PHOSPHORYLATION OF RIBOSOMAL PROTEINS IN SARCOMA-180 TUMOR CELLS

by

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A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy
June 1973

APPROVED: [redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

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Dedicated

To

Dianne, Lisa and Rachel

Acknowledgements

I gratefully acknowledge the exemplary efforts of Dr. David Kabat in his strict adherence to the highest of professional standards. Moreover, I greatly appreciate the personal integrity which Dr. Kabat continuously displayed throughout the course of this study. I am the beneficiary of the mentor system at its best.

Financial support through a National Institute of Health predoctoral fellowship, 5 T01 GM01200, is gratefully acknowledged.

Abbreviations

RNA, ribonucleic acid; mRNA, messenger ribonucleic acid; TCA, trichloroacetic acid; Cyclic AMP, Adenosine 3':5'-cyclic monophosphate; ATP, Adenosine 5' triphosphate; ADP, Adenosine 5' diphosphate; AMP, Adenosine 5' monophosphate.

I. INTRODUCTION

This dissertation is concerned with a special group of ribosomal proteins containing orthophosphate covalently bound to the β-hydroxyl group of constituent threonine and serine residues. These proteins are henceforth referred to as ribosomal phosphoproteins. The purpose of studying ribosomal phosphoproteins was to obtain information concerning the significance of their occurrence in nature. More specifically, four questions that were given paramount consideration during the conduct of this research are: (1) How ubiquitous to mammalian cells are ribosomal phosphoproteins? (2) Does any similarity exist between ribosomal phosphoproteins from different mammalian sources? (3) Does cyclic AMP cause any stimulation of the phosphorylation of ribosomal proteins in sarcoma-180 tumor cells? (4) What effect does the phosphorylation of ribosomal proteins have on ribosome function? Only those aspects of ribosomal structure and function that are relevant to this specialized subject of ribosomal phosphoproteins will be discussed in detail. An extensive review of the relationship between eukaryotic ribosomal structure and function is available elsewhere (1).

Ribosomes from eukaryotic cells are composed of approximately 50% ribosomal ribonucleic acid (rRNA) and 50% ribosomal proteins (1,2). The rRNA is found in three nucleic acid polymers commonly referred to by their sedimentation values of 28S, 18S and 5S (3,4,5). Ribosomal RNA forms a structural backbone surrounded by approximately 70 different ribosomal proteins (6,7,8). The intact single ribosomal particle has a sedimentation value of 76S; and it is structurally

dissociable into a larger 64S and a smaller 44S subunit (1). The larger ribosomal subunit contains the 28S and 5S rRNA and 39 ribosomal proteins while the smaller subunit contains the 18S rRNA and 30 ribosomal proteins (7,8).

Evidence that phosphoproteins occur in eukaryotic ribosomes was first obtained with rabbit reticulocytes and chick embryos (9) and with rat liver (10,11); and later with mouse sarcoma 180 tumor cells (12) and with mouse mammary glands (13). Intracellular phosphorylation in these cases was demonstrated by the incorporation of [^{32}P]-orthophosphate into ribosomal proteins. Subsequent protein hydrolysis and amino acid analysis revealed that most of the radioactivity was in o-phosphoserine with a smaller quantity in o-phosphothreonine.

Kinetic studies with rabbit reticulocytes have shown that these phosphoryl groups turn over intracellularly at the rate of approximately 3% per min (14). Thus, they are sites of an active and continuous metabolism.

Many reports have also indicated that ribosomal proteins can be phosphorylated in vitro by incubating ribosomes with γ-[^{32}P]-ATP in the presence of cyclic AMP dependent or of cyclic AMP independent protein kinases (10,13,15-22). Sources of the ribosomes used in these latter studies were rabbit reticulocytes (15,18,19,22), bovine adrenal glands (22), rat liver (10,18), chick embryo fibroblasts (17), mouse mammary glands (13) and trout testis (20).

The exact number of ribosomal phosphoproteins has not yet been established. However, present estimations for different tissues are in the range of 5 to 8 (9,11,13,21). One complication in making

a final number determination is that in vitro experiments utilizing γ-[^{32}P]-ATP and protein kinase preparations tend to produce more phosphoproteins than whole cell experiments in which extracellular [^{32}P]-orthophosphate is incorporated into ribosomal proteins. Some, and perhaps all, of the ribosomal proteins which are phosphorylated intracellularly can also be phosphorylated with γ-[^{32}P]-ATP in the cell free systems (15). However, certain ribosomal proteins which are phosphorylated in cell free systems have not been observed to be phosphorylated in whole cells (13,21). The possibility exists that some phosphoryl groups are turning over very slowly in vivo, resulting in insufficient [^{32}P]-orthophosphate incorporation for detection. However, in vitro experiments are subject to the criticism that they may lead to extraneous phosphorylation of nonfunctional sites or may fail to catalyze the phosphorylation of functional sites which become masked during isolation of the ribosomes. In spite of such uncertainties, however, we do know from the intracellular studies that there are at least 5 phosphoproteins in rabbit reticulocyte and in mouse sarcoma 180 ribosomes (9,12,15) and that reticulocyte ribosomes contain 7 to 11 exchangeable phosphoryl groups in the steady state (14).

The biological functions of ribosomal phosphoproteins are presently unknown. However, phosphorylation is the only enzymatic modification of ribosomal proteins known to occur in mammalian cells. A recent report that liver ribosomal proteins are also acetylated (23) has not yet been confirmed; it was not supported by preliminary studies

made in this laboratory (24). The finding that ribosomal phosphoproteins from rabbit reticulocytes and from mouse sarcoma 180 cells are very similar (12) suggests functions for these phosphoproteins common to mammalian cells in general, and seems to exclude the possibility that they perform specialized functions related to differentiation or to neoplasia. However, ribosome phosphorylation does not occur ubiquitously throughout nature; it has been shown that E. coli ribosomes lack phosphoproteins (25). It has been reported that addition of protein kinase from reticulocytes to a rat liver cell free protein synthesizing system causes an inhibition of protein synthesizing activity (26); however, the mechanism of this inhibition is not yet known.

