In vitro phosphorylation of rabbit reticulocyte ribosomes

Wesley Kiyoshi Tanaka

Iowa State University

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In vitro phosphorylation of rabbit reticulocYTE ribosomes

by

Wesley Kiyoshi Tanaka

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics Major: Biochemistry

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For the Graduate College

Iowa State University
Ames, Iowa

1974
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ABBREVIATIONS

ACTH  adrenocorticotropic hormone
ATP    adenosine-5'-triphosphate
BBOT   2,5-bis-2-(5-tert-butylbenzoxazolyl)-thiophene
cAMP   adenosine-3',5'-monophosphate
DEAE-cellulose  diethylaminoethyl-cellulose
DOC    deoxycholate
dibutyryl cAMP N^6-,O^2'-dibutyryl adenosine-3',5'-monophosphate
DTT    dithiothreitol
EDTA   ethylenediaminetetraacetic acid
EF-T1, EF-T2 elongation factor-transferase 1
        elongation factor-transferase 2
GDP    guanosine-5'-diphosphate
GSH    glutathione
GTP    guanosine-5'-triphosphate
IF-1, IF-2, IF-3 initiation factor-1, -2, -3
mA     milliampere
mCi    milliCurie
mRNA   messenger ribonucleic acid
Pi     inorganic phosphate
poly U  polyuridylic acid
S      Svedberg unit
TCA    trichloroacetic acid
Tris   tris-(hydroxy methyl) aminomethane
tRNA   transfer ribonucleic acid
ugm    microgram
ul     microliter
INTRODUCTION

The past few years have seen remarkable advances in our understanding of the molecular biology of cells and particularly of the mechanism of protein biosynthesis. With the advent of in vitro systems capable of carrying out protein synthesis, it first became possible to study the individual processes and components involved in translation of information from messenger RNA into protein. The deciphering of the genetic code is one of the early notable advances stemming from in vitro work (Nirenberg et al., 1966). As research has continued in various systems, the universality of the genetic code has become apparent as well as the similarities in the translational machinery between organisms widely separated on the evolutionary scale (Krisko et al., 1969).

The various requirements for in vitro protein synthesis have been studied in great detail. These requirements include an appropriate ionic environment, a sulphydryl protecting reagent, GTP, cytoplasmic protein factors necessary for initiation, elongation and termination, aminoacyl-tRNA, messenger RNA and ribosomes. Much of the current research has been directed towards the interaction of these various components in the different phases of protein synthesis. As knowledge of the mechanism of protein synthesis has advanced, it has become possible to study, in more detail, the controls which regulate translation.

This review will deal with the area of translational control of protein synthesis. Specifically it will concern itself with the topic of phosphorylation of ribosomal proteins as a possible mechanism for
controlling ribosome function and activity. A very general background on the mechanism of protein synthesis is presented. Special emphasis is placed on the initiation phase of protein synthesis, the point at which some translational controls seem to be operating. The elongation process in vitro is also presented in detail since the assays for elongation are used in the present studies for the determination of ribosomal activity.

Scheme 1 is a "simplified" flow diagram of the sequence of events leading to the synthesis of a polypeptide chain in prokaryotic organisms (Haselkorn and Rothman-Denes, 1973). There is still considerable controversy over many aspects of this process. In initiation, for example, the role of IF-1 in the formation of an initiation complex is not yet clear. In spite of early evidence for a role of IF-3 in dissociating the inactive "storage" 70S ribosomes (Subramanian and Davis, 1970; Sabol et al., 1970), IF-3 seems to act by inhibiting association of the 30S and 50S subunits (Kaempfer, 1970, 1972). IF-3 has also been suggested to function in ribosome recognition of natural messenger RNA, as opposed to synthetic mRNA (Revel et al., 1968, 1970). This natural messenger-specific role of IF-3 may be subject to some qualifications in light of recent work which shows a dependence for all 3 initiation factors, IF-1, IF-2 and IF-3, in the formation of an initiation complex using a synthetic random copolymer, poly (U,G) (Dubnoff and Maitra, 1971). Needless to say, the problem of assigning specific functions to each factor continues to be a complex one. Revel has suggested that the problem of deciding on specific roles for each factor may be in trying to consider
Scheme 1. Diagram for protein synthesis in prokaryotes. IF-1, IF-2 and IF-3 are initiation factors; R1 and R2 are termination factors; fmet-tRNA is formylated methionyl transfer ribonucleic acid; "acceptor site" designates non-reactivity with puromycin; "donor site" designates reactivity with puromycin (Haselkorn and Rothman-Denes, 1973).
each as an entity in itself. Function might be better thought of in terms of a combination of factors, as for example, IF-3 being responsible for preventing formation of 70S ribosomes (Kaempfer, 1972) and a complex of IF-2 and IF-3 being involved in mRNA-ribosome binding (Revel, 1972).

The process of initiation of protein synthesis in eukaryotes is similar to prokaryotes in general respects. Initiation occurs by sequential addition of a small 40S subunit and a large 60S subunit. There are 3 factors responsible for initiation of protein synthesis in rabbit reticulocytes, M1, M2 (made up of M2A and M2B) and M3 (Prichard et al., 1970). M3, like IF-3, is involved in recognition and translation of natural messenger. Unlike prokaryotes, however, the initiator tRNA is unformylated methionyl-tRNA (Smith and Marcker, 1970; Housman et al., 1970; Shafritz and Anderson, 1970a). More detailed comparisons of the initiation process in prokaryotic and eukaryotic systems will have to await further purification and characterization of the eukaryotic initiation factors (Haselkorn and Rothman-Denes, 1973).

The elongation phase of protein synthesis in prokaryotes and eukaryotes is somewhat similar. EF-T1, the binding enzyme, is responsible for binding aminoacyl-tRNA to the ribosomes in the presence of GTP. The EF-T1-mediated binding occurs at the A (acceptor) site on the ribosome in a reaction analogous to the reaction catalyzed by EF-Tu and EF-Ts in prokaryotes (Arlinghaus et al., 1964; Lucas-Lenard and Lipmann, 1971). EF-T2, the translocase, is responsible for GTP-dependent translocation of the peptidyl-tRNA from the A site to the D (donor) site in analogy
with the reaction catalyzed by EF-G in prokaryotes (Skogerson and Moldave, 1968a; Culp et al., 1969; Lucas-Lenard and Lipmann, 1971). In vitro systems for studying elongation have been developed. Poly U-directed polyphenylalanine synthesis in reticulocytes occurs at high magnesium ion concentrations (9 mM) in a reaction that requires EF-T1 and EF-T2 but not initiation factors (Miller and Schweet, 1968). At lower magnesium ion concentrations (5-6 mM) there is a dependence on M1 and M2 in addition to EF-T1 and EF-T2 for polyphenylalanine synthesis (Shafritz et al., 1970b). EF-T1 catalyzes binding of aminoacyl-tRNA to ribosomes in the presence of GTP, 6.7 mM magnesium and 70 mM KCl ("enzymatic binding") (Arlinghaus et al., 1964). At a high magnesium to KCl ratio (13.3 mM magnesium and 6.7 mM KCl), nonenzymatic binding occurs in a reaction which does not require GTP (Shaeffer et al., 1968).

A great deal of work remains to be done to complete our understanding of the translational process. One of the least understood areas of protein synthesis is that dealing with translational control. Advances in this area have been hampered largely by a lack of knowledge about the molecular events occurring in the translational process itself. A number of review articles have appeared in the recent literature which cover many aspects of translation and translational control in greater detail than can be covered here (Lengyel and Söll, 1969; Lucas-Lenard and Lipmann, 1971; Haselkorn and Rothman-Denes, 1973).
As yet there is little direct evidence for the existence of translational controls. Observations in a number of systems suggest control of protein synthesis at the translational level. These include sea urchin egg development (Monroy and Tyler, 1963), feather keratinization (Bell et al., 1965), protein synthesis at specific phases of the cell cycle (Tomkins et al., 1969), protein synthesis at specific phases of cell growth (Dietz et al., 1965), control of protein synthesis by epidermal growth factor (Cohen and Stastny, 1968) and processing of poliovirus protein (Jacobson and Baltimore, 1968). This discussion will deal with translational control in 3 systems. They are synthesis of virus-specific protein in bacterial cells, hemin control of protein synthesis in mammalian cells and cAMP-mediated hormone effects in various target tissues.

The evidence for translational control in bacterial cells comes from translation of viral RNA. The RNA phages, R17, f2 and MS2, have a polycistronic messenger RNA containing the information for 3 proteins: "A" (attachment) protein, coat protein and an RNA-synthesizing enzyme (or its subunit). Despite the presence of mRNA for all 3 proteins in equal quantity, the coat protein is the major in vitro translation product (Nathans et al., 1962). Restricted accessibility of ribosomal binding sites due to secondary and tertiary structure of the mRNA (Lodish, 1968; Voorma et al., 1971) and suppression of translational initiation by coat protein (Sugiyama and Nakada, 1967; Lodish, 1969) have been invoked to account for this differential translation. A
regulatory role for IF-3 has been suggested by the isolation and characterization of 2 IF-3 proteins from uninfected *E. coli* cells. IF-3α shows high selectivity toward translation of MS2, *E. coli* and early T4 RNA. IF-3β has high selectivity toward late T4 RNA and low activity with MS2, *E. coli* and early T4 RNA. Translational control of T4 protein synthesis during infection is postulated to occur via inactivation of IF-3α without inactivation of IF-3β (Lee-Huang and Ochoa, 1973); alternatively, inhibition of a particular IF-3 may occur via actions of a specific "i factor" (Haselkorn and Rothman-Denes, 1973).

The regulatory role of phosphorylation of ribosomal proteins in bacterial protein synthesis is doubtful. Phosphorylation of *E. coli* ribosomal proteins occurs only to a very low extent, if at all (Gordon, 1971; Kurek et al., 1972a, 1972b). These studies were done in uninfected cells and have not ruled out the possibility of phosphorylation of loosely bound ribosomal proteins or of initiation factors. *E. coli* ribosomal proteins can be phosphorylated by protein kinase from rabbit skeletal muscle (Traugh and Traut, 1972).

One of the most well studied cases of translational control in mammalian cells is the effect of hemin on protein synthesis. Hemin stimulates de novo synthesis of globin in intact reticulocytes (Grayzel et al., 1966; Waxman and Rabinovitz, 1966) and in cell-free preparations from reticulocytes (Adamson et al., 1968; Zucker and Schulman, 1968). Under conditions of hemin deficiency an inhibitor of globin synthesis, Q fraction, is produced from a precursor, termed proinhibitor (Maxwell et al., 1971). This inhibitor appears to block initiation of translation (Adamson et al., 1972; Waxman et al., 1967). The effect of hemin is to
retard and, to a limited extent, reverse the conversion of proinhibitor to inhibitor (Mizuno et al., 1972). The translational inhibitor is a protein with a molecular weight of about $4 \times 10^5$ daltons (Gross and Rabinovitz, 1973). Recent work has indicated that hemin can stimulate synthesis of all proteins in erythroid cells as well as some protein in nonerythroid cells (Beuzard et al., 1973; Mathews et al., 1973).

