

Phosphorylation of Ribosomal Proteins in Rabbit Reticulocytes. A Cell-Free System with Ribosomal Protein Kinase Activity*

David Kabat

ABSTRACT: Rabbit reticulocyte ribosomes isolated from low ionic strength solutions are contaminated with a ribosomal protein kinase. Washing the ribosomes in a high ionic strength solution elutes the kinase. The same ribosomal proteins previously shown to be phosphorylated within reticulocytes incubated with [32 P]orthophosphate are labeled in the cell-free system with [γ - 32 P]ATP. Radioactivity in both cases is incorporated into *o*-phosphoserine and *o*-phosphothreonine

residues of the polypeptide chains. The rate of ribosomal protein phosphorylation declines as ATP becomes depleted from the cell-free system. The phosphorylation kinetics are accordingly very strongly influenced by ATPase and also by phosphoprotein phosphatase which are present in reticulocytes. The kinase may have a broad substrate specificity since it appears to be active in phosphorylation of chicken erythrocyte histones.

It has been recently shown that several ribosomal proteins in rabbit reticulocytes are phosphoproteins (Kabat, 1970). The phosphate groups turn over within the cells and the ribosomal proteins, therefore, become radioactive during incubation of the reticulocytes in a nutrient medium containing [32 P]orthophosphate. The phosphorylation of one protein on the large ribosome subunits apparently inactivates the ribosomes since the ribosomes containing this phosphate accumulate in the cells as single ribosomes which do not participate in the ribosome cycle of protein synthesis (Kabat, 1970). It seemed important to study in a cell-free system the enzymes and factors which control the ribosome phosphorylation, and my initial studies of such a system are described here. This work indicates that the same ribosomal proteins which are labeled intracellularly can be phosphorylated in a cell-free system by transfer of the γ -phosphate group of ATP.

Materials and Methods

Solutions. Two buffers with different ionic strengths were used for preparation of ribosome fractions as described previously (Kabat, 1970). Buffer A is 0.01 M Tris-HCl-0.01 M KCl-0.0015 M MgCl₂ (pH 7.4) and buffer B is 0.01 M Tris-HCl-0.25 M KCl-0.01 M MgCl₂ (pH 7.4).

Preparation of Materials. Reticulocytes were obtained from anemic rabbits and were washed to remove leukocytes as pre-

viously described (Kabat, 1970). The preparation of cell lysates with buffer A and the method used for pH 5.0 precipitation of ribosomes are described by Warner and Rich (1964). The crude ribosome fraction resulting from pH 5.0 precipitation was redissolved in buffer A and the pH was readjusted to pH 7.4. Buffer A (1 ml) was used to redissolve the pH 5.0 precipitate obtained from 1 ml of reticulocytes. Ribosomes were further purified by sedimentation through a cushion of 15% sucrose. A 1-ml solution containing ribosomes was diluted to 9 ml with either buffer A or B. This was layered over a 2.0-ml solution of 15% sucrose dissolved in the same buffer in a centrifuge tube for the Spinco 40 rotor. Centrifugation was at 40,000 rpm for 80 min. The clear pellet of ribosomes was used as a substrate for phosphorylation. The ribosomes sedimented in buffer A will be referred to as A ribosomes whereas those sedimented in buffer B will be referred to as B ribosomes. In some preparations, the ribosomes in buffer B were adjusted to 0.5% sodium deoxycholate before sedimentation through the buffer B sucrose cushion. Such a preparation is referred to as D ribosomes.

Phosphorylation in the Cell-Free System. The phosphorylations were performed routinely in buffer A at 37°. The quantities of ribosomes and of [γ - 32 P]ATP were varied and are separately indicated for each experiment. The [γ - 32 P]ATP (660 mCi/mmol) was purchased in several lots from Amersham-Searle Corp. and was used within 1 month of delivery. In some experiments reticulocyte protein fractions were added to the incorporation mixture. These were prepared as described in the text and were assayed for protein concentration by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Assay for phosphoprotein phosphate was initially performed by the method of

* From the Department of Biochemistry, University of Oregon Medical School, Portland, Oregon 97201. Received August 24, 1970. This research was supported by U. S. Public Health Service Grant CA 11347.

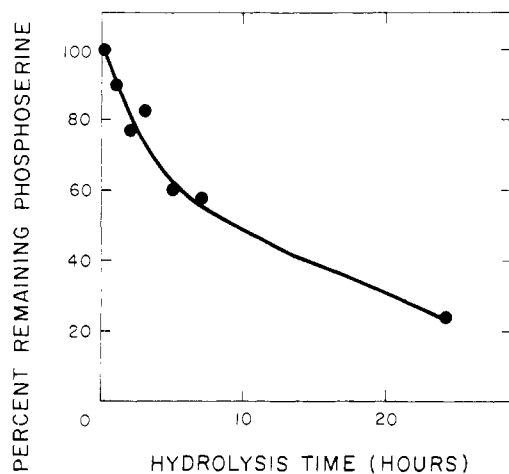


FIGURE 1: Kinetics of *o*-phosphoserine hydrolysis in 6 M HCl at 105°.