Many of the effects of cyclic AMP on eukaryotic systems have been shown to be due to influence on the phosphorylation of proteins (27,28). Hence, it is reasonable to ask whether cyclic AMP is involved in the control of ribosomal protein phosphorylation. Blat and Leob (11) have shown that glucagon injected into rats stimulates the incorporation of [^{32}P]-orthophosphate into liver ribosomal proteins. This suggests that the intracellular ribosomal kinase may have been activated by cyclic AMP. However, increased incorporation of [^{32}P]-orthophosphate into ATP could also have produced the observed stimulation; it is known that cyclic AMP causes an increase in orthophosphate transport into liver and heart (29-31). Therefore, the studies of Blat and Leob are inconclusive.

Cyclic AMP stimulation of ribosomal protein phosphorylation

has also been demonstrated in vitro when cyclic AMP dependent kinases are incubated with highly purified ribosomes (10,15,16,18,21,22). However, eukaryotic ribosomes isolated with low ionic strength buffers (below 250 mM KCl) have protein kinase associated with them that is unresponsive or is only weakly stimulated by cyclic AMP (16-18). Also, Majumder and Turkington (32) have observed that when both cyclic AMP dependent and independent protein kinases are present in a preparation, a cyclic AMP effect is not observed. Furthermore, the ribosomal sites phosphorylated appear to be the same regardless of which of these two kinases is employed (21). Thus, these in vitro experiments have also failed to indicate whether the intracellular phosphorylation of ribosomal proteins is affected by cyclic AMP.

The foregoing introduction to the field of eukaryotic ribosomal phosphoproteins was written using all current literature pertinent to this subject. However, the studies described in this dissertation were initiated when investigations were in a more rudimentary stage of development. At that time, the only detailed information concerning ribosomal phosphoproteins was from studies with rabbit reticulocytes (9). In vitro studies with that system indicated that their phosphorylation was not stimulated with cyclic AMP (15). Therefore, one of the major goals of the research described herein was to perform a comparative study of ribosomal phosphoproteins from a cell type distinct from rabbit reticulocytes both by evolution and by function. Also, I wished to investigate the effects of cyclic AMP on ribosomal protein phosphorylation when added to whole cell cultures.

Rabbit reticulocytes are highly specialized cells. They are non-nucleated and their protein synthesis is directed almost exclusively toward producing hemoglobin. Mouse sarcoma 180 tumor cells were selected for comparative studies because of their species distinction, and because they are nucleated and are rapidly undergoing mitotic division. Furthermore, Lee and Brawerman (33) had recently reported that protein synthesis in previously starved sarcoma 180 cells was rapidly reinitiated when amino acids were added to their culture media. They reported that the amino acids stimulated a rapid shift (within 10 min) of all single ribosomes into polyribosomes.

One result of the early studies with rabbit reticulocyte ribosomal phosphoproteins was that the phosphorylation pattern of single ribosomes was distinctly different from that of polyribosomes which are involved in peptide elongation (9). Single ribosomes in eukaryote cells constitute an inactive stagnant pool of ribosomes which do not rapidly equilibrate with polyribosomes or with native ribosomal subunits (34,35). Hence, it was proposed that the phosphorylation of ribosomal proteins might control the activity of ribosomes (9); the switch of active ribosomes to inactive ones might be caused by changes in ribosome phosphorylation. I had hoped that a detailed study of S-180 ribosomal phosphoproteins commensurate with rapid shifting of single ribosomes into polysomes would test that proposal.

A comparative analysis of mammalian ribosomal phosphoproteins from rabbit reticulocytes and sarcoma-180 tumor cells has been obtained

in this study. The results prove conclusively that ribosomal phosphoproteins are real ribosomal constituents and that they are not derived from the specialized functions of differentiation or neoplasia. The studies with cyclic AMP demonstrate the complexity of intracellular events which occur following cyclic nucleotide addition to cultured cells. One result of cyclic AMP is the stimulation of incorporation of [^{32}P]-orthophosphate into ribosomal proteins. My original expectation of evaluating ribosomal phosphoprotein patterns during rapid protein synthesis activation (i.e., accompanying the shift of single ribosomes into polyribosomes) was not realized for reasons that will be explained later.

II. MATERIALS AND METHODS

A. Labeling of Ribosomal Phosphoproteins with [^{32}P]-Orthophosphate

(Methods for Reticulocytes and for Sarcoma 180 Cells.)

1. Principles

Nearly all phosphorus in mammalian ribosomes is present in the constituent RNA molecules (approximately 6.5 x 10^3 P-atoms/ribosome, with only a small number occurring in the proteins (approximately 7-11 P-atoms/ribosome) (15). Consequently, it is desirable to label the phosphoryl groups on proteins with [^{32}P]-orthophosphate under conditions in which ribosomal RNA synthesis is absent. This has been accomplished by utilizing reticulocytes (which lack a nucleus and are accordingly inactive in RNA synthesis) (9,15), and by culturing tumor cells in the presence of actinomycin D (an inhibitor of RNA synthesis) (36).

