Phosphorylation of Ribosomal Proteins in Rabbit Reticulocytes. Characterization and Regulatory Aspects*

David Kabat

ABSTRACT: Rabbit reticulocyte suspensions extensively incorporate [^{32}P]phosphate into covalent linkage with ribosome structural proteins. Only a minor fraction of the incorporation is into ribonucleic acids or their terminal nucleotides (less than 1–2%) or is into phospholipids (less than 10%). At least 75% of the ribosome-bound phosphate is released from the ribosomes by digestion with *Escherichia coli* alkaline phosphomonoesterase. The phosphate occurs in the ribosomes as *o*-phosphoserine and *o*-phosphothreonine residues of polypeptides. A similar incorporation into reticulocyte ribosomal phosphoproteins occurs *in vivo* after injection of rabbits with [^{32}P]phosphate. All tested chick embryo tissues (heart, liver, muscle) also contain phosphoproteins in their ribosomes. The radioactive ribosomal phosphoproteins have been analyzed by electrophoresis in polyacrylamide gels containing 0.6% sodium dodecyl sulfate. The ribosomes fully dissociate in the gels and all of the macromolecular components migrate toward the anode. The mobilities of protein–dodecyl sulfate complexes are inversely proportional to the logarithms of the polypeptide chain molecular weights. All of the major radioactive components are converted into rapidly migrating low molecular weigh material by proteolytic digestion with pronase. The following conclusions can be made about the phosphorylated components of ribosomes. (1) Approximately 40% of the radioactivity associated with ribosomes is present in about 10 different loosely associated phosphoproteins. These components are probably contaminants since they can be removed by washing the ribosomes in high ionic strength solution. Furthermore, these components are present on polyribosomes, single ribosomes, subribosomal particles, and also in the supernatant fraction. (2) The "P band" of the gels contains a phosphorylated compound which is specifically and firmly associated only with polysomal ribosomes and which has a rapid electrophoretic mobility. The P component is released quantitatively from polysomal ribosomes as they are converted into single ribosomes during reticulocyte incubation with 0.03 M NaF. Dissociation of ribosomes into subunits with EDTA also causes release of P.

The molecular nature of P is uncertain. (3) The "Si band" is a protein (mol wt ~ 21,000 daltons) which is present on the larger ribosomal subunits. Although present also on polysomes and on subribosomal particles, the Si protein is only phosphorylated on single ribosomes. Phosphorylation of Si protein is not obligatorily coupled to the formation of single ribosomes which occurs when reticulocytes are incubated with 0.03 M NaF. The kinetics of Si phosphorylation suggest that the phosphate is turning over only slowly and that single ribosomes containing phosphorylated Si protein are excluded from participating in the ribosomal subunit–polyribosome cycle of protein synthesis. They are a class of inactive particles which may be a storage form of ribosomes. (4) The "Su band" is a large phosphoprotein (mol wt ~ 70,000 daltons) which is specifically associated only with the small 44S native subribosomal particles. It is absent from the supernatant suggesting that it is not a contaminant of ribosomes. However, the unphosphorylated Su protein is also absent from polyribosomes and single ribosomes. The Su protein can be eluted from the subribosomal particles by washing them in high ionic strength solutions. (5) The "F band" is a phosphoprotein (mol wt ~ 33,000 daltons) which is firmly associated with all ribosome fractions and which is absent from the supernatant fraction. The F protein is on the small ribosomal subunits. Phosphorylation of F is specifically stimulated when the cells are incubated with 0.03 M NaF, even when polyribosome disaggregation is blocked with cycloheximide. This effect of NaF is unrelated to the well-known enhancement by fluoride of the intracellular concentration of cyclic 3′,5′-adenosine monophosphate. It is concluded that eukaryote ribosomes are heterogeneous and are subject to modification by protein phosphorylation. This modification appears to regulate their ability to participate in protein synthesis.

Protein phosphorylations have been increasingly implicated in the regulation of cellular metabolism. For example, a phosphorylase kinase stimulated by cyclic AMP[1] is involved in regulation of glycolysis in skeletal muscle cells and in liver (DeLange *et al.*, 1968; Sutherland and Robison, 1969). Similarly, phosphorylation regulates the activity of the pyruvate dehydrogenase enzyme complex of beef kidney (Linn *et al.*, 1969). Protein kinases dependent upon cyclic AMP have been found in all examined tissues and in microorganisms (Kuo and Greengard, 1969a,b) and they have been purified from adipose tissue (Corbin and Krebs, 1969), skeletal muscle (Walsh *et al.*, 1968), brain (Miyamoto *et al.*, 1969), liver (Langan, 1968a,b), and *Escherichia coli* (Kuo and Greengard, 1969b). Insulin stimulates a protein kinase in mammary epithelial cells (Turkington and Riddle, 1969). Prolactin stimulates the phosphorylation of histone and of

* From the Department of Biochemistry, University of Oregon Medical School, Portland, Oregon 97201. *Received May 22, 1970.* This research was supported in part by Grant P-539 from the American Cancer Society and by U. S. Public Health Service Grant CA 11347.

[1] Abbreviation used is: cyclic AMP, adenosine 3′,5′-monophosphate.

nonhistone nuclear proteins in these mammary cells and induces the synthesis of specific milk proteins (Turkington and Riddle, 1969). Similarly, glucagon and insulin stimulate histone phosphorylation in rat liver cells (Langan, 1968a). As these latter examples would indicate, the possible involvement of chromosomal protein phosphorylation in gene regulation has been suspected for some time and there are many reports of regulatory phosphorylation of chromosomal proteins including protamines (for example, Marushige *et al.*, 1969; Langan, 1968b; Kleinsmith *et al.*, 1966a,b; Benjamin and Goodman, 1969).

Because protein phosphorylations do play such a central role in metabolic regulation, it seemed important to analyze for phosphoproteins on eukaryote ribosomes. Rabbit reticulocytes were chosen for this investigation because they lack nuclei and are not engaged in nucleic acid synthesis. These cells, therefore, provide a simple system for analysis of ribosome phosphorylation with [^{32}P]phosphate. It is demonstrated here that several of the reticulocyte ribosomal proteins are indeed phosphoproteins and that phosphoproteins occur also in the ribosomes of various other eukaryote cells. This work provides evidence that eukaryote ribosomes are heterogeneous and are subject to chemical modifications after their assembly into functional units. Furthermore, it appears that this chemical modification performs a regulatory role in controlling protein synthesis.

Materials and Methods

Solutions. Two buffers with different ionic strengths were used for preparation of ribosome fractions: buffer A is 0.01 M Tris-HCl–0.01 M KCl–0.0015 M $MgCl_2$, pH 7.4; and buffer B is 0.01 M Tris-HCl–0.25 M KCl–0.01 M $MgCl_2$, pH 7.4.

The reticulocytes were washed with cold physiological salt solution which contains 0.13 M NaCl–0.005 M KCl–0.0075 M $MgCl_2$.

Enzymes. Pronase was obtained from Calbiochem, electrophoretically pure pancreatic ribonuclease from Worthington, and *E. coli* alkaline phosphomonoesterase (APF) from Worthington.

Preparation of Materials. Rabbits were made anemic by 7 daily injections of 15 mg of phenylhydrazine and were bled 1 day after the final injection. The blood cells, examined by staining with brilliant cresyl blue, routinely contained over 80% reticulocytes. The cells were washed three times by centrifugation with ice-cold saline solution, the buffy coat containing most of the leukocytes was removed, and the cells were resuspended at 1×10^9 cells/ml in nutrient medium as described by Hori and Rabinovitz (1968). The medium was supplemented with 5% normal rabbit serum and 5 μg/ml of actinomycin D (Calbiochem) and was sterilized by filtration through a Millipore membrane.

The cell suspension was rotated gently in a water bath at 37° for 15 min before the addition of 50 μCi/ml of carrier-free [^{32}P]phosphate (New England Nuclear Corp. NEX-054). Incorporation at 37° was continued for 60 min unless otherwise mentioned. The cells were then chilled by dilution with 4 volumes of cold saline solution.

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. One milliliter of buffer A was used to redissolve the ribosomes obtained from 1 ml of reticulocytes. This ribosome precipitation procedure does not cause any breakdown or loss of polyribosomes (Warner and Rich, 1964) and it was routinely performed to increase the ribosome concentration and to remove hemoglobin and other contaminating materials. Control experiments showed also that the precipitation procedure does not cause any artifactual association of phosphorylated compounds with ribosomes.

In some experiments, ribosomes were further purified from the resulting crude ribosome fraction by sedimentation through a cushion of 15% sucrose, rather than through a sucrose gradient. In this case, a 1-ml solution containing ribosomes was diluted to 9 ml with either buffer A or B. This was layered over a 1.5-ml solution of 15% sucrose dissolved in the same buffer and placed into a centrifuge tube for the Spinco 40 rotor. Centrifugation was at 40,000 rpm for 80 min. The clear pellet of ribosomes was used for further studies.

Sucrose gradient centrifugation at 2° was used for preparation of polyribosomes, single ribosomes, subribosomal particles, and supernatant fractions, as described elsewhere (Kabat, 1970). Ribonuclease-free sucrose (Schwartz Bio-Research) was dissolved in buffer A or B. In a few experiments, where indicated, polysome and single ribosome fractions were obtained after sedimentation into 29-ml linear 15–30% sucrose gradients for 2.5 hr at 25,000 rpm in the SW 25.1 Spinco rotor. However, the single ribosomes prepared in this manner are contaminated with subribosomal particles. In all experiments unless otherwise mentioned, the ribosome fractions were prepared by centrifugation into 29-ml isokinetic (Noll, 1967) 15–30% sucrose gradients for 19 hr at 18,000 rpm in the SW 25.1 Spinco rotor. This method is described in detail elsewhere (Kabat, 1970). Figures 1–3 show typical examples of this isokinetic type of sucrose gradient fractionation. In the preparative conditions (Figure 3), the polyribosomes are pelleted in the tubes. However, the single ribosomes (76 S) and subribosomal particles (64 and 44 S) are well separated from each other and from the more slowly sedimenting supernatant fraction (3–30 S). After centrifugation, the gradients were pumped through a Gilford Model 222 spectrophotometer equipped with a bubble-free flow cell (designed by Mr. P. Lu, MIT, Cambridge, Mass.), and the various ribosome fractions were collected into ice-cooled flasks. The single ribosomes, subribosomal particles, and supernatant fractions were then pelleted by centrifugation for 16 hr at 40,000 rpm in the Spinco 40 rotor.

Measurement of Radioactivity. In some cases the gradients were collected into 25-drop fractions which were assayed for radioactivity. In this case the fractions were given 50 μg/ml of carrier bovine serum albumin and were then precipitated at 0° for 30 min by adjusting them to 10% trichloroacetic acid. Unless otherwise mentioned, the fractions were heated to 90° for 20 min to hydrolyze nucleic acids and then chilled in ice for 30 min. The fractions were then filtered onto 0.45 μ Millipore membranes, and the membranes were washed thoroughly with 5% trichloroacetic acid before they were glued onto planchets for radioactivity measurement in a low-background gas-flow counter. The background was 1.5 cpm.