DeLange *et al.* (1968). However, a simpler procedure which gave identical results in this system was to precipitate the macromolecular components of the 0.1-ml sample with 2 ml of 10% trichloroacetic acid at 0° for 30 min. The nucleic acids were then hydrolyzed by warming the tube to 90° for 20 min. After chilling at 0° for 30 min, the samples were filtered onto Millipore membranes (0.45 μ), and the membranes were thoroughly washed with 5% trichloroacetic acid before they were glued onto aluminum planchets. The samples were then assayed for radioactivity in a low-background gas-flow counter.

In some experiments the cell-free incorporation mixtures were layered onto 29-ml isokinetic 15–30% sucrose gradients in buffer B and were sedimented in the Spinco SW 25.1 rotor at 15,000 rpm for 18 hr at 2°. The sedimented gradients were scanned continuously for absorbance at 260 m μ and the fractions were assayed for phosphoprotein radioactivity after heating in 10% trichloroacetic acid at 90°. In other experiments, ribosomes were purified from the cell-free system by diluting 1 ml of incubation mixture with 9 ml of buffer B and preparing B ribosomes, as described above. Electrophoresis of the phosphorylated constituents of the radioactive B ribosomes in 4% polyacrylamide gels containing 0.6% sodium dodecyl sulfate and the autoradiographic analysis of the distribution of radioactive materials in the gels were described previously (Kabat, 1970).

Analysis of ^{32}P Compounds by Thin-Layer Chromatography. During the incorporation, samples containing 1 μ l of the cell-free incorporation mixtures were periodically applied onto anion-exchange thin-layer plates (PEI-cellulose, Brinkman Instruments) under infrared lamps at 55°. The chromatograms were developed for 2 hr at room temperature with 1 M LiCl as described by Randerath and Randerath (1967). They were then dried in an oven at 75°. The location of the radioactive compounds was determined by autoradiography using X-ray film (Kodak SB-54). The radioactive regions of the chromatograms were cut out and were placed into Bray's solution for radioactivity measurement in a liquid scintillation counter (Bray, 1960).

Hydrolysis of Radioactive Ribosomes. Ribosome fractions were hydrolyzed in 6 M HCl at 105° in evacuated ampoules for 7 hr. The samples were then dried *in vacuo*, dissolved in electrophoresis solution (2.5% formic acid–7.8% acetic acid, pH 1.85), and analyzed by high-voltage paper electrophoresis

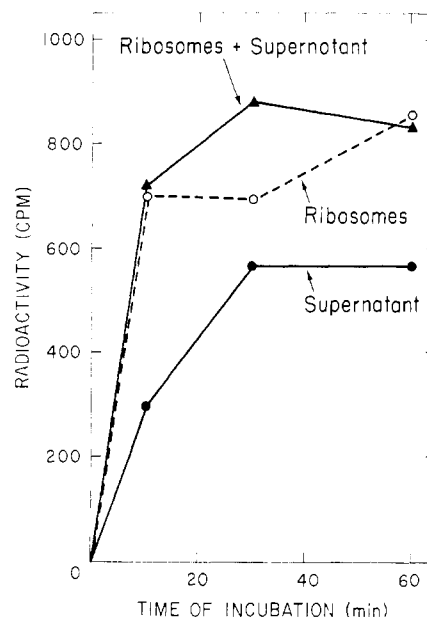


FIGURE 2: Kinetics of protein phosphorylation in the cell-free system. The incubation tubes all contained 2 $\mu\text{Ci/ml}$ of [γ - ^{32}P]ATP. The concentration of A ribosomes was 0.13 mg/ml of incubation mixture, whereas the concentration of supernatant enzymes was 0.1 mg/ml. The supernatant enzyme preparation was obtained from a reticulocyte lysate after sedimentation in buffer A at 105,000g for 3 hr. The ribosomes and supernatant enzyme preparations were incubated either alone or together. At various times after beginning the incubation at 37°, 0.1-ml samples were assayed for radioactive phosphoprotein (see Methods). A background of 210 cpm was subtracted from all radioactivity measurements. This amount of radioactivity was present in samples from a control solution which contained only ATP. (O-O) Ribosomes, (●-●) supernatant enzymes, and (▲-▲) ribosomes + supernatant enzymes.

as previously described (Langan, 1968; Kabat, 1970). Control samples of [^{32}P]orthophosphate, phosphoserine, and phosphothreonine were also analyzed. After electrophoresis the paper was stained for amino acids with a cadmium–ninhydrin stain which develops a stable absorbance at 500 m μ . This absorbing material can be eluted with methanol and is proportional to the amount of the amino acid (Dreyer and Bynum, 1967). The kinetics of hydrolysis of *o*-phosphoserine was studied with this method, and the results are shown in Figure 1. Seven hours was selected as the hydrolysis time for the ribosomes in order to maximize peptide-bond hydrolysis while limiting hydrolysis of the phosphorylated amino acids.