2. Solutions used

a. Physiological salt solution (0.13 M NaCl, 0.005 M KCl, 0.0015 M $MgCl_2$) was used for washing mammalian cells.

b. Buffer A (0.01 M KCl, 0.0015 M $MgCl_2$, 0.01 M Tris-HCl, pH 7.4) is a low ionic strength buffer in which ribosomes are stable.

c. Buffer B (0.25 M KCl, 0.01 M $MgCl_2$, 0.01 M Tris-HCl, pH 7.4) is a high ionic strength buffer in which single ribosomes may partially dissociate into subunits.

d. Buffer C (0.5 M KCl, 0.005 M $MgCl_2$, 0.05 M Tris-HCl, pH 7.4) is a high ionic strength buffer used to dissociate ribosomes.

e. Buffer D (0.1 M KCl, 0.04 M NaCl, 0.005 M MgAc, 0.02 M Tris-HCl, pH 7.6) is an intermediate ionic strength buffer in which

ribosomes are stable.

f. Sodium deoxycholate. A 10% solution is stored at room temperature.

g. New methylene blue stain [5.0 g methylene blue NN (Allied Chemical, Morristown, N.J.), 8.5 g NaCl and 4.0 g Na citrate·2 H_2O were mixed with 1 liter of H_2O followed by filtration].

h. Toluidine blue stain. A 1% solution in physiological salt solution.

i. Nutritional medium for reticulocytes [modified from Hori and Rabinovitz (37)]. The modified medium contains physiological salt solution supplemented with 1 mg/ml glucose, 0.4 mg/ml $NaHCO_3$, 2×10^{-4} M ferrous ammonium sulfate, and 5% fetal calf serum. Amino acids were present in the following concentrations, expressed as mM/liter: L-glutamine 0.096, L-histidine 0.116, L-leucine 0.20, L-lysine 0.090, L-phenylalanine 0.080, L-serine 0.086, L-tryptophan 0.015, L-tyrosine 0.042, L-valine 0.154, L-methionine 0.040, L-arginine 0.040, and L-isoleucine 0.031. L-cysteine, L-alanine, L-asparagine, glycine, L-proline, and L-threonine as present at 0.010 mM.

j. Nutritional medium for sarcoma 180 cells. This medium contains Krebs bicarbonate buffer lacking inorganic phosphate (0.12 M NaCl, 0.005 M KCl, 0.0025 M $CaCl_2$, 0.001 M Mg SO_4, 0.2% $NaHCO_3$, pH 7.8 to 8.0) supplemented with 1 mg/ml glucose, with 10% fetal calf serum and with L-amino acids in the final concentrations recommended by Lee et al. (33).

3. Preparation of reticulocytes

Rabbits were made anemic by injecting them subcutaneously for at least 7 days with 10 mg per kg body weight of phenylhydrazine hydrochloride. A neutralized solution containing 25 mg/ml was prepared daily just before use. We generally made a new rabbit anemic each week and used it throughout the following week. The blood was collected into heparinized beakers which were kept chilled in ice. The cells were sedimented by centrifugation at 800 x g for 6 min and were washed three times with cold physiological salt solution. Blood cells were routinely stained with new methylene blue stain in order to determine the proportion of reticulocytes. The washed cells were resuspended in one volume of physiological salt solution. Four drops of cell suspension and three drops of stain were mixed in a tube and were incubated at 37° for one hour. A smear of cells was then made on a slide which was examined in the microscope. We routinely obtained by these methods at least 95% reticulocytes.

4. Incubation and lysis of reticulocytes

The washed cells were resuspended at 7×10^8 cells/ml in the nutritional medium for reticulocytes. Generally, we added 5 µg/ml actinomycin D to suppress any RNA synthesis in contaminating leukocytes. The cell suspension was swirled in a water bath at 37° for 15 min before addition of 50 µCi/ml [^{32}P]-orthophosphate (New England Nuclear Corp., Boston, Mass.). After incorporation for various time periods, the cell suspension was diluted 5-fold with ice-cooled physiological salt

solution and the cells were sedimented at 800 x g for 6 min. All subsequent procedures were at 2°. The packed cells were lysed by the addition of 4 volumes of Buffer A. After centrifugation at 10,000 x g for 10 min, the supernatant was collected into an ice-cooled beaker. A solution of 0.1 M acetic acid was added dropwise with swirling until the pH was reduced to pH 5.1; this causes the ribosomes and many cellular enzymes to coprecipitate, leaving the hemoglobin in solution. Ribosomes were purified for assay of radioactive ribosomal phosphoproteins by one of the isolation procedures described under "Purification and assay of ^{32}P-labeled ribosomes."

5. Preparation of sarcoma 180 cells

The S-180 cell culture was maintained by transferring 0.2 ml of ascites fluid (1×10^7 cells) from the peritoneum of infected mice into the peritoneum of new 28-35 g female Swiss Webster mice. Cells were harvested for experimentation between 5-7 days postinoculation and were then washed three times by centrifugation with cold physiological salt solution. Generally, I obtain between 2-6 ml of ascites fluid (with 0.1-0.4 ml of packed cells per ml of ascites fluid) from each mouse. Cultures of ascites cells occasionally change their growth characteristics or karyotype. Consequently, in beginning work with such cultures it is desirable to store aliquots of the cells in a liquid N_2 freezer. I routinely used the procedure of Hauschka et al. (38) for S-180 cells except that dimethyl sulfoxide (10% of the final cell suspension volume) is used instead of glycerol.

6. Incubation of sarcoma 180 cells

For isotopic incorporation the cells were suspended in the nutritional medium for S-180 cells in the ratio of 1 ml of packed cells to 29 ml of medium; and the flasks were incubated at 37° in a rotary shaking water bath. Actinomycin D (10 micrograms/ml) was added to the cell suspension to inhibit RNA synthesis 10 min prior to adding [^{32}P]-orthophosphoric acid (50 microcuries/ml of cell suspension). After incorporation for various time periods, the cells were chilled by dilution with four volumes of cold physiological salt solution and were collected by centrifugation at 600 x g for 5 min at 2-4°. The cells were then washed twice by centrifugation in cold physiological salt solution.

