S particles, obtained by zonal centrifugation. Sedimentation conditions as in figure 4.1.

1. 80 S peak. Solvent: standard 5.10^{-4} M $MgCl_2$ buffer.
2. 80 S peak, after dialysis against standard buffer for 3 hours. Solvent: standard buffer.

3. 60 S peak. Solvent as in 1.

4. 60 S peak, after dialysis against standard buffer for 3 hours. Solvent: standard buffer.

5. 50 S peak. Solvent as in 1.

6. 50 S peak, after dialysis against standard buffer for 3 hours. Solvent: standard buffer. The last pair of patterns (no 5 and 6) was obtained from a preparation different from that used in the other pairs. Since more material was available here, these peaks appear to be disproportionately high. The relative amount of material, however, was similar to that in peak 3 in the zonal centrifugation pattern, shown in figure 4.11. For further explanations see text.

tion, which had been maintained at $5 \cdot 10^{-4}$ M during the whole procedure, and subsequently were run in the analytical ultracentrifuge. The 80 S material had hardly changed after this procedure, except that the few 60 S particles present in this preparation (1) were converted into 80 S ribosomes again (2). A fair amount of the 60 S material (3) had changed into 80 S ribosomes after dialysis (4). Some of the 40 S and 50 S particles (5) had also been converted to 80 S particles after this treatment (6), but most of the material had been broken down during dialysis against standard buffer, as pointed out before.

Unfortunately the 60 S particles were never completely transformed into 80 S ribosomes, as they did in a crude mixture (figure 4.8.8). This may be due to a conversion of 60 S into 50 S and 40 S particles during the zonal centrifugation and further treatments. Thus, at the moment that dialysis against standard buffer was started, only part of the original amount of 60 S particles was remaining, which resulted in only a partial conversion into 80 S particles, whereas the 50 S and 40 S material was broken down. In addition, protein may be lost during the transformation of 80 S into 60 S material, which was centrifuged off during the subsequent zonal centrifugation. Finally, the ribosomes could be damaged during the whole procedure resulting in a partial breakdown to material of low molecular weight.

Since the 60 S particles could be formed from 50 S and 40 S particles as well as from 80 S particles, it is unlikely that 60 S particles are subunits. As can be seen in figure 4.11, no material was recovered from the bottom or the top of the gradient, indicating that during the transformation from 80 S to 60 S and subsequently to 50 S and 40 S particles essentially no material was lost. This strongly indicates that all these changes occurred without proteins being split off. Thus, possibilities 4b and 5b of scheme 4.1 can be excluded as well. The remaining three possibilities will be discussed hereafter.

4.4. RNA FROM 70 S AND 80 S RIBOSOMES

The RNA content of both 70 S and 80 S ribosomes was determined with the orcinol method, as described in chapter 2.10. In parallel tests the protein content was determined according to LOWRY et al. The ribosomes used for these experiments were purified on sucrose gradients, as described in chapter 2.3.c. The sedimentation pattern depicted in figure 4.1.b (1 and 2) show their degree of purity.

The results of several determinations are shown in table 4.1. No essential difference appeared to exist in RNA and protein content between 70 S and 80 S ribosomes, which both consist of about 40% RNA and 60% protein.

RNA was isolated from both 70 S and 80 S ribosomes as described in chapter 2.11. Samples of these RNAs were run in the analytical ultracentrifuge; the sedimentation patterns are represented in figure 4.13. Both 70 S and 80 S ribosomal RNA usually showed two components. For the 70 S ribosomal RNAs both peaks were mostly equal in height and the sedimentation coefficients were calculated as 17 S and 23 S. The sedimentation coefficients of the

TABLE 4.1. RNA and protein content of 70 *S* and 80 *S* ribosomes. The RNA and protein contents were determined as described in 2.10 and the values obtained were expressed in per cent.

	Ribosomes	Experiment				Average
		1	2	3	4	
RNA content in %	70 <i>S</i>	41	43	39	41	41
Protein content in %		59	57	61	59	59
RNA content in %	80 <i>S</i>	41	38	40	39	39.5
Protein content in %		59	62	60	61	60.5

80 *S* ribosomal RNAs proved to be 17 *S* and 25 *S*. The 25 *S* component seemed more stable than the 17 *S* component, for in some preparations the latter was hardly noticeable. However, no preparations in which both peaks were equal in height could be obtained.

Thus ribosomes could be characterized by the sedimentation patterns of their RNA. Therefore, it seemed of interest to analyze the RNA isolated from the particles obtained by dialyzing 80 *S* ribosomes against 5.10^{-4} M Mg^{2+}



FIG. 4.13. Sedimentation patterns of RNA from 70 *S* and 80 *S* ribosomes. The RNA was prepared as described in 2.11. The solvent was 0.01 M phosphate buffer pH 7.0, containing 0.1 M NaCl. Sedimentation is from right to left. Speed 50,740 rpm. Temperature 20°C.

1. RNA from 70 *S* ribosomes.
2. RNA from 80 *S* ribosomes.

FIG. 4.14. Sedimentation pattern of RNA from 60 *S* particles. 80 *S* ribosomes were dialysed against 5.10^{-4} M Mg^{2+} for 14 hours and then centrifuged in the zonal rotor as described in 4.3 (see figure 4.11). From the purified 80 *S* and 60 *S* particles RNA was prepared. Solvent was 0.01 M phosphate buffer pH 7.0, containing 0.1 M NaCl. Sedimentation is from right to left. Speed 50,740 rpm. Temperature 20°C.

1. RNA from 80 *S* particles.
2. RNA from 60 *S* particles.

in the ultracentrifuge. Dialysed 80 S ribosomes were centrifuged in the zonal rotor and the 60 S and 50 S and 40 S particles were then collected and concentrated as described in 4.3 (see figure 4.11). From these components RNA was prepared and the various RNAs were analysed in the analytical ultracentrifuge. The results are shown in figure 4.14. Both 80 S and 60 S material appeared to contain 2 RNA components. This constitutes additional evidence for our suggestion that upon the removal of Mg^{2+} ions 60 S particles appear which are not 80 S subunits. However, the ratio between the 25 S and the 17 S peak was not the same in both cases. Since the 17 S RNA seemed rather unstable, part of it might have been broken down during isolation of the 60 S particles. Only degraded RNA could be obtained from the 50 S and 40 S particles. This made it impossible to decide whether the 40 S and 50 S particles are real subunits.

The presence of two RNA components, of 25 S and 17 S respectively, in 80 S ribosomes as well as in the 60 S particles, strongly favours the transformation of one 80 S ribosome into one 60 S particle. This excludes a dissociation into two 60 S particles. (scheme 4.1, line 3). The presence of 4 RNA molecules per ribosome required in the latter case, would not agree with the size of the RNA molecules and of the ribosomes, and with the RNA content of the ribosomes.

4.5. DISCUSSION AND CONCLUSIONS

In this chapter the isolation and purification of several classes of ribosomes from tobacco leaves is described. Some of their properties were investigated.

The ratio between 70 S and 80 S ribosomes observed appeared to be extremely dependent on the conditions under which the tobacco plants were grown. Even under controlled conditions, this ratio was not always constant, i.e., in winter always a smaller amount of 70 S ribosomes was found in the leaves than during the summer season. It proved to be of advantage to use isolated intact chloroplasts for the preparation of 70 S ribosomes. Sucrose gradient centrifugation was found to be a useful method to obtain reasonably pure 70 S and 80 S ribosomes. The isolation of 70 S ribosomes from the sucrose gradient tubes was always difficult because of the low concentration of these ribosomes and the very small distance between the 70 S and 80 S bands. The use of a zonal rotor greatly improved the separation. Moreover, it resulted in a much higher yield of pure 70 S ribosomes.

The presence of polyribosomes could be demonstrated, but it was not possible to isolate large amounts of them. In general this has proved to be difficult with plant material. In most cases nuclease inhibitors have been used for this purpose and only from *Chenopodium album* a fair amount of polyribosomes could be isolated without using nuclease inhibitors (LYTTLETON, 1967) (see further 1.1). In our material polyribosomes were found consisting of 80 S ribosomes but no further attention was paid to the polyribosome fraction.

The instability of 70 S ribosomes in high salt concentrations is probably due to their rather pronounced Mg^{2+} requirements. They may be more stable when

at high salt concentration the Mg^{2+} concentration is also increased. This was not studied, however, for the following reason. It is known that 80 S ribosomes precipitate irreversibly at higher Mg^{2+} concentrations (VAN KAMMEN, 1963), so that the increased Mg^{2+} concentration during the salt washing might stabilize the 70 S ribosomes but precipitate the 80 S ribosomes. If only pure 80 S ribosomes were required the NH_4Cl treatment at low Mg^{2+} concentration would be a good method to eliminate the 70 S ribosomes. Another method for eliminating the 70 S ribosomes is a short (2–4 hours) dialysis against 10^{-4} M Mg^{2+} . Unfortunately, such a convenient method was not available for preparing pure 70 S ribosomes.

Upon removal of Mg^{2+} ions, the 70 S ribosomes very easily dissociate reversibly into their subunits, i.e., 50 S and 35 S particles. Our results do not differ from those obtained by BOARDMAN, FRANCKI and WILDMAN (1966). However, our results differ substantially from theirs as far as the 80 S ribosomes are concerned. BOARDMAN et al. (1966) have reported that 80 S ribosomes dissociate irreversibly into 58 S and 35 S particles. Ts'o, BONNER and VINOGRAD (1958) have investigated 80 S pea ribosomes, which, according to these authors, dissociate into 60 S and 40 S particles. They have also found a 26 S particle. BAYLEY (1964), also studying pea ribosomes, has presented similar data. He constructed a model, showing an 80 S ribosome consisting of two 40 S and two 26 S particles. Some of his patterns are rather similar to ours, viz. those in figures 4.5.4 and 4.6.5, which clearly demonstrates that during removal of Mg^{2+} ions, the 80 S peak decreased to be replaced by three other peaks, i.e., a 60 S, a 50 S and a 40 S peak. From our own experiments we conclude that 80 S ribosomes first transformed into 60 S particles when Mg^{2+} ions were removed. This process was largely reversible. The removal of more Mg^{2+} led to a transformation into 40 S and 50 S particles, which process was hardly reversible. Since no intact RNA could be isolated from 40 S and 50 S particles it is likely that these particles were at least partly degraded. In this connection it was not too unexpected that they could hardly be reorganized into 80 S particles.

Since the 60 S particles were shown to contain both 25 S and 17 S RNA, the transformation of 80 S ribosomes into 60 S particles is a kind of unfolding of the ribosomes. GESTELAND (1966) and GAVRILOVA, IVANOV and SPIRIN (1966) have reported similar data for unfolding of the 50 S and 30 S *E. coli* ribosomal subunits to particles of lower sedimentation value upon removal of Mg^{2+} ions. These processes were reversible. SUZUKA (1967) was able to unfold the 70 S *E. coli* ribosomes to 50 S particles by treating them with 0.015 M EDTA containing 0.02 M magnesium acetate. No dissociation occurred under these conditions, and the process was reversible. This process seems to be very similar to the transformation of tobacco 80 S ribosomes into 60 S particles. Similar data have been obtained with 80 S *Paramecium* ribosomes by treatment with EDTA (REISNER, ROWE and MACINDOE, 1968). In all these cases the ribosomal particles appear to unfold without detectable loss of material. Such structural changes may be of great use for elucidating the precise structure and functions of the ribosomes, about which little is known, as yet. Instability,

however, is a great drawback in handling the 60 S particles. A specific Mg^{2+} ion concentration is required for equilibrium. Under our conditions, this was about 5.10^{-4} M Mg^{2+} . Even at this concentration they were not completely stable, while at higher concentrations the unfolding process was too slow.

There is another, indirect, indication which makes us prefer our model to that of Ts'o et al. (1958) and BAYLEY (1964). According to Ts'o et al. the molecular weight of 80 S pea ribosomes is about 4.2×10^6 . CLICK and TINT (1967) have compared ribosomal RNAs of different origins with *E. coli* ribosomal RNAs. For the 80 S plant ribosomal RNAs they came to the conclusion that both components have molecular weights of 5.6×10^5 and 1.3×10^6 , respectively. The relative amounts of these RNAs, taking into account their sizes, were in agreement with the idea that ribosomes consist of two subunits each containing one molecule of RNA. Indeed, these values for ribosomal RNAs and the molecular weight of 4.2×10^6 given by Ts'o et al. (1958) roughly agree with the assumed composition of plant ribosomes: 40% RNA and 60% protein. According to Ts'o et al. and BAYLEY (1964) each ribosome would consist of 4 subunits (2×40 S and 2×26 S), each containing an equal percentage of RNA. This concept is not in agreement with the finding of two RNA molecules with molecular weights of 5.6×10^5 and 1.3×10^6 .

HERSH and PHILLIPS (1964) have also studied the dissociation of plant ribosomes. Working with pea ribosomes (80 S), they found, after dissociation in low Mg^{2+} concentrations, 60 S and 40 S particles in a ratio varying from 3.5 to 5.3. In their recombination experiments, the concentration of 60 S particles was taken so high that all the 40 S particles should have recombined. This did not occur and they concluded that the 80 S ribosome is composed of two 60 S particles. However, it is not very likely that this conclusion is valid. The 80 S ribosomes contain two RNA molecules, different from each other. Thus, the 60 S particles found by HERSH and PHILLIPS are probably similar to ours.

One could still visualize that 60 S particles dissociate into 50 S and 40 S particles or first unfold into 50 S and subsequently into 40 S particles (i.e., possibilities 4a and 5a of scheme 4.1). There is no definite evidence for either of these possibilities, as 40 S and 50 S particles could not be separated from each other and no intact RNA could be isolated from them. However, we tend to conclude that 40 S and 50 S particles are not subunits, but unfolded particles, because during dialysis against 10^{-4} M Mg^{2+} the ratio between these particles changed, i.e., first more 50 S particles were present and these were replaced successively by 40 S particles (see figure 4.5, pictures 3 and 4, figure 4.6, pictures 5 and 7).

It has become clear from the RNA studies that both 70 S and 80 S ribosomes contain two RNA components. The 70 S chloroplast ribosomal RNA shows peaks in the analytical ultracentrifuge which are about equal in height. This indicates a molar excess of the smaller RNA component, assuming that the larger component is about twice as large as the smaller one. Since both RNA molecules are supposed to occur in equimolar amounts, some of the larger

RNA may be lost during preparation, suggesting that this RNA is rather unstable or that extraction of this RNA is more difficult.

As far as the 80 *S* cytoplasmic ribosomal RNAs are concerned, their stability is the opposite to that of the 70 *S* ribosomal RNAs, i.e., the larger component proved to be the most stable one in our preparations, whereas the smaller RNA seemed to be broken down. Similar data have already been discussed by LOENING and INGLE (1967). An alternative explanation, although less likely, is dimerization of the 17 *S* RNA.

The sedimentation coefficients of 25 *S* and 17 *S* found by us for the RNAs from 80 *S* ribosomes and of 23 *S* and 17 *S* for the RNAs from 70 *S* ribosomes are in fair agreement with the values reported by LOENING and INGLE. We were not able to distinguish the smaller RNAs from 70 *S* from those of 80 *S* ribosomes by means of the analytical ultracentrifuge and with Schlieren optics.

The occurrence of two distinct RNA molecules in 80 *S* ribosomes indicated the existence of two subunits. The presence of subunits themselves, however, could not be demonstrated directly.

In summary, both 70 *S* chloroplast and 80 *S* cytoplasmic ribosomes occur in tobacco leaves. Those ribosomes can be separated on sucrose gradients. Their stability in high salt and low Mg^{2+} concentrations is highly different. Both types contain two different RNA molecules. The 70 *S* ribosomes dissociate reversibly into two subunits. The 80 *S* ribosomes are not dissociable into subunits under the conditions studied, suggesting a more complicated structure of these ribosomes. Removal of Mg^{2+} ions causes a successive unfolding of the 80 *S* ribosomes into 60 *S*, and 50 *S* and 40 *S* particles. A further study of these unfolded particles may lead to a better understanding of the multifunctional properties of the 80 *S* ribosomes.

5. AMINO ACID INCORPORATION BY 70 S AND 80 S RIBOSOMES

Chapter 4 describes the results of studies on the composition of the ribosome preparations. The 70 S and 80 S ribosomes were separated. In addition, two fractions containing polyribosomes could be distinguished. The stability of the 70 S and 80 S ribosomes proved to be rather different at low Mg^{2+} and high NH_4^+ ion concentrations. This prompted us to investigate whether there are any differences in amino acid incorporation between these two types of ribosomes.