Results

Phosphorylation of Ribosomal Proteins in the Cell-Free System. To initially investigate the system, ribosomes isolated from reticulocyte lysates by sedimentation in low ionic strength buffer A (A ribosomes) were incubated with [γ - ^{32}P]ATP in the presence or absence of supernatant enzymes at 37°. Samples were removed at various times for assay of ^{32}P -labeled phosphoproteins. As can be seen in Figure 2, the A ribosomes are phosphorylated in the cell-free system, even in the absence of supernatant enzymes. However, the cell-free system is obviously complex. For example, the extent of protein phosphorylation in the tube containing supernatant fraction plus A ribosomes is not equal to the sum of the phosphorylation which occurs when the two fractions are sepa-

TABLE I: Solubility Characteristics of Ribosomal Phosphorylated Constituents.

Solubilization Procedure	Ribosomes Phosphorylated within Reticulocytes ^a (% of Radioactivity)	Ribosomes Phosphorylated in Cell-Free System ^b (% of Radioactivity)
1. 90° for 20 min in 10% trichloroacetic	18	22
2. Nonaqueous solvent extracts	14	6
3. Final residue	68	72

^a These ribosomes were from reticulocytes which had been labeled for 60 min with [³²P]orthophosphate in nutrient medium as described previously (Kabat, 1970). ^b A ribosomes were phosphorylated in the cell-free system with [γ -³²P]ATP for 30 min (see text). The ribosomes were then purified from the solution by centrifuging in buffer B. Both ribosome preparations were washed several times with cold 10% trichloroacetic acid and were analyzed at the same time. Nucleic acids were extracted from the precipitates in step 1 by heating in 10% trichloroacetic acid at 90° for 20 min. The remaining precipitates were then extracted in step 2 with a series of nonaqueous solvents as suggested by Davidson *et al.* (1961). Approximately 70% of the radioactivity on both ribosome preparations was in the residual fraction not solubilized by these procedures.

ately incubated. A background radioactivity of 210 cpm caused by a small amount of contamination from the [γ -³²P]ATP was subtracted from all radioactivity measurements (see Figure 2).

More conclusive evidence that the ribosome structural proteins are phosphorylated in the cell-free system containing only [γ -³²P]ATP and A ribosomes was obtained by sucrose gradient analysis of the radioactive incubation mixtures (*e.g.*, Figure 3). The gradients were made with high ionic strength buffer B since this is known to extract contaminating proteins and phosphoproteins from ribosomes (Warner and Pene, 1966; Kabat, 1970). A few polyribosomes were generally present in the solutions following the incorporation at 37° and they were partially pelleted to the bottom of sucrose gradient shown in Figure 3. However, approximately 70% of the ribosomes are present as 76S monoribosomes which are clearly resolved. It is clear from Figure 3 that some of the incorporated phosphoprotein radioactivity sediments in the gradients with the ribosomal material. In fact the distribution of radioactivity is almost identical with the distribution which was obtained when the ribosomes were labeled by whole cell incubation with [³²P]orthophosphate (Kabat, 1970). A large portion of the radioactivity which sediments near the top of the tubes is due to contamination from the [³²P]ATP which was previously mentioned. As a result of several experiments, I conclude that 30–50% of the radioactivity incorporated by the A-ribosome preparation goes into material which cosediments with the ribosomes in sucrose gradients. The remainder is incorporated into proteins which contaminate

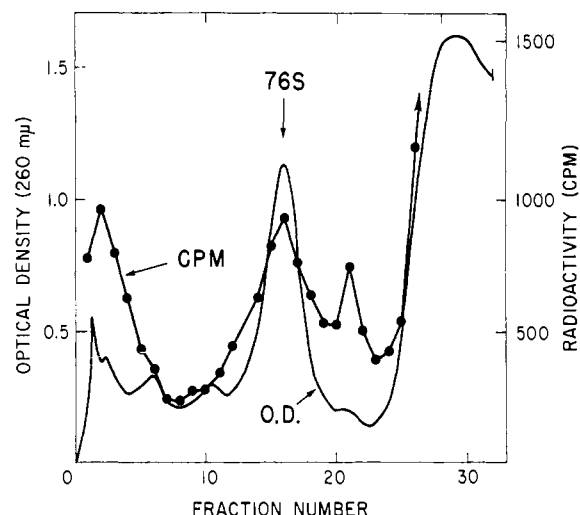


FIGURE 3: Sucrose gradient sedimentation of ribosomes from a cell-free incorporation solution. The A ribosomes (1.3 mg) were incubated at 37° for 20 min with 5 μ Ci of [γ -³²P]ATP before the solution was chilled to 0° and sedimented at 15,000 rpm for 18 hr at 2° in a 29-ml 15–30% isokinetic sucrose gradient in buffer B. Sedimentation is toward the left. The sedimentation position of the single ribosomes (76 S) is indicated with an arrow. The peak of material sedimenting behind the single ribosomes contains subribosomal particles.

the A ribosomes and which are eluted in the buffer B sucrose gradients. Negligible radioactivity becomes associated with the ribosomes if the incubations are performed at 0°.

Ribosomes which had been phosphorylated in the cell-free system were next purified away from contaminating materials by sedimentation through buffer B, and the radioactive components were then characterized by various methods. As can be seen in Table I, only a minor portion of the radioactivity in the purified ribosomes is solubilized by heating at 90° for 20 min in 10% trichloroacetic acid or is extractable with nonaqueous solvents. Thus, it is clear that the incorporation is neither into nucleic acids nor into phospholipids. Rather, the majority of the radioactivity is associated with the residual protein fraction which is not solubilized by these treatments. The labeling of ribosomes in the cell-free system is very similar in this respect to the labeling which occurs within reticulocytes incubated with [³²P]orthophosphate (Table I).