The involvement of ribosomal protein phosphorylation in hemin-mediated translational control is doubtful. The regulatory element in this system is a supernatant protein which can be converted to an inhibitor by incubation of a ribosome-free lysate. This precludes involvement of ribosomal phosphorylation.

The most promising line of evidence implicating phosphorylation in translational control is cAMP-mediated effects of hormones. Assuming for the moment that these hormones regulate translation, a reasonable mechanism can be postulated. Certain hormones activate the membrane-bound enzyme, adenyl cyclase, stimulating the production of cAMP. In response to elevated levels of cAMP, a cAMP-dependent protein kinase could carry out phosphorylation of particular ribosomal proteins. This phosphorylation could cause a change in ribosome activity or specificity for a particular messenger RNA (Garren et al., 1971).

Although it has not been shown directly that cAMP-mediated phosphorylation of ribosomal proteins causes a change in ribosome function, the model has several appealing features. It has been shown: 1) that phosphorylation can serve as a regulatory control; 2) that hormone
action on target cells is mediated, in some cases, through cAMP; 3) that hormones can control protein synthesis at the translational level, and 4) that cAMP-dependent phosphorylation occurs.

Evidence for phosphorylation operating as a control mechanism has been found in a number of systems such as glycogen breakdown (Soderling et al., 1970), lipolysis (Huttunen et al., 1970) and regulation of pyruvate dehydrogenase activity (Linn et al., 1969). In addition phosphorylation may exert some regulatory function via phosphorylation of histones (Langan, 1969) and sigma factor of RNA polymerase (Martelo et al., 1970).

Evidence for cAMP-mediated hormone effects has been cited in a number of review articles on cAMP (see Hardman et al., 1971 for references). Needless to say, the role of cAMP as a "second messenger" has been well established.

The conclusion that certain hormones are exerting translation level controls is open to some question. Many experiments rely heavily upon inhibition of RNA synthesis by Actinomycin D to determine if RNA synthesis is necessary for hormone-mediated effects. Since some RNA species show differences in their sensitivity to Actinomycin D (Reich and Goldberg, 1964), the possibility of a species of RNA which is refractory to inhibition complicates interpretation of experiments which measure stimulation of synthesis of one or a small number of proteins. Complications also arise from possible side effects of antibiotics such as inhibition of enzyme degradation (Kenney, 1967; Reel and Kenney, 1968) and nonspecific poisoning of metabolic processes (Tata, 1966).
In spite of these considerations, there are a number of systems in which hormones have been suggested to influence protein synthesis at the translational level. Two of the most well-studied cases are adrenocorticotropic hormone (ACTH)-stimulated steroidogenesis in the adrenal cortex (Garren et al., 1971) and glucagon-corticosteroid stimulation of liver enzyme synthesis (Wicks, 1971; Tomkins et al., 1972). Translation level controls have also been suggested to operate in cAMP stimulated protein synthesis in the anterior pituitary (Labrie et al., 1971), epinephrine stimulation of protein synthesis in parotid glands (Grand and Gross, 1970) and insulin effects on protein synthesis in muscle (Martin and Wool, 1968).

ACTH produced by the hypophysis stimulates steroid synthesis by the adrenal gland via its effects on intracellular levels of cAMP (Garren et al., 1971). This stimulation is not affected by the presence of Actinomycin D in concentrations which inhibit adrenal RNA synthesis by 90%, but is inhibited by puromycin and cycloheximide, both inhibitors of protein synthesis (Garren et al., 1965). ACTH stimulates corticosteroid secretion within 3 minutes after administration (Pearlmutter et al., 1973). Continued presence of ACTH and protein synthesis is necessary to maintain elevated rates of corticosteroid synthesis. Addition of cycloheximide after ACTH-stimulation results in a rapid decrease in corticosteroid synthesis. This decrease displays first order kinetics with a half-life of approximately 10 minutes (Garren et al., 1965). These observations along with other antibiotic data suggest that ACTH participates in
translational control of a protein with a rapid rate of turnover which is involved in the rate limiting step of corticosteroid biosynthesis (Garren et al., 1971).

Glucagon, via cAMP, and corticosteroid stimulate the synthesis of a number of liver enzymes. The most well-studied of these is tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5). The differential effects of corticosteroids and cAMP have led to the suggestion that they may act at sequential sites in protein synthesis. Administration of dibutyryl cAMP, a nonmetabolized cAMP analog, stimulates the in vivo rate of transaminase synthesis in 20-40 minutes. Hydrocortisone stimulation occurs after 90 minutes (Wicks et al., 1969). Sequential addition of one inducer after maximal induction by the other shows that hydrocortisone markedly amplifies the subsequent effect of dibutyryl cAMP. Reversing the order of addition did not lead to any marked increase of stimulation (Wicks, 1971). Removal of dibutyryl cAMP during or after maximal induction has been attained leads to a rapid cessation of induction or drop in activity. Removal of hydrocortisone did not stop elevation of transaminase for 1½ to 2 hours (Wicks, 1971). Addition of Actinomycin D at the time of removal of corticosteroid even leads to activation ("super induction") of transaminase levels (Tomkins et al., 1969, 1972). These observations have led to the suggestion that glucagon, via its effects on cAMP, stimulates translation while corticosteroids prevent inactivation of the transaminase mRNA (Wicks, 1969; Tomkins et al., 1969). "Super induction" of transaminase could
be explained by the concomitant synthesis of a repressor molecule which has a rapid rate of turnover. This repressor prevents translation of message and aids in its degradation. In the presence of Actinomycin D repressor RNA synthesis is shut down and the concentration of repressor quickly drops. This results in an increase in active transaminase mRNA which leads to "super induction".

It should be noted, however, that conflicting data exists on the nature of the glucagon-corticosteroid stimulation. The basic issue is whether stimulation is inhibited by Actinomycin D (Wicks et al., 1969). In particular, the existence of the "super induction" phenomena has been questioned (Kenney et al., 1973). Some of these contradictory results stem from different systems being examined, different dosages employed, timing of administration of inhibitor and nutritional conditions (Wicks, 1971; Auricchio et al., 1969). Although translational control of tyrosine aminotransferase is indirect, one can argue that "there is no compelling evidence against such a mechanism" (Wicks, 1971).

There are numerous tissues in which ribosomal protein phosphorylation have been observed. These include rabbit reticulocytes (Kabat, 1970), rat liver (Loeb and Blat, 1970), anterior pituitary (Barden and Labrie, 1973), adrenal gland (Walton et al., 1971), mouse sarcoma 180 tumor cells (Bitte and Kabat, 1972) and chick embryo fibroblast cells (Li and Amos, 1971). The evidence for in vivo phosphorylation in all but rat liver, mouse tumor cells and reticulocytes has been questioned because of complications arising from contamination of ribosomes by
phosphorylated supernatant factors (Eil and Wool, 1973a). With reticulocyte ribosomes there are approximately 11 phosphorylated sites per single ribosome, at least 2 on the 40S subunit and 9 on the 60S subunit (Kabat, 1972; Traugh et al., 1973). Three protein kinase species have been isolated from reticulocyte ribosomes on DEAE-cellulose and phosphocellulose chromatography. Each shows a different substrate specificity with respect to phosphorylation of ribosomal proteins (Traugh et al., 1973). Two of these kinases are cAMP-dependent and the third cAMP-independent. In rat liver 2 cAMP-dependent protein kinases have been isolated which catalyze the phosphorylation of at least 4-40S and 10-60S ribosomal proteins in an in vitro system (Eil and Wool, 1973a). In vitro phosphorylation of adrenal cortex ribosomes yields 2 and 10 phosphate groups per 40S and 60S subunit, respectively (Walton and Gill, 1973).

The present evidence that phosphorylation of ribosomal proteins affects function is somewhat disappointing in view of promising preliminary observations. Early observations showed that phosphorylation in rat liver is stimulated by cAMP in vitro (Loeb and Blat, 1970) and that in vivo phosphorylation is stimulated by glucagon (Blat and Loeb, 1971). Thyroidectomy in rats results in diminished activity of liver polyribosomes. This diminished activity is accompanied by a drop in phosphate content of liver ribosomal proteins (Correze et al., 1972). The rate and extent of in vitro phosphorylation of chick embryo fibroblast ribosomes varies with the growth conditions of the cells from
which the ribosomes were isolated. The significance of this observation, as far as phosphorylation of ribosomal proteins is concerned, is questionable since a high salt wash, to eliminate contaminating supernatant proteins, eliminates the observed differences (Li and Amos, 1971). Isolation of single ribosomes and polysomes from reticulocytes labeled for short times with $^{32}$P inorganic phosphate showed a difference in phosphorylation pattern of the proteins. This suggested that phosphorylation might participate in the interconversion of inactive single ribosomes and ribosomes actively engaged in protein synthesis (Kabat, 1970). Further work with reticulocyte ribosomes have shown that the phosphate groups turnover at a rate too slow (3% of the phosphate groups per minute) to be an obligate step in the synthesis of the globin molecule. In addition, the differences in the phosphorylation patterns between single ribosomes and polysomes were not found to be reproducible (Kabat, 1972). In vitro phosphorylated rat liver ribosomes were assayed for various in vitro functions such as polyphenylalanine synthesis at high and low magnesium, EIF-1 (M1) catalyzed binding of phenylalanyl-tRNA to the 40S subunit and ability to translate encephalomyocarditis virus RNA (which requires all 3 initiation factors). No differences in activity were found between phosphorylated and nonphosphorylated ribosomes with respect to these activities (Eil and Wool, 1973b).

This research was initiated before the activity study with phosphorylated rat liver ribosomes was published. The purpose of this research is to examine the effect of in vitro phosphorylation on in vitro
ribosome activity to determine whether phosphorylation can effect function. Although the physiological significance of this phosphorylation could be questioned because of the use of muscle protein kinase, the correlation of change of activity and phosphorylation of particular proteins might yield data on ribosomal protein function as it relates to elongation. Secondly phosphorylation might be useful as a probe of ribosome structure. Potential sites for phosphorylation, normally hidden in the ribosome interior, could become exposed upon unfolding. Changes in phosphorylation patterns might provide clues as to structural changes in the ribosome.
# MATERIALS AND METHODS

## Materials

Listed below are the chemicals used in this research and their commercial source.

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<td>Research Products International, Inc.</td>
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ammonium sulfate (enzyme grade) sucrose

(enzyme grade), Tris (enzyme grade),

yeast tRNA, $^{14}$C L-phenylalanine (Stan Star)........Schwarz/Mann

protamine sulfate (salmon, grade 1), Dowex-1-Cl,

coomassie blue R ..................................Sigma Chemical Company

bacterial alkaline phosphatase.......................Worthington Biochemical

(Code: BAPF) .................................................Corp.