In this chapter we shall report our experiments on the amino acid incorporation of the various components of the ribosome mixture. In particular, the incorporation by 70 S and 80 S ribosomes will be compared in more detail. The ribosomes proved to be different in several respects, such as the effect of variations in Mg^{2+} , pH and temperature, and the kinetics of the incorporation.

5.1. AMINO ACID INCORPORATION BY 70 S AND 80 S RIBOSOMES AND POLYRIBOSOMES

Ribosome mixtures were separated into 4 fractions by means of sucrose gradient centrifugation as described in chapter 4.1. These fractions consisted of 70 S and 80 S ribosomes and smaller and larger polyribosomes. The amino acid incorporation by each of these fractions was determined by incubation in the standard incubation mixture. The results given in table 5.1 show that there is no considerable difference in incorporating activities between these fractions under the conditions given. Figure 4.1.b has already shown the composition of a crude ribosome mixture.

Each fraction was present in different amounts. Table 5.1 gives the amount of each fraction obtained from the gradient. Since the amount of polyribosomes

TABLE 5.1. Yield and amino acid incorporating activity of different ribosome fractions

Ribosomes	70 S	80 S	Polyribosomes 1	Polyribosomes 2
Exp. 1 mg ribosomal RNA	—	2.2	1.7	0.1
activity in cpm/mg	—	161	167	200
Exp. 2 mg ribosomal RNA	0.7	2.4	1.0	—
activity in cpm/mg	236	400	595	—
Exp. 3 mg ribosomal RNA	0.9	2.5	1.5	0.3
activity in cpm/mg	143	175	185	160

The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 7.8, 11 μ moles $MgCl_2$, 75 μ moles NH_4Cl , 6 μ moles MCE, 0.003 μ mole ^{14}C -leucine (150 mC/mmmole), 25 μ g tRNA, 100 μ l enzymes and ribosomes (0.1–0.7 mg ribosomal RNA). The incubation time was 45 minutes.

which could be isolated was small and the activities of the various fractions did not differ greatly, it seemed of little use to study extensively the properties of the polyribosome fractions in this stage of this investigation.

The amount of 70 S ribosomes, isolated in this way, was small. For this reason, 70 S ribosomes were isolated from the chloroplast fraction as described in chapter 2.2.2. Usually the yield was considerably higher when this method was used. Yield and activity of these 70 S ribosomes were checked and compared to those of 80 S ribosomes. In addition, the amino acid incorporation by whole chloroplasts was determined, as they were thought to be highly active (SPENCER and WILDMAN, 1964). The results of some of these experiments are given in table 5.2. The activity in cpm seemed to be relatively low for the whole chloroplasts as compared to that of the 70 S ribosomes, taking into account that it was started from the same amount of ribosomes in both cases. The 70 S ribosome fraction was more active and therefore no further attention was paid to the whole chloroplasts. Under the given conditions the 70 S ribosomes were less active than the 80 S ribosomes, and there was essentially no difference between the activities of the 70 S ribosomes which were prepared either from a whole homogenate or from isolated chloroplasts. So the isolation from purified chloroplasts was useful because of the simpler isolation procedure and the higher yield (about 2 mg). For this reason, 70 S ribosomes were isolated from chloroplasts as described in chapter 2.2.2 in the following experiments.

TABLE 5.2. A comparison between the amino acid incorporating activity of whole chloroplasts, 70 S ribosomes from chloroplasts and 80 S ribosomes.

Ribosomes	Conditions	Amino acid incorporation in cpm/mg	
		exp. 1	exp. 2
80 S	Complete	282	290
	Complete - energy	10	21
70 S	Complete	207/241 ¹	120/290 ¹
	Complete - energy	4/5 ¹	5/11 ¹
Whole chloroplasts	Complete	169 ¹	53 ¹
	Complete - energy	57 ¹	50 ¹

The complete incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 7.8, 11 μ moles $MgCl_2$, 75 μ moles NH_4Cl , 6 μ moles MCE, 0.003 μ mole ^{14}C -leucine (150 mC/mmole), 25 μ g tRNA, 100 μ l enzymes and ribosomes (0.3-0.7 mg ribosomal RNA). The incubation time was 45 minutes.

¹ Activities given in cpm.

5.2 INFLUENCE OF THE MAGNESIUM ION CONCENTRATION ON THE AMINO ACID INCORPORATION BY 70 S AND 80 S RIBOSOMES

The amino acid incorporation by non-purified ribosomes did not show a strong dependence on the Mg^{2+} concentration (chapter 3). We supposed that this was due to the presence of different types of ribosomes in the incubation mixture. Since the Mg^{2+} concentration was critical for the physical properties

of 70 *S* and 80 *S* ribosomes (chapter 4.3), the influence of Mg^{2+} ions on the amino acid incorporation by both classes of ribosomes was studied. The results of these experiments are shown in figure 5.1.

The 70 *S* ribosomes appeared to have a very distinct optimum at 17 mM Mg^{2+} as shown by curve a in figure 5.1, which is characteristic for the Mg^{2+} dependence of all our 70 *S* ribosome preparations.

The 80 *S* ribosomes did not seem to have such a distinct Mg^{2+} optimum, even when purified in 0.5 M sucrose. We often obtained curves having optima at 5 mM as well as in the range of 11–17 mM Mg^{2+} like curve b in figure 5.1. The curves were not very reproducible from preparation to preparation, which may be due to varying amounts of 70 *S* ribosomes contaminating the 80 *S* ribosome preparations. Therefore, 80 *S* ribosomes were purified by sucrose gradient centrifugation as described in chapter 2.3.c to eliminate the 70 *S* ribosomes. Such purified 80 *S* ribosomes showed a Mg^{2+} dependence as given by curve c in figure 5.1. Thus, the optimum Mg^{2+} concentration for amino acid incorporation by 80 *S* ribosomes is 5 mM. In cases where this optimum could not be established contamination with 70 *S* ribosomes was assumed. With decreasing amounts of 70 *S* ribosomes the optimum varied from curve b to c. Figure 5.1 further shows that under optimal conditions 70 *S* ribosomes were more active (generally 2–3 times) in amino acid incorporation than 80 *S* ribosomes.

Knowing that 70 *S* ribosomes were rather unstable at low Mg^{2+} concentrations (chapter 4.3), it became desirable to check at which Mg^{2+} concentration they would show greatest stability under our experimental conditions. Therefore,

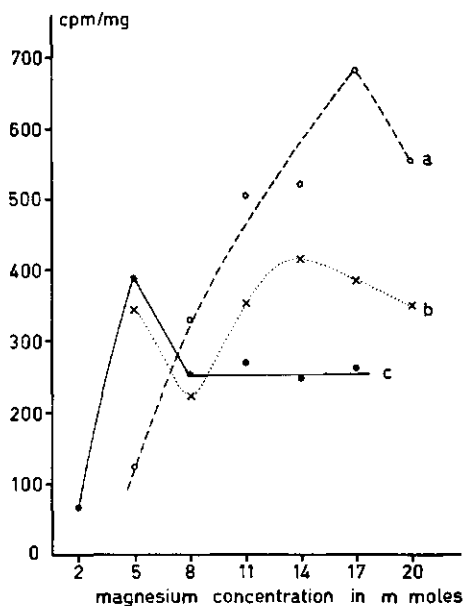


FIG. 5.1. Magnesium ion dependence of 70 *S* and 80 *S* ribosomes. The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 7.8, $MgCl_2$ (2–20 μ moles), 75 μ moles NH_4Cl , 6 μ moles MCE, 0.002 μ mole ^{14}C -leucine (250 mC/mmmole), 25 μ g tRNA, 100 μ l enzymes and ribosomes. The incubation time was 45 min. a. 70 *S* ribosomes (0.19 mg ribosomal RNA). b. 80 *S* ribosomes, purified on 0.5 M sucrose (0.58 mg ribosomal RNA). c. 80 *S* ribosomes, purified on a sucrose gradient (0.40 mg ribosomal RNA).

samples were incubated for 45 minutes at different Mg^{2+} concentrations in an incubation mixture as used for the incorporation experiments, and then analysed in the analytical ultracentrifuge. The results are shown in figure 5.2. The 70 S ribosomes were highly contaminated with 80 S ribosomes (5–8). The 80 S ribosomes contained a small amount of 70 S ribosomes (1–4). At every Mg^{2+} concentration used, the 80 S ribosomes proved to be stable, i.e., no dissociation or breakdown of 80 S ribosomes was perceptible after incubation (1–4). The 70 S ribosomes appeared to be stable only at 17 mM Mg^{2+} when compared to the unincubated sample. This agrees with the optimal Mg^{2+} concentration for incorporation. At lower concentrations, particularly below 12 mM, a large part of the 70 S ribosomes had dissociated into 50 S and 35 S

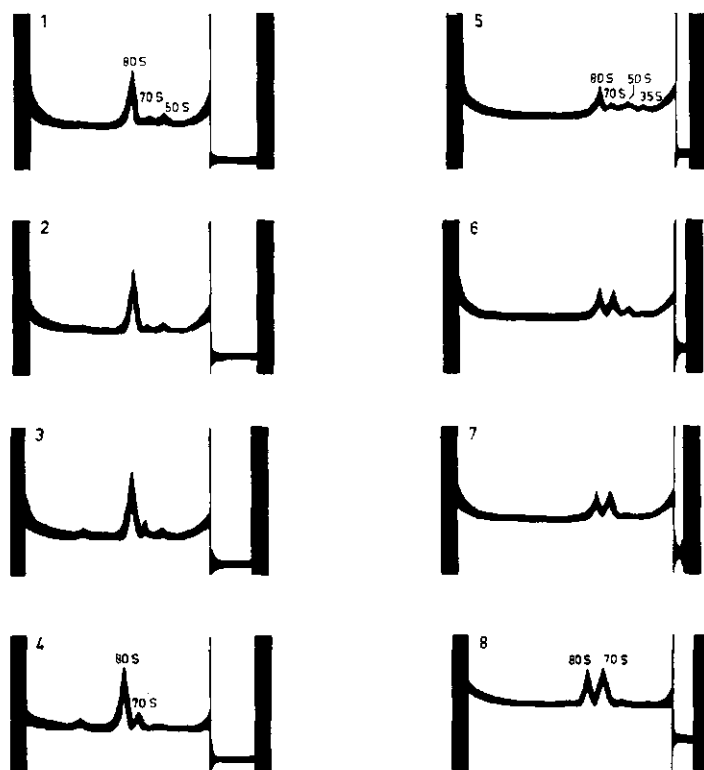


FIG. 5.2. Effect of the magnesium ion concentration on 70 S and 80 S ribosomes during incubation. The reaction mixture was as indicated in figure 5.1, except for Mg^{2+} . The incubation time was 45 minutes. Sedimentation is from right to left. Speed 37,020 rpm. Temperature 20°C.

80 S ribosomes.

1. incubated at 5 μ moles Mg^{2+}
2. incubated at 11 μ moles Mg^{2+}
3. incubated at 17 μ moles Mg^{2+}
4. untreated.

70 S ribosomes.

5. incubated at 8 μ moles Mg^{2+}
6. incubated at 12.5 μ moles Mg^{2+}
7. incubated at 17 μ moles Mg^{2+}
8. untreated.

particles during incubation. The critical concentration seemed to be 12 mM, because at 12.5 mM there was only slight dissociation (6), whereas at 11 mM most of the 70 S ribosomes had dissociated, as can be seen in (2). The latter effect is more clearly demonstrated by unpublished results.

The dissociation of 70 S ribosomes during incubation at low Mg^{2+} concentrations was dependent on the conditions of incubation. After incubation in standard buffer only, pictures similar to 5.2.8 were obtained. This indicates that amino acid incorporation was required for this dissociation and that it was not caused by keeping the ribosomes at 30°C and 10 mM Mg^{2+} alone. Hence, for stability during the process of amino acid incorporation a higher Mg^{2+} concentration was required than during isolation. Some Mg^{2+} might have been bound by the phosphate derived from ATP, but at the concentrations used it is not likely that this drastically lowered the Mg^{2+} concentration.

In all the following experiments optimal Mg^{2+} concentrations were used, i.e., 5 mM for the 80 S and 17 mM for the 70 S ribosomes. At these concentrations the contaminating class of ribosomes did not contribute greatly to the total amino acid incorporation as will be discussed in 5.6.

5.3. KINETICS OF THE AMINO ACID INCORPORATION BY 70 S AND 80 S RIBOSOMES

In this section the kinetics of the incorporation for separated 70 S and 80 S ribosomes was studied (figure 5.3.a). A characteristic difference between the two curves thus became apparent. The incorporation rate by 80 S ribosomes

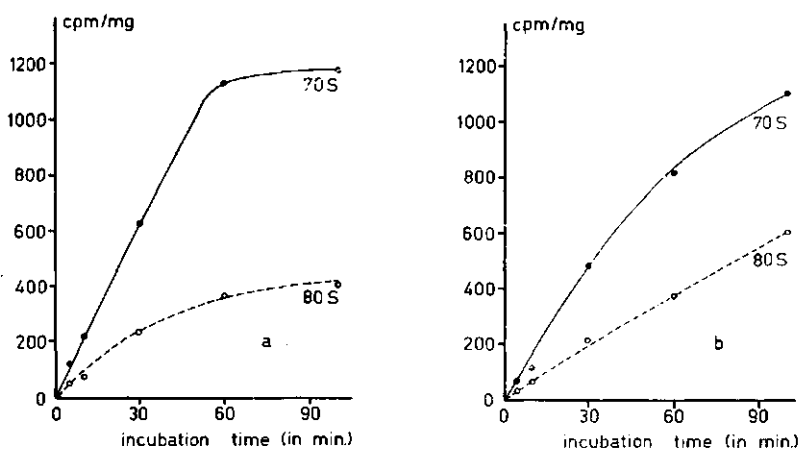


FIG. 5.3. Kinetics of amino acid incorporation by 70 S and 80 S ribosomes. The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 2 μ mole ATP, 100 μ moles Tris, pH 7.8, 75 μ moles NH_4Cl , 6 μ moles MCE, 0.003 μ mole ^{14}C -leucine (150 mC/mmmole), 25 μ g tRNA, 100 μ l enzymes and ribosomes (0.55 mg and 0.13 mg ribosomal RNA for the 80 S and 70 S ribosomes respectively). The Mg^{2+} concentration was 5 mM for the 80 S and 17 mM for the 70 S ribosomes. a. ribosomes purified on 0.5 M sucrose in standard-N-buffer. b. ribosomes purified on 0.5 M sucrose in standard-N-buffer, containing 0.5 M NH_4Cl .

decreased gradually as was shown previously (chapter 3), whereas the 70 *S* ribosomes displayed a constant incorporation rate during the first 40–60 minutes, abruptly dropping thereafter to almost zero.

As seen in figure 5.2 no 70 *S* ribosomes had dissociated after an incubation at 17 mM Mg^{2+} for 45 minutes. No dissociation could be observed either after incubation for two hours. Thus, dissociation of the 70 *S* ribosomes could not be responsible for the abrupt decrease in the rate of incorporation after about 60 minutes (figure 5.3.a). At lower Mg^{2+} concentrations, e.g. 11 mM, the incorporation stopped abruptly at the time when the ribosomes had dissociated. In this case the decrease of rate of incorporation may be correlated with the process of dissociation, whereas at optimal conditions the read-out of the messenger fragments may be responsible.

It was checked whether the 70 *S* ribosomes remaining after the NH_4Cl treatment had remained active. In figure 5.3.b the kinetics of 70 *S* and 80 *S* ribosomes purified in the presence of 0.5 M NH_4Cl is shown. For 80 *S* ribosomes the results were as found previously (3.3.2). However, 70 *S* ribosomes showed quite a different picture. First the ribosomes not degraded by NH_4Cl treatment were less active than the untreated ones (compare a to b). Secondly, the rate of incorporation decreased gradually and not abruptly as was the case with untreated ribosomes. We can only speculate about these differences.

Since 70 *S* ribosomes were partly broken down during centrifugation through 0.5 M NH_4Cl (chapter 4.2) no such treatment was used for the following experiments.