It was shown previously that the [³²P]phosphate on intracellularly labeled ribosomes is present in *o*-phosphoserine and *o*-phosphothreonine residues and is approximately 75% hydrolyzed by incubation with *E. coli* alkaline phosphomonoesterase (Kabat, 1970). As can be seen in Figure 4, a similar proportion is sensitive to phosphatase when the ribosomes are labeled in the cell-free system. Furthermore, it appears that the incorporation in the cell-free system is indeed into phosphorylated amino acid residues of ribosomal polypeptide chains. Figure 5 shows that an acid hydrolysate of these labeled ribosomes contains radioactive compounds which coelectrophorese with *o*-phosphoserine and with *o*-phosphothreonine. The hydrolysate also contains a relatively high level of [³²P]orthophosphate. Although some of this phosphate certainly was produced by hydrolysis of the phosphorylated amino acids (see Figure 1), it is possible that some was produced by hydrolysis of other compounds.

That phosphorylation in the cell-free system containing A ribosomes and [γ -³²P]ATP is very similar to that which occurs within reticulocytes is further supported by an electrophoretic analysis of the phosphorylated ribosome constituents

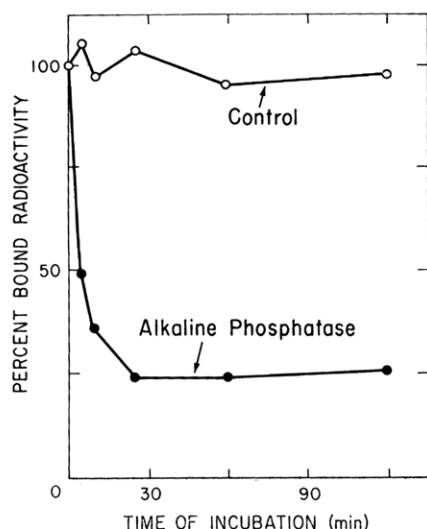


FIGURE 4: Removal of [32 P]phosphate from cell-free labeled ribosomes with *E. coli* alkaline phosphomonoesterase. The conditions of digestion and of assay are described elsewhere (Kabat, 1970). (●-●) alkaline phosphatase (1 mg/ml) in 0.1 M Tris-HCl-0.4 M NaCl (pH 7.6). (○-○) 0.1 M Tris-HCl-0.4 M NaCl (pH 7.5) without enzyme.

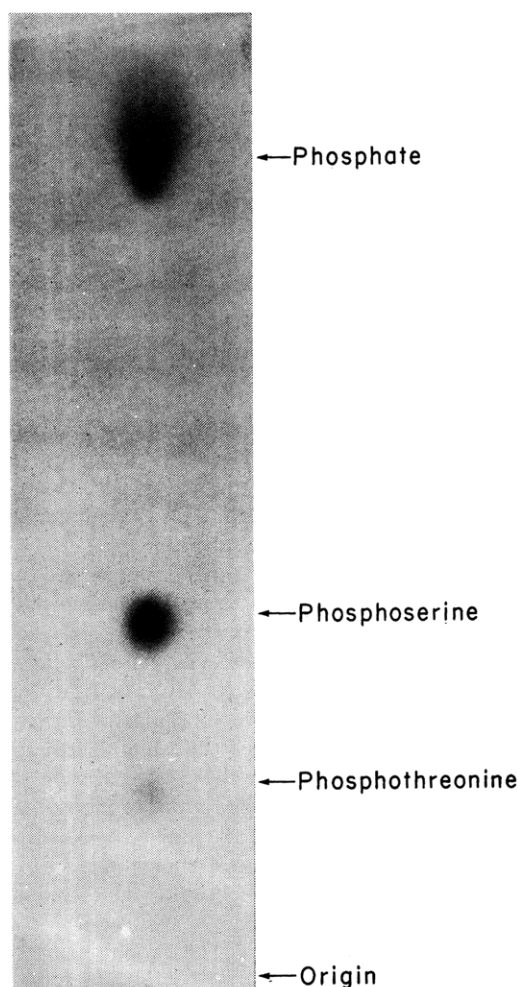


FIGURE 5: Paper electrophoresis of hydrolysate of ribosomes labeled with [γ - 32 P]ATP in the cell-free system. The radioactive spots are located by autoradiography on the exposed X-ray film. After the ribosomes were labeled, they were washed by sedimentation through high ionic strength buffer B. The ribosome pellet was then hydrolyzed in 6 M HCl for 7 hr at 105° (see Methods).