Solutions

KTM no. 2 contains: 200mM Tris-HCl, pH 7.5, 400mM KCl, 40 mM MgCl$_2$. KfM no. 7 contains: 200 mM Tris-HCl, pH 7.5, 40mM KCl, 80mM MgCl$_2$. All the solutions used are made up in deionized, distilled water and adjusted to neutrality except when pH is specified. All buffers are adjusted at 4°C except Tris which is adjusted at 23°C. Urea is deionized by stirring two liters of 8M urea with 100 grams of Amberlite MB-3 for at least 2 hours. The counting fluid used in the polyphenylalanine assay and the nonenzymatic binding assay contains 12 gm BBOT, 150 gm napthalene dissolved in 600 ml toluene and 2400 ml p-dioxane. To this is added 90 ml absolute ethanol and 108 gm Cab-O-Sil (Heintz et al., 1968). The counting fluid used for the disc gel samples and for counting paper samples contains 5.5 gm Permablend no. 1™ per liter toluene.
Rabbit muscle protein kinase is prepared by a modification of the method of Reimann et al. (1971). Muscle from 4 New Zealand white rabbits (1.5 kg) is ground and homogenized in 4mM EDTA, pH 7.0 (2.5 liters/kg muscle) for 1 minute in a Waring Blendor at 4°C. All subsequent steps were also carried out at 4°C. The homogenate is centrifuged at 10,000 x g for 30 minutes, and the supernatant is adjusted to pH 5.5 by adding 1N acetic acid. The precipitate which forms is removed by centrifugation at 10,000 x g for 30 minutes and the supernatant adjusted to pH 6.8 by addition of 1M potassium phosphate, pH 7.2. The enzyme is precipitated by slow addition of 39 gm ammonium sulfate per 100 ml. The suspension is stirred for 30 minutes at 4°C and then centrifuged for 40 minutes at 10,000 x g. The precipitate is suspended in 5mM potassium phosphate-1mM EDTA, pH 6.5 (final volume about 130 ml) and dialyzed against several changes of the same buffer overnight. The enzyme is clarified by centrifugation at 30,000 rpm for 90 minutes, and the precipitate discarded. Protein concentration is determined by the absorbance at 260nm and 280nm (Warburg and Christian, 1942). Hydroxyapatite (250 mg per ml) equilibrated with dialysis buffer is added to the supernatant (approximately 4 gm hydroxyapatite/gm protein). The suspension is stirred for 20 minutes and the hydroxyapatite removed by centrifugation at 5,000 x g for 10 minutes. The enzyme-containing supernatant is mixed with 100 ml calcium phosphate gel (30 mg/ml) (Singer and Kearney, 1950), equilibrated with the dialysis
buffer, to adsorb enzyme. The suspension is stirred for 30 minutes and then centrifuged at 5,000 x g for 10 minutes. The gel is washed with a series of phosphate buffers composed of dibasic and monobasic potassium phosphate (9:1) containing 1mM EDTA. The volume and phosphate concentration of the washes are as follows: 100ml of 0.05M, 20 ml of 0.05M, 15 ml of 0.1M, 15 ml of 0.2M and 15 ml of 0.3M. The final two washes are combined and passed through a Sephadex G-25 column (2.5 x 40 cm) equilibrated with 5mM Tris-HCl, pH 7.5 - 1mM EDTA. The eluted enzyme (approximately 65 ml) is concentrated overnight by vacuum dialysis (Richardson and Kornberg, 1964) to approximately 25 ml and then applied to a DEAE-cellulose column (1.25x15 cm) equilibrated with 5mM Tris-HCl, pH 7.5 - 1mM EDTA. The column is washed with the starting buffer and then eluted with a step gradient of 0.1M Tris-HCl, pH 7.5-1mM EDTA and 0.3M Tris-HCl, pH 7.5-1mM EDTA. Enzyme eluting at 0.1M Tris is stored at -20°C and is referred to as fraction 1 protein kinase. Enzyme eluting at 0.3M Tris is dialyzed against 0.1M Tris-HCl, pH 7.5-1mM EDTA before freezing and is referred to as fraction 2 protein kinase. The yield of enzyme varied greatly but averaged 5-10 mg for each fraction. The enzyme was usually used within 2 months after preparation.

Ribosomes, crude AS70 enzyme fraction and purified EF-T1 and EF-T2 are obtained from reticulocytes collected from the blood of rabbits made anemic by subcutaneous injections of phenylhydrazine (Allen and Schweert, 1962). The reticulocytes are lysed by osmotic shock. The lysate is centrifuged at low speed to remove cell debris and then at
high speed to pellet the ribosomes. The supernatant fraction is saved for preparation of AS70 enzyme fraction, EF-T1 and EF-T2. The pelleted ribosomes are further purified for 3XDOC ribosomes.

Ribosomes from the high speed centrifugation of the lysate are re-suspended in sucrose, incubated in a high salt solution containing 46mM Tris-HCl, pH 7.5, 92 mM KCl, 18mM GSH & 8.7 mg/ml ribosomes and then incubated in a complete protein synthesizing system. The ribosomes are collected by centrifugation at 78,000 x g for 90 minutes and re-suspended in 0.25M sucrose. They are then incubated briefly in a solution made 1% in deoxycholate, collected by centrifugation and re-suspended in 0.25M sucrose to a final concentration of 15 mg/ml. These ribosomes, termed 3XDOC ribosomes (3 times centrifuged deoxycholate-treated), are stored at -20°C. They are essentially free of mRNA, EF-T1 and EF-T2 (Arlinghaus et al., 1968b).

AS70 enzyme fraction is prepared from the supernatant fraction. Contaminating tRNA is precipitated with protamine sulfate and centrifuged down. The supernatant is buffered with 0.1M Tris-HCl, pH 7.5, and the solution is brought to pH 6.5 by the slow addition of 1M acetic acid. Enzyme is precipitated by ammonium sulfate fractionation. Protein which precipitates between 40% and 70% saturation with ammonium sulfate is taken, dissolved in a solution containing 0.10M Tris-HCl, pH 6.5, 1mM GSH and 0.10mM EDTA, reprecipitated with 70% ammonium sulfate and dialyzed against a buffer containing 0.02M Tris-HCl, pH 7.5, 0.5mM GSH and 0.1mM EDTA. The dialyzed fraction is clarified by centrifugation (10,000 x g for 10 minutes) and made 1mM in EDTA. This enzyme
preparation is stored at -20°C and is designated the AS70 enzyme frac-
tion (Arlinghaus et al., 1968a).

EF-T1 and EF-T2 are both prepared from the AS70 enzyme fraction. Both enzymes are adsorbed to calcium phosphate gel equilibrated with 100mM Tris-HCl, pH 7.5. The EF-T2 fraction is eluted at 0.1M potassium phosphate, pH 7.0 and the EF-T1 fraction is eluted at 0.3M potassium phosphate, pH 7.5. Both fractions are dialyzed against their respective buffers and then chromatographed on DEAE-cellulose columns. EF-T2 is eluted at 0.1M NaCl. The peak fractions are pooled and dialyzed against 20mM Tris-HCl, pH 7.5, 0.5mM GSH and 0.1mM magnesium ammonium EDTA. After dialysis the enzyme is made 25mM in GSH and 1mM in magnesium ammonium EDTA and stored at -20°C. EF-T1 is eluted at 0.25M NaCl. The peak fractions are pooled, dialyzed against 20mM Tris-HCl, pH 7.5 and stored at -20°C (Arlinghaus et al., 1968a). Both EF-T1 and EF-T2 are free of contaminating phosphatase activity using phosphorylated ribosomes as a substrate.

The tRNA from yeast, used in the ribosome assays, is charged by yeast aminoacyl-tRNA synthetases, which are purified by the method of Hoskinson and Khorana (1965). The charging reaction is carried out in a 60 ml reaction mixture containing 4mM ATP, 8mM MgCl2, 40mM Tris-HCl, pH 7.5, 0.02mM each of the 18 common amino acids (except phenylalanine and asparagine), 0.02mM 14C-L-phenylalanine (specific activity 50mCi/m mole), 0.5 mg/ml yeast tRNA, 0.4 mg/ml yeast aminoacyl tRNA synthetases and 0.4mM EDTA. Incubation is carried out for 20 minutes at 37°C.
Aminoacylated tRNA is isolated as described by Heintz et al. (1968).

Gamma labeled $^{32}$P-ATP is prepared by a modification of the method of Glynn and Chappell (1964). The exchange reaction catalyzed by 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase is carried out as described in a 10 ml reaction mixture using 50mCi carrier-free $^{32}$P-inorganic phosphate. This is incubated at 30°C for 1 hour. The incubation mixture is then applied to a prewashed Dowex-1-Cl column (volume about $1\frac{1}{2}$ ml) equilibrated with water. Sample application and elution require a slight air pressure to speed flow rate. The column is washed successively with 20ml water, 40 ml of a solution containing 20mM ammonium chloride and 20mM HCl and a final wash with 40 ml water. Gamma-$^{32}$P-ATP is eluted from the column at 0.25N HCl and collected in 15 ml fractions. The first and second fractions, which contain the bulk of the activity, are buffered by the addition of 1M Tris (pH unadjusted) to a final concentration of 50mM and adjusted to pH 7.0 with careful addition of 1N KOH. The 2 fractions, treated separately, are frozen, lyophilized and redissolved in a small volume of water (5-10 ml). The first 15 ml fraction contains 80-90% of the recovered activity and is used without further purification. Overall recovery of $^{32}$P-inorganic phosphate counts is approximately 70%.

Isolation of phosphorylated ribosomes from the phosphorylation reaction mixture is done by centrifugation. The phosphorylation reaction is stopped by addition of 8 ml cold 1:6 KTM no. 2 (1 part KTM no. 2 diluted with 5 parts deionized water), made 2% in sucrose. This is carefully pipetted onto a double-layered sucrose cushion in a 40 rotor
tube. The bottom layer consists of 1.5 ml of 1:6 KTM no. 2, made 15% in sucrose. The upper layer consists of 1.5 ml of 1:6 KTM no. 2, made 8% in sucrose (0.25M) and 10mM in ATP (unlabeled). After addition of the diluted reaction mixture, 1:6 KTM no. 2 (no sucrose) is added carefully to bring the final volume up to the proper level. Centrifugation is carried out at 40,000 rpm for 2 hours. The pelleted ribosomes are rinsed with 0.25M sucrose and resuspended in about 0.3 ml of 0.25M sucrose. The rationale for centrifuging through a double cushion is to: 1) reduce the background counts by exchange of non-specifically bound radioactive ATP with the unlabeled ATP in the upper cushion, and 2) reduce inclusion of ATP in the pellet in the lower cushion. The use of 1:6 KTM no. 2 (no sucrose) for a final volume adjustment is an attempt to reduce contamination of the rotor and centrifuge with radioactive $^{32}$P.