5.4. INFLUENCE OF THE INCUBATION TEMPERATURE ON THE AMINO ACID INCORPORATION BY 70 *S* AND 80 *S* RIBOSOMES

In preliminary experiments it was found that varying the incubation temperature did not greatly influence the amino acid incorporation by ribosomes not purified to a high extent. The influence of temperature was reinvestigated with purified 70 *S* and 80 *S* ribosomes. Both types of ribosomes were incubated at their optimal Mg^{2+} concentration and at four different temperatures during two different periods of time. The results are shown in figure 5.4. The amino acid incorporation by both 70 *S* and 80 *S* ribosomes now proved to be very dependent on the incubation temperature. For the 70 *S* ribosomes the optimum temperature was 30°C. This was also observed for the 80 *S* ribosomes when incubation was for 60 minutes. The initial rate of incorporation seemed to be faster at 35°C, but the reaction apparently stopped after 30 minutes, probably because of degradation of the 80 *S* ribosomes at that temperature. Furthermore, the curves of the 80 *S* ribosomes showed the characteristics of an enzyme reaction: The optimum temperature is determined by the balance between the effect of temperature on the reaction rate and its inactivating effect, resulting in an apparent optimum temperature. In our experiments we found 30°C to be the most convenient temperature, because at that temperature the highest incorporation was obtained without too much inactivation.

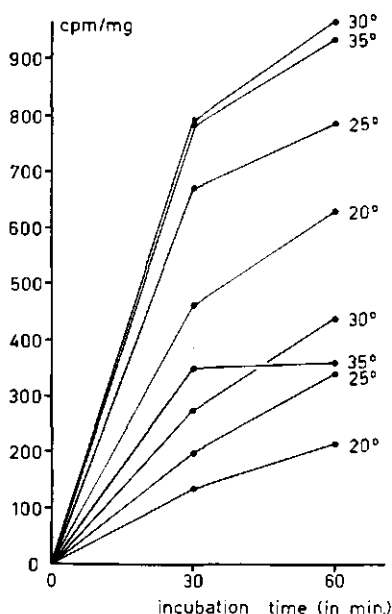


FIG. 5.4. Influence of the temperature on the amino acid incorporation by 70 *S* and 80 *S* ribosomes. The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 7.8, 5 and 17 μ moles $MgCl_2$ respectively for 80 *S* and 70 *S* ribosomes, 75 μ moles NH_4Cl , 6 μ moles MCE 0.002 μ mole ^{14}C -leucine (250 mC/mmole), 25 μ g tRNA, 100 μ l enzymes and ribosomes. The incubation time was as indicated. The values are averages of two experiments. The 70 *S* ribosomes were used at concentrations of 0.19 and 0.26 mg ribosomal RNA per ml and the 80 *S* ribosomes of 0.55 and 0.65 mg, respectively.

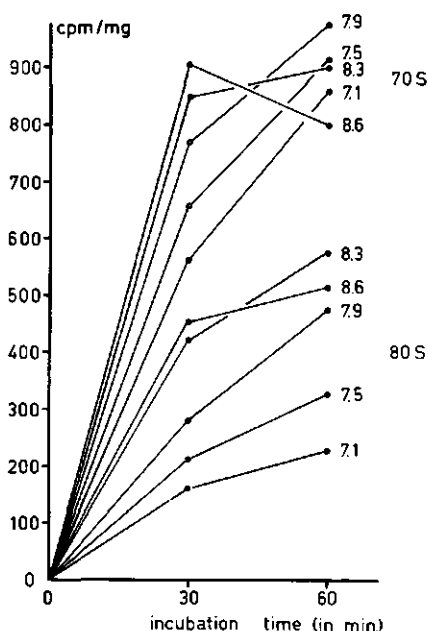
The 70 *S* ribosomes showed a different, more complicated picture. When inspecting their curves, one should compare these with the 70 *S* time curve of figure 5.3.a. There appeared to be an optimum temperature of 30°C when it is assumed that at each of the chosen temperatures the initial rate was constant for at least the first 30 minutes. At higher temperatures there was a slight inactivation of the system. When assuming that the incorporation stops when the messenger fragment is read out, it would be expected that at any of the chosen temperatures the incorporation would reach the same final level if the messenger fragments were not degraded. However, this final incorporation level seemed to be temperature dependent. This means that the decrease in the rate of incorporation must be due to two independent processes, i.e., read out of the messenger fragments and some kind of inactivation.

The results of the above experiments did not seem to make necessary a change in the incubation temperature.

5.5. INFLUENCE OF THE pH ON THE AMINO ACID INCORPORATION BY 70 *S* AND 80 *S* RIBOSOMES

Varying the pH did not seem to affect the amino acid incorporation by non-purified ribosomes (unpublished results). We have reinvestigated the pH dependence, using purified 70 *S* and 80 *S* ribosomes. The pH's were checked in the final incubation mixtures. The results of such an experiment are shown in figure 5.5. The amino acid incorporation by both 70 *S* and 80 *S* ribosomes proved to be highly dependent on the pH, but this dependence seemed to be rather

FIG. 5.5. Influence of the pH on the amino acid incorporation by 70 S and 80 S ribosomes. The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris (pH as indicated), 5 μ moles and 17 μ moles $MgCl_2$ for the 80 S and 70 S ribosomes respectively, 75 μ moles NH_4Cl , 6 μ moles MCE, 0.002 μ mole ^{14}C -leucine (250 mC/mmole), 25 μ g tRNA, 100 μ l enzymes and ribosomes. The incubation time was as indicated. The values are averages of three experiments. The 70 S ribosomes were used at concentrations of 0.17, 0.21 and 0.11 mg ribosomal RNA per ml and the 80 S ribosomes of 0.53, 0.63 and 0.61 mg, respectively.



complicated. In general, a pH optimum is determined by several effects, such as a truly reversible effect on the reaction rate, an effect of the pH on the affinity between enzyme and substrate, and an effect of the pH on the stability of the enzyme proteins. A combination of these effects can make the pH optimum dependent on the incubation time. Our experimental data (figure 5.5) show such a shift in the pH optimum.

In order to get some information on the stability of both classes of ribosomes at different pH's, samples of the incubation mixtures, described above, were investigated in the analytical ultracentrifuge. For this experiment three different pH's were chosen, viz. 7.1, 7.9 and 8.6. The results are shown in figure 5.6. The particle stability of both classes of ribosomes did not prove to differ significantly at the three pH's used. Only the few 70 S ribosomes present in the 80 S preparation dissociated (figure 5.6, 6, 7 and 8), but this was due rather to the Mg^{2+} concentration which was 5 mM. Thus, any difference in activity at a different pH could not be due to dissociation of the ribosomes.

5.6. DISCUSSION AND CONCLUSIONS

As shown in chapter 4, the leaf homogenate could be separated into four fractions by sucrose gradient centrifugation. Under the conditions used there was not much difference in activity between the 70 S ribosomes, the 80 S ribosomes and the polyribosomes. As polyribosomes are supposed to be the active structures in vivo, it was expected that they would be more active in vitro than 70 S or 80 S ribosomes. This was not found and was possibly due to

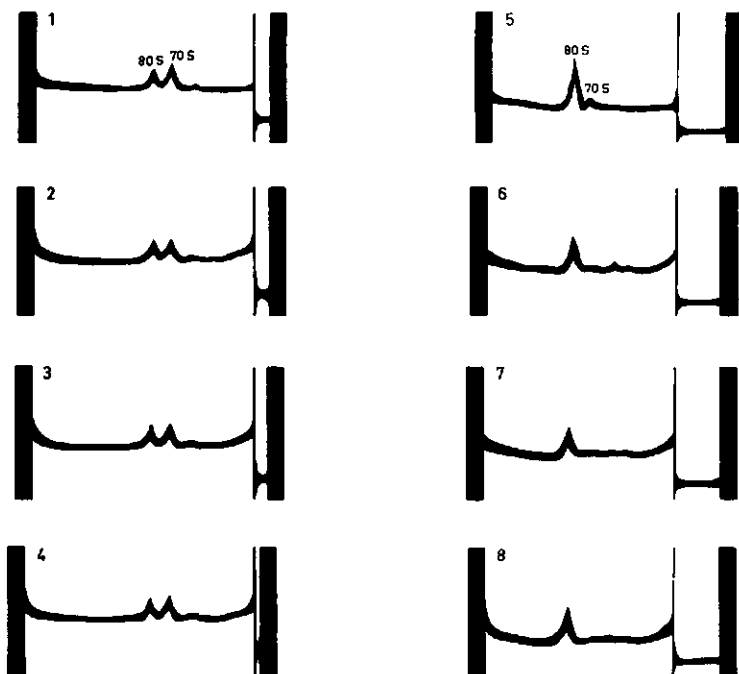


FIG. 5.6. Influence of the pH on the stability of 70 *S* and 80 *S* ribosomes. The incubation mixture was as in figure 5.5, except for Mg^{2+} and pH. The sedimentation was from right to left. Speed 37,020 rpm. Temperature 20 °C.

70 *S* ribosomes.

1. untreated.

2. incubated for 60 minutes at 30 °C in 17 mM Mg^{2+} and at pH 7.1.

3. as in 2, but at pH 7.9.

4. as in 2, but at pH 8.6.

80 *S* ribosomes.

5. untreated.

6. incubated for 60 minutes at 30 °C in 5 mM Mg^{2+} and at pH 7.1.

7. as in 6, but at pH 7.9.

8. as in 6, but at pH 8.6.

a breakdown of the polyribosomes during preparation of the ribosomes. This left fragments of messenger on the ribosomes of sufficient length to allow at least the production of acid precipitable peptides which, however, could not be released from the ribosomes. Whole chloroplasts did not prove to be highly active, although SPENCER and WILDMAN (1964) reported that they are. Under the given conditions the 70 *S* ribosomes were only a little less active than the 80 *S* ribosomes. So there was no reason to look for any shortcomings of the whole chloroplast system.

The 70 *S* and 80 *S* ribosomes required magnesium ions for their activity, but the optimum concentrations differed, viz. 17 mM for the 70 *S* and 5 mM

for the 80 *S* ribosomes. The optimum was very distinct for the 70 *S* ribosomes, whereas seemingly the incorporation by 80 *S* ribosomes was less dependent on the Mg^{2+} concentration. Only highly purified 80 *S* ribosomes showed a true optimum at 5 mM. No magnesium ion dependence curves for plant ribosomes were found in the literature, except those in the publication by BOARDMAN, FRANCKI and WILDMAN (1966). These, however, were not very distinct. The present results roughly agree with theirs.

The 70 *S* ribosomes were 2 to 3 times more active than the 80 *S* ribosomes when both were incubated at their optimum Mg^{2+} concentration. Figure 5.1 suggests that 70 *S* ribosomes were 1.5 to 2 times as active as 80 *S* ribosomes, but the different curves of that Figure were not obtained in experiments with the same leaf material. More characteristic therefore is figure 5.3.a.

When studying either class of ribosomes at their optimum Mg^{2+} concentration, contamination with the other class did not affect materially the results obtained in studies on the effect of time, temperature or pH of incubation. In the experiments reported in this chapter 70 *S* ribosomes were contaminated with 80 *S* ribosomes in amounts varying from 10–30 % with an average of 20 %. The activity of the 70 *S* ribosomes was about 2.5 times as high as that of the 80 *S* ribosomes at optimum Mg^{2+} concentrations. The 80 *S* ribosomes were 1.5 times as active at 5 mM Mg^{2+} as they were at 17 mM. So the incorporating activity (expressed in cpm/mg) of the 20 % 80 *S* ribosomes present in the 70 *S* ribosome preparation, was only about 6 % of the total activity of the 70 *S* ribosome preparation at 17 mM Mg^{2+} . The 80 *S* ribosomes were less than 10 % contaminated with 70 *S* ribosomes. The 70 *S* ribosomes were 2.5 times more active at optimum Mg^{2+} concentrations, but at 5 mM they had only 0.2 of their activity at 17 mM. So these 10 % or less 70 *S* ribosomes had only an incorporating activity (expressed in cpm/mg) which was 5 % or less of that of the 80 *S* ribosome preparation at 5 mM Mg^{2+} .

At 11 mM Mg^{2+} the contribution to total activity of each class of ribosomes can hardly be calculated, even if 'simultaneous equations' are used (BOARDMAN et al., 1966). At this Mg^{2+} concentration a contamination of 20 % 80 *S* and 10 % 70 *S* ribosomes will result in a contribution to the total activity of 10 % and 22.5 % respectively. From this it can be concluded that, in particular, a contamination with 70 *S* ribosomes at higher Mg^{2+} concentrations will give confusing results (see 5.2). By incubating the two types of ribosomes at their optimum Mg^{2+} concentrations this confusion can be largely prevented.

The NH_4Cl treatment did not seem to result in either loss or inactivation of the 80 *S* ribosomes. Although the initial incorporation rate did not show any difference, the incorporation reaction continued for a longer time to reach a higher final level. The 70 *S* ribosomes were inactivated by the NH_4Cl treatment. The yield was 20–40 % less and the remaining 70 *S* ribosomes were less active. This effect differed from that on 80 *S* ribosomes. It was expected that messenger degrading enzymes would be eliminated by the NH_4Cl treatment (see chapter 3.3.2), resulting in a higher final level of incorporation. This was not observed. It is possible that the ribosomes which apparently withstood the

NH₄Cl treatment nevertheless were damaged in some way.

Obviously, the messenger degradation process, discussed in chapter 3.6, did not influence the incorporation rate by 70 S ribosomes, at least not during the first 40–60 minutes. The optimum incubation temperature appeared to be 30°C. On the other hand, the optimum incubation pH was dependent on the incubation time (figure 5.5).

The 80 S ribosomes showed the common temperature effect, i.e., with increasing temperature both the rate of incorporation and inactivation increased. This resulted in a more rapid incorporation and a more rapid slowdown of this. From the experimental data it could not be determined whether the ribosomes or an enzyme were inactivated. To this problem we shall return in the next chapter. The pH dependence was rather complicated. Surprisingly, the incubation mixture could withstand a pH as high as 8.6. Moreover, the differences in activity at pH 7.9 and pH 8.3 were always very remarkable. Only at pH 8.6 and with longer incubation periods relatively more inactivation seemed to occur, but this again, will be discussed further in the next chapter.

It can be concluded that there are several characteristic differences between the properties of the 70 S and the 80 S ribosomes with respect to their amino acid incorporating activity. Their Mg²⁺ requirements are remarkably different and under optimal conditions, 70 S ribosomes are about 2–3 times more active than 80 S ribosomes. Also, the time curves are characteristically different and 70 S ribosomes are unstable at high salt concentrations. Both types of ribosomes have their own, specific temperature and pH dependence.

6. EFFECT OF EXOGENOUS MESSENGERS UPON THE AMINO ACID INCORPORATION BY 70 S AND 80 S RIBOSOMES

In this chapter the influence of exogenous messengers on the amino acid incorporating activity of 70 S and 80 S ribosomes will be reported. In studying this problem, two facts have to be taken into account. First, a binding site must be available on the ribosome for the added messenger, i.e., endogenous messengers have to be adequately removed. Second, the added messenger has to attach to the ribosome in the right way. The removal of the endogenous messengers from the ribosomes proved to be a very critical step in the whole procedure and much attention was paid to this removal. For both classes of ribosomes methods will be described to remove, at least in part, the endogenous amino acid incorporating activity. Finally, some of the properties of the complex between the ribosome and an exogenous messenger will be described.

6.1. THE EFFECT OF POLY U ON THE AMINO ACID INCORPORATION

It was first investigated whether the addition of poly U to the incubation mixtures as described in the chapters 3 and 5 would have any effect on the incorporation. Experiments were carried out with non-purified ribosomes, purified 70 S and 80 S ribosomes and with polyribosome fractions (see chapter 4.1). The results are shown in table 6.1. The incorporation by ribosome mixtures appeared to be stimulated 2 to 3 times by the addition of poly U (table 6.1, part a). Similar results were obtained with purified 70 S and 80 S ribosomes. Poly U had no effect on the polyribosome fractions, as was to be expected (table 6.1, part b). With 70 S ribosomes the results were not always reproducible.

Compared with *E. coli* ribosomes (MATTHAEI and NIRENBERG, 1961) a sti-

TABLE 6.1. Effect of poly U on the ^{14}C -phenylalanine incorporation.

		^{14}C -phenylalanine incorporation in cpm/mg					
		a. ribosome mixture			b. 70 S	80 S	polys. polys.
		0.60	0.37	0.70			1 2
		exp. 1	exp. 2	exp. 3	0.07	0.07	0.07 0.07
mg rib. RNA							
Conditions							
Complete minus ATP, GTP, PEP and PK		32	8	13	—	—	— —
Complete		155	78	72	250	190	140 130
Complete + poly U (100 μg)		378	116	153	535	270	130 115

The incubation mixture contained 10 μmoles PEP, 20 μg PK, 0.03 μmole GTP, 1 μmole ATP, 100 μmoles Tris, pH 7.8, 11 μmoles MgCl_2 , 75 μmoles NH_4Cl , 6 μmoles MCE, 0.003 μmole ^{14}C -phenylalanine (200 mC/mmmole), 25 μg tRNA, 100 μl enzymes, ribosomes and poly U as indicated. The incubation time was 45 minutes.

mulation by poly U of 2 to 3 times is very low. This may be due to several factors:

1. Impurities in the ribosome preparations may inhibit the amino acid incorporation.
2. The fraction of active ribosomes may be very low.
3. Endogenous messengers may be bound very tightly to the ribosomes, resulting in a low percentage of ribosomes available for poly U.
4. Poly U may bind very poorly to the ribosomes.