Cell lysis and ribosome preparation were performed at 2°. Washed cells were suspended in three volumes of Buffer D. Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) was added at a concentration of 0.25% and lysis was accomplished by drawing the cell suspension into a Pasteur pipette six times. A small sample of the cell lysate was stained with toluidine blue and was observed with a phase contrast microscope in order to ensure that cell lysis was complete with no disruption of nuclei. The cell lysate was then centrifuged at 12,000 x g for 10 min to produce a supernatant fraction which will be referred to as the cell extract. Ribosomes were precipitated from this cell extract by titration to pH 5.1 and were further treated as is described in "Purification and assay of ^{32}P-labeled ribosomes."

B. Purification and Assay of ^{32}P-Labeled Ribosomes

1. Principles

The above methods yield solutions containing partially purified ^{32}P-labeled reticulocyte and sarcoma 180 ribosomes. Further purification is needed in order to obtain preparations suitable for analysis of phosphorylated ribosomal components. However, there are no rigorous criteria for defining the purity of mammalian ribosomal preparations. Some true intracellular constituents may be only loosely bound and may be lost readily; conversely, many contaminants adsorb strongly to ribosomes. Multiple cycles of ribosome sedimentation and resolution is an ineffective means of removing such adsorbed contaminants. Furthermore, it is generally recognized by workers in this field that highly purified mammalian ribosomes are very unstable, especially when they have been repeatedly sedimented from solution and dissolved in fresh buffers.

Accordingly, I have used preparative procedures which do not require multiple cycles of ribosome sedimentation and resolution. The ribosomes were sedimented through sucrose solutions in buffers of differing ionic strengths. Although the phosphoprotein content of the preparations is highly dependent on the ionic strength used during the centrifugation, certain of the phosphoproteins cannot be extracted from the ribosomes with high ionic strength buffers, even in the presence of 0.5% sodium deoxycholate. Components in reticulocyte and in S-180 ribosomes are very similar and since they occur in ribosomes in a reasonable

stoichiometry (14), I have concluded (12) that they are true ribosome constituents rather than contaminants.

2. Purification of ribosomes

Following precipitation at pH 5.1, ribosomes were purified by one of the following isolation methods:

Isolation Method A. The precipitate at pH 5.1 was dissolved in 7 ml of low salt buffer (Buffer A, Section II, A, 2, b) by agitation with a Pasteur pipet. The dissolved ribosomes were centrifuged at 12,000 g for 10 min and the supernatant was layered over 2 ml of 15% sucrose dissolved in Buffer B. The ribosomes were then pelleted by centrifugation at 65,000 rpm in a Beckman Spinco 65 rotor for 2-1/2 hr.

Isolation Methods B, C and D. These methods were identical to Method A except that Buffers B, C, and D (Section II, A, 2, c, d, e), respectively, were substituted for Buffer A.

Isolation Method E. The precipitated ribosomes were dissolved in distilled water and were then dissociated into subunits by the method of Blobel and Sabatini (39). Ribosome dissociation was accomplished by incubation with 0.5 mg/ml puromycin (Nutritional Biochemical, Cleveland, Ohio) at 0° for 15 min followed by warming to 37° for 10 min. Dissociated ribosomes were fractionated into subunits in linear 10% to 30% sucrose density gradients made with Buffer C. Sucrose gradient purification and collection of subunits is described in the following section (Section II, B, 3).

Isolation Method F. The ribosomes precipitated at pH 5.1 were

dissolved in a low salt buffer (Buffer A) and were precipitated from this solution by adjusting the magnesium concentration to 50 mM. Ribosomes precipitated in the magnesium solution upon standing in an ice bath for 20 min and were collected by centrifugation at 12,000 g for 20 min.

Isolation Method G. Magnesium precipitated ribosomes were prepared as described in Method F. These magnesium precipitated ribosomes were dissolved in 2 ml distilled water containing 0.01 M EDTA, pH 7.4 (Matheson, Coleman and Bell, Norwood, Ohio) and the solution was centrifuged at 12,000 g for 10 min. The supernatant from this centrifugation was diluted to 7 ml with Buffer C and was layered over 2 ml of 15% sucrose in Buffer C. The ribosomes were then pelleted by centrifugation at 65,000 rpm in a Spinco 65 rotor for 2-1/2 hr. When this method was used to prepare ribosomes for electrophoresis on polyacrylamide gels, sodium deoxycholate (0.5%) was added to the dissolved ribosomes prior to sedimentation.

3. Sucrose gradient purification

Solutions containing ribosomes dissolved in Buffers A, B, C, or D (Section II, A, 1) were fractionated by centrifugation in sucrose gradients made in the same buffers. Frequently, ribosomes in Buffer B were adjusted to 0.5% sodium deoxycholate 5 min before layering onto the sucrose gradients. The sedimentation conditions for the Spinco SW 27 rotor are described in the legends to Figures 1 and 2. Polyribosomes were obtained from gradients like those in Figure 1, whereas

single ribosomes and subribosomal particles were prepared from gradients like those in Figure 2. After centrifugation, the gradients were pumped directly through a flow cell in a Gilford spectrophotometer and the absorbance at 260 nm was plotted on a recorder. The appropriate regions of the sucrose gradient were collected directly into ice-cooled flasks and the ribosome fractions were then pelleted by centrifugation (Section II, B, 2). The ^{32}P-labeled ribosome pellets can then be stored in a freezer.

4. Assay of radioactivity in ribosomal phosphoproteins

Ribosomal phosphoproteins were assayed for radioactivity in a low background gas flow counter following precipitation with 10% TCA at 0°. Contamination with radioactive RNA was excluded firstly by incubating cells with [^{32}P]-orthophosphate in conditions where RNA synthesis is greatly inhibited (Section II, A, 1). As an additional precaution, RNA was quantitatively removed from the precipitates by heating in 10% TCA at 90° for 20 min (40). This procedure results in a 10-20% loss of protein and a 23-27% loss of radioactivity from the precipitates which were then collected onto membrane filters and washed with cold 5% TCA before being assayed. Radioactive analysis of multiple samples from one preparation of ribosomes produced results with a maximum deviation of 4% from their mean value. No radioactive decay calculations were made since all comparative samples from an experiment were assayed at the same time.