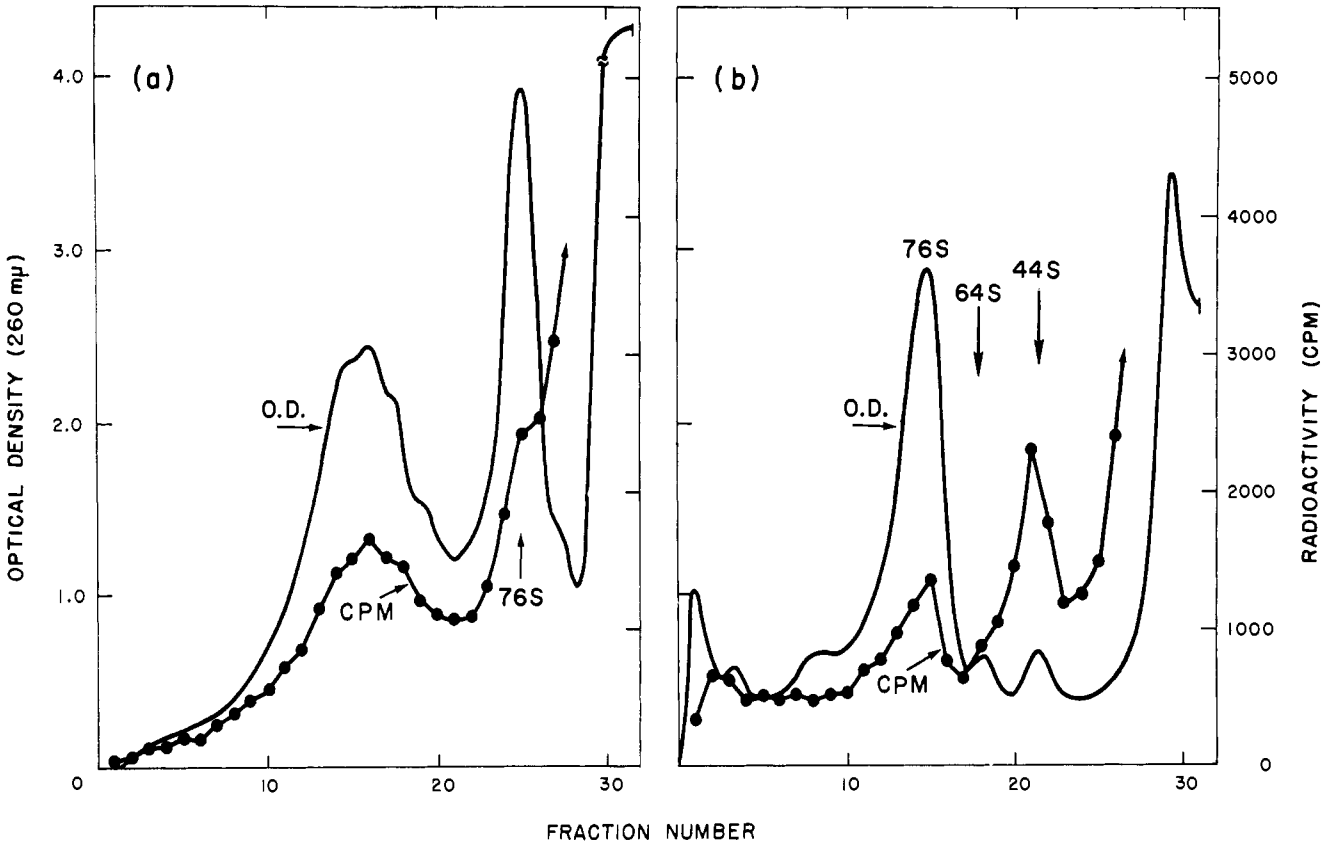

FIGURE 1: Sucrose gradient sedimentation of [^{32}P]phosphate-labeled reticulocyte ribosomes. Incorporation into the reticulocyte suspension was for 60 min. In a, the crude ribosome extract was sedimented in a 29-ml linear 15–30% sucrose gradient in buffer A in the SW 25.1 rotor at 25,000 rpm for 2.5 hr. In b, the extract was sedimented in the SW 25.1 rotor in a 29-ml isokinetic 15–30% sucrose gradient at 17,500 rpm for 20 hr. The gradient fractions were precipitated with cold 10% trichloroacetic acid and were then filtered onto membranes for radioactivity measurement.

Electrophoresis of Ribosome Constituents. RNA was extracted in high yield from ribosome preparations as described by Oda and Joklik (1964) and was analyzed by electrophoresis in polyacrylamide gels (Loening and Ingle, 1967; Kabat, 1970). Polyacrylamide gels were all 6 cm long × 6 mm internal diameter and were prepared at 4 or 8% concentrations as described by Loening (1967). Gels to be used for transverse sectioning were prepared with 10% glycerol as described by Weinberg *et al.* (1967). The glycerol facilities sectioning of frozen gels.

The electrophoresis buffer was 0.036 M Tris–0.030 M NaH_2PO_4–0.001 M EDTA–0.6% sodium dodecyl sulfate (Loening and Ingle, 1967; Kabat, 1970). The ribosome fractions were dissolved in electrophoresis buffer containing 5% sucrose to give an optical density at 260 mμ of 60 units/ml, and aliquots containing 10 OD_{260} of ribosomes were applied to the polyacrylamide gels. This amount of the ribosome fractions contained approximately 35 μg of protein. On the other hand, 60 μg of supernatant fraction protein was used for electrophoretic analysis of this fraction. Protein concentrations were measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The supernatant fraction was only occasionally analyzed. Electrophoresis of 4% gels was for 85 min, whereas the 8% gels were run for 90 min at 5 mA/gel.

After electrophoresis, gels were scanned for absorbance at 260 mμ with a Gilford Model 222 spectrophotometer equipped with a Gilford linear transport apparatus. A Brinkmann gel slicer was used for transversely sectioning gels into 1-mm sections. Pairs of adjacent gel sections were dried together onto aluminum planchets and were used for radioactivity measurement.

Four longitudinal sections for autoradiography were cut from 4 or 8% polyacrylamide gels as described by Fairbanks *et al.* (1965). The two central longitudinal gel sections from 4% gels were placed onto high-wet strength paper (Schleicher and Schuell, 497) which were dried in an oven at 70°. The central longitudinal slices from 8% gels were dried onto blotter paper (Rochester Paper Co., Rochester, Mich.) using a special vacuum apparatus (Fairbanks *et al.*, 1965) under infrared lamps at 55°. The longitudinal gel sections dried onto the paper backing were covered with single-coated X-Ray film (Kodak SB-54) until the films were sufficiently exposed (3–30 days). The exposed film was then developed with X-ray developer.

Gels were occasionally stained for protein with coomassie blue dye (Mann Research Laboratories) before they were longitudinally sectioned for autoradiography. The gels were washed at 25° for 15 hr with several changes of 12.5% trichloroacetic acid. This removed the sodium dodecyl sulfate

from the gels. They were next stained for 10 hr with 0.2% coomassie blue dye dissolved in 10% acetic acid–20% methanol in H_2O. The gels were destained by rinsing with 10% acetic acid–20% methanol in H_2O.

Hydrolysis of Ribosome Fractions. Ribosome fractions were hydrolyzed in 6 M HCl at 105° in sealed evacuated ampules for 7 hr. The samples were then dried *in vacuo*. The dried hydrolysates were dissolved in the paper electrophoresis solution (2.5% formic acid–7.8% acetic acid, pH 1.85) and were applied to prewet Whatmann No. 3MM paper. Control samples of [^{32}P]orthophosphate, phosphoserine, and phosphothreonine were also analyzed. The high-voltage paper electrophoresis was carried out as described by Langan (1968b) in a water-cooled, flat-plate apparatus, kindly made available by Dr. M. Litt. After electrophoresis for 90 min, the paper was dried in an oven at 70° and was then stained for amino acids with a cadmium–ninhydrin stain (Dreyer and Bynum, 1967). The paper was covered with single-coated X-Ray film (Kodak SB-54) in the darkroom for 20 days before film development.

Results

Phosphorylation of Rabbit Reticulocyte Ribosomes. During incubation of rabbit reticulocyte suspensions at 37° with [^{32}P]phosphate, there occurs an extensive labeling of the polyribosomes, single ribosomes, and subribosomal particles. Actinomycin D has no influence on the labeling; however, it was routinely included in the incubation medium to suppress any DNA-dependent RNA synthesis occurring in leukocytes. The buffy coat containing most of the leukocytes was routinely removed from the reticulocytes before the incubation with the [^{32}P]phosphate. Figure 1 shows a sucrose gradient sedimentation analysis of a ^{32}P-labeled reticulocyte lysate. The cell lysis and the sedimentation were carried out in low ionic strength buffer A (see Methods). All of the ribosome fractions are highly radioactive, and there is a pronounced peak of labeling on the native 44S subribosomal particle.

Evidence That the Phosphorylated Ribosome Constituents Are Phosphoproteins. To determine whether the incorporated phosphate was associated with RNA, with phospholipids, or with proteins, ^{32}P-labeled polyribosomes were precipitated with cold 10% trichloroacetic acid. The precipitate was washed with cold trichloroacetic acid and it was then subjected to a sequence of procedures which remove RNA and phospholipids. As can be seen in Table I, only 14% of the radioactivity was converted into soluble material by heating in 10% trichloroacetic acid at 90° for 20 min. On the contrary, 98–100% of purified RNA or DNA is solubilized by this procedure. Furthermore, extraction of the remaining precipitate with a series of nonaqueous solvents also solubilized only a small percentage (11%) of radioactivity. At least 75% of the phosphate incorporated into polyribosomes is associated with the residue protein fraction. This is likely to be a minimum estimate of the phosphoprotein radioactivity because of the losses occurring during the solubilization procedure (see Table I).

Warner and Pène (1966) have shown that some proteins adhere nonspecifically to ribosomes in low ionic strength solutions but can be removed by washing in solutions containing 0.20 M NaCl. Studies were therefore done to determine whether some of the radioactive phosphorylated material was extracted from ribosomes in a high ionic strength solution. In the experiment shown in Figure 2, the sucrose gradients were centrifuged long enough to pellet a portion of the polysomes and to partially resolve the subribosomal region of the gradient. Figure 2a shows the sedimentation into low ionic strength buffer A, whereas Figure 2b shows the sedimentation into the higher ionic strength buffer B. The higher ionic strength buffer caused a reduction of the specific activity (cpm/OD) of the polysomes and single ribosomes by approximately 40%. Sedimentation for a longer time is shown in Figure 3a,b, and it is apparent from these gradients that a large proportion of the radioactivity associated with the native 44S subribosomal particles is extracted with the B buffer. It is shown below that certain of the radioactive materials on ribosomes are eluted with buffer B, whereas other

TABLE I: Solubility Characteristics of Ribosomal Phosphorylated Constituents.[a]

Solubilization Procedure	Solubilized Radioactivity (cpm)	Percentage of Total
1. 10% Trichloroacetic acid at 90° for 20 min	6,860	14.0
2. Nonaqueous solvent extracts	5,280	10.9
3. Final residue	36,980	75.1

[a] Ribosomes were from reticulocytes which had been labeled for 60 min with [^{32}P]phosphate. After pH 5 precipitation, the ribosomes were dissolved in high ionic strength buffer B and they were then sedimented through a 1.5-ml sucrose cushion in the Spinco 40 rotor (see Methods). The radioactive ribosomes (2 mg) were dissolved in buffer B at 0° and were precipitated by adjusting the solution to 10% trichloroacetic acid. The precipitate was washed several times by sedimentation in 10% trichloroacetic acid at 0°. The supernatant resulting from the final sedimentation contained negligible radioactivity. In step 1, the precipitate suspended in 10% trichloroacetic acid was heated at 90° for 20 min. After chilling the tube to 0° for 30 min, the precipitate was again sedimented. The resulting supernatant was removed and a portion was dried onto a glass fiber filter for radioactivity measurement. In step 2, the precipitate resulting from step 1 was extracted with a series of nonaqueous solvents as described by Davidson *et al.* (1951). The nonaqueous solvent extracts were pooled and an aliquot was used for radioactivity measurement. The final residual precipitate was solubilized in step 3 by hydrolyzing for 2 hr with 6 M HCl at 105° in a sealed ampule. An aliquot was then plated for radioactivity measurement. The recovery of radioactivity in this experiment was 86% of the 10% trichloroacetic acid precipitable radioactivity initially estimated to be present in the ribosome solution. The percentages of the recovered radioactivity are indicated in the Table. The small radioactivity loss occurs during the washing steps and is likely to be greatest from the final residue fraction since this is the last step in the procedure.