Since ribosomes, purified on a Sephadex G200 column, have proved to be very pure (chapter 3.3.3), such ribosomes were used to find out whether impurities would be a limiting factor for poly U stimulation. As can be seen in table 6.2, part a, this treatment did not improve the incorporation obtained by adding poly U. If the intrinsic activity of plant ribosomes was low, endogenous activity first had to be eliminated before any stimulating effect by poly U could be demonstrated. Ribosomes were preincubated for 45 min. in an incubation mixture as used in the other experiments according to a method similar to that described for the *E. coli* system by MATTHAEI and NIRENBERG (1961) and VOORMA (1965). Ribosomes were sedimented from this mixture by centrifugation at $105,000 \times g$ for 3 hours on a sucrose layer containing 0.5 M NH_4Cl . The pelleted ribosomes were incubated in a fresh mixture. The results of these experiments are shown in table 6.2, part b. It is clear that the stimulating effect of poly U was hardly greater after preincubation, which means, that this method was not suitable to eliminate endogenous messengers. The high incorporating activity of the control preparation after preincubation could not be explained.

From the experiments described above it thus appeared that no impurities inhibit the amino acid incorporation, but that endogenous messengers could

TABLE 6.2. The effect of purification on Sephadex G200 (a) and preincubation (b) on the poly U directed ^{14}C -phenylalanine incorporation by 80 S ribosomes.

Conditions	mg. rib. RNA	^{14}C -phenylalanine incorporation in cpm/mg			
		a. ribosomes		b. ribosomes + Seph	
		— Seph. 0.22	+ Seph. 0.20	— pre 0.56	+ pre 0.34
Complete minus ATP, GTP, PEP and PK		25	14	30	112
Complete		184	133	78	76
Complete + poly U (100 μg)		317	264	136	169

The incubation mixture was as that described in table 6.1. The incubation time was 45 minutes.

- a. — Seph: non-purified ribosomes; + Seph.: ribosomes purified by Sephadex G200 chromatography.
b. Ribosomes purified by Sephadex G200. — pre: non-preincubated; + pre: preincubated.

not be removed easily from the ribosomes. Under these conditions it could hardly be expected that poly U would be bound to the ribosomes.

6.2. THE EFFECT OF PREINCUBATION UNDER VARIOUS CONDITIONS ON THE STIMULATION OF THE AMINO ACID INCORPORATION BY POLY U

Plant ribosomes could not be treated similarly to *E. coli* ribosomes to eliminate endogenous activity. It appeared from various time curves that endogenous messengers were still active after 40 minutes of incubation. In chapter 5.5 it was demonstrated, however that the incorporation rate depended on the pH. This rate increased with increasing pH, while incorporation stopped more rapidly. In view of these results, ribosomes were preincubated at pH 8.6, then concentrated at $105,000 \times g$ and incubated again in a fresh incubation mixture. In a preliminary experiment the results were rather promising, when preincubation was carried out for 3 hours. Therefore, more extensive experiments were done in order to investigate the optimal conditions for preincubation. This was done with both 80 S and 70 S ribosomes.

6.2.1. 80 S ribosomes

Table 6.3 shows the activity of 80 S ribosomes at various pH's and Mg^{2+} concentrations after preincubation for 3 hours at pH 8.6 and 5 mM Mg^{2+} , as compared to the activity of non-preincubated ribosomes. Relatively, the best results were obtained with incubation at pH 8.6 and 11 mM Mg^{2+} , after preincubation.

In a next series of experiments the most favourable conditions for preincubation were investigated in order to obtain maximal stimulation. Since the

TABLE 6.3. Effect of the Mg^{2+} concentration and the pH on the ^{14}C -phenylalanine incorporation by preincubated 80 S ribosomes stimulated by poly U.

Conditions	^{14}C -phenylalanine incorporation in cpm/mg			
	5 mM Mg^{2+} pH 7.8	11 mM Mg^{2+} pH 7.8	5 mM Mg^{2+} pH 8.6	11 mM Mg^{2+} pH 8.6
Complete	105	161	275	490
Complete + poly U	197	414	373	728
Complete (pre)	47	90	56	108
Complete + poly U (pre)	101	159	203	412

The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ mole ATP, 100 μ moles Tris (pH as indicated), $MgCl_2$ (as indicated), 75 μ moles NH_4Cl , 6 μ moles MCE, 0.003 μ mole ^{14}C -phenylalanine (200 mC/mmmole), 25 μ g tRNA, 100 μ l enzymes, 20 μ g poly U when indicated, and ribosomes (0.4 mg ribosomal RNA). The incubation time was 50 minutes.

(pre): ribosomes preincubated in complete incubation mixture (5 mM Mg^{2+} and pH 8.6) without ^{14}C -phenylalanine, centrifuged for 2 hours at $105,000 \times g$ and resuspended in standard buffer.

amount of ribosomes available for one experiment was too low, not all conditions chosen could be investigated in one and the same experiment.

It was not known whether conditions optimal for incubation were also optimal for preincubation. Therefore, preincubation was carried out at several Mg^{2+} concentrations as summarized in table 6.4. It appeared that preincubation at 3 and 5 mM Mg^{2+} resulted in similar effects. As the ribosomes were broken down in part during preincubation at 3 mM Mg^{2+} , 5 mM proved to be the most favourable concentration. This meant that the pH, optimal for incubation with endogenous mRNA, was also optimal for preincubation. Incubation in the presence of poly U was done at 7, 9, 11 or 13 mM Mg^{2+} . At 9 or 11 mM the best results were obtained and therefore Mg^{2+} concentrations of 9 to 11 mM were always used in the following experiments.

Finally, the most suitable preincubation time was determined. Preincuba-

TABLE 6.4. Effect of the Mg^{2+} concentration during preincubation and incubation on the ^{14}C -phenylalanine incorporation by 80 S ribosomes stimulated by poly U.

	Conditions			^{14}C -phenylalanine incorporation in cpm/mg	
				Complete	Complete + poly U
	mM Mg^{2+} during preincubation ¹	mM Mg^{2+} during incubation ¹	mg. rib. RNA		
Exp. 1	— ²	11	0.66	246	161
	1	11	0.06	103	168
	5	9	0.27	88	379
	5	11	0.27	98	326
	5	13	0.27	112	312
Exp. 2	— ²	11	0.59	113	120
	3	7	0.16	52	214
	3	9	0.16	66	432
	3	11	0.16	71	324
	5	7	0.29	43	162
	5	9	0.29	60	352
	5	11	0.29	55	386
Exp. 3	— ²	11	0.61	214	408
	5	9	0.25	114	620
	6.5	9	0.67	74	194

The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 8.6, $MgCl_2$ (as indicated), 75 μ moles NH_4Cl , 6 μ moles MCE, 0.003 μ mole ^{14}C -phenylalanine (200 mC/mmole), 25 μ g tRNA, 100 μ l enzymes, poly U (20 μ g when indicated) and ribosomes (as indicated). The incubation time was 50 minutes.

Preincubation was carried out in the above complete incubation mixture without ^{14}C -phenylalanine, at pH 8.6. Subsequently the ribosomes were sedimented by running them for 2 hours at $105,000 \times g$ and then they were suspended in standard buffer.

¹ preincubation for 3 hours at pH 8.6; incubation for 50 min. at pH 8.6.

² no preincubation.

TABLE 6.5. Effect of preincubation time on the ^{14}C -phenylalanine incorporation by 80 S ribosomes stimulated by poly U.

	^{14}C -phenylalanine incorporation in cpm/mg			
	Preincubation time			
Conditions	no	2 hours	3 hours	4 hours
mg rib. RNA	0.38	0.51	0.62	0.51
Complete	285	85	55	70
Complete + poly U	395	225	210	145

The incubation mixture contained 10 μmoles PEP, 20 μg PK, 0.03 μmole GTP, 1 μmole ATP, 100 μmoles Tris, pH 8.6, 11 μmoles MgCl_2 , 75 μmoles NH_4Cl , 6 μmoles MCE, 0.003 μmole ^{14}C -phenylalanine (200 mC/mmole), 25 μg tRNA, 100 μl enzymes, poly U (20 μg when indicated) and ribosomes (as indicated). The incubation time was 50 minutes.

Ribosomes were preincubated in the above complete mixture without ^{14}C -phenylalanine (containing 5 mM Mg^{2+} and at pH 8.6) for 0, 2, 3 and 4 hours, respectively, sedimented and resuspended in standard buffer prior to incubation.

tion should be prolonged sufficiently to remove endogenous messengers, but no breakdown of the ribosomes should take place. Ribosomes were preincubated for different periods under identical conditions and incubated subsequently under the same conditions. The results of these experiments are shown in table 6.5. Apparently, the best results were obtained when ribosomes were preincubated for 3 hours. The ribosomes were broken down in part after preincubation for longer periods.

In summary it may be said that optimal stimulation by poly U of the incorporation by 80 S ribosomes is obtained after preincubation of the ribosomes at pH 8.6 and 5 mM Mg^{2+} for 3 hours, sedimentation of the latter in the ultracentrifuge and subsequent incubation at pH 8.6 and 9 to 11 mM Mg^{2+} .

The question arose what was happening with the ribosomes during preincubation. In order to get some information on this process, ribosomes were preincubated, and subsequently, one sample was used for amino acid incorporation experiments. Another sample was run in the analytical ultracentrifuge, and a further sample was used for the preparation of RNA, which was also run in the analytical ultracentrifuge. The results of this experiment are shown in table 6.6 and figure 6.1 (1, 2, 4 and 5). It is clear indeed, that the 80 S particles were intact after preincubation and concentration, and that these particles could be influenced by poly U. Both RNA molecules remained also intact, as appears from figure 6.1 (4 and 5). In the above described experiment (see table 6.6, 1 and 2, and figure 6.1, 1 and 2) the Mg^{2+} concentration was 5 mM during preincubation and concentration, while it was 11 mM during incubation. To determine whether the 80 S ribosomes were still intact immediately after preincubation, preincubated 80 S ribosomes were run in the analytical ultracentrifuge directly after preincubation. These ribosomes were compared with ribosomes which had been concentrated as usual at 5 mM Mg^{2+} and $105,000 \times g$. In both cases the amino acid incorporation was determined as a control for activi-

TABLE 6.6. ^{14}C -phenylalanine incorporating activity of preincubated 80 S ribosomes

Conditions		^{14}C -phenylalanine incorporation in cpm/mg	
		Complete	Complete + poly U
Untreated	(1)	156	371
Preincubated	(2)	34	445
Preincubated	(3)	9	29

The incubation mixture was as described in table 6.5. Ribosomes were added at a concentration of 0.6 mg ribosomal RNA and the incubation time was 50 minutes.

- (1) Ribosomes untreated.
- (2) Ribosomes preincubated, sedimented at $105,000 \times g$ and resuspended in standard buffer, containing 5 mM MgCl_2 , prior to incubation.
- (3) Ribosomes preincubated and then incubated directly after this treatment in a fresh incubation mixture.

ty. The results are shown in figure 6.1, 2 and 3, and table 6.6, 2 and 3. It is clear that during preincubation 80 S ribosomes were transformed into 60 S, 50 S and 40 S particles, which also occurred when ribosomes were dialysed against 10^{-4} M Mg^{2+} (Chapter 4.3). During the concentration process these particles reverted to 80 S ribosomes (figure 6.1, 2) which means that formation of the 80 S ribosomes was necessary for the binding of the messenger. The unfolded particles were hardly stimulated by the addition of poly U (table 6.6, 3).

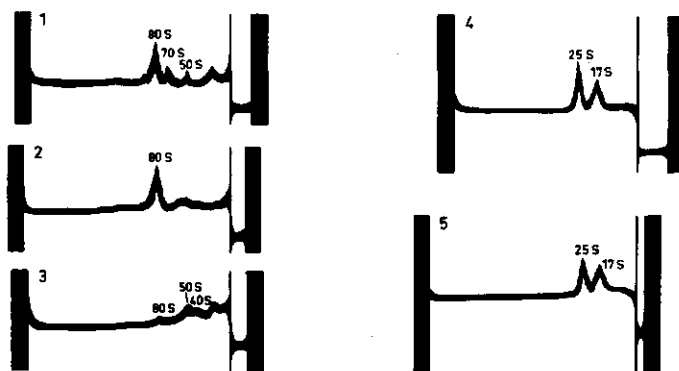


FIG. 6.1. Sedimentation patterns of 80 S ribosomes before and after preincubation and of their RNAs. Preincubation as is described in table 6.6. Sedimentation from right to left. Speed 37,020 rpm (1, 2 and 3) and 50,740 rpm (4 and 5). Temperature 20°C.

1. Untreated ribosomes.
2. Preincubated ribosomes, sedimented at $105,000 \times g$ and resuspended in standard buffer, containing 5 mM MgCl_2 .
3. Preincubated ribosomes, immediately after preincubation without prior sedimentation.
4. RNA from untreated ribosomes.
5. RNA from ribosomes, preincubated and sedimented as in 2.

6.2.2. 70 S ribosomes

Treatments, similar to those given to the 80 S ribosomes were given to the 70 S ribosomes. In preliminary experiments it appeared that preincubation at optimal Mg^{2+} concentrations (17 mM) had no effect on the stimulation of the amino acid incorporation by poly U. As the effect of preincubation of the 80 S ribosomes was similar to that of dialysis against low Mg^{2+} concentrations, 70 S ribosomes were preincubated at 5 mM Mg^{2+} , at which concentration the 70 S ribosomes dissociated during the preincubation (figure 5.2).

The results of these experiments are summarized in table 6.7. Two conclusions can be drawn from the results:

1. Preincubation under the given conditions has a doubtful, poorly reproducible effect on the poly U dependent amino acid incorporation.
2. Incubating the 70 S ribosomes at pH 8.6, without preincubation, already makes them susceptible to stimulation by poly U. However, this effect appeared similarly to be poorly reproducible.

The above conclusions imply that this method which is successful with 80 S

TABLE 6.7. Effect of preincubation on the ^{14}C -phenylalanine incorporation by 70 S ribosomes directed by poly U

	Conditions			^{14}C -phenylalanine incorporation in cpm/mg	
				Complete	Complete + poly U
	mM Mg^{2+} during preincubation ¹	mM Mg^{2+} during incubation ¹	mg rib. RNA		
Exp. 1	— ²	5	0.127	235	254
	— ²	11	0.127	720	965
	5	5	0.127	155	166
	5	11	0.127	220	908
Exp. 2	— ²	11	0.075	547	530
	5	11	0.143	55	140
Exp. 3	— ²	11	0.087	243	702
	5	11	0.067	146	205
Exp. 4	— ²	11	0.108	122	297
	5	11	0.131	64	137
Exp. 5	— ²	11	0.082	350	2530
	5	11	0.095	202	585

The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 8.6, $MgCl_2$ (as indicated), 75 μ moles NH_4Cl , 6 μ moles MCE, 0.003 μ mole ^{14}C -phenylalanine (200 mC/mmole), 25 μ g tRNA, 100 μ l enzymes, poly U (20 μ g, when indicated) and ribosomes (as indicated). The incubation time was 50 minutes.

When indicated, ribosomes were preincubated for 3 hours in the above complete mixture without ^{14}C -phenylalanine (5 mM Mg^{2+} and pH 8.6), sedimented at 105,000 $\times g$ and resuspended in standard buffer, prior to incubation.

¹ Preincubation for 3 hours at pH 8.6, incubation for 50 min. at pH 8.6.

² No preincubation.

ribosomes, is not reliable with 70 S ribosomes. The latter require another method to remove endogenous messengers.

6.3. THE EFFECT OF DIALYSIS AGAINST 10^{-4} M Mg^{2+} ON THE STIMULATION OF THE AMINO ACID INCORPORATION BY POLY U

Figure 6.1 suggests that 80 S ribosomes unfolded during the preincubation process. Much of the endogenous messenger proved to be removed after preincubation. In chapter 4.3 a method was described, which, in a simple way, leads to the unfolding of 80 S ribosomes and the dissociation of 70 S ribosomes, i.e., dialysis against 10^{-4} M Mg^{2+} . If endogenous messengers were removed during preincubation, this may happen also during dialysis against 10^{-4} M Mg^{2+} .