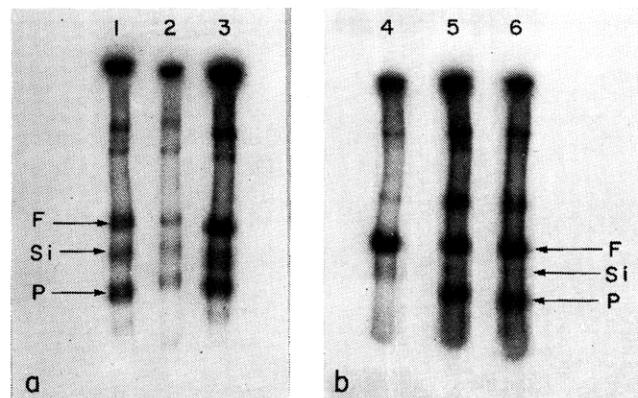


FIGURE 6: Electrophoresis in sodium dodecyl sulfate polyacrylamide gels of 32 P-labeled ribosomes, and autoradiographic visualization of the radioactive bands. The ribosomes in panel a were labeled in the cell-free system with [γ - 32 P]ATP (4 μ Ci/ml) for 30 min at 37° as follows: (gel 1) no additions, (gel 2) with 0.02 M cyclic AMP, and (gel 3) with 0.02 M NaF. These ribosomes had specific radioactivities of 6.6×10^4 , 6.3×10^4 , and 9.3×10^4 cpm per mg, respectively. The ribosomes in panel b were labeled intracellularly, as described elsewhere (Kabat, 1970). The labeled cell lysates were used for purification of D ribosomes (gel 4) and of B ribosomes (gels 5 and 6). The two latter gels illustrate the reproducibility obtained. The positions of electrophoresis of the major phosphorylated constituents (the P, Si, and F bands) are indicated with arrows. These bands for the cell-free labeled ribosomes were identified on the basis of coelectrophoresis with ribosome components labeled intracellularly and analyzed simultaneously. The samples shown in panels a and b were electrophoresed on different days with different gel preparations.

in polyacrylamide gels containing 0.6% sodium dodecyl sulfate. Portions of the cell-free incorporation mixtures were also incubated with 0.02 M NaF or with 0.02 M cyclic AMP.¹ The ribosomes were sedimented through buffer B before the electrophoresis. Figure 6a shows the autoradiographic results obtained by allowing the gels to expose X-ray film. The patterns are very similar to patterns obtained using ribosomes labeled within intact cells (Figure 6b). This type of analysis has been repeated several times and has lead to the following conclusions: (i) The major ribosome components labeled in whole cells (the P, Si, and F components) are also labeled in the cell-free system. The Si and F components were previously shown to be phosphoproteins; however, the P component is apparently not a macromolecule and has not yet been identified (Kabat, 1970). (ii) The protein phosphorylations are slightly stimulated by 0.02 M NaF (generally by 20–50%) and are usually inhibited by 0.02 M cyclic AMP (generally by about 10%, but occasionally by up to 50%). Many other tissues contain protein kinases dependent upon cyclic AMP (Kuo and Greengard, 1969), but even dialyzed kinase preparations from reticulocytes are inhibited or unaffected by this cyclic nucleotide. The preparation of a crude protein kinase extract from A ribosomes is described below. NaF specifically stimulates F-protein phosphorylation in whole cells, whereas it stimulates protein phosphorylation in the cell-free system without apparent specificity. It is likely that this NaF effect is due to inhibition of the reticulocyte ATPase (Farias *et al.*, 1970). Also in the cell-free system, NaF seems to stimulate the phosphorylation of a ribosome constituent which

¹ Abbreviations used are: cyclic AMP, adenosine 3',5'-monophosphate; ribosomal protein kinase, ATP:ribosomal protein phosphotransferase; ATPase, ATP phosphohydrolase (EC 3.6.1.4); phosphoprotein phosphatase, phosphoprotein phosphohydrolase (EC 3.1.3.16).

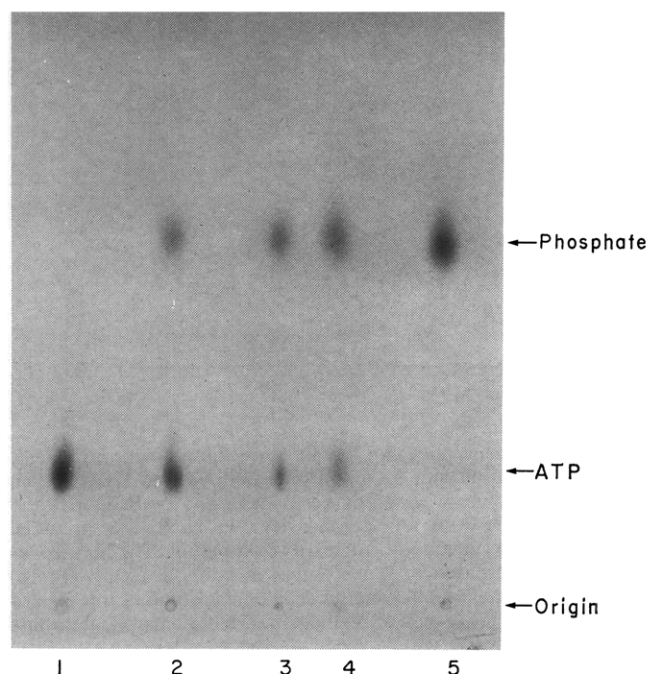


FIGURE 7: Thin-layer chromatography showing ATP hydrolysis in the cell-free system. The A ribosomes (0.36 mg/ml) were incubated at 37° with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (12 $\mu\text{Ci/ml}$). Samples containing 1 μl were spotted on to thin-layer chromatograms at the following times after beginning the incubation: (1) 0 min, (2) 5 min, (3) 10 min, (4) 11.5 min, (5) 30 min.

migrates just in front of the Si phosphoprotein (Figure 6). Labeling of this component has not been observed intracellularly.