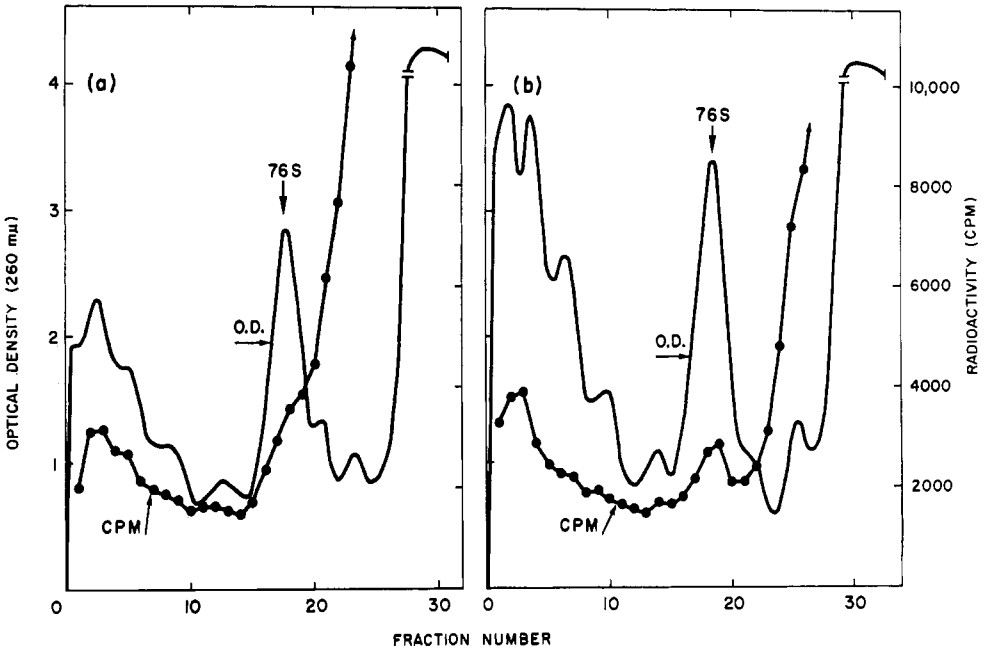

FIGURE 2: Sucrose gradient sedimentation of [^{32}P]phosphate-labeled ribosomes in low ionic strength buffer A and in higher ionic strength buffer B. The isokinetic gradients were centrifuged at 15,000 rpm for 20 hr in the SW 25.1 rotor. After adjusting to 10% trichloroacetic acid, the fractions were heated at 90° for 20 min before filtering. In a, 0.7 ml of crude extract was sedimented in the buffer A sucrose gradient. In b, approximately 1.2 ml of the same extract was sedimented in the buffer B sucrose gradient.

specific components remain firmly attached to the ribosomes in these conditions. Thus, we are observing a quantitative extraction of certain components rather than a partial extraction of one or more materials. Furthermore, one highly radioactive phosphoprotein is eluted from the 44S subribosomal particles by washing in buffer B.

The phosphorylated components of ribosomes were next analyzed by electrophoresis in polyacrylamide gels containing 0.6% of the anionic detergent sodium dodecyl sulfate. As shown also by Warner (1966), the ribosomal constituents

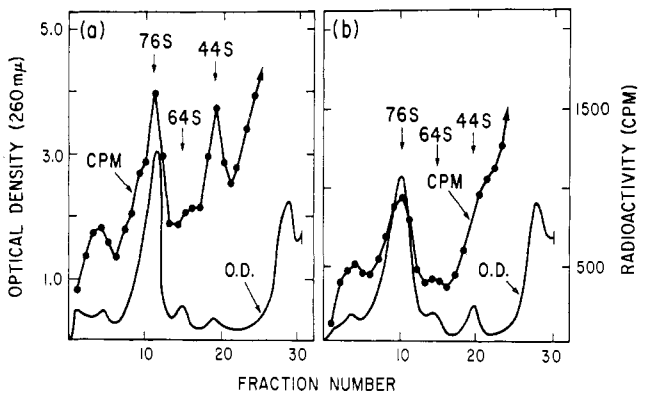

FIGURE 3: Sucrose gradient sedimentation of [^{32}P]phosphate-labeled ribosomes in low ionic strength buffer A and in higher ionic strength buffer B. The isokinetic gradients were centrifuged at 18,000 rpm for 19 hr in the SW 25.1 rotor. The fractions were heated in 10% trichloroacetic acid at 90° for 20 min before filtering. Crude cell extract (1 ml) was sedimented in both gradients. (a) The gradient is in low ionic strength buffer A. (b) The gradient is in high ionic strength buffer B.

dissaggregate fully in these conditions and they electrophorese independently in the gels. The protein–dodecyl sulfate complexes are polyanionic, like RNA, and they migrate toward the positive electrode. No detectable radioactivity migrates to the negative electrode. Various workers have shown that the electrophoretic mobilities of protein–dodecyl sulfate complexes are inversely proportional to the logarithms of the polypeptide chain molecular weights (Dunker and Reuckert, 1969; Shapiro *et al.*, 1967; Kiehn and Holland, 1970) and this has been confirmed in the conditions of these experiments.

The radioactive components in purified RNA were compared with the components of the unextracted ribosomes (Figure 4). The ribosomes had been sedimented through high ionic strength buffer B to remove any nonspecific contaminants. The same amount of RNA was applied to both gels. After electrophoresis the gels were scanned for absorbance at 260 mμ and were then sectioned for radioactivity measurement. The absorbance tracings show the distribution of RNA components in the gels and the 4S and 5S RNA peaks are identified in Figure 4b. The absorbance patterns are very similar to those previously reported by Loening (1967). The 18S and 28S ribosomal RNA molecules penetrate only slightly into the gels and are not resolved. Comparison of the optical density tracings in Figure 4a,b shows clearly that the nigration of RNA was the same in the two gels. Therefore, the migration of RNA must be independent of protein, suggesting that the ribosome dissociation in the electrophoresis solution is complete. Ribonuclease digestion did not alter the migration of the radioactive components. Furthermore, as seen in Figure 4, the radioactive components are almost completely absent from the extracted RNA. There are four main radioactive peaks in the ribosomes (P, Si, F,

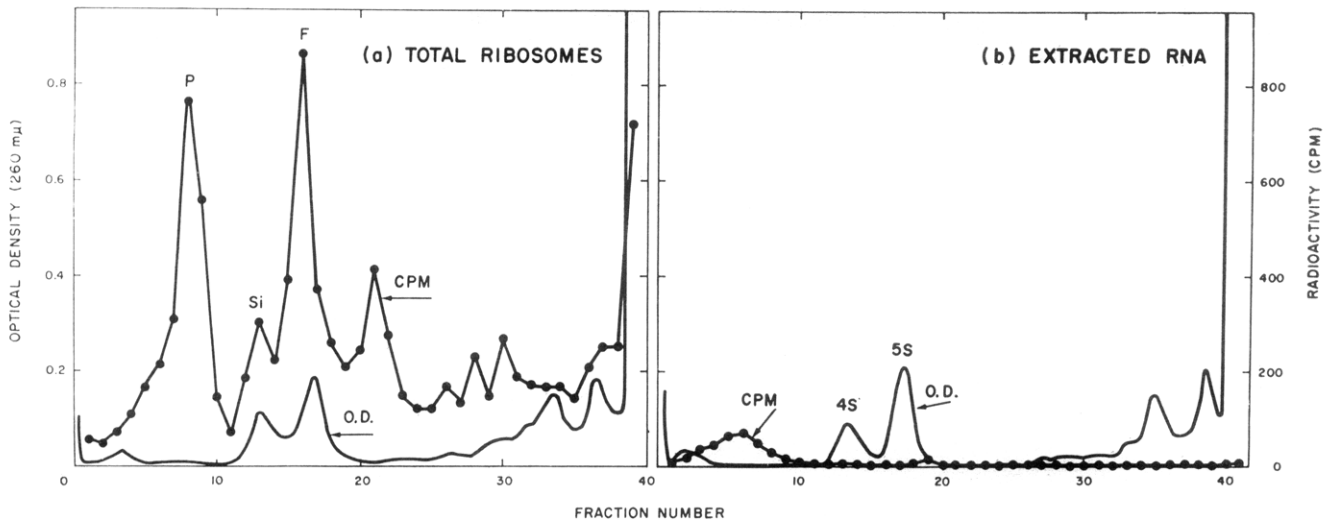

FIGURE 4: Polyacrylamide gel electrophoresis of ^{32}P-labeled total ribosomes and of extracted RNA. The ribosomes were purified by sedimentation through a sucrose cushion (see Methods) in buffer B. The gel concentration is 4%. After electrophoresis, the gels were scanned for absorbance at 260 mμ and were then sectioned into 1-mm slices for radioactivity measurement. Two adjacent gel slices were counted together on the same planchet. The same amount of RNA (30 μg) was analyzed on each of the gels. Electrophoresis was toward the left: (a) total ribosomes; (b) extracted RNA. The P, Si, and F radioactive components are present in the total ribosomes but are absent from the extracted RNA.

and a trailing component) and they are all absent from extracted RNA.

Although the phosphorylated ribosome constituents are separated by greater distances after electrophoresis in more concentrated polyacrylamide gels, no additional radioactive components were resolved in these denser gels. Some results obtained with more concentrated 8% gels will be described below. The dilute 4% gels were used in most of these experiments because all of the radioactive components were adequately resolved in them. Furthermore, longitudinal sections of the 4% gels can be more readily processed for autoradiographic analysis since they adhere strongly to high wet strength paper during drying and they dry as a thin film. On the contrary, longitudinal sections of the more concentrated gels tend to crack, shrink, and to curl badly during drying.

In Figure 5 is shown an autoradiographic analysis of the phosphorylated ribosome components, resolved by electrophoresis in 4% polyacrylamide gels. The radioactive lysates were separated into two portions and the ribosomes were sedimented through either low ionic strength buffer A or high ionic strength buffer B. A number of radioactive bands are extracted from the ribosomes by washing in buffer B. However, the P, Si, and F components are present in similar levels in both ribosome preparations. Several additional radioactive molecules also remain bound to the ribosomes prepared in buffer B.