Since the preincubation method was not reliable with 70 S ribosomes, it would be of interest, in particular, with respect to these ribosomes, to learn whether this is actually the case. Moreover, the dissociation of 70 S ribosomes occurred without any further complications, in contrast to the unfolding process of the 80 S ribosomes (chapter 4.3). Therefore, first the effect of dialysis on the incorporation was investigated with 70 S ribosomes.

6.3.1. 70 S ribosomes

In chapter 4.3 it was shown that 70 S ribosomes dissociated reversibly into 50 S and 35 S particles during dialysis against 10^{-4} M Mg^{2+} for 2 to 4 hours. In order to find out whether during such treatment endogenous messengers were removed and the ribosomes became capable of accepting poly U as a messenger, a series of experiments was carried out using ribosomes which had been treated as described above. The results of these experiments are given in table 6.8. The hypothesis that by such a treatment endogenous messengers were

TABLE 6.8. The effect of poly U on the incorporation of ^{14}C -phenylalanine by 70 S ribosomes before and after dialysis against 10^{-4} M Mg^{2+}

Conditions		^{14}C -phenylalanine incorporation in cpm/mg		
		Exp. 1	Exp. 2	Exp. 3
No dialysis	Complete	487	450	392
	Complete + poly U	569	410	485
After dialysis against 10^{-4} M Mg^{2+}	Complete	195	61	185
	Complete + poly U	578	304	1345

The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 7.8, 11 μ moles $MgCl_2$, 75 μ moles NH_4Cl , 6 μ moles MCE, 0.003 μ mole ^{14}C -phenylalanine (200 mC/mmmole), 25 μ g tRNA, 100 μ l enzymes, poly U (20 μ g, when indicated) and ribosomes (0.3 mg ribosomal RNA). The incubation time was 50 minutes.

When indicated, ribosomes were dialysed for 4 hours against standard buffer containing 10^{-4} M Mg^{2+} . The dialysed ribosomes were added to the incubation mixture and then the Mg^{2+} concentration was increased to 11 μ moles.

eliminated appeared to be correct. This decrease in endogenous activity did lead to a stimulation by poly U, which was about 5 to 7 times.

As only a very small amount of 70 *S* ribosomes was obtained by means of the conventional sucrose gradient centrifugation method, no extensive experiments could be done. As already mentioned, however, during the last stages of these investigations, a zonal rotor became available, by means of which an excellent separation of 70 *S* from 80 *S* ribosomes could be obtained (see chapter 2.5 and chapter 4.3, figures 4.9 and 4.10). When using these very pure 70 *S* ribosomes, even a greater stimulation was obtained and the results were also reproducible. This confirmed the hypothesis that dissociation and reassociation of the 70 *S* ribosomes led to removal of endogenous messengers. These results will be described in 6.4 and 6.5, together with those, obtained with the 80 *S* ribosomes.

6.3.2. 80 *S* ribosomes

As endogenous messengers could be removed from 70 *S* ribosomes by dialysing them against 10^{-4} M Mg^{2+} , we tried if this method, which was simpler than the preincubation procedure, could also be used for 80 *S* ribosomes. However, 80 *S* ribosomes did not dissociate in a simple way into two subunits during this treatment, but unfolded into several particles (chapter 4.3). Since 80 *S* ribosomes could be reformed better from 60 *S* particles than from 50 *S* and 40 *S* particles, ribosomes were dialysed against 10^{-4} M Mg^{2+} for about 14 hours because most of the particles were then in the 60 *S* form.

This procedure proved to have some effect, but the stimulation was still not very pronounced. Since the pH had a very pronounced effect on the amino acid incorporation by 80 *S* ribosomes (chapter 5.5), these experiments were

TABLE 6.9. The effect of poly U on the ^{14}C -phenylalanine incorporation by 80 *S* ribosomes before and after dialysis against 10^{-4} M Mg^{2+}

Conditions		^{14}C -phenylalanine incorporation in cpm/mg			
		Incubation at pH 7.8		Incubation at pH 8.6	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
No dialysis	Complete	34	40	81	244
	Complete + poly U	47	52	161	405
After dialysis against 10^{-4} M Mg^{2+}	Complete	55	23	52	22
	Complete + poly U	90	120	963	505

The incubation mixture was as described in table 7.8, except for the pH, which was as indicated. Ribosomes were added at a concentration of 0.50 mg ribosomal RNA. The incubation time was 50 minutes.

The ribosomes were dialysed for 14 hours against standard buffer containing 10^{-4} M Mg^{2+} prior to incubation, when indicated. The dialysed ribosomes were added to the incubation mixture and then the Mg^{2+} concentration was increased to 11 μ moles.

repeated at pH 8.6. A summary of both series of experiments is given in table 6.9. When comparing these experiments it becomes clear that, indeed, the increased pH of the incubation mixture had a considerable stimulatory effect on the incorporation. When the other conditions were the same, the stimulatory effect of poly U was about 20 times at pH 8.6 while it was only 2 to 5 times at pH 7.8.

In different experiments (for example table 6.9, the experiments 1 and 2 at pH 8.6), varying results were obtained. Sometimes only little endogenous activity was removed, but by means of poly U the activity was increased to about 6 times the original one (exp. 1). In exp. 2 only about 10% of the endogenous activity was left after treatment and the original activity could be restored by means of poly U. In both cases the stimulatory effect was similar, but the ratios between the original and the final activity were different, viz., 6 and 1 for the experiments 1 and 2 respectively. We shall return to this in 6.7.

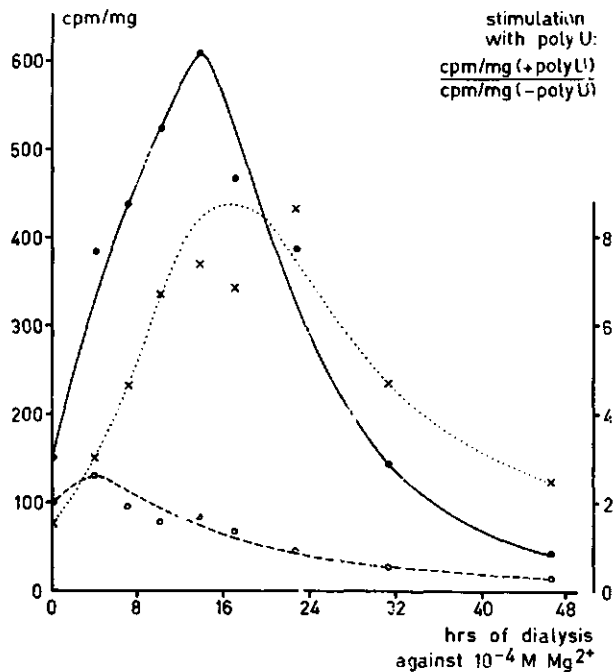


FIG. 6.2. Effect of the dialysis time against 10^{-4} M Mg^{2+} on the poly U-dependent ^{14}C -phenylalanine incorporation by 80 S ribosomes. The incubation mixture was as in table 6.9, the pH was 8.6. Ribosomes were added at a concentration of 0.6 mg ribosomal RNA. The incubation time was 50 minutes. Before incubation the ribosomes were dialysed against 10^{-4} M Mg^{2+} in standard buffer for different periods, as indicated in the figure. Dialysed ribosomes were added to the incubation mixture and subsequently the Mg^{2+} concentration was increased to 11 μ moles. ●—● incorporation in the presence of poly U, ○—○ in the absence of poly U. × × stimulation by poly U expressed as $\frac{\text{cpm/mg (+ poly U)}}{\text{cpm/mg (- poly U)}}$.

In the next experiment samples were dialysed against 10^{-4} M Mg^{2+} during different periods, in order to determine the optimal dialysis period. The results are shown in figure 6.2. In this figure the amino acid incorporation stimulated by poly U is compared with the incorporation without poly U, whereas a third curve indicates the degree of stimulation. The stimulatory effect of poly U on the amino acid incorporation reached optimal values after 14 hours of dialysis and this time was chosen for the following experiments. The maximal activity at about 14 hours of dialysis confirmed the hypothesis that 60 S particles can easily revert back into 80 S ribosomes. After more than 14 hours of dialysis the activity decreased rather rapidly, indicating that 60 S particles when unfolding further to 50 S and 40 S particles were broken down, at least in part. This then, would be in agreement with the finding that hardly any 80 S ribosomes could reform from these particles (see figure 4.6). Furthermore, it is remarkable that during the first 14 hours of dialysis the stimulatory effect of poly U increased rapidly without an appreciable loss of endogenous activity. This will be discussed further in 6.7.

6.4. THE DEPENDENCE ON THE Mg^{2+} CONCENTRATION OF POLY U-STIMULATED AMINO ACID INCORPORATION BY 70 S AND 80 S RIBOSOMES

Knowing the optimal conditions for preincubation and dialysis, the magnesium ion dependence of the amino acid incorporation of both 70 S and 80 S ribosomes as stimulated by poly U was investigated. The results presented in figure 6.3 show that both 70 S and 80 S ribosomes had a distinct Mg^{2+} optimum, which was 10–12 mM for the 80 S and 17–18 mM for the 70 S ribosomes. Comparing these optima with those obtained when endogenous messengers were still present (see chapter 5.2, figure 5.1), it was striking that for the 70 S ribosomes these optima were very similar (17–18 mM), whereas the poly U dependent amino acid incorporation by 80 S ribosomes required a higher Mg^{2+} concentration (11 mM) than the incorporation by endogenous messengers (5 mM). Again, in these Mg^{2+} curves the difference between 70 S and 80 S ribosomes was reflected in that 70 S ribosomes required a higher Mg^{2+} concentration for stability and activity than the 80 S ribosomes.

6.5. THE KINETICS OF POLY U-STIMULATED AMINO ACID INCORPORATION BY 70 S AND 80 S RIBOSOMES

6.5.1. 80 S ribosomes

The kinetics of the amino acid incorporation by 80 S ribosomes can be seen in figure 6.4. Such a time curve is given for both preincubated ribosomes and ribosomes dialysed against 10^{-4} M Mg^{2+} . It is clear that the way in which the endogenous messengers were removed had no significant effect on the kinetics, since both curves are very similar. Strikingly, the rate of incorporation did not decrease, but was very constant for at least two hours. The incorporation could hardly be investigated for still longer incubation periods, since it was

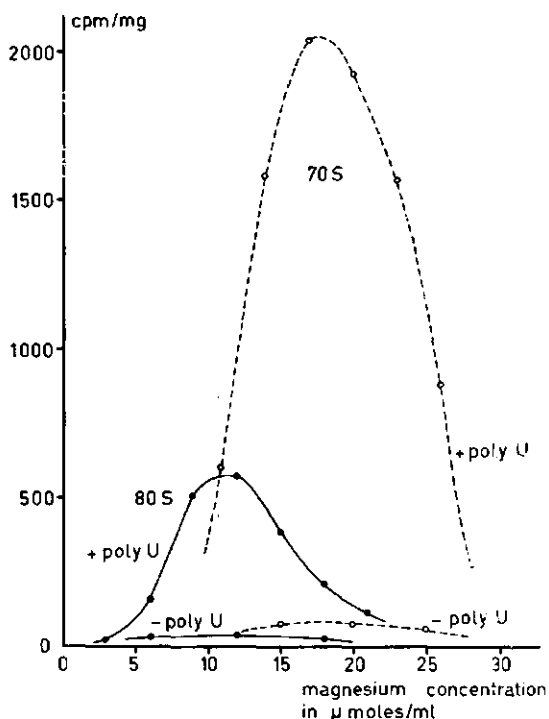


FIG. 6.3. The effect of the Mg^{2+} concentration on the poly U-stimulated ^{14}C -phenylalanine incorporation by 70 S and 80 S ribosomes. The incubation mixture contained 10 μ -moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 8.6, $MgCl_2$ (as indicated), 75 μ moles NH_4Cl , 6 μ moles MCE, 0.003 μ mole ^{14}C -phenylalanine (200 mC/mmole), 25 μ g tRNA, 100 μ l enzymes, poly U (20 μ g, when indicated) and ribosomes (0.24 and 0.41 mg ribosomal RNA for 70 S and 80 S ribosomes respectively). The incubation time was 50 minutes. Prior to incubation, the 70 S ribosomes and 80 S ribosomes were dialysed against 10^{-4} M Mg^{2+} in standard buffer for 3 hours and 14 hours, respectively.

difficult to prevent bacterial growth. In other experiments there were some indications, however, that the incorporation continued for some time after two hours. In these cases, the rate still remained constant, whereas the controls only had a low activity. In cases of bacterial growth the incorporation rate of the samples in the presence as well as in the absence of poly U increased rapidly.

From these results it can be concluded that the 80 S ribosomes-poly U-complex was very stable. The incorporation rate was rather low. After 100 minutes of incubation 72 μ moles of phenylalanine were incorporated, corresponding with 216 μ moles equivalents of U. Poly U was added in the amount of 20 μ g and assuming a MW of about 300 for UMP, this means that 67.10^3 μ moles U were added. Apparently only about 0.3% of the added information was translated. Provided that no degradation of poly U occurred, the incorporation process could continue for an extensive period of time until all poly U molecules would have been read only once.

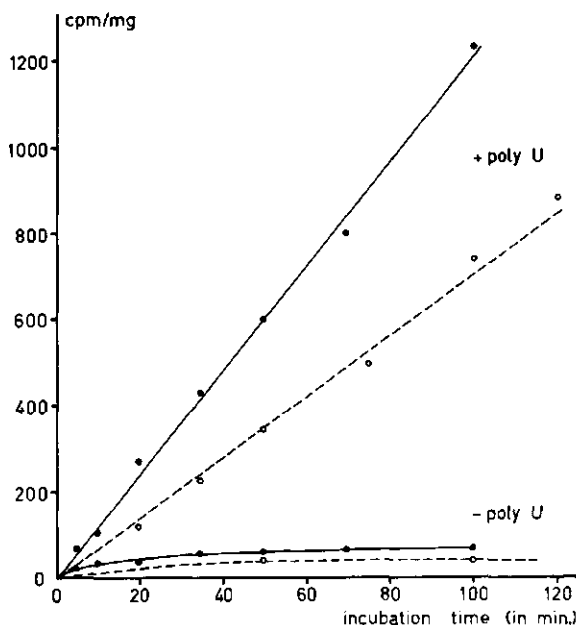


FIG. 6.4. Kinetics of the poly U-stimulated ^{14}C -phenylalanine incorporation by 80 S ribosomes. The incubation mixture was as described in figure 6.3, except for the Mg^{2+} concentration, which was 11 μmoles . ●—● Prior to incubation ribosomes were preincubated for 3 hours at pH 8.6 and 5 μmoles Mg^{2+} , sedimented at $105,000 \times g$ and resuspended in standard buffer. Ribosomes were added at a concentration of 0.44 mg RNA. ○-----○ Prior to incubation ribosomes were dialysed against 10^{-4} M Mg^{2+} for 14 hours. Then they were added to the incubation mixture and the Mg^{2+} concentration was raised to 11 μmoles . Ribosomes were added at a concentration of 0.67 mg RNA.

6.5.2. 70 S ribosomes

Figure 6.5 shows the kinetics of the amino acid incorporation by the 70 S ribosomes. Two time curves are shown, which are similar in part to those of the 80 S ribosomes (figure 6.4). Especially when high activities were measured, the incorporation rate decreased after 60–90 minutes, whereas at lower activities the rate remained constant. Although no further investigations were carried out on this point, the decrease of the incorporation rate at high activities was due probably to a deficiency in one or more components necessary for incorporation or to a breakdown of the 70 S ribosomes, which are known to be less stable than 80 S ribosomes. The decrease in incorporation rate could not be due to read out of the messenger, since only about 1 % of the added poly U was read in these experiments (compare 6.5.1).

Thus, 70 S and 80 S ribosomes are rather similar in their time curves, although the activity of 70 S ribosomes is higher than that of 80 S ribosomes, i.e., 30–45 $\mu\mu\text{moles}$ phenylalanine/mg/hour for the 80 S ribosomes and 70–140 $\mu\mu\text{moles}$ /mg/hour for the 70 S ribosomes.