5. Extraction of phospholipids

The quantity of radioactivity in contaminating phospholipids

was estimated by measuring the loss of radioactivity when the TCA-precipitate was extracted with a series of lipid solvents. The solvents and sequence of extractions used were suggested by Davidson et al. (41). The precipitate was extracted sequentially; once with acetone, ethanol, and chloroform; twice with ethanol-ethyl ether (3:1); and then once with ethyl ether.

One method used to extract the TCA precipitated ribosomal proteins with lipid solvents was in test tubes following the hot acid treatment (see Section B). The precipitate was sedimented by centrifugation and the solvents were removed by aspiration. However, this procedure produced variable losses of protein from the precipitate and is especially difficult for the chloroform extraction in which the precipitate floats on the solvent surface.

Consequently, I have more recently used a method in which the TCA precipitation was done in a glass fiber filter (Reeve Angel, 934A-A) and the filter was then extracted with the series of nonaqueous solvents. I observed no loss of radioactivity when this procedure was used for samples of ^{32}P-labeled ribosomes from reticulocytes and from S-180 cells. This is true regardless of the isolation procedure used for ribosome preparation (Section II, B, 2). Hence, I concluded that phospholipid contamination does not contribute significantly to the radioactivity which remains in the ribosomal precipitates after the hot acid treatment.

6. Other contaminants

Greenaway (42) has shown that Mg-ATP complexes can contaminate proteins precipitated with cold 10% TCA. Dissolving the precipitates in cold 0.1 M NaOH and then reprecipitating with TCA was suggested as a method for removing this source of contamination. Other workers (43) have also used a 0.1 M NaOH resolution step as a means to remove contaminants from phosphoproteins precipitated with cold 10% TCA. However, such contamination is apparently not significant when the precipitates of ^{32}P-labeled ribosomal phosphoproteins are heated in 10% TCA at 90° for 15-20 min. Resolution of such treated precipitates in cold 0.1 M NaOH does not cause any further reduction in measured radioactivity.

C. Electrophoresis of ^{32}P-Labeled Ribosomal Components in Sodium Dodecyl Sulfate-Polyacrylamide Gels

1. Principles

The ^{32}P-labeled constituents of mammalian ribosomes were conveniently analyzed by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS). Many of the basic techniques have been recently described (44). The advantages of this method are the following: (a) Ribosomes and most other subcellular assemblages of macromolecules are fully dissolved and dissociated into their components in the presence of the SDS buffer. The sample can be layered directly onto the gel without prior separation of the RNA and protein constituents. The method is therefore very convenient and simple. (b) The fractionation of proteins in SDS-gels is on the basis of their

molecular weights; a plot of the mobility of proteins versus the logarithm of their molecular weights falls approximately on a straight line (45,46). (c) The resolution of constituents is excellent; it is at least as good as other electrophoretic methods of fractionating mammalian ribosomal proteins (7).

2. Reagents and solutions

a. Acrylamide-bisacrylamide stock solutions. These are stable at 4° for several months. The acrylamide and N,N'-Methylene-bisacrylamide was obtained from Eastman Organic Chemicals (Rochester, N.Y.) and were recrystallized before use (47). Acrylamide was dissolved in $CHCl_3$ at 50° and the solution was filtered. Crystallization occurs during storage overnight at -20°. The crystals were washed with heptone. Methylenebisacrylamide was recrystallized from water and then from acetone. After dissolving in H_2O at 90° and filtering, the solution was stored overnight at 4° and the crystals were collected by filtration. Ten g of crystals were then dissolved in acetone at 50° and were recrystallized at -20°.

Stock 1 (for gels containing less than 5% acrylamide). This contains 15 g acrylamide and 0.75 g of N,N'-Methylenebisacrylamide in 100 g of solution.

Stock 2. This contains 20 g of acrylamide and 0.5 g of bisacrylamide in 100 g of solution.

b. Ammonium persulfate. A 1.6% solution was kept in the refrigerator and was prepared fresh each week.

c. Stock electrophoresis buffer (10 x). It is 0.36 M Tris, 0.30 M NaH_2PO_4, and 0.01 M EDTA. The actual electrophoresis buffer contains 100 ml of stock buffer, 900 ml of H_2O, and 6.0 g of sodium dodecyl sulfate (Matheson, Coleman and Bell).

d. TEMED. N,N,N'-Tetramethylethylenediamine (Eastman Organic Chemicals, Rochester, N.Y.).

e. Gel dissolving solution (48). It must be freshly made. It contains 1% NH_4OH dissolved in 30% H_2O_2.

f. Scintillation fluid. This was made by mixing 3 g of 2,5-diphenyloxazole (PPO), 0.3 g of 1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene(dimethyl POPOP), 400 ml of toluene, and 800 ml of Triton X-100.

3. Gel preparation

Eight cm gels were made in 6 mm (I.D.) plastic tubes. For preparation of 4% gels, 6.67 ml of stock 1 acrylamide-bisacrylamide and 1.25 ml of 1.6% ammonium persulfate were diluted to 25 ml with electrophoresis buffer lacking SDS. Twenty microliters of TEMED was added. The solution was then quickly mixed and placed into the plastic tubes, and the menisci were overlain with 100 microliters of H_2O. Polymerization was complete in 30 min at room temperature. Eight percent gels were made identically, except that we used 10 ml of the stock 2 acrylamide-bisacrylamide solution. Before use, the gels in the plastic tubes were soaked overnight at room temperature in electrophoresis buffer containing 0.6% SDS.