That the major phosphorylated ribosome constituents are proteins is further supported by the following experiment. A reticulocyte suspension was incubated with [^{32}P]phosphate for 60 min and the polysomes, single ribosomes, subribosomal particles, and supernatant fractions were purified from the cell lysate in sucrose gradients containing low ionic strength buffer A. A portion of the cells was incubated for the last 20 min with 0.03 M NaF and single ribosomes were isolated from these cells. Fluoride ion is a reversible inhibitor of protein synthesis which causes an accumulation of single ribosomes due to conversion from polysomes and from subribosomal particles (Colombo *et al.*, 1968). Each of these ribosome fractions was then dissolved in the electrophoresis buffer at 3 mg/ml and aliquots containing 50 μg of RNA were

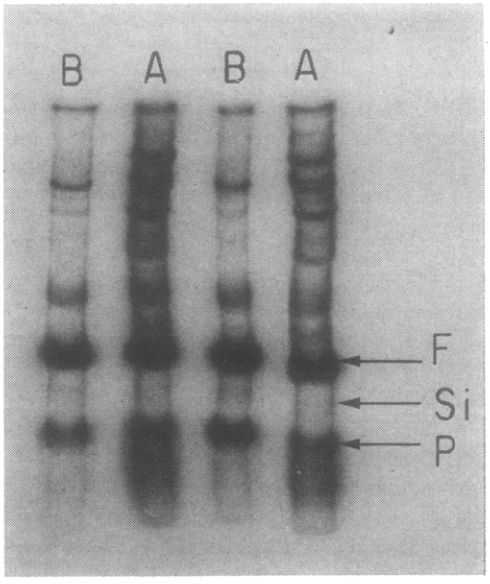

FIGURE 5: Autoradiographic analysis of polyacrylamide gel electrophoresis of ^{32}P-labeled ribosomes which had been purified either in low ionic strength buffer A or in high ionic strength buffer B. The gel concentration is 4%. The migration is toward the bottom. After electrophoresis, the gels were longitudinally sectioned into 4 slices and the two internal sections were dried on high wet strength paper. The dried gel sections were used for autoradiographic development of X-Ray film. Both of the internal gel sections are shown in order to illustrate the reproducibility obtained. Several radioactive bands are extracted from the ribosomes with the high ionic strength buffer B. The P, Si, and F bands are indicated with arrows. The Si band is only weakly labeled in this experiment.

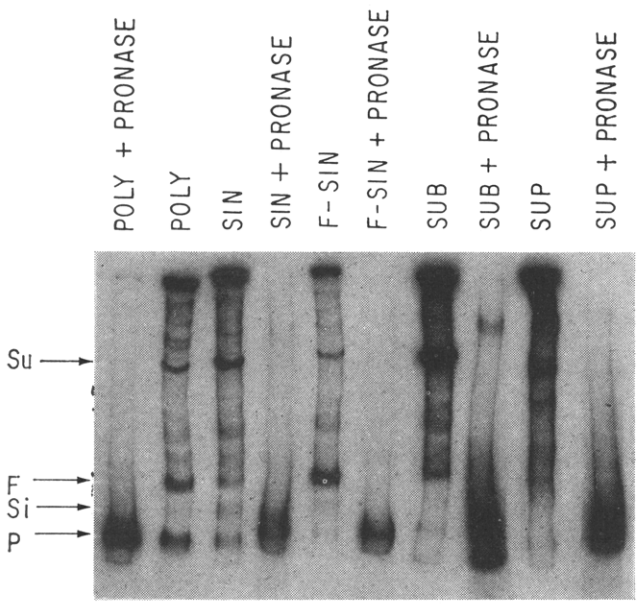

FIGURE 6: Electrophoresis in polyacrylamide gels of ^{32}P-labeled ribosome fractions and the proteolytic degradation of the radioactive components with pronase. The digestion conditions are described in the text. The migration positions of the P, Si, F, and Su radioactive components are indicated with arrows. Electrophoresis was toward the bottom. The gel concentration is 4%. The ribosome fractions were purified in buffer A sucrose gradients. The ribosome fractions are indicated above the gels and are in the following order, from left to right: polysomes treated with pronase; polysomes; single ribosomes; single ribosomes treated with pronase; single ribosomes from NaF-treated cells; single ribosomes from NaF-treated cells digested with pronase; subribosomal particles; subribosomal particles digested with pronase; supernatant fraction (60 μg); supernatant fraction treated with pronase.

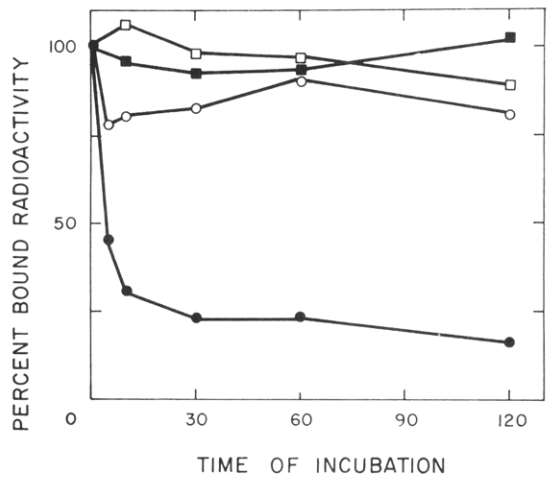

FIGURE 7: Removal of [^{32}P]phosphate from ribosomes with *E. coli* alkaline phosphomonoesterase. The ^{32}P-labeled ribosomes were purified by sedimentation through a sucrose cushion in buffer B (see Methods). The graph shows the radioactivity which remains as a precipitate in 10% trichloroacetic acid acid after heating at 90° for 20 min. Before precipitation the ribosomes were incubated at 1 mg/ml at 37° in the following conditions: (—●—●—) 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 7.6 not containing any enzyme; (—■—■—) 1 M hydroxylamine–1 M succinic acid, pH 5.5; (—□—□—) 1 M succinic acid, pH 5.5.

treated with 10 μg of pronase. It is shown elsewhere that pronase is very active in sodium dodecyl sulfate solutions and that RNA is not degraded in these conditions (Kabat, 1970). Control portions of the ribosomes were incubated without the protease. The samples were then subjected to electrophoresis. The autoradiographic analysis of the gels is shown in Figure 6 and leads to the following conclusions. (i) All of the major phosphorylated components on all of the ribosome fractions are converted by pronase digestion into rapidly migrating materials, suggesting that they are proteins. This conclusion is not clear for the Si and P components because the phosphopeptides resulting from pronase digestion electrophorese in this region of the gels. However, a similar analysis in 8% gels showed clearly that Si is a protein since it is definitely degraded by proteolysis. The Si band also stains with protein dyes as shown below. However, as will be seen below, it is likely that the P material is not a protein. (ii) The various ribosome fractions all contain the F phosphoprotein as well as several other phosphoproteins. However, each of the ribosome fractions contains a distinguishing phosphorylated component which is generally present only to a minor extent in the other fractions. The polysomes, single ribosomes, and subribosomal particles contain specifically the P, Si, and Su phosphorylated constituents, respectively. Further studies of this specificity are described below. (iii) The supernatant fraction contains several phosphoproteins, but it is lacking in the major ribosome phosphorylated components (the P, Si, F, and Su bands).

Other workers have often found phosphate combined with proteins, either as phosphate esters of serine and threonine residues (Langan, 1968b) or in combination with carboxyl groups as acyl phosphates (Martonosi, 1969). Acyl phosphates are anhydrides and are susceptible to aminolysis at pH 5.5 with hydroxylamine, whereas phosphate esters are hydrolyzed with alkaline phosphatase (Ingles and Dixon, 1967). Ribosomes labeled with [^{32}P]phosphate were therefore incubated with these reagents and the results are shown in Figure 7. Incubation with alkaline phosphatase caused a rapid loss of radioactivity from the ribosomes, suggesting that at least 75% of the phosphate was present as phosphate esters. It is possible that additional phosphate esters in ribosomes are inaccessible to the monoesterase. On the contrary, the studies with hydroxylamine indicate that acyl phosphates are present in only negligible levels on the ribosomes. The ribosomal radioactivity is also completely hydrolyzed by heating at 90° for 15 min in 1 M NaOH, as would be expected for phosphate esters.

Experiments were done to directly search for *o*-phosphoserine and *o*-phosphothreonine residues by hydrolyzing the radioactive ribosomes with HCl and then analyzing the hydrolysate by paper electrophoresis. As is indicated in Figure 8, all of the ribosome fractions contained radioactive molecules which coelectrophoresed with *o*-phosphoserine and *o*-phosphothreonine. The hydrolysates also contained considerable levels of radioactive orthophosphate. Although some of this would certainly have arisen from hydrolysis of phosphoserine, it is possible that some orthophosphate was produced from another compound which hydrolyzed during the HCl treatment. In any case, these results strongly indicate that *o*-phosphoserine and *o*-phosphothreonine residues occur in the polypeptide chains of reticulocyte ribosomes.

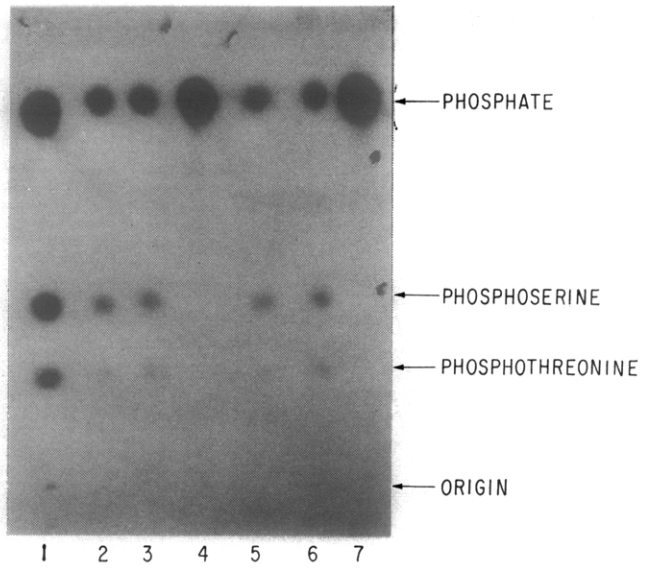

FIGURE 8: Paper electrophoresis of hydrolysates of ^{32}P-labeled ribosome fractions. The radioactive spots are located by autoradiography on the exposed X-Ray film. The ribosome fractions were purified in sucrose gradients made with high ionic strength buffer B, and were hydrolyzed in HCl (see Methods). The samples analyzed are as follows: (1) polysomes, (2) single ribosomes, (3) single ribosomes from NaF-treated cells, (4) [^{32}P]orthophosphate control, (5) subribosomal particles, (6) supernatant fraction, (7) [^{32}P]orthophosphate control. All of the hydrolysates contain radioactive compounds which coelectrophorese with phosphoserine and phosphothreonine. Approximately 12,000 dpm of polysomes were analyzed, whereas approximately 4,000 dpm of the other fractions were analyzed. The direction of electrophoresis is toward the positive electrode.