Ribosomal proteins for use in disc gel electrophoresis are prepared by the method of Spitnik-Elson (1965). Ribosomal RNA is precipitated by addition of a solution containing 6M LiCl and 8M urea to an equal volume of resuspended ribosomes (approximately 0.25 ml of 5 mg ribosome per ml). The suspension is allowed to stand at 4°C overnight. The RNA precipitate is removed by centrifugation for 30 minutes at 20,000 x g. The supernatant is then dialyzed against 8M urea (deionized) in a continuous flow dialysis cell (Chemical Rubber Company), to reduce sample loss upon recovery, for 6 hours.

Disc gel electrophoresis of ribosomal proteins at pH 4.5 in 8M urea is carried out as described by Leboy et al. (1964). Pyrex glass
tubes (18 cm in length with an inside diameter of 6 mm) are prepared by washing with hot nitric acid, rinsing extensively with distilled water, air drying and then immersing in 0.5% (v/v) Photo-flo (Eastman Kodak Co.) (Gabriel, 1971). This procedure facilitates removal of gels after electrophoresis. The gels consist of a 0.5 cm stacking gel on a 14 cm separating gel which is 7.5% (w/v) in acrylamide and 0.11% (w/v) in bisacrylamide. Electrophoresis is carried out using a constant current of 2.5 mA/tube at 4°C for 12-14 hours. The gels are stained for 1 hour in a fresh 1:5 dilution of a 0.5% (w/v) coomassie blue solution (50% methanol-9.2% acetic acid) using 12.5% (w/v) TCA for dilution. Destaining is carried out by diffusion in 7.5% acetic acid-15% methanol for 48-72 hours. Under the conditions used all proteins migrate toward the anode. No stainable bands migrated toward the cathode upon reversal of the terminals.

For determination of radioactivity in the protein bands, the gels are sliced into 3 mm sections. Each section is placed in a glass scintillation vial containing 1 ml of a Protosol:water:toluene (9:1:10) mixture. The vials are tightly capped, incubated at 60°C for 5 hours, frozen for 1 hour and then thawed. Samples are prepared for counting by addition of 10 ml of the Permablend no. 1-toluene scintillation cocktail. Recovery of ^32P-counts by this procedure is 84%.

Counting of samples is done in a Packard TriCarb Model 577 or Model 526 Liquid Scintillation Spectrometer. ^32P is counted at 1% gain with an open window setting (50-1000 on the Model 577 and 50-infinity on the Model 526). ^14C is counted at 10% gain with an open window setting.
For samples containing both $^{32}\text{P}$ and $^{14}\text{C}$, $^{32}\text{P}$ is counted on one channel at 1% gain with a window setting of 400-1000. No $^{14}\text{C}$ is counted at this setting. $^{14}\text{C}$ is counted on a second channel at 10% gain with an open window setting and corrected for $^{32}\text{P}$ counts. Controls for background, $^{14}\text{C}$ efficiency and $^{32}\text{P}$ counts, at the various settings and in the various counting systems, are routinely run with each set of samples.

Protein concentrations are determined either on the basis of the absorbance at 260 nm and 280 nm (Warburg and Christian, 1942) or by the method of Lowry et al. (1951).

**Assays**

The basic reaction mixture for the phosphorylation of ribosomes contained in a volume of 0.03 ml: 3mM MgCl$_2$, 29mM Tris-HCl, pH 7.0, 42mM KCl, 12mM DTT, 0.06mM cAMP (when indicated), 0.22-1.18 mg/ml rabbit muscle protein kinase, 2.5 mg/ml 3XDOC ribosomes and 3.5mM ATP. Reagents are listed in the order of their addition. Water, if needed, is added first. Tris, KCl, DTT and cAMP (when indicated) are added together as part of a concentrated stock solution. Reaction mixtures were incubated at 37°C for various times as specified. Determination of protein-bound $^{32}\text{P}$ is by a method similar to that described by Reimann et al. (1971). Reactions were stopped by pipetting a 20-50 µl aliquot of the reaction mixture onto a 2 cm x 2 cm piece of Whatman ET 31 Chromatography paper and placing it into a beaker of cold 10% TCA for 30 minutes. The beaker is equipped with a magnetic stirring bar covered
by a stainless steel wire mesh to prevent the bar from coming into contact with the paper. The samples were then transferred into gently boiling 10% TCA for 10 minutes and then washed twice in 5% TCA at room temperature for 30 minutes each. The samples were rinsed briefly in ethanol:ether (1:1), dried and counted in 10 ml of Permablend-toluene cocktail. The final ethanol:ether wash facilitates drying of the paper samples and does not affect the number of counts bound. Under the conditions described free ATP is completely washed off. Background is usually around 70 dpm. After counting, the filter papers are discarded, and the scintillation cocktail pooled and reused.

Calculation of the number of picomoles of $^{32}$P bound to ribosomal protein is done by calculating the specific activity of the ATP. An aliquot of the $^{32}$P-ATP is counted on a piece of filter paper in the Permablend no. 1-toluene cocktail. Concentration of ATP is determined from the optical density at 259nm using a molar extinction coefficient of $15.4 \times 10^3$ (PL Biochemicals, Inc. catalog number 101). Calculation of the number of picomoles of ribosome, for use in determining phosphate groups per ribosome, is done using $4.1 \times 10^6$ daltons as the molecular weight of the ribosome (Dintzis et al., 1958). 3XDOC ribosome concentration is determined from optical density at 260nm using an extinction coefficient of 11.2 optical density units per milligram (Arlinghaus et al., 1968b).

Polyphenylalanine synthesis is measured by a modification of the method described by Gregg and Heintz (1972). The reaction mixture contains in a volume of 0.3 ml: 67 uM L-phenylalanine, 1.33mM GTP, 1.33mM
DTT, 6.7mM MgCl$_2$, 67mM KCl, 33mM Tris-HCl, pH 7.5, 0.6 mg/ml AS70 enzyme fraction, 67 ugm poly U, 70 ugm radioactively labeled phenylalanyl-tRNA/ml and 0.042 mg/ml 3XDOC ribosomes. After incubation for 15 minutes at 37°C, the reaction is terminated by the addition of 2 ml of cold 5% TCA containing 1mM L-phenylalanine. The precipitated reaction mixture is allowed to stand for 30 minutes in an ice bath, heated at 90°C for 20 minutes in a hot water bath and then put into an ice bath for 30 minutes. They are then filtered through nitrocellulose membrane filters prewashed with 2 ml of the cold 5% TCA solution. The membrane is then washed 3 times with 2 ml of the cold 5% TCA each time. The membrane is placed in a liquid scintillation vial (plastic), 0.5 ml of 1N NaOH is added, followed by 15 ml of the Cab-O-Sil counting fluid. The vials are shaken vigorously to dissolve the filter and then counted.

The nonenzymatic binding of phenylalanyl-tRNA to ribosomes is carried out as described by Heintz et al. (1968). The reaction mixture contains in a total volume of 0.3 ml: 67 uM L-phenylalanine, 0.67mM DTT, 13.3mM MgCl$_2$, 6.7mM KCl, 33mM Tris-HCl, pH 7.5, 67 ugm poly U/ml, 70 ugm radioactively labeled phenylalanyl-tRNA/ml and 0.17 mg 3XDOC ribosomes/ml. After gentle swirling of the reaction mixture, it is incubated for 20 minutes at 37°C. The reaction is stopped by chilling the tubes and adding 3 ml of cold 1:6 KTM no. 7 (1 part KTM no. 7 diluted with 5 parts water). The diluted reaction mixture is filtered through nitrocellulose filters premoistened with 3 ml cold 1:6 KTM no. 7. The filters are then washed 3 times with 3 ml of 1:6 KTM no. 7 each. Filters are counted by the same method used for the polyphenylalanine assay.
Assay of activity of phosphorylated ribosomes by polyphenylalanine synthesis is usually done by taking a 5 μl aliquot of the phosphorylation reaction mixture containing the ribosomes and adding it directly to 0.295 ml of the polyphenylalanine assay mixture. Incubation for polyphenylalanine synthesis is normally started immediately after the addition of ribosomes. Assay of phosphorylated ribosome activity by nonenzymatic binding is carried out similarly to polyphenylalanine synthesis except that a 20 μl aliquot of the phosphorylation reaction mixture is added to 0.28 ml of the nonenzymatic binding mixture.
RESULTS

Rabbit muscle protein kinase was chosen as the enzyme for carrying out in vitro phosphorylation for a number of reasons. Before the initiation of these studies, rabbit muscle protein kinase had been used to phosphorylate *E. coli* ribosomal proteins in vitro (Traugh and Traut, 1972). It is a well-characterized enzyme with respect to purification, reaction conditions and phosphorylation of various substrates. Preliminary studies indicated that a crude rabbit muscle extract could catalyze phosphorylation of purified rabbit reticulocyte ribosomes. From the extract a purified preparation of rabbit muscle protein kinase was obtained and a series of studies undertaken to determine the conditions necessary for optimal phosphorylation of ribosomal proteins.

In order to study the effect of phosphorylation on ribosome function, it was necessary to modify the phosphorylation conditions. This is done to prevent inactivation of ribosomal activity due to buffer effects. The modifications mean that for the studies on the effect of phosphorylation on ribosomal function, phosphorylation is being carried out under nonoptimal conditions.

Before the phosphorylation reaction itself could be studied, a suitable assay method had to be found for determining the levels of phosphorylation. The filter paper procedure, described in the Methods section, proved to be a simple reliable assay. Assays of the phosphorylation reaction by the filter paper method are in general agreement with those obtained by millipore filtration of hot TCA precipitable...
counts. The millipore filtration assay is carried out as described for polyphenylalanine synthesis except that 1 mg bovine serum albumin is added to the phosphorylation reaction mixture just before precipitation with 5% TCA. The filter paper assay gives about 63% of the counts which appear in hot TCA precipitable material. The millipore filtration assay has the added disadvantage of higher background activity and tends to give somewhat nonreproducible results.