4. Sample preparation

The pellets containing purified ribosomes were dissolved at room temperature in a few drops of 0.6% SDS-electrophoresis buffer adjusted to 5% sucrose. Ten microliters of the resulting solution was added to 1 ml of H_2O and the absorbance at 260 nm was measured. The concentrated ribosome solution was then adjusted to 1% 2-mercapto-ethanol and was heated at 60° for 30 min to reduce disulfide bonds.

5. Electrophoresis

The gels were prerun at 5 ma/gel for 1 hr before 1 $O.D._{260}$ unit of the ribosome sample in a volume of 10-30 microliters was layered onto the upper surface of the gel. Electrophoresis was at room temperature at 5 ma/gel for approximately 2 hr.

6. Autoradiographic detection of ^{32}P-labeled constituents

Radioactive components in the gels can be visualized by autoradiography. The cylindrical gels were cut into 4 longitudinal sections with the apparatus described by Fairbanks et al. (49). They were then dried onto high-wet strength paper (Schleicher and Schuell, #497) or onto Whatman 3 mm paper. Gels with 4% polyacrylamide were dried without special apparatus on the high-wet strength paper; the gel slices dry smoothly without shrinking or cracking in an oven at 75°. However, more concentrated gels require a special drying technique (44,49). The dried gels on the paper backing were then pressed together with X-ray film (Kodak, single coated type SB-54) for varying time periods before the film was developed.

7. Transverse sectioning of gels for direct measurement of radioactivity

Gels were frequently transectioned with a commercially available sectioning apparatus (Brinkman Instruments, Westbury, N.Y.). Gels for this purpose were made as described above except that they also contained 10% glycerol; this facilitated the accurate sectioning of frozen gels (50). The 1 mm gel sections were dissolved by incubation at room temperature in 0.8 ml of gel dissolving solution. The incubation was for 24-48 hr in tightly stoppered scintillation vials. After addition of 15 ml of scintillation fluid, radioactivity was measured in a liquid scintillation spectrometer.

D. Other Methods

1. Proteolytic digestion of ribosomal phosphoproteins

Pronase (Streptomyces griscus protease) is highly active in solutions containing 0.6% sodium dodecyl sulfate, whereas ribonuclease is markedly inhibited in these conditions (51). This forms the basis of a simple procedure for proteolysis of phosphoproteins in the absence of any degradation of nucleic acids.

Sedimented pellets of ^{32}P-labeled ribosomes were dissolved in electrophoresis buffer containing 0.6% sodium dodecyl sulfate as described above (Section II, C, 4). After the absorbance of the solution at 260 nm was adjusted to 50 units/ml, an aliquot containing 20 microliters was placed into a second tube. Two microliters of a freshly prepared pronase solution [5 mg/ml Pronase (Calbiochem, Los

Angeles, Calif.) dissolved in the 0.6% SDS-electrophoresis buffer] was added to this tube. After 30 min at room temperature, the pronase-treated sample was layered onto the surface of a polyacrylamide gel for electrophoresis. Twenty microliters of the untreated control sample was layered onto another gel. Electrophoresis was then performed as described above (Section II, C).

2. Detection of o-phosphoserine and o-phosphothreonine in ribosomal hydrolysates

Radioactive o-phosphoserine and o-phosphothreonine can be detected in acid hydrolysates of ^{32}P-labeled ribosomal proteins. This is possible because the hydrolysis of the phosphomonoester bonds is considerably slower in acid than the hydrolysis of peptide bonds. After hydrolysis of the protein, the amino acids were fractionated by high voltage paper electrophoresis at pH 1.85; only the phosphorylated amino acids migrate toward the cathode in these conditions (52).

Solutions containing ^{32}P-labeled ribosomes were precipitated with 5% trichloroacetic acid at 0°. After heating at 90° for 15 min to hydrolyze nucleic acids and subsequent chilling at 0° for 30 min, the radioactive precipitate was collected by centrifugation. It was then washed with ethanol-ethyl ether (3:1) and then with acetone. The precipitate from 1 mg of ribosomes was suspended in 2 ml of 6 M HCl in a sealed hydrolysis tube which was then heated to 105° for 7 hr. The hydrolysate was evaporated to dryness in a vacuum dessicator and the residue was dissolved in 100 microliters of paper electrophoresis buffer

(see below). The quantity of o-phosphoserine and of o-phosphothreonine was analyzed following electrophoretic separation of the amino acids.

Separation of amino acids by electrophoresis was in a water-cooled flat plate apparatus on Whatman 3 MM paper (52). The paper strip (10" wide x 20" long) was wet with paper electrophoresis buffer (2.5% formic acid, 7.8% acetic acid) and was blotted so that it was only slightly damp. Ten microliters of radioactive sample was applied at the origin. Ten microliter control samples containing 2 µg of o-phosphoserine and of o-phosphothreonine were applied to adjacent positions on the origin. A control sample containing [^{32}P]-orthophosphate was also analyzed. A voltage of 3000 volts was then applied across the electrodes for 120 min. After drying in an oven at 75°, the paper was sprayed thoroughly with a Cd-ninhydrin stain (53) (made by mixing 200 ml acetone, 20 ml H_2O, 4.0 ml acetic acid, 200 mg Cd acetate and 2.0 g of ninhydrin; this stain is stable in dark bottles at 4°); and it was then heated at 75° for 3 hr. Positions of the marker phosphoserine and phosphothreonine were circled with a pencil and the paper was placed together with X-ray film for autoradiographic localization of radioactive compounds. Such regions of the paper can be cut out and analyzed quantitatively for radioactivity in a liquid scintillation spectrometer.

The color which develops on the paper after Cd-ninhydrin staining can be quantitatively eluted with methanol. Its absorbance at 500 mµ is proportional to the quantity of the amino acid (53). However,

the actual percentages of phosphoserine and phosphothreonine cannot be determined by this method because some phosphomonoester hydrolysis undoubtedly occurs during the protein hydrolysis treatment.