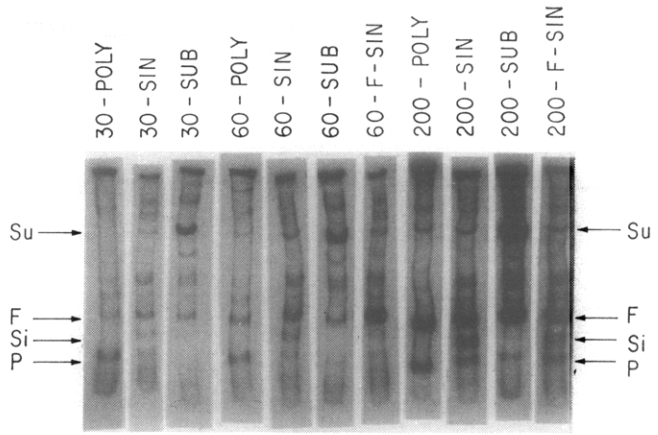

FIGURE 9: Electrophoresis in polyacrylamide gels of ribosome fractions from cells labeled for various times with [^{32}Pp]hosphate. The radioactive bands were visualized by autoradiography. The gel concentration is 4%. The ribosome fractions were purified in buffer A sucrose gradients. The ribosome fractions are written above the gels. The reticulocytes were incubated with ^{32}P-phosphate for 30 min, 60 min, or 200 min at 37° before chilling the cells. At 40 min a portion of the cells was adjusted to 0.03 M NaF and single ribosomes were isolated from this cell suspension at 60 min and 200 min. The latter single ribosomes are indicated in the figures as 60-F-sin and 200-F-sin, respectively. The direction of electrophoresis is toward the bottom. The dried gel sections were placed together under the same X-Ray film. The photograph of the autoradiogram was cut into separate gels which were aligned more accurately to facilitate comparison of phosphoproteins.

Correlation of Ribosome Phosphorylation with the Ribosomal Subunit–Polyribosome Cycle of Protein Synthesis. The purpose of the next experiments was to determine whether the phosphorylation of ribosomes is related to their function in protein synthesis. Labeled [^{32}P]ribosomes were fractionated in sucrose gradients into polysomes, single ribosomes, and subribosomal particles, and the phosphorylated components were analyzed by electrophoresis in polyacrylamide gels. Occasionally, a supernatant fraction was also utilized. Routinely, one portion of labeled reticulocyte suspension was treated briefly with 0.03 M NaF in order to convert polysomes and subribosomal particles into single ribosomes. The assumption of these experiments is that any distinctive localization of phosphorylated constituents on only certain of these ribosome fractions is likely to be of functional significance. On the other hand, any phosphoproteins nonspecifically adsorbed to the ribosomes or unrelated to ribosome function are likely to be randomly distributed among the ribosome fractions at all stages of the ribosome cycle of protein synthesis. A typical experiment was presented in Figure 6 where it was shown that each of the ribosome fractions has a distinguishing phosphorylated constituent. The P, Si, and Su phosphorylated components occur selectively in polysomes, single ribosomes, and subribosomal particles, respectively. The absence of these materials (and of the F phosphoprotein) from the supernatant is evidence that they are true ribosome constituents.

A time course for the incorporation is shown by autoradiography in Figure 9. However, transverse sectioning of gels was also done to get a quantitative check of the results. At various times (30, 60, and 200 min) after beginning the reticulocyte incubation with [^{32}P]phosphate, samples were removed for the preparation of the ribosome fractions. One portion of cell suspension was adjusted to 0.03 M NaF at 40 min. Single ribosomes were isolated from this latter portion of cells at 60 and 200 min after beginning the incorporation. As can be seen in Figure 9, the radioactivity associated with all of the ribosome components continues to increase throughout the incubation. The following conclusions, some of which are apparent also in Figure 6 and in other studies described below, are demonstrated in this experiment: (i) the P band is a specific polysome constituent. After long times of cell incubation there is usually some breakdown of polysomes, presumably due to nucleolytic degradation of mRNA, so that some P-band material sediments in the single ribosome fraction. However, this breakdown occurs to a minor extent. It is also known that single ribosomes in reticulocytes are contaminated with a low level of monoribosomes (*i.e.*, mRNA-ribosome complexes which contain by chance only one ribosome) (Lamfrom and Knopf, 1964), and presumably these particles would also contain the P component. The single ribosomes which accumulate in NaF-treated reticulocytes lack the P band. Therefore, conversion of polysomes into single ribosomes by NaF is coupled with a concerted loss of P band radioactivity from the ribosomes. (ii) It can be clearly seen that the Si phosphoprotein is specifically localized on the single ribosomes. Furthermore, phosphorylation of Si protein does not obligatorily accompany the single ribosome accumulation which accurs in NaF. Rather, the Si band in the fluoride-treated cells has an approximately twofold reduced specific radioactivity (cpm/OD

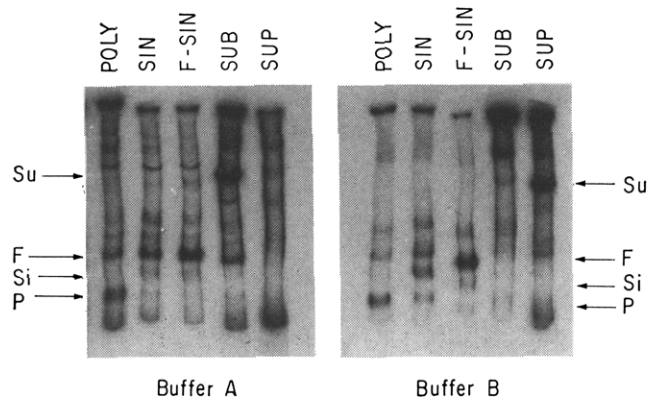

FIGURE 10: Polyacrylamide gel electrophoresis of ^{32}P-labeled ribosome fractions isolated in buffer A or in buffer B sucrose gradients. Radioactive bands are visualized by autoradiography. The experiments were done on separate days using different rabbits. The labeling times were 60 min. The Si band is more highly labeled in the experiment done in buffer B whereas the F protein is only weakly labeled in these ribosomes. This variation is due to the use of different reticulocyte samples. The Su protein is eluted from the subribosomal particle in buffer B and sediments in the supernatant region of the sucrose gradients.

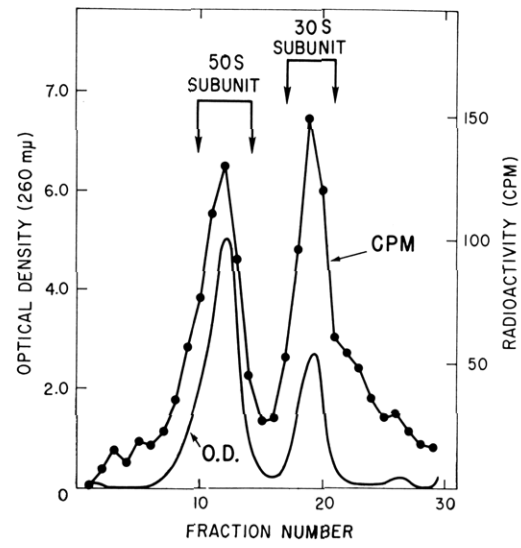

FIGURE 11: Sucrose gradient sedimentation of ^{32}P-labeled ribosome subunits. The reticulocytes were labeled with 10 μCi/ml of [^{32}P]-phosphate for 60 min. Ribosomes were pelleted through a sucrose cushion in buffer B (see Methods) The ribosomes were then dissociated into subunits in 0.01 M Tris-HCl–0.01 M NaCl–0.01 M EDTA, pH 7.4, and were sedimented in 29-ml 15–30% linear sucrose gradients in this same buffer at 25,000 rpm for 17 hr at 5° in the Spinco SW 25.1 rotor (Warner and Pène, 1966; Knight and Darnell, 1967). Sedimentation is toward the left. The fractions were treated with 10% trichloroacetic acid at 90° for 20 min before they were filtered onto membranes.

of ribosomes) compared with the control single ribosomes. This is undoubtedly due to dilution of the radioactive single ribosomes with ribosomes unlabeled in Si which are converted from polysomes. (iii) The Su phosphoprotein is specifically localized on subribosomal particles and is heavily radioactive. (iv) The F phosphoprotein is present on all ribosome fractions. Its phosphorylation is increased severalfold in the single ribosomes from the cells treated with 0.03 M NaF. Further study of this increase is described below. (v) Although the phosphorylations of the different bands tends to increase in parallel during incubation, the P band is slightly anomalous in that its phosphorylation level is relatively high after the shortest incubations. These conclusions are all based both on the autoradiographic results (Figure 9) and on the results obtained by counting the slices of transversely sectioned gels.

The previously described experiments with ribosomal fractions (Figures 6 and 9) were done with ribosomes isolated in low ionic strength buffer A. The same specific localization of phosphorylated components has been fully substantiated in many independent experiments. The only variability observed is that the extents of Si and F phosphorylations differ in different reticulocyte samples. In ribosomes sedimented into high ionic strength buffer B, however, the Su phosphoprotein is reproducibly absent from the subribosomal particles. Figure 10 shows a comparative electrophoretic analysis of ribosome fractions isolated from sucrose gradients made either with A buffer or with B buffer. These two experiments were done independently using different rabbits. The subribosomal particle fraction from the B buffer lacks the Su phosphoprotein which is now shifted into the supernatant fraction. This result agrees well with the sedimentation analysis (Figure 3) which showed that the radioactivity on the 44S subribosomal particle is greatly reduced after sedimentation into buffer B. It is concluded that Su phosphoprotein binds firmly to the 44S subribosomal particles in buffer A, but is extracted by gradient sedimentation in buffer B.

A study was also performed which suggests that the ribosomes with phosphorylated Si protein are prevented from attaching to polyribosomes. Single ribosomes which accumulate in 0.03 M NaF contain obviously some phosphorylated Si protein and some ribosomes lacking this radioactive component (Figures 6, 9, 10). Upon removal of NaF, the polysomes again increase in quantity as protein synthesis is resumed, and these "new" polysomes also completely lack the phosphorylated Si protein. Thus, the single ribosomes do not randomly reattach to mRNA after NaF is removed. Rather, the ribosomes with phosphorylated Si protein remain inactive. It will be shown below that Si protein is present in all ribosomes although it is only phosphorylated in single ribosomes. Furthermore, the Si protein is very strongly bound to the larger ribosome subunits.

Localization of Different Phosphoproteins on the Two Subunits of Ribosomes. The following experiments analyze the distribution of the various ribosomal phosphoproteins on the two subunits of ribosomes. Reticulocyte ribosomes which had been labeled for 80 min with [^{32}P]phosphate were pelleted through high ionic strength buffer B. They were then dissolved in a ribosome dissociating buffer which contains EDTA and sedimented in sucrose gradients containing this same buffer (Figure 11). The ribosome subunits sediment in this solution as 50S and 30S particles (Warner and Pène, 1966; Knight and Darnell, 1967). As can be seen in Figure 11, both ribosomal subunits contain radioactive phosphoproteins. Some radioactive material is eluted from the ribosome subunits by the dissociation procedure and sediments near the top of the tubes.

Gradients sedimented in parallel with the gradients of Figure 11 were used for the preparation of 50S and 30S ribosomal

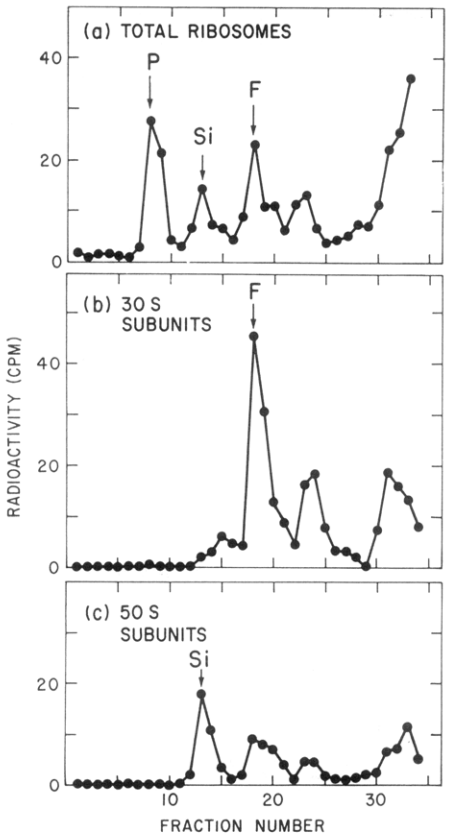

FIGURE 12: Electrophoresis in polyacrylamide gels of ^{32}P-labeled ribosome subunits. The ribosomes were isolated from sucrose gradients (see Figure 11) and were then analyzed by electrophoresis in 8% polyacrylamide gels; 10 OD_{260} of ribosomes was applied to each gel: (a) total ribosomes control; (b) 30S subunits; (c) 50S subunits.