Further Characterization of the Cell-Free System Active in Ribosome Phosphorylation. The following experiments were done to further characterize the factors which influence the cell-free phosphorylation of ribosomal proteins. When A ribosomes are incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, only a minor portion (approximately 0.1%) of the radioactivity is present in phosphoproteins when net incorporation has ceased. The vast majority of the radioactive ATP is hydrolyzed during incubation to form $[\text{P}^{32}]\text{orthophosphate}$. Apparently this hydrolysis is catalyzed by an ATPase which is present in the A ribosomes. Figure 7 shows a thin-layer chromatographic analysis of the cell-free system. Almost all of the radioactivity is converted during incubation into $[\text{P}^{32}]\text{orthophosphate}$, with negligible amounts being transferred into other compounds. The phosphoprotein radioactivity is relatively low and remains at the origin. No significant ATP hydrolysis occurs in the absence of the A ribosomes.

As would be expected from this relatively extensive ATPase activity, the kinetics of phosphoprotein formation in the A-ribosome system are highly dependent upon the concentrations of A ribosomes and of ATP. Figure 8 shows the kinetics of phosphoprotein formation in the cell-free system at two different concentrations of A ribosomes. Although the extent of protein phosphorylation is initially greater when the A-ribosome concentration is raised, the incorporation also ceases more rapidly at the higher ribosome concentration. Generally, there also occurs a subsequent slow decline of phosphoprotein radioactivity, indicating that a phosphoprotein phosphatase is present in the solutions. The kinetics of ATP hydrolysis was followed in this same experiment and the results are

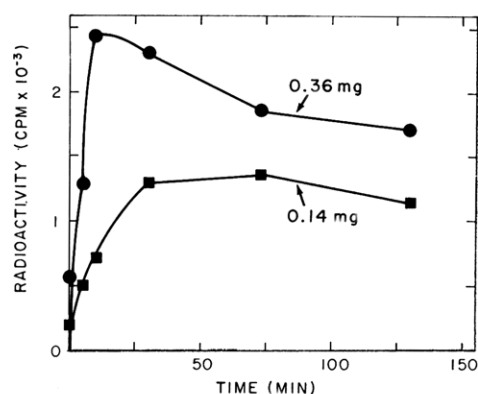


FIGURE 8: Kinetics of protein phosphorylation in the cell-free system at two different ribosome concentrations. The A ribosomes were incubated at 37° with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (12 $\mu\text{Ci/ml}$). At various times after beginning the incubation, 0.1-ml samples were assayed for radioactive phosphoprotein. (■-■) 0.14 mg/ml of A ribosomes and (●-●) 0.36 mg/ml of A ribosomes.

shown in Figure 9. The hydrolysis occurs more rapidly at the higher A ribosome concentration, as expected. Comparison of Figures 8 and 9 indicates that the protein phosphorylation kinetics closely reflect the availability of ATP and that the kinetics are also influenced by the phosphoprotein phosphatase activity. These studies indicate that the γ -phosphate of ATP is very likely the direct precursor of the ribosomal phosphoprotein phosphate.

Extraction of Protein Kinase from the Ribosomes. In contrast to ribosomes isolated in low ionic strength buffer A (A ribosomes), ribosomes sedimented through high ionic strength buffer B (B ribosomes) or through solutions containing 0.5% sodium deoxycholate (D ribosomes) are relatively deficient in ribosome protein kinase activity. Figure 10 shows that D ribosomes are inactive in protein phosphorylation. In other

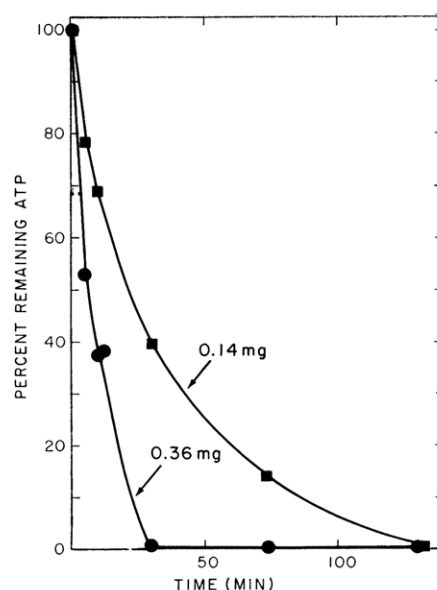


FIGURE 9: Kinetics of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysis in the cell-free system at two different ribosome concentrations. The experiment is the same as described in Figure 8, except that $[\text{P}^{32}]\text{ATP}$ and $[\text{P}^{32}]\text{orthophosphate}$ were analyzed on thin-layer chromatograms. The graph shows the percentage of radioactivity in ATP. (■-■) 0.14 mg/ml of A ribosomes and (●-●) 0.36 mg/ml of A ribosomes.