Protein kinase, cAMP and magnesium ion are the principal components for in vitro phosphorylation of rabbit reticulocyte ribosomal protein. As outlined in the Methods section, there are 2 fractions of protein kinase which are characterized by their elution from DEAE-cellulose. Fraction 1 protein kinase, eluted at 0.1M Tris, is used in the studies on the characterization of the phosphorylation reaction. Fraction 1 protein kinase usually has a higher specific activity and shows somewhat lower levels of background phosphorylation than fraction 2 protein kinase. Generally background levels of phosphorylation (controls in which ribosomes are omitted) in the presence of cAMP represent less than 5% of the total incorporation levels for fraction 1 protein kinase and 15-20% for fraction 2 protein kinase. Background levels in the absence of cAMP reflect overall low levels of incorporation in the complete system. For fraction 1 protein kinase the background is about 10% of total incorporation in the absence of cAMP and for fraction 2 protein kinase about 50% of total incorporation. No incorporation of activity, above background levels, is detected in the absence of added protein kinase.
This observation indicates the absence of endogenous ribosome-associated protein kinase activity under the phosphorylation reaction conditions. Figure 1 shows a time course of phosphorylation in the presence of cAMP. The rapid initial phosphorylation followed by a slower rate which never quite levels out, is an unusual feature of the phosphorylation time courses. In this experiment, 120 picomoles of phosphate is equivalent to approximately 10 phosphate groups per ribosome. Fraction 1 protein kinase is free of contaminating phosphatase activity under the conditions of the phosphorylation reaction. This is determined by using ribosomes, previously phosphorylated with fraction 1 protein kinase, as a substrate and determining the amount of radioactivity solubilized in an incubation with fraction 1 protein kinase in the absence of ATP.

The rate of phosphorylation is stimulated 8-fold by the addition of saturating levels of cAMP (2.3 µM). Figure 2 shows incorporation of $^{32}$P at various concentrations of cAMP. The slight decrease in incorporation seen at high cAMP concentrations (117 µM) may or may not be significant. Up to 60 µM cAMP, the concentration normally used in the phosphorylation reaction, the incorporation levels show no decrease. The cAMP-independent phosphorylation is significant and depends on addition of both protein kinase and ribosomes.

The phosphorylation reaction has a narrow magnesium ion optimum which depends on the concentration of ATP in the reaction mixture. Figure 3 shows the magnesium optima at 2 concentrations of ATP in the presence of saturating levels of cAMP. cAMP-independent
Figure 1. Time course of phosphorylation with fraction 1 protein kinase in the presence of cAMP. Phosphorylation is carried out and assayed as described in the Methods section, except for the following components in the phosphorylation reaction, 0.06mM cAMP, 1.18 mg/ml fraction 1 protein kinase, 12mM KF and 5mM ATP.

Complete reaction mixture (●—●—●)
Minus 3XDOC ribosomes (▲—▲—▲)
Minus protein kinase (□—□—□)
Figure 2. Phosphorylation as a function of cAMP concentration. Phosphorylation is carried out and assayed as described in the Methods section, except for the following components in the phosphorylation reaction, 1.18 mg/ml fraction 1 protein kinase, 12mM KF, 5mM ATP and varying concentrations of cAMP as indicated on abscissa (final concentration in reaction mixture). Incubation time is 10 minutes at 37°C.

Complete reaction mixture (●—●—●)
Minus 3XDOC ribosomes (□—□—□)
Minus protein kinase (▲—▲—▲)
Phosphorylation as a function of magnesium ion concentration at two different ATP concentrations. Phosphorylation is carried out and assayed as described in Methods section except for the following components in the phosphorylation reaction, 1.18 mg/ml fraction 1 protein kinase, 12mM KF, 0.06mM cAMP and 2 concentrations of ATP, 3.5mM and 5.0mM (final concentration in reaction mixture). Incubation time is 10 minutes at 37°C.

Phosphorylation in 3.5mM ATP (■——■——■)
Phosphorylation in 5.0mM ATP (●——●——●)
phosphorylation shows similar magnesium ion optima. Dependence of the magnesium ion optimum on ATP concentration may reflect formation of a magnesium : ATP complex which serves as the substrate for the phosphorylation reaction (Reimann et al., 1971). The inhibition of phosphorylation at higher magnesium ion concentrations is puzzling in view of the requirement for high magnesium ion concentrations (10mM) in the phosphorylation of other substrates by rabbit muscle protein kinase (Reimann et al., 1971). The present observation is in agreement with the results of Eil and Wool (1973a), who have suggested that the inhibition at high magnesium ion might be due, in part, to a more compact ribosome structure and decreased accessibility of phosphorylation sites.

As a note, magnesium is always added separately to the reaction mixture. Mixing magnesium chloride (17.5mM) in a concentrated stock solution containing 175mM Tris-HCl, pH 7.0, 70mM DTT, 250mM KCl and 0.35mM cAMP and freezing it, caused the magnesium ion to be bound up in a form which could not be utilized in the phosphorylation reaction. The presence of magnesium ion in the concentrated stock solution did not make any difference in the amount of magnesium ion which had to be added separately in order to get optimal phosphorylation.

KCl is inhibitory to both cAMP-dependent and cAMP-independent phosphorylation. Both are inhibited about 15% by 42mM KCl, the concentration used in the phosphorylation reaction mixture. This inhibition increases to about 50% at 140mM KCl. Phosphorylation of rat liver ribosomal protein is also inhibited by KCl (Eil and Wool, 1973a).
The inhibitory effect of KCl in their system appears to be on the enzyme itself rather than due to a change in ribosome structure. Evidence for this comes from the observations that KCl decreases labeling of all bands equally on polyacrylamide gels and inhibits rat liver protein kinase catalyzed phosphorylation of histones (Eil and Wool, 1973a). Figure 4 shows KCl inhibition of cAMP-dependent phosphorylation. KCl is included in the reaction mixture because of a KCl requirement by mammalian ribosomes for stabilization of structure and activity (Näslund and Hultin, 1970; Eil and Wool, 1973b). The inhibition of phosphorylation at the KCl concentration used in the phosphorylation buffer (42mM), is not a significant problem.

Potassium fluoride, often used to inhibit phosphatase activity, did not stimulate either cAMP-dependent or cAMP-independent phosphorylation. Because of the inhibitory effect of fluoride ion on protein synthesis (Lin et al., 1966), fluoride is not included in the phosphorylation reaction used to study the effect of phosphorylation on ribosome activity.

Phosphorylation is relatively insensitive to changes in Tris concentration as well as changes in pH from 6.5 to 8.0. DTT has no effect on phosphorylation, but is included in the reaction mixture to protect the ribosome during the incubation and subsequent isolation steps. The ribosome concentration is 2.5 mg/ml and is not limiting in the phosphorylation reaction.

The time course of the phosphorylation reaction, as noted before, does not level off at time points up to 60 minutes (Figure 1). It
Figure 4. Effect of KCl on phosphorylation. Phosphorylation is carried out and assayed as described in the Methods section except for the following components in the phosphorylation reaction, 1.18 mg/ml fraction 1 protein kinase, 12 mM KF, 0.06 mM cAMP, 5 mM ATP and KCl as indicated on abscissa. Incubation is 10 minutes at 37°C.

Complete reaction mixture (●——●——●)
Minus 3XDOC ribosomes (□——□——□)
Minus protein kinase (▲——▲——▲)
seemed possible that this slow phosphorylation might reflect unfolding of the ribosome which could result in exposure of additional sites for phosphorylation. The idea of hidden phosphorylation sites has been mentioned by others. The differences in extent of phosphorylation of proteins on single ribosomes and polyribosomes may be due to a difference in accessibility of sites to protein kinase (Kabat, 1972). Inhibition of phosphorylation by the 0.5M KCl wash fraction has been suggested to be due to masking of phosphorylation sites (Traugh et al., 1973). Eil and Wool have similarly found that a smaller number of proteins are phosphorylated on the 90S ribosome than on the 40S and 60S subunits separately. This suggested that the proteins which were phosphorylated on the subunits but not on the intact 80S ribosome might occupy the interface between ribosomal subunits (Eil and Wool, 1973a).

There is some experimental evidence supporting the idea that disruption of ribosome structure can lead to increased phosphorylation. Ribosomes, exposed for 1 hour at 37°C, to a buffer containing no KCl or magnesium, are completely inactive for polyphenylalanine synthesis. These ribosomes showed a marked disruption of structure as determined from ultracentrifugation studies. The incubated ribosomes sediment in a broad, slow moving peak in contrast to the untreated control ribosomes, which sediment in a sharper, more well-defined faster sedimenting peak. As seen in Figure 5, the incubated ribosomes are also characterized by a very rapid initial rate of phosphorylation. Note that phosphorylation is being carried out by fraction 2 protein kinase. The control ribosomes
Figure 5. Time course of phosphorylation of ribosomes pre-incubated in a KCl-free, magnesium-free buffer. Preincubated ribosomes are incubated in 0.1M Tris-HCl, pH 7.5, 0.2M ammonium sulfate, 0.11M sucrose for 1 hour at 37°C. The solution is then diluted with 12 ml cold 1:6 KTM no. 2, centrifuged for 2 hours at 40,000 rpm and resuspended in 0.25M sucrose to a final concentration of 6.84 mg/ml.

Control ribosomes (3XD)(C-untreated) are not incubated but diluted with 1:6 KTM no. 2, centrifuged and resuspended to 6.84 mg/ml.

Preincubated and control ribosomes are both phosphorylated under the conditions described in the Methods section except for the following changes in the phosphorylation reaction mixture: 0.9 mg/ml fraction 2 protein kinase, 0.06mM cAMP and 2.7 mg/ml 3XDOC ribosomes. A 30 ul aliquot of the reaction mixture is used in the filter paper assay of bound phosphate.

Preincubated ribosomes (●—●—●)
Control ribosomes (▲--▲--▲)
PICOMOLES PHOSPHATE

TIME (MIN)

40 80 120 160 200
eliminate the possibility that this increased rate of phosphorylation is due to the centrifugation and resuspension steps used in isolating the ribosomes. This preliminary observation suggests that phosphorylation might be a useful probe of gross ribosome structure. Further work is necessary to determine whether phosphorylation might also be useful in defining specific proteins which become exposed in the unfolding ribosome.

A problem of using phosphorylation as a probe of conformation lies in the amount of phosphate which is already present on ribosomes when they are first isolated. Levels of incorporation, initial rates and patterns of labeling could reflect preexisting levels of phosphate rather than changes in ribosome structure. The experiments of Li and Amos (1971) support the idea that the extent of \textit{in vivo} phosphorylation of ribosomal proteins can change.