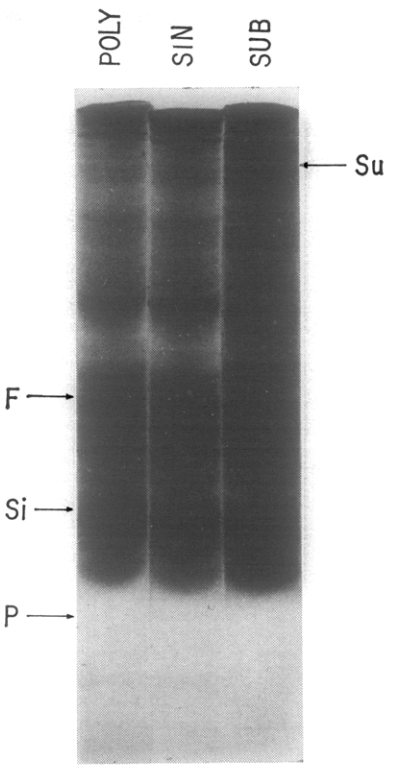

FIGURE 13: Electrophoresis of ribosome proteins in 8% polyacrylamide gels. The ribosome fractions were isolated in buffer A sucrose gradients. The proteins are stained with coomassie blue dye. After photography of the stained gels, they were processed for autoradiography of the ^{32}P-labeled components. The positions of the F, Si, P, and Su bands are indicated with arrows. The P band is extracted during the gel washing with 12.5% trichloroacetic acid (see text). Its migration position was determined on a gel which was not subjected to the washing procedure. Visual observation of the stained gels was necessary to see all of the resolved bands. The photographic visualization is incomplete because of the narrow depth of focus in the camera used and the unavailability of a color filter. The Si and F bands were very clearly seen as sharp bands. The ribosome fractions are indicated above the gels in the following order, from left to right: polysomes, single ribosomes, subribosomal particles.

subunit fractions. The radioactive preparations were then analyzed by electrophoresis in 8% polyacrylamide gels. The gels were transversely sectioned after electrophoresis. A control gel was made using a portion of the ribosomes which had not been separated into subunits. As can be seen in Figure 12, the Si phosphoprotein is specifically localized on the large ribosomal subunit whereas the F-phosphoprotein is specifically located on the small ribosomal subunit. The P band is absent from the purified subunits and presumably it dissociates from the ribosomes during the subunit separation in the EDTA buffers. Similar results were obtained when 0.20 M NaCl was present in the sucrose gradients.

The gels shown in Figure 12 were scanned for absorbance at 260 mμ before they were sectioned for radioactivity measurement. Contrary to the results obtained by Knight and Darnell (1967) with HeLa cell ribosomes, it was very clear that the 5S RNA molecules were almost completely absent from the purified 50S subunits of reticulocyte ribosomes. Knight and Darnell reported that one 5S RNA remains bound to each 50S particle. In my experiments, however, the dissociation of ribosomes in EDTA buffers and their sedimentation in sucrose gradients is sufficient to extract most of this 5S RNA. Transfer RNA is also largely extracted by this procedure. These observations indicate that ribosomal subunits isolated in EDTA buffers lack several of their normal constituents. Accordingly, the absence of P band from the purified subunits (Figure 12) does not indicate that P component is not a physiological ribosome constituent. Warner and Pène (1966) have proposed that all true ribosome constituents are present in isolated subunits sedimented through EDTA buffers, even when the gradients contain 0.20 M NaCl. On the contrary, it is very likely that a number of normal ribosome constituents in addition to 5S RNA are absent from the isolated subunits.

Staining of [^{32}P]Ribosomal Proteins Fractionated in Polyacrylamide Gels. Studies were done in which the [^{32}P]ribosomal proteins, separated in 8% polyacrylamide gels, were stained with dye before they were analyzed for radioactivity by autoradiography. Typical results of gel staining with coomassie blue dye are shown in Figures 13 and 14, and the positions of the major radioactive bands are indicated by arrows. Figure 13 shows the staining analysis of ribosomes isolated in low ionic buffer A, whereas Figure 14 shows analysis of the ribosomes isolated in high ionic strength buffer B. There is a higher background level of staining on the ribosomes isolated

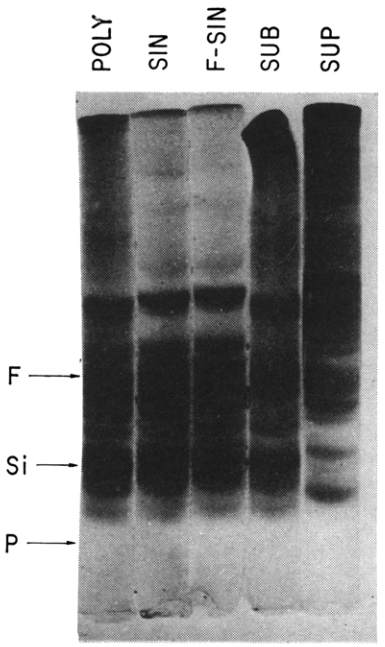

FIGURE 14: Electrophoresis of proteins from ribosome fractions in 8% polyacrylamide gels. The fractions analyzed were isolated from buffer B sucrose gradients. The gels were analyzed as described in Figure 13. The samples analyzed on the gels are in the following order, from left to right: polysomes, single ribosomes, single ribosomes from NaF-treated cells, subribosomal particles, and supernatant fractions.

in buffer A, presumably due to adsorbed proteins in these conditions (Warner and Pène, 1966). As was shown by Warner (1966), a large number of the eukaryote ribosomal proteins are resolved by electrophoresis in sodium dodecyl sulfate–polyacrylamide gels. The subribosomal particle fraction contains all of the proteins which are present in the other ribosome fractions, but the proportions of the different bands are altered because the subunits are not present in 1:1 ratio in the subribosomal fraction. The number of resolved proteins which can be seen by careful visual inspection of the gels is approximately 25 when the utilized ribosomes are isolated in low ionic strength buffer A, whereas about 18–20 protein bands are observed when the utilized ribosomes are isolated from buffer B sucrose gradients. This is approximately as many resolved protein bands as have been obtained using other methods of gel electrophoresis to fractionate eukaryote ribosomal proteins but is fewer than is generally believed to exist in the ribosomes (Wool *et al.*, 1968; Clegg and Arnstein, 1970; Reboud *et al.*, 1969). In agreement with previous results (DiGirolamo and Commarano, 1968), I have also found little if any difference between the stained protein constituents of reticulocyte polysomal and single ribosomes.

The Si and F phosphoproteins appear to coelectrophorese with darkly staining ribosomal proteins. The Su phosphoprotein coelectrophorses with a weakly staining protein present on subribosomal particles (Figure 13) which have not been exposed to high ionic strength solutions. The coelectrophoresis appears to be exact. The autoradiograms were placed on top of the stained longitudinal gel slices after film development and the contours and thicknesses of the stained and autoradiographic bands were seen to correspond precisely. Interpretation of this coelectrophoresis is possible because the electrophoretic mobilities of protein–dodecyl sulfate complexes depend almost exclusively only on the polypeptide chain molecular weights (Dunker and Reuckert, 1969; Shapiro *et al.*, 1967; Kiehn and Holland, 1970). The electrophoretic mobility of a phosphoprotein would be expected not to differ significantly in this gel system from that of the nonphosphorylated polypeptide chain. The following preliminary conclusions, therefore, seem to follow from the gel staining experiments. (a) Although it is more heavily phosphorylated after reticulocyte incubation with NaF (e.g., Figures 6, 9, 10), the F protein appears to be present on all ribosome fractions. The incubation with NaF stimulates the phosphorylation of a constant amount of F protein rather than to cause any increase in quantity of this protein attached to ribosomes. (b) The Si protein also appears to be present on all ribosomes although it is only phosphorylated on single ribosomes (see Figures 6, 9, 10). (c) The Su protein appears to be absent from single ribosomes and from polyribosomes. It is present only in subribosomal particles isolated in buffer A sucrose gradients. (d) The P component migrates faster than any stained ribosome proteins, indicating that it is not a ribosome structural protein.

All of these conclusions based on coelectrophoresis should be regarded as tentative because it is likely that some of the stained protein bands in Figures 13 and 14 are mixtures of several different polypeptides which have the same molecular weights. If the [^{32}P]phosphoproteins were minor components in such a mixed electrophoretic band, then these conclusions would be subject to error. However, none of the major conclusions of this paper rely heavily on these data. These staining results are emphasized here because they help to characterize the ribosome protein fractionation system and because they lead to preliminary conclusions which can be tested by further experiments.

The gel electrophoresis system was also calibrated for approximate molecular weight determinations as described by Dunker and Reuckert (1969), using as standards bovine serum albumin (67,000 daltons), chymotrypsinogen (25,700 daltons), trypsin (23,800 daltons), and hemoglobin (17,500 daltons). The molecular weights of the major ribosomal phosphoproteins are 21,000 daltons, 33,000 daltons, and approximately 70,000 daltons for the Si, F, and Su phosphoproteins, respectively.

Further Studies of the Polysome-Specific Phosphorylated Constituent (P Band). Although the other phosphorylated ribosome constituents are clearly phosphoproteins, none of the cited experiments indicate that P is a protein. It migrates in electrophoresis in the position of pronase-digested phosphoproteins (Figure 6), and this is faster than any major ribosome structural protein (Figures 13 and 14). When polyacrylamide gels containing fractionated ribosome constituents are washed overnight at 25° with 12.5% trichloroacetic acid before staining them with dyes or slicing them longitudinally for autoradiography, there occurs a selective loss of P-band radioactivity from the gels. All of the other radioactive components precipitate in trichloroacetic acid and do not diffuse in the gels. This indicates either that P is soluble in 12.5% trichloroacetic acid or that the phosphate is hydrolyzed and then extracted during the washing procedure. In either case it seems clear that P is not a phosphoprotein. The possibility that the P component is GTP or GDP is currently being investigated in this laboratory.