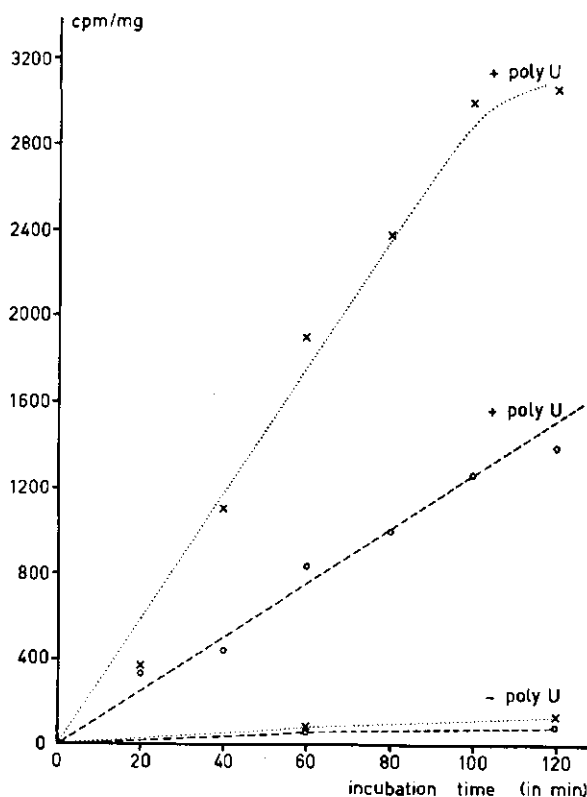


FIG. 6.5. Kinetics of the poly U-stimulated ^{14}C -phenylalanine incorporation by 70 S ribosomes. The incubation mixture was as described in figure 6.3, except for the Mg^{2+} concentration. Prior to incubation ribosomes were dialysed against 10^{-4} M Mg^{2+} for 3 hours. They were then added to the incubation mixture and the Mg^{2+} concentration was raised. o — — — o Incubation at $18\ \mu\text{moles Mg}^{2+}$, with 0.13 mg ribosomal RNA. x x Incubation at $18\ \mu\text{moles Mg}^{2+}$, with 0.30 mg ribosomal RNA.

6.6. THE EFFECT OF VARIOUS VIRAL RNAs ON THE AMINO ACID INCORPORATION BY 70 S AND 80 S RIBOSOMES

In the previous sections methods have been described which were developed to remove endogenous messengers from 70 S and 80 S ribosomes. In this section the effect on the amino acid incorporation will be described of naturally occurring messengers, viz., viral RNAs.

First the template function of several viral RNAs was tested in the *E. coli* system. For this purpose a preincubated S_{30} fraction (iS_{30}) of *E. coli* was used. The effect of both poly U and various viral RNAs was investigated. In the case of the RNAs, a mixture of 6 amino acids was used for labeling, i.e., ^{14}C -leucine, ^{14}C -phenylalanine, ^{14}C -isoleucine, ^{14}C -valine, ^{14}C -threonine and ^{14}C -arginine, at a concentration of $0.0005\ \mu\text{mole}$ ($200\ \text{mC/mmole}$) each. The

TABLE 6.10. The effect of various exogenous messengers on the amino acid incorporation by *E. coli* ribosomes

Conditions	Amino acid incorporation in cpm/mg
Complete (1)	80
Complete + poly U (1)	29,000
Complete (2)	187
Complete + TMV-RNA (2)	2,330
Complete + TYMV-RNA (2)	8,620
Complete + CPMV-RNA (2)	443
Complete + AMV-RNA (top a) (2)	1,335

The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 7.8, 17 μ moles $MgCl_2$, 75 μ moles NH_4Cl , 6 μ moles MCE, *E. coli* ribosomes (iS_{30} fraction, 0.50 mg ribosomal RNA) and 0.003 μ mole ^{14}C -phenylalanine (200 mC/mmole) (1), or 0.0005 μ mole of each of a mixture of ^{14}C -phenylalanine, ^{14}C -leucine, ^{14}C -isoleucine, ^{14}C -valine, ^{14}C -threonine and ^{14}C -arginine (200 mC/mmole) (2). The incubation time was 40 minutes.

results are shown in table 6.10. It is clear that the RNAs used, were all able to stimulate the amino acid incorporation by *E. coli* ribosomes, although to a different extent. The relative inactivity of CPMV-RNA may be due to a poor quality of the RNA preparation.

In the next series of experiments the effect of viral RNAs on the amino acid incorporation by 80 S plant ribosomes was investigated. The results of these experiments are summarized in table 6.11. Only in some cases a small stimulation occurred, but the effect was too small to be significant. This meant that ribosomes, which had been treated in such a way that they were able to accept poly U as a messenger, were not able to accept naturally occurring messengers, such as viral RNAs. This may have been due to inhibitors or factors lost during the preparation of the ribosomes.

In order to investigate whether the enzyme preparation used in all experiments thus far, contained an inhibitor, the $S_{105\ 000}$ normally used for the preparation of the enzyme fraction, was centrifuged at $105,000 \times g$ again, but now for 14 hours. (VOORMA, 1965). Further preparation of the enzymes was as usual. The results of this experiment are summarized in table 6.12. Hardly any effect of the treatment given could be observed. It can be concluded that if any inhibitor was present in the enzyme fraction, this inhibitor could not be removed in this way.

Next, a lack of initiating factors, which are not needed by poly U was considered (REVEL and GROS, 1966; EISENSTADT and BRAWERMAN, 1966 and 1967; BRAWERMAN and EISENSTADT, 1966; REVEL, HERZBERG, BECAREVIC and GROS 1968; BROWN and DOTY, 1968). In the *E. coli* system the initiation of protein synthesis is dependent on N^{10} -formyltetrahydrofolic acid and a transformylating enzyme, resulting in the synthesis of *N*-formyl-methionyl-tRNA_f. The formyl-group can also be provided by means of leucovorin (MARCKER and SANGER, 1964; NAKAMOTO and KOLAKOFSKY, 1966; CAPECCHI, 1966 and 1967;

TABLE 6.11. The effect of various messengers on the amino acid incorporation by 80 S ribosomes

Conditions	Amino acid incorporation in cpm/mg				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Complete (1)	36	42	—	—	242
Complete + poly U (1)	1150	634	—	—	4430
Complete (2)	76	74	97	225	900
Complete + poly U (2)	—	470	202	725	—
Complete + TMV-RNA (2)	77	52	96	256	—
Complete + TYMV-RNA (2)	—	77	—	245	—
Complete + AMV-RNA (top a) (2)	—	103	112	245	—
Complete + CPMV-RNA (M) (2)	—	97	133	283	1220
Complete + CPMV-RNA (B) (2)	—	85	—	354	1010

The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 8.6, 11 μ moles $MgCl_2$, 75 μ moles NH_4Cl , 6 μ moles MCE, 25 μ g tRNA, 100 μ l enzymes, ribosomes and ^{14}C -amino acids. The incubation time was 50 minutes.

Exp. 1 Ribosomes were preincubated at pH 8.6 prior to incubation (see 6.2) and were then and 2 added at a concentration of 0.45 mg (1) and 0.43 mg (2) ribosomal RNA.

Exp. 3 Ribosomes were dialysed against 10^{-4} M Mg^{2+} , as described in 6.3, prior to incubation and were then added at a concentration of 0.67 (3) and 0.70 (4) mg ribosomal RNA.

Exp. 5 Ribosomes were purified with the use of the zonal rotor and dialysed against 10^{-4} M Mg^{2+} prior to incubation. They were added at a concentration of 0.55 mg ribosomal RNA.

(1) labeled with ^{14}C -phenylalanine.

(2) labeled with a mixture of 6 ^{14}C -amino acids (see table 6.10).

TABLE 6.12. The effect on the ^{14}C -phenylalanine incorporation by 80 S ribosomes of centrifuging the soluble enzyme fraction for 14 hours at $105,000 \times g$.

Conditions	^{14}C -phenylalanine incorporation in cpm/mg	
	Exp. 1	Exp. 2
Complete + ribosomes	252	198
Complete + ribosomes + poly U	732	475
Complete + ribosomes ¹	273	124
Complete + ribosomes + poly U ¹	822	173
Complete + preincubated ribosomes	57	50
Complete + preincubated ribosomes + poly U	396	257
Complete + preincubated ribosomes ¹	33	25
Complete + preincubated ribosomes + poly U ¹	336	133

The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris, pH 8.6, 11 μ moles $MgCl_2$, 75 μ moles NH_4Cl , 6 μ moles MCE, 25 μ g tRNA, 100 μ l enzymes, ribosomes (0.43 and 0.57 mg ribosomal RNA for preincubated and untreated ribosomes respectively) and 0.003 μ mole ^{14}C -phenylalanine (200 mC/mmmole). The incubation time was 50 minutes.

¹ Enzymes prepared from $S_{105,000}$ centrifuged for 14 hours.

BRETSCHER and MARCKER, 1966; ADAMS and CAPECCHI, 1966; CLARK and MARCKER, 1966a and 1966b; SCHWARTZ, MEYER, EISENSTADT and BRAVERMAN, 1967; NOMURA and LOWRY 1967; HILLE, MILLER, IWASAKI and WAHBA, 1967; STANLEY, SALAS, WAHBA and OCHOA, 1966; CLARK, 1967; SALAS, HILLE, LAST, WAHBA and OCHOA, 1967; LUCAS-LENARD and LIPMANN, 1967).

We have performed some preliminary experiments on the effect of leucovorin as a formyl-donor on the CPMV-RNA dependent incorporation by 70 S and 80 S ribosomes and the results are shown in table 6.13. Different pH's and Mg^{2+} concentrations were used, but under none of these conditions leucovorin had any effect. Although it cannot be concluded from these results that plant ribosomes use an initiation mechanism different from that of *E.coli*, this mechanism did not seem to be the limiting factor under our conditions. However, more extensive experiments will have to be performed to elucidate this mechanism.

TABLE 6.13. The effect of leucovorin on the CPMV-RNA dependent amino acid incorporation.

		Amino acid incorporation in cpm/mg		
		Exp. 1	Exp. 2	Exp. 3
80 S	Complete (1) (4)	59	35	-
	Complete + poly U	260	153	-
	Complete (2) (3)	-	128	475
	Complete + RNA	-	206	316
	Complete + lv (2) (3)	230	131	450
	Complete + lv + RNA	257	220	336
	Complete (2) (5)	-	129	339
	Complete + RNA	-	86	444
	Complete + lv (2) (5)	120	206	394
	Complete + lv + RNA	242	173	615
70 S	Complete (2) (5)	110	128	-
	Complete + RNA	169	154	-
	Complete + lv (2) (5)	133	150	-
	Complete + lv + RNA	210	170	-

The incubation mixture contained 10 μ moles PEP, 20 μ g PK, 0.03 μ mole GTP, 1 μ mole ATP, 100 μ moles Tris (80 S: pH 7.8; 70 S: pH 8.6), $MgCl_2$ (as indicated), 75 μ moles NH_4Cl , 6 μ moles MCE, 25 μ g tRNA, 100 μ l enzymes, ribosomes (80 S: 0.55, 0.56 and 0.47 mg ribosomal RNA for the exp. 1, 2 and 3 respectively and 70 S: 0.133 and 0.295 mg for the exp. 1 and 2. The 80 S ribosomes were dialysed against 10^{-4} M Mg^{2+} in standard buffer for 14 hours and the 70 S ribosomes for 3 hours, prior to incubation), and ^{14}C -amino acids ((1) phenylalanine and (2) mixture of 6 amino acids, as in table 6.10). The incubation time was 50 minutes.

The ribosomes were purified by means of the zonal rotor. When indicated 20 μ g leucovorin (lv) was added and also 20 μ g of poly U or CPMV-RNA.

Incubation was carried out at various Mg^{2+} concentrations, since it was not known at what Mg^{2+} concentration leucovorin would show its optimal effect.

(3) 5 μ moles Mg^{2+}

(4) 11 μ moles Mg^{2+}

(5) 15 μ moles Mg^{2+}

EISENSTADT and BRAWERMAN (1967) found that purified *E. coli* ribosomes lacked a factor required for viral RNA-dependent polypeptide synthesis. As poly U did stimulate the amino acid incorporation in the absence of this factor, the conclusion was drawn that this missing factor was an initiation factor. They have shown that this factor is present on the 30 S ribosomal subunit and they have isolated this subunit from the high speed supernatant. Similarly, we have tried to isolate such a factor from our high speed supernatant in the following way. Ribosomes were prepared as described in chapter 2.2.1. After centrifugation at $105,000 \times g$ the supernatant was removed from the tubes by pipetting, except for the one ml on top of the pellet (bottom fraction of the supernatant). This bottom fraction was then removed from the tubes and dialysed overnight against standard buffer. The dialysed supernatant was used as 'factor f'.

Washing *E. coli* ribosomes with 0.5 M NH_4Cl causes most of the factors to be washed off. (SALAS, SMITH, STANLEY, WAHBA and OCHOA, 1965). These factors can be isolated from the remaining supernatant. Therefore, we centrifuged ribosomes through 0.5 M sucrose containing standard buffer (3 ml) and 0.5 M NH_4Cl , at $105,000 \times g$. After centrifugation, the supernatant was removed except for the bottom fraction, consisting of about 4 ml on top of the pellet. This fraction was dialysed against standard buffer overnight and was then used as 'factor fN'.

Some experiments were carried out on the effect of leucovorin and the 'factors f' and 'fN' on the incorporation by 70 S and 80 S ribosomes. The results showed that leucovorin had no stimulating effect on the amino acid incorporation. Both 'factors f' and 'fN' seemed to have had little effect on the poly U directed amino acid incorporation. 'Factor f' tended to inhibit this, whereas 'factor fN' seemed to cause a slight stimulation, but these few results were inconclusive. The incorporation dependent on endogenous messengers appeared to be stimulated by both factors. The addition of CPMV-RNA did not seem to have any further influence.

The isolation of some fractions having a stimulatory effect on the amino acid incorporation directed by the endogenous mRNA is a strong support for the hypothesis that some initiating factors are missing in our system which are required for the viral RNA-directed polypeptide synthesis, but which are not needed for the poly U-directed amino acid incorporation. The whole high speed supernatant fraction should be examined in detail for the purification of such factors.

6.7. DISCUSSION AND CONCLUSIONS

In this chapter we have described our efforts to stimulate the amino acid incorporation by exogenous messengers. When ribosomes were isolated in the usual way, poly U stimulated the incorporation of phenylalanine up to 3 times. Similar data were obtained by BOARDMAN et al. (1966) and VAN KAMMEN (1967a). No impurities or inhibitors seemed to be responsible for this poor stimulation,

since purifying the ribosomes on Sephadex G200 had no significant effect. It was further found that endogenous activity could not be removed by preincubation according to MATTHAEI and NIRENBERG (1961) and VOORMA (1965), i.e., preincubation for 45 minutes in the standard incubation mixture. After such treatment the endogenous activity was about equal to that before the treatment. Thus, methods had to be found leading to a better removal of the endogenous activity.

In view of the rather pronounced pH dependence discussed in the previous chapter one could ask whether the active complex would be broken down at prolonged incubation at higher pH's. This question could not be answered at that time. Because of the high initial rate at a higher pH endogenous activity might be eliminated by preincubation at a higher pH. This was successfully attempted with the 80 S ribosomes (6.2.1). The best results were obtained when the 80 S ribosomes were preincubated for 3 hours at pH 8.6 and 5 mM Mg^{2+} , pelleted and then incubated at pH 8.6 and 11 mM Mg^{2+} . During preincubation the 80 S ribosomes proved to transform into 60 S, 50 S and 40 S particles, but intact 80 S ribosomes could be reformed after the treatment. Both rRNA molecules were also intact, which indicates that the ribosomes were not damaged during this treatment. This means that the decrease of the incorporation rate at a higher pH (chapter 5) was not due to inactivation of the ribosomes. These results suggest that during the transformation into 60 S, 50 S and 40 S particles, the messenger fragments are released from the ribosomes.

Another method to transform 80 S ribosomes into 60 S, 50 S and 40 S particles was by dialysing them against 10^{-4} M Mg^{2+} . In this way it was also possible to eliminate endogenous activity. Again, the pH of incubation proved to be very important as the effect was much greater at pH 8.6 than at pH 7.9 (table 6.9). Further it appeared that the stimulation by poly U was highly dependent on dialysis time (figure 6.2). These results indicate that the highest activity was obtained when ribosomes had been dialysed until most of them were in the 60 S form. With dialysis for longer periods, the activity decreased rapidly.

In most of the experiments with 80 S ribosomes, either preincubated or dialysed, two phenomena could be observed. First, after either of these treatments, the endogenous activity decreased to 20–50% of the original one. Secondly, the poly U-directed activity increased up to tenfold. This suggests that part of the ribosomes had an attached, but already read out messenger fragment after isolation, preventing poly U from being attached and therefore blocking the ribosomes. These fragments were largely removed by the subsequent preincubation or dialysis. Apparently the number of such blocked ribosomes varied strongly from experiment to experiment. In some cases, the poly U-directed activity was about equal to that prior to treatment (preincubation or dialysis). The endogenous activity was then largely removed during preincubation or dialysis. In other cases, only half of the endogenous activity was removed, but then the final activity with poly U after treatment was up to 10 times the original activity. In the first case, hardly any ribosomes were blocked whereas in the latter case most of them were.