3. ATP assay

ATP was extracted and analyzed only from sarcoma-180 cells. Cells used for assay of ATP were incubated, washed, and collected by sedimentation as described previously (Methods II, A, 6). Sedimented cells were frozen immediately upon removal from the centrifuge and were kept frozen until all samples were collected and preparations were completed for the extraction of ATP.

The frozen cells were thawed at room temperature by dilution with 0.6 ml of 0.4 M $HClO_4$ per 0.2 ml of packed cells. This procedure lysed the cells and formed aggregates of precipitate which were broken up by vigorous stirring. After 30 min at 2° the precipitate was removed by centrifugation at 10,000 x g for 10 min and the supernatant was neutralized with a solution of 0.72 M KOH and 0.16 M KH CO_3 (0.3 ml per sample which initially contained 0.2 ml packed cells). The samples were chilled for 15 min at 0° and the precipitates of $KClO_4$ were removed by sedimentation at 5,000 x g for 10 min. This centrifugation produced a supernatant fraction that was assayed for ATP concentration by the firefly luciferase method (54). The quantity of radioactivity in ATP was measured after its purification by two-dimensional thin layer chromatography (55).

Firefly lantern extract (Sigma Chemical Co., St. Louis) was purchased in lyophilized form and was reconstituted with distilled

water as per instructions. 100 µl of the supernatant sample containing ATP was added to 1 ml of the firefly extract at 0° and the fluorescence at 560 nm was measured 60 sec after adding the sample at room temperature. The quantity of ATP was determined by comparing the fluorescence yield with a standard curve obtained by measuring the fluorescence produced by weighed quantities of ATP.

Two µl of the sample containing ATP was spotted on a polyethyleneimine-impregnated cellulose plate (PEI-cellulose plates, Brinkman Instruments) and to this spot was added 1 $O.D._{260}$ of control ATP (Calbiochem, Los Angeles). The thin layer plate was developed for approximately 2 hr with solvent 1 (1 N lithium chloride/1 N acetic acid), washed in methanol and then was redeveloped in the perpendicular direction with solvent 2 (3 N ammonium acetate/5% boric acid). Development was continued until the solvent fronts had migrated 13 cm. Following development and drying, the U.V. adsorbing carrier ATP was located with a Mineral Light (Ultra-violet Products, Gabriel, Calif.); and it was outlined with a pencil. The thin layer plates were then pressed together with X-ray film for autoradiographic location of radioactive areas. A single radioactive spot was found to coincide in position with the U.V. adsorbing ATP spot. This area was removed from the thin-layer plates and was analyzed quantitatively for radioactivity with a liquid scintillation spectrometer.

4. Protein synthesis

The rate of protein synthesis in sarcoma-180 cell suspensions

was determined by measuring the incorporation into proteins of a mixture of uniformly ^{14}C-labeled L-amino acids (New England Nuclear Corp., NEC-445). Two µCi of radioactive amino acid mixture was added per ml of sarcoma-180 cell suspension (Section II, A, 6). When cyclic-AMP was also added to a suspension, the radioactive amino acids and cyclic-AMP were added together and that addition constituted time 0. 100 µl aliquots of the incubation mixture were removed at 10 min intervals and were added to test tubes containing 1 ml of 10% TCA at 0°. This procedure precipitated the proteins.

The precipitated protein was washed twice with cold 5% TCA. The washed precipitate was then collected onto membranes and was assayed for protein radioactivity as described for ribosomal phosphoproteins (Section II, B, 1).

III. RESULTS

A. Phosphorylation of Ribosomal Proteins in Sarcoma-180 Cells

1. Conditions of cell lysis

Lysis of rat liver or rabbit muscle cells in the presence of 1% Triton X-100 is known to solubilize certain membrane constituents (56-58). Furthermore, when this lysis is carried out in the presence of low ionic strength buffers, there occurs an artifactual adsorption of polyribosomes onto the modified membranes and released membranous proteins, resulting in a lowering of polysome yields. Such adsorption does not occur if the monovalent cation concentration is elevated to 150 mM (56). Accordingly, I have used as low a Triton X-100 concentration as possible for complete cell lysis (0.25%) and a lysis buffer 140 mM in monovalent cations. A higher monovalent cation concentration was avoided because S-180 single ribosomes are partially dissociated into subunits in 150 mM KCl (33). When isolated in my conditions, S-180 polysomes are routinely obtained in high yields as compared with previous reports (33,34,58-60) and are undegraded as judged by the absence of nascent polypeptide chains from single ribosomes. Furthermore, the polysome size distribution is unaffected by dissolution of membranes in the cell extract with 0.5% sodium deoxycholate. This implies that the polysomal clusters in the extracts are not significantly aggregated onto membranes (56).

2. Phosphorylation of ribosomal proteins

During incubation of S-180 cell suspensions with

[^{32}P]-orthophosphate there occurs an extensive labeling of the ribosomes. Figure 1 shows a sucrose gradient sedimentation analysis of ^{32}P-labeled ribosomes, isolated from cells labeled for 60 min in the presence of 10 µg per ml of actinomycin D. Clearly, there is a co-sedimentation of radioactive material with the ultraviolet absorbing polyribosomes and single ribosomes. Figure 2 shows a similar sedimentation analysis which indicates that the subribosomal particles are also highly radioactive, and that there is a pronounced peak of labeling on the 44S native subribosomal particles. These results are all closely similar to those previously obtained with rabbit reticulocytes (9).

That this incorporation is not into nucleic acid components of the ribosomes is shown by several lines of evidence. Firstly, the incorporation was done in the presence of 10 µg per ml of actinomycin D which suppresses the synthesis of both ribosomal RNA and messenger RNA within 10 min of addition to the culture (36). Secondly, the sucrose gradient fractions were incubated with 10% trichloroacetic acid at 90° for 20 min before the precipitates were filtered onto membranes for radioactivity measurement. This treatment efficiently hydrolyzes any nucleic acids which are present in gradient fractions.