Effects of NaF on Ribosome Phosphorylation and Investigation of Possible Involvement of Cyclic 3',5'-Adenosine Monophosphate. Because NaF is a widely used inhibitor of protein synthesis which is believed to interfere specifically with initiation of synthesis of the polypeptide chain (Colombo *et al.*, 1968), the specific stimulation of F-protein phosphorylation by NaF (Figures 6, 9, and 10) was analyzed in more detail. Figure 15 shows that NaF causes an accumulation of single ribosomes in reticulocytes which had been labeled with [^{32}P]phosphate. However, as seen in Figure 15c, this accumulation is blocked by 3×10^{-4} M cycloheximide which inhibits translation of the mRNA. The cycloheximide was added 5 min before addition of the NaF, but the same results are observed if cycloheximide is added after the NaF. The ^{32}P-labeled polysome and single ribosome fractions from these sucrose gradients were isolated and analyzed by electrophoresis in polyacrylamide gels, and the data is shown in Figure 16. The results verify that the single ribosomes accumulated in NaF contain much more heavily labeled F protein than do the control ribosomes. However, the polysomes in the cells treated with both NaF and cycloheximide also contain extremely radioactive F protein. Thus, the stimulation of F-protein phosphorylation by NaF can be dissociated from the NaF-induced dissaggregation of polyribosomes. It seems likely that the NaF inhibition of protein synthesis and the associated accumulation of single ribosomes are therefore not the same molecular event as F protein phosphorylation. Since all of the ribosome fractions in control cells contain F phosphoprotein in approximately equal amounts (Figures 6, 9, and 10), it also seems clear from this standpoint that this phosphorylation does not prevent the ribosomes from attaching to and translating the mRNA. If the phosphorylation of F protein inhibited ribosome attachment to mRNA, the polysomes would not be labeled to a significant extent.

Several protein kinases which have been isolated are stimulated by cyclic AMP (*e.g.*, Delange *et al.*, 1968; Kuo and Greengard, 1969b; Langan, 1968a) and it was therefore important to investigate the role of cyclic AMP in ribosome phosphorylation. The possibility of cyclic AMP involvement was initially suggested by the NaF stimulation of ribosome phosphorylation. NaF is a potent stimulator of the enzyme adenyl cyclase in all studied tissues (Sutherland and Robison, 1969). It now appears that reticulocytes do contain a significant level of adenyl cyclase (Sheppard and Burghardt, 1969), whereas the level of the cyclic AMP phosphodiesterase in reticulocytes has been reported as being extremely low (Malkin and Lipmann, 1969). Reticulocytes incubated with 0.03 M cyclic AMP, 0.03 M dibutyryl cyclic AMP, or 0.03 M theophylline had the same rate of ribosome protein phosphorylation as did control cells. Nor did these compounds alter the pattern of the protein phosphorylations. These compounds also had only a slightly inhibiting effect on protein synthesis by reticulocytes. Although these results are negative, they do not rule out a role for cyclic AMP in ribosome phosphorylation since the protein kinase(s) might be already saturated with this nucleotide.

Ribosome Phosphorylation in Vivo. Previous workers have injected [^{32}P]phosphate into anemic rabbits and have studied the subsequent appearance of radioactivity in the reticulocyte ribosomes. It has been assumed that all of this radioactivity is incorporated into RNA (*e.g.*, Marbaix *et al.*, 1970; DeBellis, 1969). An anemic rabbit was injected with 1 mCi of [^{32}P]phosphate and blood obtained after 16 hr and after 38 hr. The ribosome fractions isolated from sucrose gradients were treated with 10% trichloroacetic acid at 90° for 15 min to hydrolyze the RNA portion of the radioactivity. After the 16-hr labeling, approximately 10–15% of the radioactivity remained in the precipitate after the hydrolysis treatment. On the contrary, approximately 100% of the radioactivity on extracted RNA was hydrolyzed in a control experiment. After the 38-hr labeling, when more labeled cells have entered

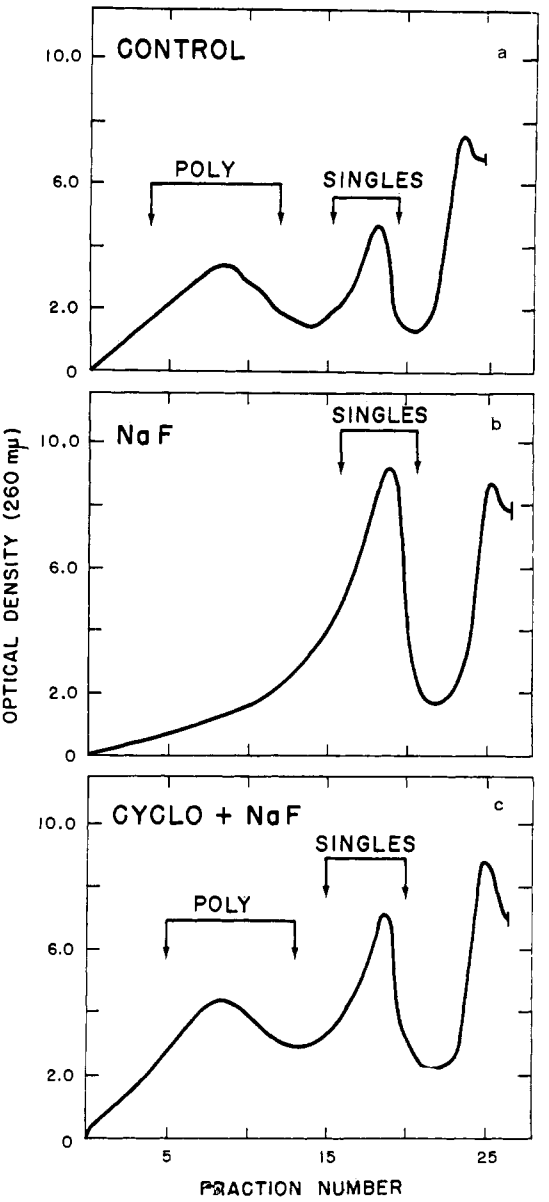

FIGURE 15: Effects of NaF and of cycloheximide plus NaF on polysome profiles in reticulocytes. The ribosomes were sedimented for 2.5 hr in linear sucrose gradients in buffer A at 25,000 rpm (see Methods). The reticulocytes were incubated with [^{32}P]-phosphate for 70 min. At 45 min one portion was adjusted to 3×10^{-4} M cycloheximide. At 50 min this portion of cells was given 0.03 M NaF. Another portion of cells was given only the 0.03 M NaF at 50 min. The polysomes are converted into single ribosomes in 0.03 M NaF but this disaggregation is blocked with cycloheximide. Polysomes and single ribosomes were isolated from these gradients for further analysis (see Figure 16).

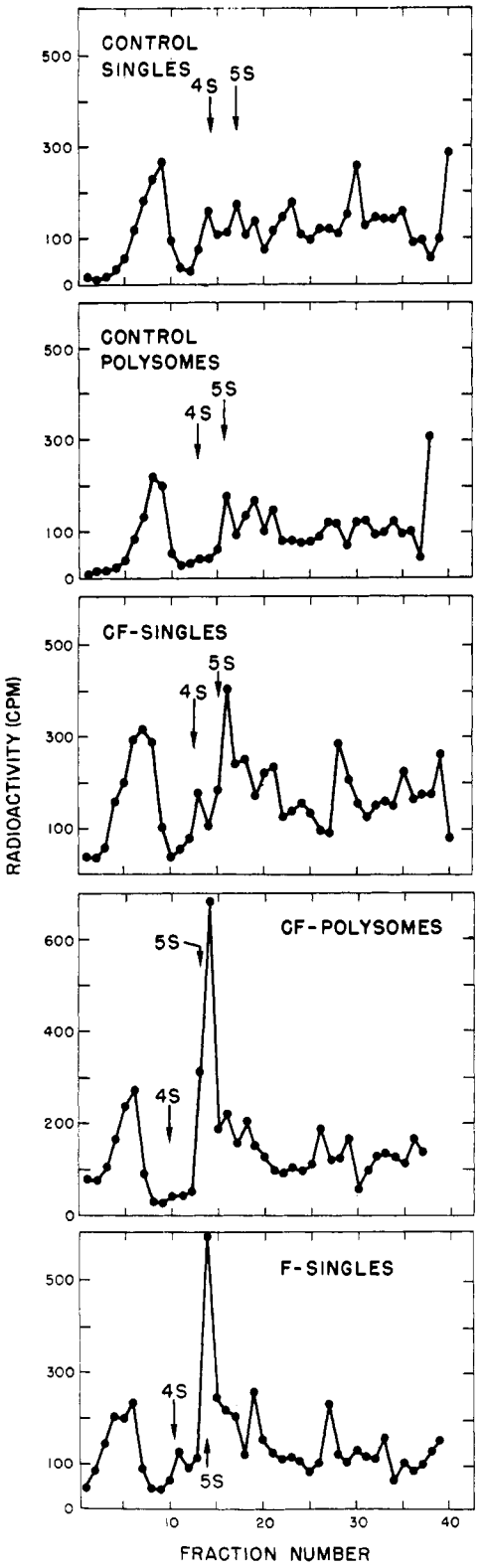

FIGURE 16: Polyacrylamide gel electrophoresis of ^{32}P-labeled ribosome fractions and the effects of NaF on the ribosome labeling. The ribosome fractions were isolated from the buffer A sucrose gradients shown in Figure 16. The 4% gels were scanned for absorbance at 260 mμ before sectioning for radioactivity measurement. The migration positions of 4S and 5S RNA are indicated with arrows. The ribosomes isolated in buffer A are contaminated with adsorbed radioactive components (Figure 5) and most of the components are not well resolved by gel transverse sectioning. However, the F protein can be clearly seen at fractions 14–16. The frames are in the following order, from top to bottom: control single ribosomes, control polysomes, single ribosomes from cells treated with cycloheximide plus NaF, polysomes from the latter cells, single ribosomes from cells treated only with NaF.

the blood from the bone marrow, the proportion of the nonhydrolyzed radioactivity was only about 1%. It seems very likely that phosphoprotein radioactivity indeed contributes to the labeling results which have been obtained, especially when the labeling times are short, and this should be considered in future *in vivo* experiments.

Ribosome Protein Phosphorylation in Other Tissues. In order to determine whether other tissues engage in ribosome protein phosphorylation, 14-day chick embryo heart, liver, and skeletal muscles were incubated with [^{32}P]phosphate in nutrient medium at 37°. The conditions were the same as used with reticulocytes except that a higher concentration of actinomycin D (10 μg/ml) was used in order to fully suppress DNA-dependent RNA synthesis. The tissues were homogenized as described by Heywood *et al.* (1968) and the ribosomes were isolated by sedimentation through 15% sucrose in high ionic strength buffer B. Approximately 50% of the trichloroacetic acid precipitable radioactivity in these ribosome preparations was resistant to the hot trichloroacetic acid treatment described in Table I. The resistant radioactivity in these fractions also cosedimented with the ribosomes in buffer B sucrose gradients which were analyzed as described in Figure 2. Furthermore, radioactive components in these ribosome preparations were fractionated by electrophoresis in sodium dodecyl sulfate–polyacrylamide gels, and all of the major phosphorylated components were degraded by proteolytic digestion with pronase. Aspects of this work are continuing and will be reported elsewhere.

Discussion

Phosphoproteins in Ribosomes. These results demonstrate that several reticulocyte ribosome proteins are subject to modification by phosphorylation of their serine and threonine residues. Furthermore, protein phosphorylation occurs in ribosomes of other tissues. As far as the author is aware, this is the first reported evidence that eukaryote ribosomes are chemically heterogeneous and that they are subject to modification after their protein and nucleic acid constituents have been assembled. The results discussed below indicate that the phosphorylations may regulate ribosome participation in protein synthesis. This is perhaps not surprising since phosphorylation of several complex enzyme aggregates regulates their activity (DeLange *et al.*, 1968; Linn *et al.*, 1969; Turkington and Riddle, 1969). Although the author has no evidence about this, the phosphorylation of ribosomes might also control their ability to recognize certain specific mRNA, their passage through membranes, their attachment to membranes of the endoplasmic reticulum, or their recognition by degradative enzymes. Ribosomes are turning over in eukaryotes (Hirsch and Hiatt, 1966) and are being degraded rapidly during reticulocyte maturation (Glowacki and Millette, 1965).