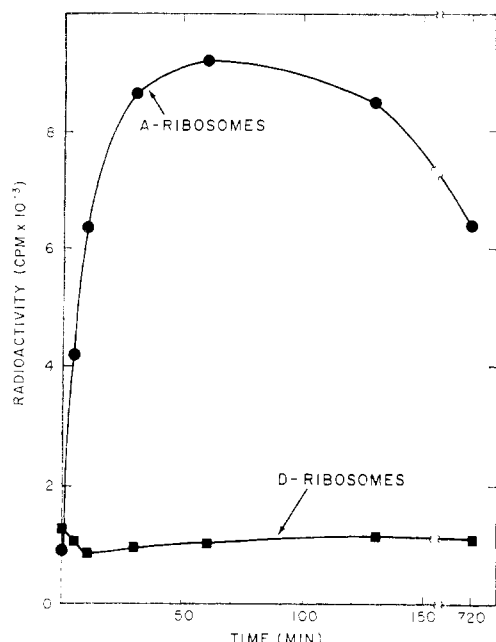


FIGURE 10: Kinetics of protein phosphorylation using A- or D-type methods to prepare ribosomes. The ribosomes were incubated at 0.28 mg/ml at 37° with 20 μ Ci/ml of [γ - 32 P]ATP, and 0.1-ml samples were periodically removed for radioactive phosphoprotein assay. The percentage of remaining ATP was measured at various times during incubation of the A ribosomes and was found to be: 100% (0 min), 80% (5 min), 71% (10 min), 33% (30 min), and 9.4% (60 min). The maximum labeling observed here in the A-ribosome preparation corresponds to approximately 2.8 moles of phosphate/mole of ribosomes. Approximately one to two of these phosphates are actually on ribosomal proteins, the remainder being on contaminating proteins (see Figure 3 and text). (●-●) A ribosomes and (■-■) D ribosomes.

experiments I found that B ribosomes are approximately 10% as active as A ribosomes. The inactivity of D ribosomes in phosphoprotein formation is not simply due to extraction of the protein substrates from the ribosomes by deoxycholate. As can be seen in Figure 6b, the major ribosomal phosphoproteins labeled within reticulocytes (the Si and F proteins) are not extracted with deoxycholate. The P band, which is not a phosphoprotein, is extracted with deoxycholate. In addition, the Si and F proteins also remain firmly bound to B ribosomes (Figure 6b) (see also Kabat, 1970). Furthermore, evidence described below shows that an active protein kinase is extracted from the A ribosomes with buffer B. Therefore, I believe that the inactivity of B and D ribosomes is mainly due to extraction from the ribosomes of protein kinase, rather than due merely to extraction of the protein substrates or to enzyme inactivation.

Although B ribosomes only retain 10% of the total protein kinase activity as compared with A ribosomes, they are nevertheless capable of ribosome protein phosphorylation. However, the relative phosphorylation of different proteins is altered after the ribosomes are sedimented through buffer B. The Si protein is phosphorylated relatively extensively on the B ribosomes, whereas the F protein is labeled relatively less. It is possible that an inhibitor of Si-protein phosphorylation is eluted from ribosomes with buffer B, or that the ribosomes are structurally altered by buffer B extraction so that the Si protein becomes relatively more accessible to remaining kinase. The idea that an inhibitor may be involved is supported by the preliminary finding that reticulocyte lysates contain

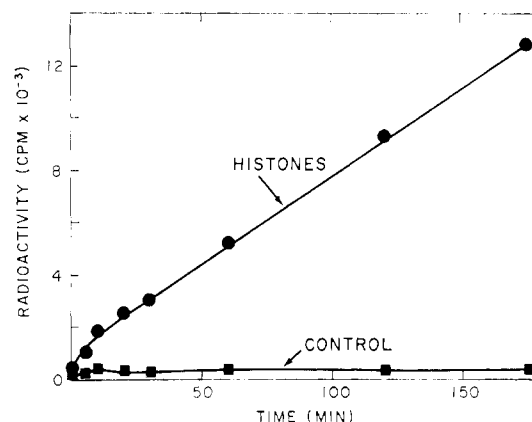


FIGURE 11: Histone phosphorylation using the reticulocyte kinase. The incubation was with 2 μ Ci/ml of [γ - 32 P]ATP at 37°. Chicken erythrocyte histones were prepared as described by Sadgopal and Kabat (1969) and were not phosphorylated in the absence of added kinase. The histone concentration was 0.95 mg/ml. The kinase was obtained by extraction of A ribosomes in buffer B (see Methods) and was incubated at 0.044 mg/ml. The control tube in this experiment contained the extract of A ribosomes, but did not contain histones.

a material which specifically inhibits Si-protein phosphorylation. This material is in the pH 5.0 soluble fraction and is precipitated by 40% $(\text{NH}_4)_2\text{SO}_4$.

The extract obtained by sedimenting A ribosomes through buffer B indeed contains protein kinase. The extracted kinase is able to phosphorylate the F and Si ribosomal proteins when it is added to D ribosomes, whereas no P-band labeling is observed in this system. However, the extracted kinase appears to have a rather low substrate specificity since it is active also in phosphorylation of chicken erythrocyte histones, as is shown in Figure 11.