It proved to be impossible to eliminate by preincubation the endogenous activity from 70 S ribosomes. Occasionally a good stimulation by poly U was observed with or without preincubation. This was not reproducible and no explanation can be given. However, after dissociation into subunits the 70 S ribosomes could be stimulated by poly U. Apparently in this case endogenous activity was also eliminated by dialysis against 10^{-4} M Mg^{2+} . Again, 70 S ribosomes seemed to be partly blocked, as described for the 80 S ribosomes.

Several conclusions can be drawn from the time curves. First, the active complex was rather stable, because the rate of incorporation remained constant for at least two hours. Second, ribosomes were not degraded during incubation, which indicates that in cases where 70 S ribosomes were dissociating or 80 S ribosomes were transforming into 60 S particles during incubation, this was due to a read out of the messenger and not to breakdown of the ribosomes. Furthermore, an activity of about 100 $\mu\mu$ moles/mg/hour means that the incorporation rate of plant ribosomes, at least in vitro, but probably also in vivo, is comparatively low. *E.coli* ribosomes do incorporate about 20,000 $\mu\mu$ moles phenylalanine/mg/hour (MATTHAEI and NIRENBERG, 1961), which suggests that *E.coli* ribosomes are about 2000 times as active as plant ribosomes.

The ability of 70 S and 80 S ribosomes to accept exogenous messengers did not extend to viral RNAs. Possible initiation factors were tried in a preliminary investigation, but they did not give any improvement. Some of the supernatant fractions contained stimulating factors, but the results obtained so far did not allow a conclusion to be drawn as to which factors were lacking. For this purpose the whole supernatant fraction should be examined in detail.

In summary it can be said that both 70 S and 80 S ribosomes can be activated in such a way that they accept poly U as an exogenous messenger. Each system shows characteristics of its own in polyphenylalanine synthesis and the active complex is very stable. Under our experimental conditions no effect could be obtained with viral RNAs as a messenger.

7. GENERAL DISCUSSION

7.1. EFFECT OF PURIFICATION ON THE AMINO ACID INCORPORATING ACTIVITY OF THE RIBOSOMES

There were several reasons why we purified our amino acid incorporating system. First, purifying the ribosomes resulted in a dependence of the incorporation on the addition of soluble factors, such as tRNA and enzymes. In this way the process could be better controlled *in vitro*. Second, purified and non-purified ribosomes had different time curves, so that certain conclusions could be drawn with respect to the degradation of the messengers. Endogenous messengers seemed to be degraded during incorporation, even when purified ribosomes were used (section 3.5). Finally, a proper separation of 70 S and 80 S ribosomes proved to be necessary for investigating the properties of both types of ribosomes. These properties gave rise to the development of methods to eliminate endogenous messengers.

During the purification of enzymes, the activity per mg of protein (specific activity) increases. Our purification of the ribosomes did not result in an increase in their specific activity. The non-purified system described in chapter 3 incorporated 10–20 μmoles of leucine per mg of protein per hour and the purified system described in chapter 4 (still a mixture of 70 S and 80 S ribosomes) had a similar activity. These experiments were performed at 11 mM Mg^{2+} . Only incubation at the optimal Mg^{2+} concentration for each class (5 and 17 mM for separated 80 S and 70 S ribosomes, respectively) resulted in an increase in the activity (24 and 66 μmoles leucine per mg per hour for the 80 S and 70 S ribosomes respectively; see figure 5.3), which therefore was not due to the purification as such. Our purification was not aimed at a single enzyme but rather at the whole complex of ribosome and factors. Lack of an increase in the specific activity during the purification process could be ascribed to the partial loss of one or more factors during purification. In this case a further increase in activity of the ribosomes themselves would not imply an increase in the specific activity of the system as a whole. An alternative interpretation is the following. The contribution of the ribosomes to the mass of the whole active complex is considerable. When eliminating impurities, no large increase in the proportion of ribosomal material present will be expected. This means that the specific activity will hardly increase during the purification process. It can thus be said that with each purification step the incorporating capacity becomes more dependent on the addition of special and specific fractions, whereas the specific activity of the complete system remains at about the same level.

7.2. COMPARISON OF THE ACTIVITIES OF RIBOSOMES FROM DIFFERENT ORIGINS

As mentioned in section 3.6, the amino acid incorporating system from tobacco leaves is not very active, compared with other systems, on the basis

TABLE 7.1. Amino acid incorporating activities of *E. coli* and tobacco ribosomes.

Ribosomes	Amino acid incorporation in $\mu\mu\text{moles/mg/hour}$			
	With endogenous messengers (a)	After treatment ¹		(c)/(a)
		- poly U (b)	+ poly U (c)	
<i>E. coli</i> ² 70 S	500-1000 ³	20	20,000 ⁶	20-40
Tobacco ⁴ 70 S	66 ⁵	5	70-140 ⁶	1-2
80 S	24 ⁵	5	30-45 ⁶	1-2
Ratio <i>E. coli</i> /tobacco ⁷	ca. 20	ca. 6	ca. 250	

¹ *E. coli* ribosomes: preincubation (MATTHAEI and NIRENBERG, 1961)

70 S tobacco ribosomes: dialysed against 10^{-4} M Mg^{2+} for 2 hours.

80 S tobacco ribosomes: dialysis against 10^{-4} M Mg^{2+} for 14 hours, or preincubation at pH 8.6 for 3 hours.

² MATTHAEI and NIRENBERG (1961).

³ With ^{14}C -valine.

⁴ The present article.

⁵ With ^{14}C -leucine.

⁶ With ^{14}C -phenylalanine.

⁷ Ratio in amino acid incorporation in $\mu\mu\text{moles/mg/hour}$ between ribosomes from *E. coli* and tobacco.

of $\mu\mu\text{moles}$ incorporated per mg per hour. The endogenous *E. coli* system is about 50 times as active (MATTHAEI and NIRENBERG, 1961), whereas rat liver polyribosomes are even about 350 times as active (BLOEMENDAL et al., 1964).

We shall compare our results with those obtained in the *E. coli* system. Table 7.1 summarizes the activities of 70 S *E. coli* ribosomes as found by MATTHAEI and NIRENBERG (1961), and of 70 S and 80 S tobacco ribosomes as determined in the present work. From these data the following conclusions can be drawn. *E. coli* ribosomes have an endogenous activity, which, on the average, is about 20 times as high as that of 70 S and 80 S tobacco ribosomes. In both cases endogenous messengers can be removed and the activity can be restored by the addition of exogenous messenger, in this case, poly U. Preincubated *E. coli* ribosomes have retained only 2-4% of their activity, but preincubated or dialysed 80 S and 70 S tobacco ribosomes about 8%. Poly U is able to stimulate such *E. coli* ribosomes about 250 times as much as tobacco ribosomes, assuming that initiation on poly U occurs at the same rate with plant and *E. coli* ribosomes. When programmed with poly U, *E. coli* ribosomes are 20-40 times as active as in the presence of endogenous messengers. Keeping in mind that the specific activity of polyphenylalanine is much higher than that of the polypeptides synthesized under the direction of endogenous messengers, the poly U-directed incorporation is of the same order of magnitude as the incorporation with endogenous messengers. Since plant ribosomes are only 1-2 times as active with poly U, their original specific activity is not completely restored by poly U. As pointed out in 6.7, a certain proportion of the ribosomes bears a read messenger fragment and thus is inactive in the incorporation process. The

preincubation or dialysis procedure used in the present investigations may be insufficient to remove all read messenger fragments. This explains the relatively low stimulation by poly U. Thus, our procedure of eliminating endogenous messengers from tobacco leaf ribosomes is much less efficient than simple preincubation of *E. coli* ribosomes. Since the stimulation by viral RNAs seems to be lower than that by poly U (table 6.10), the relatively high endogenous activity is probably one of the reasons which prevent an observable stimulation by viral RNAs.

7.3. COMPARISON OF 70 S AND 80 S RIBOSOMES WITH 70 S *E. coli* RIBOSOMES

In chapter 1 we described the current ideas on the mechanism of protein synthesis in *E. coli*. Both ribosomal subunits play an important role. In this mechanism the protein synthesizing process in vivo appears to continue efficiently at a proper Mg^{2+} concentration and with the aid of several factors. By changing the Mg^{2+} concentration and by isolating such factors this process could be reproduced in vitro and the role of 2 subunits could be demonstrated (JOKLIK and BECKER, 1965a, b; NOMURA and LOWRY, 1967; KAEMPFER, MESELSON and RASKAS, 1968).

The behaviour of 70 S plant ribosomes might be similar as they could be dissociated into subunits very easily and reversibly. Moreover, after incubation they appeared to be largely in the subunit form. Thus, the theory of attachment of the messenger to the 30 S subunit may apply to 70 S ribosomes in general.

On the other hand, 80 S plant ribosomes showed a totally different picture. None of our treatments, such as dialysis against 10^{-4} M Mg^{2+} for different periods, addition of EDTA, resuspension in phosphate buffer or preincubation for 3 hours, was able to dissociate those ribosomes, although some of them being rather rigorous. The only change observed was unfolding and, after prolonged treatment, breakdown. With this in mind, it is difficult to imagine that protein synthesis in vivo on 80 S ribosomes takes place by recycling through subunits (JOKLIK and BECKER, 1965a, b; KAEMPFER, MESELSON and RASKAS, 1968). On this ground, it seems reasonable to assume that there is not one general mechanism for protein synthesis, but that there may exist at least two, i.e., one for 70 S and another for 80 S ribosomes.

If several systems exist, depending e.g. on the size of the ribosomes involved, these systems might differ in the rate of protein synthesis and in the life time of their messengers. In bacteria messengers have a half-life of only about 90 seconds, in contrast to those in higher organisms for which half-lives of over 1 hour have been reported (c.f. MAHLER and CORDES, 1967). Endogenous messengers active with 80 S ribosomes might involve mechanisms different from those active with bacterial ribosomes.

7.4. SPECIFICITY IN PROTEIN SYNTHESIS

As 70 S and 80 S plant ribosomes differed in several respects (chapters 4, 5 and 6), one may wonder if both types of ribosomes might have their own specific protein synthesizing system. Some indications for this have already been obtained. BARNETT and EPLER (1966) have isolated two different phenylalanyl-tRNA synthetases from *Neurospora crassa*, each reacting with a different phenylalanyl-tRNA. The same situation was found to occur with aspartyl-tRNA synthetases and these two synthetases were thought to belong to the cytoplasmic (80 S) and mitochondrial (70 S) system, respectively.

Specificity seems to exist also in chain-elongating factors. PARISI et al. (1967) have studied protein synthesis with ribosomes belonging to the two classes, i.e. 70 S ribosomes from *E. coli* and *B. subtilis* and 80 S ribosomes from *S. cerevisiae*, castor bean seedlings and rat liver. Enzymes were also isolated from each organism and the poly U dependent polyphenylalanine synthesis was studied in a mixture of ribosomes and enzymes isolated from the different sources. It appeared that within each class of ribosomes, ribosomes and enzymes could be interchanged, but not between the two classes. This incompatibility was not due to tRNA or synthesis of phenylalanyl-tRNA, to degradation of heterologous phenylalanyl-tRNA, binding of phenylalanyl-tRNA to the ribosomes or the presence of inhibitors. PARISI et al. came to the conclusion that the size of the ribosomes might be decisive for the success of the interaction between certain factors and the ribosomes. These findings suggest that also in plants the 70 S and 80 S ribosomes might have their own, specific factors. This would agree with the concept that chloroplasts are autonomous cell organelles. Keeping this in mind, it would be of interest to isolate from tobacco factors specific for either the 70 S or the 80 S ribosomes. Since contamination of one class of ribosomes by the other is difficult to avoid, the isolation of such factors raises several practical difficulties.

7.5. STABILITY OF THE RIBOSOMES

Because of the rapidly decreasing incorporation rate it was first suggested that the amino acid incorporation system of tobacco ribosomes was rather unstable. It appeared that one of the factors causing this decrease was the degradation of the messenger fragments left on the ribosomes (chapter 3). We were not able to decide at that point whether this was the only reason or that there was also a breakdown of ribosomes during the incubation process. For the 80 S ribosomes similar conclusions could be drawn from the temperature and pH curves (chapter 5), whereas the 70 S ribosomes were not thought to be attacked by endonucleases because the kinetics followed a linear time curve. The abrupt decrease in the latter case was thought to be due to read out of the messenger fragments. Some information on the stability of the ribosomes, however, could be obtained when the effect was studied of exogenous messengers on the amino acid incorporation. First, both 70 S and 80 S ribosomes being still active after preincubation at pH 8.6 or dialysis against 10^{-4} M

Mg²⁺ for several hours, indicates that plant ribosomes are quite stable. In addition to being resistant to the above treatments, plant ribosomes could be stored for several days, as mostly several days elapsed between the isolation of the ribosomes and the final experiment. Soluble enzymes, however, had to be freshly prepared each day. Second, when preincubated or dialysed ribosomes were incubated with poly U as a messenger, the incorporation continued at a constant rate for over 2 hours. Unfortunately, we were not able to investigate how long the rate would remain constant, since we stopped the reaction after two hours to prevent bacterial growth which happened to occur upon longer incubation. So it seems that both 70 S and 80 S ribosomes can remain active in vitro for several hours, unless the messenger has been read out or the medium is exhausted. This would make these ribosomes very suitable for the study of long term processes in vitro, such as the translation of polycistronic messengers.

7.6. DIFFERENCES BETWEEN POLYPEPTIDE SYNTHESIS DIRECTED BY ARTIFICIAL AND NATURAL MESSENGERS

The question remains why in our system poly U is accepted as a messenger whereas no clear results could be obtained with viral RNAs. One possibility is that these RNAs are acceptable as a messenger, but that due to the insufficient elimination of endogenous messengers (see 7.2.) no effect could be measured. The differences between natural and artificial messengers may be another reason. The main difference between an artificial messenger, such as poly U and natural messengers is that the known natural messengers use a special initiating mechanism involving a specific triplet. For *E. coli* such a mechanism has been found and the codon AUG seems to be a general initiation codon in this organism. This causes the incorporation of N-formyl-methionine as the N-terminal amino acid in all polypeptide chains. Several other proteins have an N-substituted terminal amino acid (e.g. TMV protein: N-acetyl-serine, and TYMV: N-acetyl-methionine; KNIGHT, 1963), but no special initiating mechanism has been detected in the systems which synthesize these proteins. Artificial messengers, however, lack a special initiation codon, but some, e.g. poly U, are read easily all the same. So when poly U is used as a messenger, several initiation factors can be lacking or be substituted for by high Mg²⁺ concentrations. Among these factors are those needed for the binding of N-formyl-methionyl-tRNA to the ribosomes, F₁ and F₂ (SALAS et al., 1967) and factor F₃, needed for the binding of the ribosome to the messenger (BRAWERMAN and EISENSTADT, 1966; REVEL and GROS, 1966). It is not likely that the chain elongation factors T and G (NISHIZUKA and LIPMANN, 1966a; 1966b; LUCAS-LENARD and LIPMANN, 1966) will be different for artificial and natural messengers. Thus, one of the causes for the lack of sufficient stimulation of the amino acid incorporation by natural messengers, such as viral RNAs in our system may be the lack of some of these initiation factors or of other factors involved in messenger binding or chain initiation. EISENSTADT and BRAWERMANN (1967) have demonstrated that the purified *E. coli* ribosomes lacked a

factor required for viral RNA-dependent polypeptide synthesis, which factor proved to be an initiation factor, occurring on the 30 S ribosomal subunit. Our preliminary attempts failed to isolate such a factor. This may be due to a difference between these factors in *E.coli* and tobacco plants or because the method of isolation was not appropriate. It is to be expected that such an isolation from tobacco leaves will be difficult because many of such factors must be present in tobacco leaves at very low concentrations. Therefore, the whole high speed supernatant fraction should be thoroughly examined. In addition, the elimination of endogenous messengers in the 70 S and 80 S tobacco systems is less efficient than in an *E.coli* system (see 7.2). So even after isolation of the proper factors, a smaller stimulation by viral RNAs is to be expected than that obtained in the *E.coli* system, unless a better method for elimination of endogenous messengers can be developed. However, the present method should enable the demonstration of such a stimulation and full attention will have to be paid to the isolation of the missing factor(s).