It seems very clear that certain of the reticulocyte ribosome structural proteins are phosphoproteins. For example, the F

and Si proteins (as well as P band and several other components) remain bound to the ribosomes in buffer B which has an ionic strength appreciably higher than physiological (Figures 5 and 10). Furthermore, these phosphoproteins are associated with specific subunits of the ribosomes (Figures 11 and 12) and they are absent from the reticulocyte supernatant fraction (Figures 6 and 10). F is bound to the small subunit, whereas Si is attached to the large subunit. Finally, each of these phosphoproteins appears to coelectrophorese in sodium dodecyl sulfate–polyacrylamide gels with a major ribosome structural protein (Figures 13 and 14) and it is therefore unlikely that they are minor components (see Results, "Staining of [^{32}P]Ribosomal Proteins Fractionated in Polyacrylamide Gels").

The permissible conclusions concerning the Su phosphoprotein are uncertain, since it is not clear whether Su is a normal constituent of native 44S subribosomal particles. Although Su binds firmly to 44S particles in buffer A and is absent from the supernatant fraction, it is eluted from the 44S particles by sedimentation through the higher ionic strength buffer B (Figures 3, 6, 9, and 10). Although the latter evidence implies that the Su–44S particle association might not occur in physiological conditions, it should be recognized that the intracellular concentrations of ribosomes and Su are much higher than their concentrations in the sucrose gradients. Furthermore, the 44S subunits are continuously extracted during gradient sedimentation. The Su phosphoprotein could therefore bind rather strongly but still be eluted during the sedimentation. Finally, it is important to realize that the mole fraction of water is much less than unity in the physiological environment and it must therefore be considered in the equilibrium expression. If several water molecules are released upon association of a 44S particle with Su (as is known to occur for electrostatic binding), the association reaction would be strongly favored in the cellular environment. For all of these reasons, it is proposed that the Su phosphoprotein is indeed a physiological constituent of the native 44S subunits. If so, the Su protein must dissociate from the 44S subunits when they combine with mRNA because the polysomes lack Su (Figures 6, 9, 10, and 13). Su might, then, play a role in protein synthesis as an initiation factor. If the Su protein proves indeed to be involved in initiation of protein synthesis, it will be interesting to learn whether its activity is regulated by its phosphorylation.

Although these data indicate that certain ribosome proteins are subject to phosphorylation, they do not provide any information about the extent of phosphorylation. For example, we do not know whether all single ribosomes have phosphorylated Si protein or whether only a few contain this phosphate. Furthermore, the slow rate of apparent protein phosphorylation with [^{32}P]phosphate (occurring to an increasing extent for at least 200-min incubation as seen in Figure 9) could reflect either a slow turnover of the various phosphate esters or else a slow labeling of the γ-phosphate of ATP which is the precursor of the phosphoprotein phosphate (manuscript in preparation). However, results of Judah *et al.* (1962) indicate that this γ-phosphate group of ATP attains a constant specific activity approximately 30 min after adding [^{32}P]phosphate to erythrocyte suspensions. In addition, it has been found that the radioactivity in [^{32}P]ribosome proteins is not decreased by a 30-min incubation of reticulocytes with a large excess of unlabeled orthophosphate, suggesting that the esters may be turning over only slowly. However, this author believes that this phosphate "chase" experiment is inconclusive because labeled intracellular phosphate is in phosphorylated compounds which are not in rapid equilibrium with orthophosphate and which might continue to be incorporated into phosphoproteins. Other results, however, also strongly imply that the Si and F phosphate esters are turning over only slowly, and are certainly not obligatorily being hydrolyzed after each cycle of protein synthesis. This evidence is discussed further below.

Inactivation of Single Ribosomes by Phosphorylation. The role of single ribosomes in eukaryotes is a mystery since in all cases studied the vast majority of them do not participate in the ribosomal subunit–polyribosome cycle which obligatorily (Grubman and Nakada, 1969) accompanies each round of protein synthesis (Joklik and Becker, 1965; Girard *et al.*, 1965; Hogan and Korner, 1968; Kabat and Rich, 1969; Adamson *et al.*, 1969). Rather they may be a storage form of ribosomes, but the molecular basis for their inactivity has not been understood. However, some workers have suggested that single ribosomes are a normal obligatory stage of ribosome cycling (Colombo *et al.*, 1968). The present study indicates that the Si protein (21,000 daltons) is present on all ribosomes but that it is only phosphorylated on single ribosomes (Figures 6, 9, 10, 13, and 14). The Si protein appears to be a normal constituent of the larger ribosomal subunits (Figure 12).

The characteristics of this specific phosphorylation of single ribosomes in reticulocytes suggests that these singles are excluded from participation in protein synthesis. If single ribosomes with phosphorylated Si protein were a normal obligatory stage in ribosome cycling during protein synthesis, we would expect that the Si phosphate would turn over very rapidly. Synthesis of a globin polypeptide chain in reticulocytes requires only 0.25–0.4 min at 37° (Knopf and Lamfrom, 1965). The Si phosphate ester would have to be hydrolyzed before the ribosome attached to mRNA because polysomes lack this phosphate (*e.g.*, Figure 9). However, it is unlikely that phosphorylation and dephosphorylation of Si protein are obligatory reactions of ribosome cycling because the Si phosphate seems to turn over only slowly (Figure 9) and because single ribosome accumulation in NaF is not accompanied by Si phosphorylation (Figures 6, 9, and 10). Upon removal of the NaF, polysomes reappear in the cells as protein synthesis resumes, and these new polysomes also lack the Si phosphate. Thus, there occurs a selective reattachment to mRNA of the ribosomes lacking Si phosphate. The simplest explanation for these results is that single ribosomes with phosphorylated Si protein are an inactive class of ribosomes which are excluded from participation in ribosome cycling. Presumably, the phosphorylation of Si protein prevents the ribosome from functioning and removes it from the ribosome cycle. Single ribosomes which lack the Si phosphate accumulate in cells treated with NaF, and this class of singles might be a normal stage of the ribosome cycle which accumulate when initiation of protein synthesis is blocked. Thus, there occur two classes of single ribosomes in reticulocytes, only one of which is a normal stage of ribosome cycling.

Obviously it would be hazardous to generalize at this time about the present results. Reticulocytes are specialized differentiated cells and the results may not be valid for all eukaryote cells. Nonetheless, previous evidence obtained with

HeLa cells (Joklik and Becker, 1965; Girard *et al.*, 1965), ascites tumor cells (Hogan and Korner, 1968), KB cells (Ristow and Kohler, 1965), liver cells (Chandhuri and Lieberman, 1968), and embryonic skeletal muscle (Kabat and Rich, 1969) are all in close agreement with the proposal that eukaryote cells generally contain some single ribosomes which are inactive storage particles. Furthermore, the available evidence indicates that most of the single ribosomes in these tissues are inactive (*e.g.*, Kabat and Rich, 1969). It is likely that the inactivation can be reversed *in vivo*, because single ribosome levels in cells depend strongly on the nutrient environment and because the level can be quickly reduced in some cases by growth factors and hormones (Cohen and Stastny, 1968; Wool *et al.*, 1968). Some recent evidence concerning activation of protein synthesis upon fertilization of sea urchin eggs (Vittorelli *et al.*, 1969) and insulin-dependent protein synthesis in rat skeletal muscles (Wool *et al.*, 1968) are also consistent with the postulate that there is an inactive storage class of single ribosomes in cells of higher organisms. It will be interesting to learn whether activation of protein synthesis in these situations is accompanied by dephosphorylation of a ribosome protein. It will be important also to determine whether the ribosome storage pathway is in any way involved in the control of tissue growth in the normal animal.

Acknowledgments

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

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Control of Hemoglobin Synthesis at the Translation Level. Nascent Polypeptide Chain Distribution on Rabbit Reticulocyte Polyribosomes*

Benedetta Luppis, Antonio Bargellesi, and Francesco Conconi†

ABSTRACT: The nascent polypeptide chains present on rabbit reticulocyte polyribosomes were labeled with [^{3}H]valine, and their relative distribution was studied by two different approaches. (a) One approach concerned the determination of the average molecular weight of the nascent chains by sucrose density gradient centrifugation and by Sephadex G-50 gel filtration. The nascent chains were released either by RNase or by alkali treatment at pH 10. (b) The other approach concerned the determination of the specific activity of the tryptic peptides obtained from the growing labeled polypeptide chains, after their completion in a nonradioactive cell-free system. The results obtained are in favor of the uniform distribution of the nascent polypeptide chains, and therefore of the ribosomes, along the α- and β-globin mRNAs, with no detectable rate-limiting steps during the phases of globin assembly and release.

Among the several possible mechanisms controlling the rate of synthesis of a protein molecule, a control at the translation level has been proposed by several authors (Itano, 1966; Ames and Hartman, 1963; Conconi *et al.*, 1966). A nonuniform rate of globin assembly has been proposed by Dintzis (1961) and by Naughton and Dintzis (1962) in rabbit reticulocytes, and by Winslow and Ingram (1966) in human bone marrow. Itano (1966) and Ames and Hartman (1963) have discussed the possibility that a particular "modulating" tRNA might act to slow the synthesis of a given polypeptide chain at some point during the growing process. On the contrary a uniform rate of α- and β-globin synthesis, in rabbit reticulocytes, has been shown by Hunt *et al.* (1968). These authors challenge the assumption of Dintzis (1961) and Winslow and Ingram (1966) that the analysis of the soluble globin, formed shortly after the addition of labeled amino acids to an intact reticulocyte system, can give an accurate picture of the distribution of ribosomes on mRNA and consequently of the rate of translation and elongation of the polypeptide chains (Englander and Page, 1965).

In an effort to establish the existence of a control mechanism for hemoglobin synthesis at the translation level, we have isolated polyribosome complexes from rabbit reticulocytes, previously incubated with labeled amino acids. The experiments have been designed following two different approaches: (1) analysis of the growing polypeptide chains after breaking the aminoacyl binding between tRNAs and polypeptide chains either by alkali treatment or RNAse hydrolysis, and (2) analysis of the nascent chains after their completion in a cell-free system.

The results obtained are in favor of the uniform distribution of the growing chains on the polyribosome complexes and therefore of the uniform rate of assembly of the globin molecules.

Materials

The disodium salt of adenosine triphosphate, sodium phosphoenolpyruvate, pyruvate kinase, and crystalline horse heart cytocrome *c* were obtained from Boehringer und Soehne, Mannheim, Germany. The disodium salt of guanosine triphosphate and crystalline bovine pancreatic ribonuclease (RNase) were purchased from Sigma Chemical Corp., St. Louis, Mo. CM-cellulose (capacity of 0.66 mequiv/g) was a product of Serva, Heidelberg, Germany. L-[3,4-^{3}H]valine (3.25 mCi/μmole) and L-[U-^{14}C]valine (0.2 mCi/μmole) were obtained from New England Nuclear Corp., Boston, Mass.

* From the Centro di Studi Biochimici sul Morbo di Cooley Cassa di Risparmio di Ferrara, Institute of Biological Chemistry of the University of Ferrara, Ferrara, Italy. *Received March 16, 1970.* This investigation was supported by a grant from the Italian Consiglio Nazionale delle Ricerche, Impresa di Enzimologia, by Grant 5 RO1 HDO 2834 from the National Institutes of Health, and by a grant from the World Health Organization.

† To whom to address correspondence.