Discussion

These experiments indicate that the γ -phosphate group of ATP is the precursor of ribosomal protein phosphate in reticulocytes. The proteins phosphorylated intracellularly (Kabat, 1970) are also phosphorylated in the cell-free system and in both cases the phosphate occurs as *o*-phosphoserine and *o*-phosphothreonine residues of polypeptide chains. In the previous paper the incorporated phosphate was conclusively shown to be present in proteins. This evidence included the demonstration that the radioactivity was on macromolecules degraded by proteolysis and was absent from purified, undegraded polyribosomal RNA. All available data indicate that the ribosomal products of cell-free labeling with [γ - 32 P]-ATP are the same as the products of whole cell labeling with [32 P]orthophosphate.

The ribosome protein phosphorylation system of reticulocytes has been difficult to analyze because the substrate is complex and because there are a variety of factors which influence the phosphorylation. All of my efforts to purify the kinase away from its substrates and inhibitors have been unsuccessful. Nonribosomal proteins are also phosphorylated in reticulocytes and there are, in addition, competing ATPase (Figure 7) and phosphoprotein phosphatase activities (Figure 8) which influence the kinetics of the ribosome protein phosphorylation. Additional preliminary evidence suggests that an inhibitor may be involved in controlling the phosphorylation of one ribosomal protein. Other workers have

previously suggested that protein kinase inhibitors and modifiers may occur within cells (Meyer *et al.*, 1964; Miyamoto *et al.*, 1969). Some purified protein kinases appear to have rather low specificity for the protein substrates (*e.g.*, Miyamoto *et al.*, 1969; see also Figure 11), and it is possible that their specificity is enhanced by intracellular materials. Although it has not been shown that all of these factors influence the intracellular process of ribosome protein phosphorylation, the present studies demonstrate the complexity of the cell-free system and should provide a useful background for further investigations.

The number of phosphate groups which can be attached to ribosomes in the cell-free system is difficult to evaluate. In the first place there is an unknown level of phosphoprotein phosphate already on the ribosomes used as substrate. Ribosomes from which the phosphate esters were removed with alkaline phosphatase have not been used as substrates. Secondly, the cell-free system utilized for these studies generally ceases incorporation before the ribosomal sites are saturated. Rather, the incorporation ceases because of depletion of ATP due to an ATPase which remains associated with the ribosomes (Figures 8 and 9). The maximum ribosome labeling which I have obtained at relatively very high ATP concentrations is approximately one to two phosphoprotein phosphate groups per ribosome (see Figure 10). For the reasons just discussed, however, this is a minimum estimate of the number of phosphorylated sites on ribosomes. There are at least two different phosphoproteins which are reproducibly found firmly bound to ribosomes, even when the ribosomes have been washed with high ionic strength solutions containing deoxycholate or have been fractionated into ribosomal subunits (Kabat, 1970).

Evidence was previously presented that phosphorylation of one ribosomal protein (the protein termed Si protein) can inactivate the ribosome. The ribosomes containing phosphorylated Si-protein accumulate within reticulocytes as 76S single ribosomes which are not participating in the ribosomal subunit-polyribosome cycle of protein synthesis. Active polysomes and subribosomal particles lack the Si-phosphate group (Kabat, 1970). The present study shows that Si-pro-

tein phosphorylation occurs in a simple cell-free system from reticulocytes by transfer of the γ -phosphate group of ATP.

Acknowledgments

The enthusiastic and excellent assistance of Mrs Pauline Gaskill is gratefully acknowledged.

References

- Bray, G. W. (1960), *Anal. Biochem.* 1, 279.
- Davidson, J. N., Fraser, S. C., and Hutchison, W. C. (1951), *Biochem. J.* 49, 311.
- DeLange, R. J., Kemp, R., Riley, W. D., Cooper, R. A., and Krebs, E. G. (1968), *J. Biol. Chem.* 243, 2200.
- Dreyer, W. J., and Bynum, E. (1967), *Methods Enzymol.* 11, 32.
- Farias, R. N., Goldemberg, A. L., and Trucco, R. E. (1970), *Arch. Biochem. Biophys.* 139, 38.
- Kabat, D. (1970), *Biochemistry* 9, 4160.
- Kuo, J. F., and Greengard, P. (1969), *Proc. Nat. Acad. Sci. U. S.* 64, 1349.
- Langan, T. A. (1968), *Regulatory Mechanisms for Protein Synthesis in Mammalian Cells*, New York, N. Y., Academic Press, p 101.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* 193, 265.
- Meyer, W. L., Fischer, E. H., and Krebs, E. G. (1964), *Biochemistry* 3, 1033.
- Miyamoto, E., Kuo, J. F., and Greengard, P. (1969), *Science* 165, 63.
- Randerath, K., and Randerath, E. (1967), *Methods Enzymol.* 12, 323.
- Sadgopal, A., and Kabat, D. (1969), *Biochim. Biophys. Acta* 190, 486.
- Warner, J. R., and Pene, N. G. (1966), *Biochim. Biophys. Acta* 129, 359.
- Warner, J. R., and Rich, A. (1964), *Proc. Nat. Acad. Sci. U. S.* 51, 1134.