Bacterial alkaline phosphatase has been shown to solubilize phosphate which has been bound \textit{in vivo} or \textit{in vitro} (Kabat, 1970, 1971). Using bacterial alkaline phosphatase a preliminary attempt was made to reduce the preexisting level of ribosomal phosphoprotein, and to determine if phosphatase-treated ribosomes can serve as a better substrate for the phosphorylation reaction. In buffer A (10mM magnesium, 87mM Tris-HCl, pH 7.5, 35mM DTT and 125mM KCl) bacterial alkaline phosphatase solubilizes, in 30 minutes, 48% of the phosphate incorporated by fraction 1 protein kinase into cold TCA precipitable counts (Figure 7).
In buffer A alkaline phosphatase is 20% active with p-nitrophenyl phosphate, the substrate normally used to assay for phosphatase activity (Garen and Levinthal, 1960), as compared with activity in 1M Tris-HCl, pH 8.0. In buffer B (0.1M Tris-HCl, pH 7.6 and 0.4M NaCl) alkaline phosphatase solubilizes, in 30 minutes, 35% of the phosphate incorporated into cold TCA precipitable counts. In buffer B, alkaline phosphatase activity is 50% of that in 1M Tris-HCl, pH 8.0. Although the solubilization of counts bound to ribosomes in 30 minutes does not strictly reflect activity of alkaline phosphatase for this substrate, the differences in the change in activity observed for p-nitrophenyl phosphate and phosphorylated ribosomes almost suggests that 2 different activities are being assayed (e.g. phosphatase activity with p-nitrophenyl phosphate and protease activity with phosphorylated ribosomes). Until the mechanism behind these differences can be established results involving pretreatment of ribosomes with bacterial alkaline phosphatase to reduce levels of preexisting phosphate should be interpreted with caution.

As described in the Methods section, ribosomes are assayed for activity by taking an aliquot of the reaction mixture and adding it directly to the appropriate assay system. Under the conditions described for the polyphenylalanine and nonenzymatic binding, the incorporation of $^{14}$C-phenylalanine into nitrocellulose-filterable material is linearly dependent on the amount of ribosomes added. As a result the incorporation of counts, measured as picomoles phenylalanine, is a measure of ribosomal activity.
Table 1 summarizes the results of an experiment which examines the effect of phosphorylation carried out using 5mM ATP and 3mM magnesium ion on ribosomal activity, as measured by polyphenylalanine.

Table 1. Effect of phosphorylation reaction on ribosomal activity

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Phosphorylation reaction conditions</th>
<th>Ribosome activity (picomoles phenylalanine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Complete</td>
<td>0.15</td>
</tr>
<tr>
<td>2</td>
<td>Minus ATP</td>
<td>11.2</td>
</tr>
<tr>
<td>3</td>
<td>Minus kinase</td>
<td>2.4</td>
</tr>
<tr>
<td>4</td>
<td>Minus kinase Minus ATP</td>
<td>12.7</td>
</tr>
<tr>
<td>5</td>
<td>Minus ribosome (Control)</td>
<td>7.7</td>
</tr>
<tr>
<td>6</td>
<td>Minus ribosome Minus ATP</td>
<td>10.9</td>
</tr>
<tr>
<td>7</td>
<td>Minus ribosome Minus kinase</td>
<td>8.4</td>
</tr>
<tr>
<td>8</td>
<td>Minus ribosome Minus kinase Minus ATP</td>
<td>11.4</td>
</tr>
</tbody>
</table>

aPhosphorylation incubation is for 10 minutes at 37°C. At the end of the incubation period a 5 ul aliquot is taken from the phosphorylation reaction mixture and assayed for polyphenylalanine synthesis as described in the Methods section.

bRibosomes omitted from phosphorylation mixture. Mixtures are incubated for 10 min at 37°C and a 5 ul aliquot of mixture placed in the polyphenylalanine assay mixture. At the same time a 5 ul aliquot of 2.5 mg/ml untreated 3XDOC ribosomes are added to polyphenylalanine assay mixture and assayed for polyphenylalanine synthesis.
synthesis. Incubation of ribosomes in the complete phosphorylation reaction system, which includes 3mM MgCl₂, 30mM Tris-HCl, pH 7.5, 42mM KCl, 12mM DTT, 0.06mM cAMP, 1.18 mg/ml fraction 1 protein kinase, 2.5 mg/ml 3XDOC ribosomes (except in minus ribosome controls), and 5.0mM ATP, results in inactivation of ribosomal activity (line 1). Background level of incorporation for this system, determined in the absence of ribosomes, is about 0.15 picomoles phenylalanine. Most of the inactivation is not dependent on phosphorylation. Even when protein kinase is omitted from the phosphorylation reaction, there is an 80% loss of activity (line 3). To examine the possibility that the inactivation might be due to inhibition of polyphenylalanine synthesis caused by carryover of other reagents from the phosphorylation reaction, "minus ribosome" controls are run. In these controls, ribosomes are not added to the phosphorylation reaction but are added to the polyphenylalanine assay mixture along with an aliquot of the incubated phosphorylation reaction mixture from which ribosomes and other reagents, as indicated, are omitted. These controls indicate that polyphenylalanine synthesis is inhibited about 40% by the carryover reagents (line 5). Most of this inhibition (35%) is due to carryover of ATP (line 7). This result indicates that although some inhibition is taking place in the polyphenylalanine assay, inactivation of ribosomes, via the effects of ATP, is occurring in the preincubation. The small amount of activity observed in line 3, in contrast to line 1, indicates the possibility of some inactivation by phosphorylation.

The ATP effect observed at 5mM ATP suggests the possibility of a nonenzymatic inactivation due to lowering of free magnesium ion by chelation with ATP. If this is true then phosphorylation carried out
at lower ATP concentrations or higher magnesium ion concentrations should circumvent this problem. Since the *in vivo* concentration of ATP in reticulocytes is around 1.4mM (Kabat, 1972), the effects of lower concentrations of ATP were examined.

The time course of inactivation of ribosomal activity, as measured by polyphenylalanine synthesis, was examined for a variety of ATP concentrations at a 3mM magnesium ion concentration. At 1.0mM and 2.5mM ATP, no inactivation of ribosomal activity occurs either in the presence or absence of protein kinase. At 3.5mM ATP and 3mM magnesium ion, ribosomes are inactivated in the presence of protein kinase. No inactivation occurs in the absence of either protein kinase or ATP (Figure 6). The activation observed at the initial part of the time course is puzzling. In some 3XDOC ribosome preparations there is a very marked activation at very short time points, amounting to levels up to 150% of the activity observed at zero time. In other preparations there is little or no activation observed at all (Figures 8 and 12). A small amount of activation occurs upon addition of ATP alone (Figure 6).

In the absence of any further data on the nature of this activation, two explanations seem plausible. The first is that phosphorylation of certain sites are responsible for ribosome activity. At very short times phosphorylation produces a net increase in active ribosomes or an increase in efficiency of those ribosomes already active. Further phosphorylation of nonessential sites would cause inactivation. Differences from preparation to preparation might be due to differences in the preexisting levels of phosphorylation. A point which should be noted
Figure 6. Time course of ribosome activity versus time of incubation in the phosphorylation reaction. Ribosomes are incubated in a phosphorylation reaction as described in the Methods section using 1.18 mg/ml fraction 1 protein kinase and 3.5mM unlabeled ATP. At zero time the phosphorylation reaction is started and at the time points indicated on the abscissa, a 5 ul aliquot is taken out and assayed immediately for polyphenylalanine synthesis. Control conditions (minus protein kinase, minus ATP) refer to components omitted from the phosphorylation reaction.

Complete reaction mixture (●---●---●)
Minus protein kinase (◆---◆---◆)
Minus ATP (□---□---□)
PICOMOLES PHENYLALANINE

TIME (MIN)

10
20
30
here is that 3XDOC ribosome preparations contain 10-15% active ribosomes. This is determined from levels of phenylalanyl-tRNA bound under enzymatic binding conditions (30-40 picomoles bound per mg ribosome).

A second explanation would suggest that the activation is due to an artifact of the low magnesium ion concentration. This causes a loosening of the structure of inactive ribosomes resulting in activation. Further disruption of structure leads to inactivation. A more detailed treatment of this mechanism is contained in the discussion. Further work is needed to establish the mechanism of this activation.

A complication in interpreting the ribosomal activity experiments is the contamination of the AS70 enzyme fraction with phosphatase-like activity. Figure 7 shows that under the conditions of the polyphenylalanine reaction, there is phosphatase-like activity associated with the AS70 enzyme fraction. In 15 minutes, the incubation period for assay of polyphenylalanine synthesis, this phosphatase-like activity is capable of solubilizing approximately 33% of the phosphate bound to ribosomal proteins by fraction 1 protein kinase. The AS70 enzyme fraction does not cleave p-nitrophenol phosphate under any of the conditions examined.

To determine whether this phosphatase-like activity has any effect on ribosomal activity determinations, controls were run using T1:T2 catalyzed polyphenylalanine synthesis. Both T1 and T2 are free of any contaminating phosphatase-like activity using phosphorylated ribosomes as a substrate. Figure 8 shows the time course for inactivation of ribosome activity as measured by polyphenylalanine synthesis using the AS70 enzyme fraction and purified T1:T2. Also indicated on the same figure is
Figure 7. Assay of phosphatase-like activity in the AS70 enzyme fraction and bacterial alkaline phosphatase using phosphorylated ribosomes as substrate. 3XDOC ribosomes are phosphorylated for 30 minutes as described in the Methods section, using 0.56 mg/ml fraction 1 protein kinase and 0.06mM cAMP. Ribosomes are isolated by centrifugation as described in the Methods section. Assay of phosphatase activity in the AS70 fraction and in the control reaction are carried out in the polyphenylalanine assay buffer (67uM L-phenylalanine, 1.33mM GTP, 1.33mM DTT, 6.7mM MgCl₂, 67mM KCl, 33mM Tris-HCl, pH 7.5) in the presence or absence (control) of 1.0 mg/ml AS70 enzyme fraction. 100% in the AS70 reaction mixture and control is 4470 cpm.

Assay of bacterial alkaline phosphatase is carried out in 10mM MgCl₂, 87mM Tris-HCl, pH 7.5, 35mM DTT and 125mM KCl using 1.0 mg/ml phosphatase. 100% represents 28,832 cpm. ³²P-counts bound represent cpm's bound to filter papers washed 3 times with 5% cold TCA.

AS70 reaction mixture (□—□—□—□)
Control (minus AS70) (▲—▲—▲—▲)
Bacterial alkaline phosphatase (●—●—●—●)
% $^{32}$P-COUNTS BOUND

TIME (min)

20  40  60  80  100

0  10  20  30  40  50  60
Figure 8. Ribosomal activities, as measured by AS70, T1:T2-mediated polyphenylalanine synthesis and non-enzymatic binding, versus time of incubation in the phosphorylation reaction.

Experimental conditions are the same as those described in Figure 6 except that the protein kinase concentration is 0.58 mg/ml.

In the T1:T2-catalyzed polyphenylalanine reaction all conditions are identical to the AS70-catalyzed reaction except that 0.3 mg/ml T2 and 0.12 mg/ml T1 are substituted for the AS70 enzyme fraction. For non-enzymatic binding a 0.02 ml aliquot of the phosphorylation reaction mixture is assayed as described in Methods.