SUMMARY

1. The interaction of exogenous messengers with 70 *S* chloroplast and 80 *S* cytoplasmic tobacco ribosomes *in vitro* was studied. The isolation of an amino acid incorporating system from tobacco leaves should enable us to study some aspects of viral protein synthesis. Tobacco 70 *S* and 80 *S* ribosomes were chosen for the following reasons: Little is known about the interaction of messenger RNA with 80 *S* ribosomes. In general, plant viral RNAs did not yield meaningful results in the *E. coli* system. The interaction of 70 *S* and 80 *S* ribosomes with messengers could be compared in a homologous tobacco leave system.

2. The amino acid incorporating system as isolated initially could be characterized by its dependence on an energy source and GTP, on the concentration of ribosomes, on the magnesium and potassium ion concentration, and on the incubation time, but not on tRNA and soluble enzymes or on unlabeled amino acids.

3. The lack of an effect of tRNA or soluble enzymes was not due to the amino acid activation reaction, but to the presence of these factors in the ribosome preparations.

4. The ribosomes could be purified by sucrose gradient centrifugation, centrifugation through 0.5 M NH_4Cl , and by chromatography on a Sephadex G200 column. Then amino acid incorporation became dependent on the addition of tRNA and soluble enzymes but not of ^{12}C -amino acids. It was calculated that even after purification about 0.0012 μmoles ^{12}C -amino acids were still present in the ribosome preparation.

5. Highly purified ribosomes were active for a longer period than nonpurified ribosomes, but nevertheless the incorporation rate decreased during incubation. The conclusion was drawn that both degradation and read out of the messenger were responsible for this decrease.

6. Some of the physical properties of 70 *S* and 80 *S* ribosomes were determined. Methods were developed to separate 70 *S* and 80 *S* ribosomes and polyribosomes from each other. The polyribosomes appeared to consist of 80 *S* ribosomes. About 5–10 times as much 80 *S* ribosomes could be isolated from leaves as 70 *S* ribosomes.

7. The 70 *S* ribosomes were not stable at elevated salt concentrations. At low Mg^{2+} concentrations they dissociated reversibly into 50 *S* and 35 *S* subunits; 80 *S* ribosomes could not dissociate, but unfolded at low Mg^{2+} concentrations. During this process 60 *S* particles first appeared, followed by 50 *S* and 40 *S* particles. When the Mg^{2+} concentration was raised again, 80 *S* particles reformed from 60 *S* particles but hardly from 50 *S* and 40 *S* particles.

8. Both 70 *S* and 80 *S* ribosomes contained two different RNA molecules, i.e., the 70 *S* ribosomes had 17 *S* and 23 *S* RNA, and the 80 *S* ribosomes 17 *S* and 25 *S* RNA.

9. The amino acid incorporating activity of the 70 *S* and the 80 *S* ribosomes

as compared to that of the polyribosomes was not essentially different, but the 70 S ribosomes were about 2.5 times as active as the 80 S ribosomes at their optimal Mg^{2+} concentrations. The optimal concentration for the 80 S ribosomes was 5 mM and for the 70 S ribosomes 17 mM.

10. In contrast to the 80 S ribosomes, the 70 S ribosomes had a constant incorporation rate for about 40 minutes, which then decreased abruptly to almost zero. It was concluded that no messenger degradation took place during incubation, but that the messengers had been read out after 40 minutes. The 80 S ribosomes behaved similarly as mentioned for the mixture of ribosomes.

11. Incubation temperature and pH had a clear effect on both 70 S and 80 S ribosomes. This effect, however, was variable and depended on the incubation time. In general, the optimum temperature and pH decreased with increasing periods of incubation.

12. The effect of exogenous messengers on the amino acid incorporation was studied. Untreated ribosomes could hardly be stimulated by such messengers.

13. Two methods were developed for the elimination of endogenous messengers from 80 S ribosomes. According to the first method 80 S ribosomes were preincubated at pH 8.6 in a complete, but unlabeled, incubation mixture for 3 hours. Then the ribosomes were pelleted at $105,000 \times g$ and incubated in a complete labeled incubation mixture. The second method consisted of dialysis against 10^{-4} M Mg^{2+} in standard buffer during 14 hours. During this dialysis the ribosomes unfolded to 60 S particles. The dissociation of 70 S ribosomes into 50 S and 35 S subunits proved to result in the elimination of endogenous messengers from these ribosomes.

14. Preincubated and dialyzed 80 S and 70 S ribosomes could be programmed by poly U as an exogenous messenger. Incorporation continued at a constant rate for over 2 hours. The optimal Mg^{2+} concentrations were 11 mM for the 80 S and 18 mM for the 70 S ribosomes, respectively. At these concentrations, a stimulation of 10–40 times was obtained. Again, the 70 S ribosomes were about 2.5 times as active as the 80 S ribosomes. The activities were 30–45 μ moles phenylalanine per mg per hour for the 80 S ribosomes and 70–140 μ moles for the 70 S ribosomes.

15. Viral RNAs did not stimulate to an appreciable extent the amino acid incorporation by either type of ribosomes. Initiation factors such as leucovorin were added, but none of these showed any effect. Some fractions, isolated from the supernatant of ribosomes centrifuged at $105,000 \times g$ however, showed a small effect on the over-all incorporation. It was concluded that in our system some special initiation factor(s) may be missing and that it might be possible to isolate from the supernatant fractions such a factor or factors.

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SAMENVATTING

In dit artikel wordt een onderzoek beschreven waarin getracht wordt, uit bladeren van 'Samsun' -tabak een aminozuur-incorporerend systeem te isoleren dat geprogrammeerd zou kunnen worden met exogene boodschappers. De isolatie van een dergelijk systeem zou de mogelijkheid kunnen bieden om enkele aspecten van de viruseiwitsynthese te bestuderen.

Om verschillende redenen is tabak als uitgangsmateriaal gekozen. Ten eerste omdat er erg weinig bekend is over de interactie van boodschapper RNA met 80 S ribosomen, die in hogere organismen voorkomen. Een tweede reden is het feit dat 70 S ribosomen, die geïsoleerd zijn uit de bacterie *Escherichia coli*, niet zonder meer te programmeren zijn met natuurlijke boodschappers zoals virus RNA's. Tenslotte vormt de mogelijkheid om in een homoloog systeem de interactie tussen boodschapper RNA en 70 S en 80 S ribosomen, die beide in planten voorkomen, te bestuderen een derde reden.

Eerst is in een overzicht van wat er uit de literatuur bekend is, het mechanisme van de eiwitsynthese in het algemeen beschreven. Omdat gebleken is dat de ribosomen een zo belangrijke en tevens ingewikkelde rol spelen bij de eiwitsynthese is getracht enig inzicht te verschaffen over de ribosomen zelf. De heterogeniteit en de specificiteit van de ribosomen worden beschreven. Hierna worden de verschillende aminozuurincorporerende systemen die uit planten zijn geïsoleerd, vergeleken en tenslotte wordt de boodschapper-activiteit van virus RNA's in systemen die niet uit planten werden geïsoleerd, beschouwd.

In het tweede hoofdstuk wordt beschreven hoe de voor dit onderzoek gebruikte planten, *Nicotiana tabacum* 'Samsun' werden opgekweekt en hoe daaruit de verschillende gebruikte fracties als ribosomen, transport RNA en oplosbare enzymen werden geïsoleerd. Verder worden een aantal algemene methoden vermeld waarvan gebruik werd gemaakt.

In het derde hoofdstuk worden de eigenschappen beschreven van een aminozuur-incorporerend systeem, zoals dit aanvankelijk werd geïsoleerd en waarin nog endogeen boodschapper RNA aanwezig was. De incorporatie in dit systeem is afhankelijk van een energiebron en GTP, van de concentratie van de ribosomen, van de aanwezigheid van magnesium- en kalium ionen en van de duur van de incubatie, maar niet van de toevoeging van tRNA, oplosbare enzymen en ongemerkte aminozuren. Het feit dat tRNA en oplosbare enzymen geen effect hebben op de aminozuur-incorporatie, is niet te wijten aan het niet of slecht verlopen van de aminozuur-activeringsreactie, maar aan de aanwezigheid van deze factoren in de ribosomenpreparaten. Zowel het geïsoleerde tRNA als de gebruikte oplosbare enzymen bezitten een hoge activiteit.

Met behulp van diverse methoden blijken de ribosomen te kunnen worden gezuiverd. Met name centrifugering op suikergradiënten, centrifugering door een laag van 0,5 M NH_4Cl in suiker en chromatografie op een kolom bestaande uit Sephadex G200 blijken in staat om veel van het aanwezige tRNA en de oplosbare enzymen te verwijderen. Ongemerkte aminozuren kunnen niet in die

mate worden uitgewassen dat de aminozuur-incorporatie afhankelijk wordt van toevoeging ervan. Na zuivering is er nog steeds 0,0012 μmol aminozuren aanwezig, wat bijna even veel is als de toegevoegde gemerkte aminozuren. De genoemde zuiveringsmethoden blijken ook invloed te hebben op het verwijderen van remmende factoren, want gezuiverde ribosomen zijn langer actief dan ongezuiverde. Toch neemt ook wanneer gezuiverde ribosomen worden gebruikt, de reactiesnelheid op den duur af. Dit blijkt veroorzaakt te worden door afbraak van de boodschapper en het bereiken van het einde van deze boodschapper door het ribosoom gedurende het lezen (uitlezen van de boodschapper).

In het vierde hoofdstuk worden enkele fysische eigenschappen van planten-ribosomen beschreven. Uit planten kunnen 70 S ribosomen, die voorkomen in de chloroplasten, 80 S ribosomen, die voorkomen in het cytoplasma, en polyribosomen, die bestaan uit 80 S ribosomen, worden geïsoleerd. Met behulp van suikergadiënten kunnen deze verschillende componenten van elkaar worden gescheiden. Uit het bladmateriaal kunnen 5 tot 10 maal zoveel 80 S ribosomen als 70 S ribosomen worden geïsoleerd. De 70 S en 80 S ribosomen blijken aanmerkelijk in hun fysische eigenschappen te verschillen. In tegenstelling tot 80 S ribosomen zijn 70 S ribosomen niet stabiel in hoge zoutconcentraties, zoals 0,5 M NH_4Cl . In lage Mg^{2+} concentraties in de orde van 10^{-4} M dissociëren ze binnen 3 uur in 2 subeenheden, die een sedimentatie-coëfficiënt van 50 S en 35 S hebben. Deze dissociatie is reversibel. De 80 S ribosomen dissociëren niet, maar ontvouwen zich tijdens een dergelijke behandeling. Na een dialyse van 8 tot 14 uur tegen 10^{-4} M Mg^{2+} in standaard buffer is een aanzienlijk deel van de 80 S ribosomen overgegaan in deeltjes met een sedimentatie coëfficiënt van 60 S. Later verdwijnen deze deeltjes weer om plaats te maken voor 50 S en 40 S deeltjes. Hoewel niet definitief uitgemaakt kan worden of deze deeltjes ontvouwen subeenheden zijn of dat zowel de 50 S als de 40 S deeltjes verder ontvouwen 60 S deeltjes zijn, zijn er enkele aanwijzingen die pleiten voor de laatste opvatting. Uit 60 S deeltjes kunnen opnieuw 80 S deeltjes worden verkregen door de Mg^{2+} concentratie te verhogen, maar dit lukt nauwelijks met de 50 S en 40 S deeltjes.

Zowel de 70 S als de 80 S ribosomen bevatten 2 RNA-componenten. Deze kunnen beide worden geïsoleerd en hebben bij de 70 S ribosomen een sedimentatie constante van 17 S en 23 S, en bij de 80 S ribosomen een van 17 S en 25 S. Het is merkwaardig dat, ondanks het feit dat er bij de 80 S ribosomen geen dissociatie in subeenheden waargenomen kan worden, er toch 2 RNA-componenten geïsoleerd zijn, hetgeen wijst op het bestaan van zulke deeltjes.

In hoofdstuk 5 worden de aminozuur-incorporerende eigenschappen van 70 S en 80 S ribosomen vergeleken. Onder gelijke condities blijkt de activiteit van polyribosomen niet te verschillen van die van 70 S en 80 S ribosomen. De optimum Mg^{2+} -concentratie voor de incorporatie door 70 S ribosomen wijkt in sterke mate af van die welke benodigd is voor 80 S ribosomen. Voor 70 S en 80 S ribosomen is deze concentratie respectievelijk 17 mM en 5 mM. Onder optimale omstandigheden zijn 70 S ribosomen ongeveer 2,5 maal zo actief als 80 S ribosomen.

De incorporatiesnelheid van 70 S ribosomen is constant gedurende de eerste 40 minuten. Daarna neemt deze snelheid zeer snel af tot bijna nul. Dit duidt er op dat hier niet of nauwelijks sprake kan zijn van afbraak van de boodschapper gedurende de incubatie, maar dat de boodschapper na 40 minuten is afgelezen. Bij de 80 S ribosomen treedt wel afbraak op en de incorporatiesnelheid neemt geleidelijk af.

De aminozuur-incorporatie door zowel 70 S als 80 S ribosomen blijkt duidelijk afhankelijk te zijn van de temperatuur en de pH tijdens de incubatie. De pH blijkt van zeer grote invloed te zijn op de activiteit en ook op de mogelijkheid om endogene boodschappers te verwijderen van 80 S ribosomen.

Zoals uit het zesde hoofdstuk blijkt, zijn de ribosomen die geïsoleerd en gezuiverd waren, zonder meer nauwelijks te stimuleren met exogene boodschappers. Er zijn twee methodes ontwikkeld om endogene boodschappers van de 80 S ribosomen te verwijderen. Deze boodschappers blijken zeer vast gebonden te zijn, en een eenvoudige preïncubatie zoals dat voor 70 S ribosomen van *E. coli* geldt, heeft niet het gewenste resultaat. Een preïncubatie van 3 uur bij pH 8,6 in een volledig incubatiemengsel, zonder gemerkte aminozuren, heeft dit wel, zoals aangetoond kan worden door poly U als exogene boodschapper te gebruiken. Hiertoe worden na preïncubatie de ribosomen opnieuw gezuiverd door ze te centrifugeren bij $105.000 \times g$. Een andere methode die hetzelfde resultaat heeft, is dialyse tegen 10^{-4} M Mg^{2+} in standaard buffer. Een dialysetijd van ongeveer 14 uur heeft het gunstigste effect en blijkbaar worden tijdens het ontvouwingsproces (door te dialyseren tegen 10^{-4} M Mg^{2+} ontstaan immers ontvouwen deeltjes van 60 S) ook endogene boodschappers verwijderd. Door middel van preïncubatie kunnen endogene boodschappers niet op efficiënte wijze van 70 S ribosomen worden verwijderd. Dialyse tegen 10^{-4} M Mg^{2+} heeft wel het gewenste resultaat, waaruit blijkt dat dissociatie van 70 S ribosomen hetzelfde effect heeft als het ontvouwen van 80 S ribosomen.

Aldus behandelde 70 S en 80 S ribosomen kunnen worden geprogrammeerd met poly U. De incorporatie van fenylalanine geschiedt gedurende meer dan 2 uur met een constante snelheid en er wordt een stimulering bereikt van 10 tot 40 maal. Voor de 70 S ribosomen is de optimum Mg^{2+} -concentratie ongeveer gelijk aan die voor incorporatie onder invloed van endogene boodschappers, namelijk 18 mM. Voor de 80 S ribosomen verschilt dit optimum en bedraagt 11 mM. Ook nu weer zijn de 70 S ribosomen ongeveer 2,5 maal zo actief als de 80 S ribosomen onder optimale omstandigheden. Door de 80 S en 70 S ribosomen worden respectievelijk 30–45 en 70–140 μ molen fenylalanine per mg eiwit per uur geïncorporeerd.

Noch de 70 S, noch de 80 S ribosomen kunnen geprogrammeerd worden met virus RNA's. Aangezien ze dit wel kunnen met poly U, lijkt het er op of bepaalde initiatiefactoren hiervoor verantwoordelijk zijn, omdat een karakteristiek verschil tussen kunstmatige boodschappers zoals poly U en natuurlijke is dat natuurlijke boodschappers een eigen initiatiemechanisme bezitten. In het onderhavige systeem kon een dergelijk mechanisme echter nog niet worden ontdekt. Aangezien er wel bepaalde enzymfracties geïsoleerd kunnen worden

die een stimulerend effect hebben op de aminozuur incorporatie, wordt de verwachting uitgesproken dat uit de supernatant fractie de ontbrekende factoren kunnen worden geïsoleerd.

In hoofdstuk 7 wordt een algemene discussie gegeven, waarin getracht wordt een verband te leggen tussen de verkregen gegevens onderling en tussen deze gegevens en hetgeen er uit de literatuur bekend is over de eiwitsynthese.

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