AS70 polyphenylalanine assay (●—●—●)
T1:T2 polyphenylalanine assay (◆—◆—◆)
Non-enzymatic binding (○—○—○)
the time course change of nonenzymatic binding activity. The differences in comparative rates of inactivation are variable. Under a variety of conditions, different magnesium ion and ATP concentrations and in the presence of fraction 1 or fraction 2 protein kinase, the inactivation rates for all three activities are about the same. The fact that the nonenzymatic binding assay, in which there are no added enzymes, parallels polyphenylalanine synthesis activity further supports the conclusion that phosphatase-like contamination of the AS70 enzyme fraction does not affect activity determinations.

Figures 9 and 10 show the relationship of rate of phosphorylation and rate of inactivation of ribosomal activity at various protein kinase concentrations. Due to the activation of ribosomal activity in the time course, it is not possible to quantitatively correlate rates of phosphorylation and rates of inactivation. Qualitatively, however, it can be seen that increased rates of phosphorylation are accompanied by increased rates of inactivation. The significance of this observation is somewhat questionable in light of the fact that the inactivation of ribosomal activity is not dependent on the presence of cAMP. In spite of an 8-fold decrease in rate of phosphorylation, the time course of inactivation of polyphenylalanine synthesis is exactly the same in the absence of cAMP as in its presence.

There are only 2 ways to explain the non-cAMP-dependence of the inactivation of ribosomal activity. It could be explained if the sites of phosphorylation, which are responsible for ribosomal function, are also those sites whose phosphorylation is cAMP-independent. Alternatively,
Figure 9. Time course of phosphorylation at various concentrations of fraction 1 protein kinase. Phosphorylation is carried out as described in the Methods section. The various concentrations of protein kinase (final concentration) are indicated below.

0.44 mg/ml (●—●—●)
0.37 mg/ml (◆---◆---◆)
0.29 mg/ml (□---□---□)
0.22 mg/ml (○--○--○)

Figure 10. Time course of inactivation of ribosomal activity at various concentrations of fraction I protein kinase. Phosphorylation is carried out as described in the Methods section. At zero time the phosphorylation reaction is started and at the time points indicated on the abscissa, a 5 ul aliquot is taken out and assayed immediately for polyphenylalanine synthesis. The various concentrations of protein kinase are indicated below and are the same used in Figure 10.

0.44 mg/ml (●—●—●)
0.37 mg/ml (◆--◆--◆)
0.29 mg/ml (□--□--□)
0.22 mg/ml (○--○--○)
PICOMOLES PHOSPHATE

TIME (MIN)
the inactivation may be an artifact of low magnesium ion concentration. This obviously would not be influenced by the presence or absence of cAMP at the low concentration used (0.06mM).

Additional evidence suggests that the cAMP-independent inactivation and, by analogy the cAMP-dependent inactivation, are artifacts due to magnesium binding by the protein kinase fraction. Fraction 1 protein kinase is able to bind magnesium. In a buffered solution containing protein kinase and ribosomes, additional magnesium ion must be supplemented in order to prevent inactivation of ribosomal activity (Figure 11). In the presence of protein kinase magnesium ion must be added to a final concentration of 2-3mM. The requirement for additional magnesium ion is not due to EDTA in the kinase buffer since control ribosomes, exposed to the kinase buffer (100mM Tris-HCl, pH 7.5-1mM EDTA), are stable in the presence of less than 1mM magnesium ion. 2mM EDTA will exactly mimic the magnesium ion binding effect of fraction 1 protein kinase. 2mM EDTA will also cause the same time course of ribosome inactivation as fraction 1 protein kinase (Figure 12). Decreasing the concentration of EDTA will cause a decrease in the rate of inactivation of ribosomal activity, much the same as was seen with decreasing the concentration of protein kinase (Figure 12 and Figure 10).

Fraction 2 protein kinase either does not bind magnesium or does not bind it as tightly as fraction 1 protein kinase. In an experiment similar to the one described in Figure 11 fraction 2 protein kinase is used in place of fraction 1 protein kinase. The magnesium ion concentration required for stabilization of ribosomal activity is the same in the
Figure 11. Stabilization of ribosomes in the presence of fraction 1 protein kinase. 3XDOC ribosomes (2.5 mg/ml) are incubated in a buffer system containing 30mM Tris-HCl, pH 7.5, 12mM DTT and 42mM KCl in the presence or absence of 0.56 mg/ml fraction 1 protein kinase at various concentrations of magnesium. Incubation is carried out for 20 minutes at 37°C. A 5 ul aliquot is then taken and assayed for polyphenylalanine synthesis.

In the control tubes, which have no protein kinase, 1mM EDTA-0.1M Tris-HCl, pH 7.5 was added in the same volume as protein kinase.

Buffer plus protein kinase (0.56 mg/ml) (●—●—●)
Buffer plus 1mM EDTA-0.1M Tris-HCl, pH 7.5 (kinase buffer) (◆--◆--◆)
Figure 12. Time course of inactivation of ribosomal activity by EDTA and fraction 1 protein kinase 3XDOC ribosomes (2.5 mg/ml) are incubated as described in the Methods section in the basic phosphorylation reaction mixture in the absence of cAMP and modified as indicated.

0.47 mg/ml protein kinase
2 mM Na⁺EDTA pH 7.0 substituted for protein kinase
1.6 mM Na⁺EDTA substituted for protein kinase
presence of fraction 2 protein kinase as in the control.

Ribosomes phosphorylated in the presence of fraction 2 protein kinase in the basic phosphorylation reaction show a slower time course of inactivation. Figure 13 shows that the patterns of phosphorylation for fraction 1 and 2 protein kinase are qualitatively very similar, indicating that the differences in rates of inactivation are not due to differences in phosphorylation sites. The observed differences between the 2 profiles may be caused by a number of reasons including: 1) contamination by phosphoproteins from the protein kinase fractions (fraction 1 has bands which would appear in the region from gel fraction 20 to 34), 2) unresolved peaks, and 3) loss of protein during the isolation process (total recovery of protein is about 30-40% after dialysis assuming a ribosome protein content of 51% (Eil and Wool, 1973a); total recovery of counts varies widely from 25% to 70%). It should be noted in this experiment that NaCl is present in addition to KCl in the phosphorylation reaction. This may explain the low phosphorylation levels obtained. Because of the complexity of the gel pattern and the variability in recoveries, it is difficult to make a quantitative assessment of phosphorylation of individual sites from this experiment.

As might be expected, phosphorylation carried out at a high magnesium:ATP ratio does not cause inactivation. Figure 14 shows the time course change of ribosomal activity in 4mM magnesium ion:2mM ATP and 3mM magnesium ion:3.5mM ATP. At the end of the incubation ribosomes
Figure 13. Phosphorylation pattern of ribosomal proteins separated on disc gel electrophoresis. Ribosomes are phosphorylated as described in the Methods section in the absence of cAMP in the presence of either 0.47 mg/ml fraction 1 protein kinase or 0.74 mg/ml fraction 2 protein kinase. The phosphorylation reaction also contains 0.1 M NaCl due to its presence in the ATP preparation. Final levels of phosphate are 45 picomoles of phosphate per mg ribosome (0.18 phosphate group/ribosome) for fraction 1 protein kinase and 476 picomoles of phosphate per mg ribosome (0.93 phosphate group/ribosome) for fraction 2 protein kinase. Fraction 1 protein kinase treated ribosomes are inactive for polyphenylalanine synthesis. Fraction 2 protein kinase treated ribosomes are 55% active.

Ribosomes are isolated and protein extracted as described in the Methods section. Electrophoresis is carried out as described. Recovery of counts originally placed on gel is 30% for fraction 1 protein kinase and 51% for fraction 2 protein kinase.

Fraction 1 protein kinase treated ribosome-TOP
Fraction 2 protein kinase treated ribosome-BOTTOM
Figure 14: Time course of ribosome activity change in the phosphorylation reaction. Ribosomes are phosphorylated and assayed as described in Figure 6. The basic phosphorylation reaction is modified as follows: 0.59 mg/ml fraction 1 protein kinase in the absence of cAMP with either 2mM ATP and 4mM magnesium (reaction 1), or 3.5mM ATP and 3mM magnesium (reaction 2). Final levels of phosphorylation are 49 picomoles phosphate per mg ribosome (0.2 phosphate group/ribosome) for reaction 1 and 314 picomoles phosphate per mg ribosome (1.3 phosphate group/ribosome) for reaction 2.

Phosphorylation in 2mM ATP-6mM Mg Cl₂ (reaction 1)  (▲—▲—▲)
Phosphorylation in 3.5mM ATP-3mM Mg Cl₂ (reaction 2)  (□—□—□—□)
PICOMOLES PHENYLALANINE

TIME (MIN)

0 10 20 30
were isolated and protein extracted for gel electrophoresis. Figure 15 shows the gel pattern for the 2 phosphorylated ribosomes. Due to low levels of phosphorylation at high magnesium:ATP, it is difficult to make a comparison. In terms of the proteins being phosphorylated, they seem to be very similar. The one notable exception is the relatively highly labeled protein which appears in fraction 10 in ribosomes phosphorylated at high magnesium:ATP. This band probably corresponds to the band in fraction 11 of the inactive ribosomes. The shift is probably due to an artifact in cutting the gels and assigning different bands to different gel slices. It is difficult to say from this experiment whether ribosomes are not inactivated at high magnesium:ATP because of the low levels of phosphorylation or because of the presence of sufficient magnesium. It seems, however, that phosphorylation can occur at the same sites under conditions in which inactivation occurs and in which inactivation does not occur.

The photograph (Figure 16) is of a typical coomassie blue stained disc gel of ribosomal proteins. Gel patterns of phosphorylated ribosomal proteins are identical with untreated ribosomal proteins. Small differences exist in patterns from different runs. The Arabic and Roman numerals refer to the peaks of radioactivity in Figures 13 and 15 which correspond to these bands. In some cases assignment of radioactivity is somewhat uncertain due to closeness of bands and variability in cutting of the gels.
Figure 15. Phosphorylation pattern of ribosomal proteins separated on disc gel electrophoresis. Ribosomes are isolated from the incubation described in Figure 14 and the protein extracted as described in the Methods section. Electrophoresis is carried out as described. Total recovery of counts originally placed on the gel is 55% for reaction 1 (2mM ATP - 4mM MgCl₂) and 68% for reaction 2 (3.5mM ATP - 3mM MgCl₂).

Ribosomal proteins phosphorylated in 2mM ATP 4mM MgCl₂

Reaction 1

Ribosomal proteins phosphorylated in 3.5 mM ATP 3mM MgCl₂

Reaction 2
Figure 16. Disc gel electrophoresis of ribosomal proteins stained with coomassie blue.
DISCUSSION

Research in animal cells, as compared to bacterial cells, has revealed a highly complex structural and metabolic system involved in regulation of protein synthesis. The existence of extensive gene duplication (Britten and Kohne, 1968), rapid nuclear RNA turnover (Aronson and Wilt, 1969), stable messenger RNA (Revel and Hiatt, 1964) and mechanisms for transport of mRNA across the nuclear membrane (Adesnik et al., 1972; Joklik and Becker, 1965) suggest possible regulation of protein synthesis at levels other than transcriptional control, the predominant regulatory mechanism for protein synthesis in microbial systems. It is perhaps significant that in vivo phosphorylation of ribosomal proteins is a unique feature of cells of higher organisms. Little, if any in vivo phosphorylation of bacterial ribosomal proteins occurs (Gordon, 1971; Kurek et al., 1972a, 1972b).

Presently there is no direct evidence for the role of phosphorylation of ribosomal proteins in the regulation of protein synthesis. The work reported here and that reported by Eil and Wool (1973b) are in agreement that there are no changes in in vitro ribosomal function which are caused by in vitro phosphorylation. Assuming for the moment that phosphorylation of ribosomal proteins does regulate protein synthesis, two reasons might be suggested for why no change in function has been detected thus far. The first reason is that the wrong functions have been examined (Eil and Wool, 1973b). Phosphorylation may regulate any of a number of processes in which ribosomes participate (Eil and Wool, 1973b; Kabat, 1970) including, recognition of specific mRNA, transport
of the mRNA-ribosome complex through the nuclear membrane, attachment of the ribosomes to the endoplasmic reticulum, recognition of ribosomes by degradative enzymes and termination of protein synthesis. There are as yet no in vitro assays for these ribosome functions. The second reason is that the in vitro situation may not adequately simulate the situation in vivo. Absence of essential factors, changes in specificity of phosphorylated proteins and differences in relative concentrations of components in the in vitro assays as compared to their concentrations in vivo could be enough to mask or lift the regulation by phosphorylation of ribosomal proteins.

As stated in the Introduction, part of the purpose of the present study was to use phosphorylation as a tool to determine the correlation between modification of a particular ribosomal protein and changes in ribosomal activity. Implied in this statement is an understanding of what constitutes a ribosomal protein. In view of supernatant phosphoprotein contamination of ribosomes (Kabat, 1970), the distinction is an important one. The problem is complicated by the fact that the ribosome is not a static entity. There are proteins entering and leaving the ribosome at different stages of protein synthesis (Dice and Schimke, 1972). Considered from an operational standpoint, contaminants would be those proteins which can be removed from the ribosome by vigorous purification without impairing function. Ribosomal proteins are those proteins which remain bound (Eil and Wool, 1973a). Under this definition, initiation and elongation factors are not ribosomal proteins. The definition is somewhat arbitrary but perhaps reasonable.
With the definition of a ribosomal protein in mind, it is important to examine the evidence that covalent binding of phosphate is occurring to ribosomal proteins in the in vitro reaction used in the present study. The available evidence suggests that 3XDOC ribosomes are relatively free of contaminating proteins. They are free of endogenous mRNA (Arlinghaus et al., 1968b) and ribosome-associated protein kinase activity (Figure 1), both of which are tightly bound to the ribosome and require rigorous treatment to remove (Crystal and Anderson, 1972) (Traugh et al., 1973). The absence of protein kinase should be interpreted with caution since the conditions used here are different than those used for reticulocyte ribosome-associated protein kinase (Traugh et al., 1973). The absence of contaminating supernatant proteins implies that the phosphorylation is occurring on only ribosomal proteins. Additional support for the conclusion that phosphorylation occurs on ribosomal proteins comes from the observations that the incorporated activity is precipitable in hot TCA, extracted along with protein in LiCl-urea and coelectrophoreses with authentic ribosomal proteins on disc gel electrophoresis (page 76). Although it has not been shown in this study that covalent binding occurs to seryl and threonyl residues of proteins, rabbit muscle protein kinase has been shown to phosphorylate E. coli ribosomal proteins at seryl and threonyl residues (Traugh and Traut, 1972).

The inactivation of ribosomal activity observed in the present
study with protein kinase and ATP (Figure 6) is apparently due to magne-
sium ion binding by the protein kinase fraction and chelation with
ATP. As yet unexplained, is the initial activation which is observed
at short time points (Figure 6). Two possible mechanisms for this ac-
tivation were alluded to in the Results section. The first mechanism
suggests that the activation is due to phosphorylation of essential
sites on the ribosome which increases the efficiency of active ribosomes
or causes the activation of some inactive ribosomes. A second mechanism
might be that the activation is due to an artifact of the low magnesium
ion concentration. Magnesium ion is an important element of ribosome
structure. Low concentrations of magnesium ion will cause E. coli
ribosomes to dissociate into subunits and eventually to unfold (Tissieres
et al., 1959; Gesteland, 1966). Mammalian ribosomes are somewhat more
resistant to unfolding, but will undergo dissociation in the absence
of magnesium ion (Ts'o and Vinograd, 1961). Mammalian ribosomes, dis-
sociated under conditions of low magnesium ion concentration, are inac-
tive in protein synthesis (Lafrom and Glowacki, 1962). Dissociation
itself does not cause inactivation since other methods are available for
obtaining subunits active in protein synthesis (Hamada et al., 1968).
It is possible that the initial activation is a transitory phase involv-
ing inactive ribosomes. In response to the low magnesium ion concen-
tration some of these inactive ribosomes dissociate into active subunits.
The observed activation would come from the subunits being assayed for
activity before they are subsequently inactivated.
Alternatively, dissociation into subunits may not be required at all. Direct binding of rat liver ribosomes to poly U, which does not require initiation factors, has been shown to occur (Falvey and Staehelin, 1970). Reticulocyte ribosomes may synthesize polyphenylalanine by a similar mechanism of direct binding. If this is the case, activation could be explained by the existence of a similar transitory active form which involves a "loosening" of the structure of inactive ribosomes. Further "loosening" would result in inactivation. In either case the reason activation is not observed with some preparations might be due to the fact that the transitory phase is reached and passed too quickly to be picked up in the assay. At this point, further work is needed to establish the mechanism for the observed activation.

Results from the present study are, in some respects, similar to results of in vitro phosphorylation of rat liver ribosomes by protein kinase from rat liver cytosol (Eil and Wool, 1973b). Inhibition of phosphorylation by high concentrations of KCl and magnesium ion is observed in both systems. KCl inhibition of rat liver ribosome phosphorylation appears to be due to an effect on the activity of the protein kinase itself. In contrast the effect of high magnesium may be mediated through a change in ribosome structure. High magnesium concentrations cause preferential inhibition of at least one band on the 40S rat liver ribosomal subunit. Due to the low levels of phosphorylation at a high magnesium:ATP ratio, it is difficult to determine if magnesium causes preferential inhibition of phosphorylation of a particular protein.
in the present study (Figure 15).

The effect of cAMP on the pattern of labeling of ribosomal proteins was not determined in this study. In agreement with other studies using rabbit muscle protein kinase and other substrates, cAMP stimulates phosphorylation (Reimann et al., 1971). Cawthon et al. (1974) have shown that in vivo phosphorylation of rabbit reticulocyte ribosomes is also stimulated by cAMP, although there is only a 1.2 to 2.0-fold stimulation of incorporation. The interesting aspect of this study is that cAMP does not seem to affect the overall level of phosphorylation. Stimulation of phosphorylation occurs either by increased turnover of phosphate at very specific sites or increased phosphorylation at one or a small number of sites relative to the total number of sites phosphorylated. In addition, in vivo phosphorylation gives only 5 different labeled phosphoproteins, although the number of phosphorylated sites on each protein is not known (Cawthon et al., 1974).

The most marked contrast between the present studies and that dealing with rat liver ribosomes is the time course of phosphorylation. Phosphorylation of the 40S and 60S rat liver ribosomal subunits increased linearly for 5-10 minutes and ceased after about 20 to 25 minutes. Final plateau level of phosphorylation is about 4 and 10 phosphate groups per 40S and 60S subunit, respectively (Eil and Wool, 1973b). No plateau level is observed with phosphorylation of reticulocyte ribosomes up to a 1 hour time point (Figure 1). Phosphorylation at 1 hour is approximately 18 phosphate groups per ribosome. It is not known how
much more time is necessary to reach a plateau level. It is perhaps significant that phosphorylated rat liver ribosomes are fully active in protein synthesis. This would suggest that they still have an integrated structure. It should be noted that phosphorylation of rat liver ribosomes is carried out at a much higher magnesium to ATP ratio (5mM magnesium:0.1mM ATP) than in vitro phosphorylation of reticulocyte ribosomes.

The significance of the in vitro phosphorylation is puzzling. It represents phosphorylation which occurs in addition to the level of phosphate which is present on ribosomes when they are initially isolated. The possibility that the in vitro phosphorylation also represents turnover has not rigorously been tested. Eil and Wool (1973a) have shown that rat liver ribosomal proteins phosphorylated in vitro do not undergo turnover. It is not known if turnover occurs with pre-existing phosphates in their in vitro reaction. The question of turnover of reticulocyte phosphoprotein in the in vitro reaction has not been examined.

Although the present study cannot rule out modification of ribosomal activity by in vitro phosphorylation, such modifications do not seem to be occurring under the conditions used here. Preliminary indications suggest that phosphorylation may be a useful tool in defining ribosome structure. Its ultimate usefulness will depend on finding conditions of phosphorylation which do not induce further changes in structure. This might involve further purification of the protein kinase fraction.
in an attempt to separate the magnesium binding protein, preincubation of the protein kinase with magnesium prior to addition to the phosphorylation reaction or even use of rat liver protein kinase to carry out phosphorylation. Usefulness of phosphorylation as a probe will also depend on further refinements in resolving individual proteins, possibly through the use of 2 dimensional gel electrophoresis.
SUMMARY

Purified rabbit reticulocyte ribosomes are phosphorylated in an in vitro reaction catalyzed by rabbit muscle protein kinase. At 5mM ATP, 3mM MgCl₂, 42mM KCl, 12mM DTT, 30mM Tris-HCl, pH 7.0 and 0.06mM cAMP, approximately 18 phosphate groups per ribosome are incorporated in a 60 minute incubation period. Under these conditions ribosomes are inactivated, as assayed by polyphenylalanine synthesis, by the presence of ATP alone. Under the same conditions, except at a lower ATP concentration (3.5mM), inactivation of ribosomal activity, as assayed by polyphenylalanine synthesis and nonenzymatic binding, occurred. This inactivation depended on the presence of both ATP and protein kinase. The time course change of ribosomal activity during incubation in the phosphorylation reaction shows an initial activation at short times followed by inactivation at longer times. The mechanism of the observed activation is not known. The subsequent inactivation, seen at longer incubation times, is due to low magnesium ion concentration caused by chelation with ATP and binding with the protein kinase fraction. No change in ribosomal function has been found which is caused by the in vitro phosphorylation.

Preliminary work suggests that phosphorylation may be useful as a probe of ribosome structure. Disruption of ribosome structure leads to increased rates of phosphorylation, possibly due to exposure of new sites for phosphorylation.
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