Figure 3 shows an electrophoretic analysis of the radioactive constituents of sarcoma-180 polyribosomes, the radioactive components being visualized by autoradiography. The electrophoresis is in polyacrylamide gels containing 0.6% sodium dodecyl sulfate. In these

Figure 1. Sucrose gradient sedimentation of ^{32}P-labeled S-180 ribosomes. S-180 cells were incubated for 1 hour in the presence of [^{32}P]orthophosphate. Ribosomes were isolated by pH 5.1 precipitation and then by $MgCl_2$ precipitation as described under Materials and Methods (Section II, B, 2, Isolation Procedure G). Sedimentation of the resulting preparation is in a 15 to 40% sucrose gradient made with Buffer D. Centrifugation is at 25,000 rpm for 3 hours in the SW 27 rotor. Proteins were precipitated from the gradient fractions with 10% trichloroacetic acid, and were then heated at 90° for 20 min before they were filtered onto membranes for radioactivity measurement.

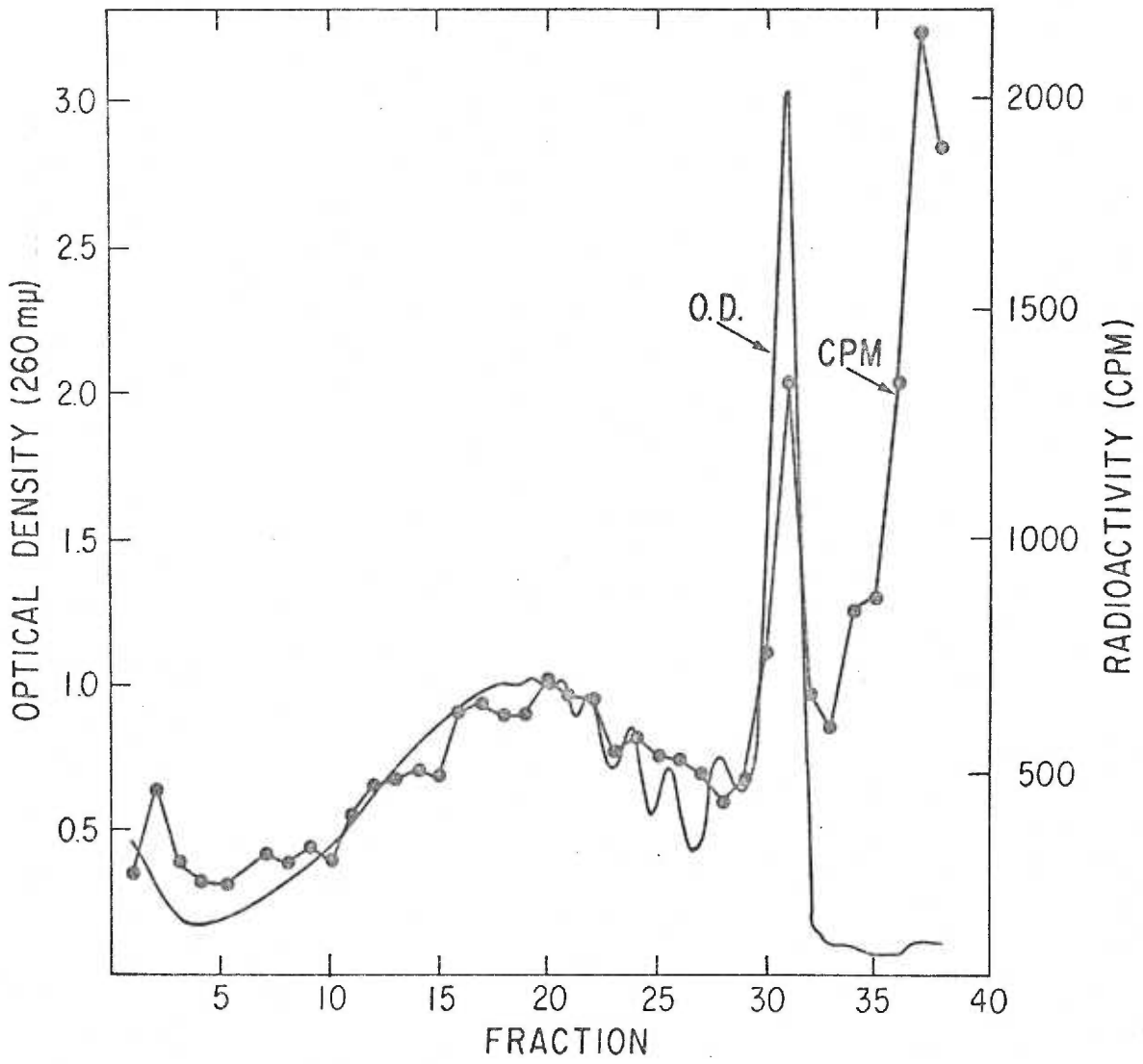
OPTICAL DENSITY (260 mμ)
RADIOACTIVITY (CPM)
FRACTION
O.D.
CPM
3.0
2.5
2.0
1.5
1.0
0.5
2000
1500
1000
500
5
10
15
20
25
30
35
40

Figure 1

Figure 2. Sucrose gradient sedimentation of ^{32}P-labeled single ribosomes and subribosomal particles from S-180 cells. With the following exceptions, experimental procedures are the same as described in Fig. 1. (a) The ribosomes were not precipitated with $MgCl_2$ since this results in a loss of subunits (Materials and Methods, II, B, 2, Isolation Procedure B). (b) The sucrose gradient was made with low ionic strength Buffer A. (c) Centrifugation is at 25,000 rpm for 17 hours.

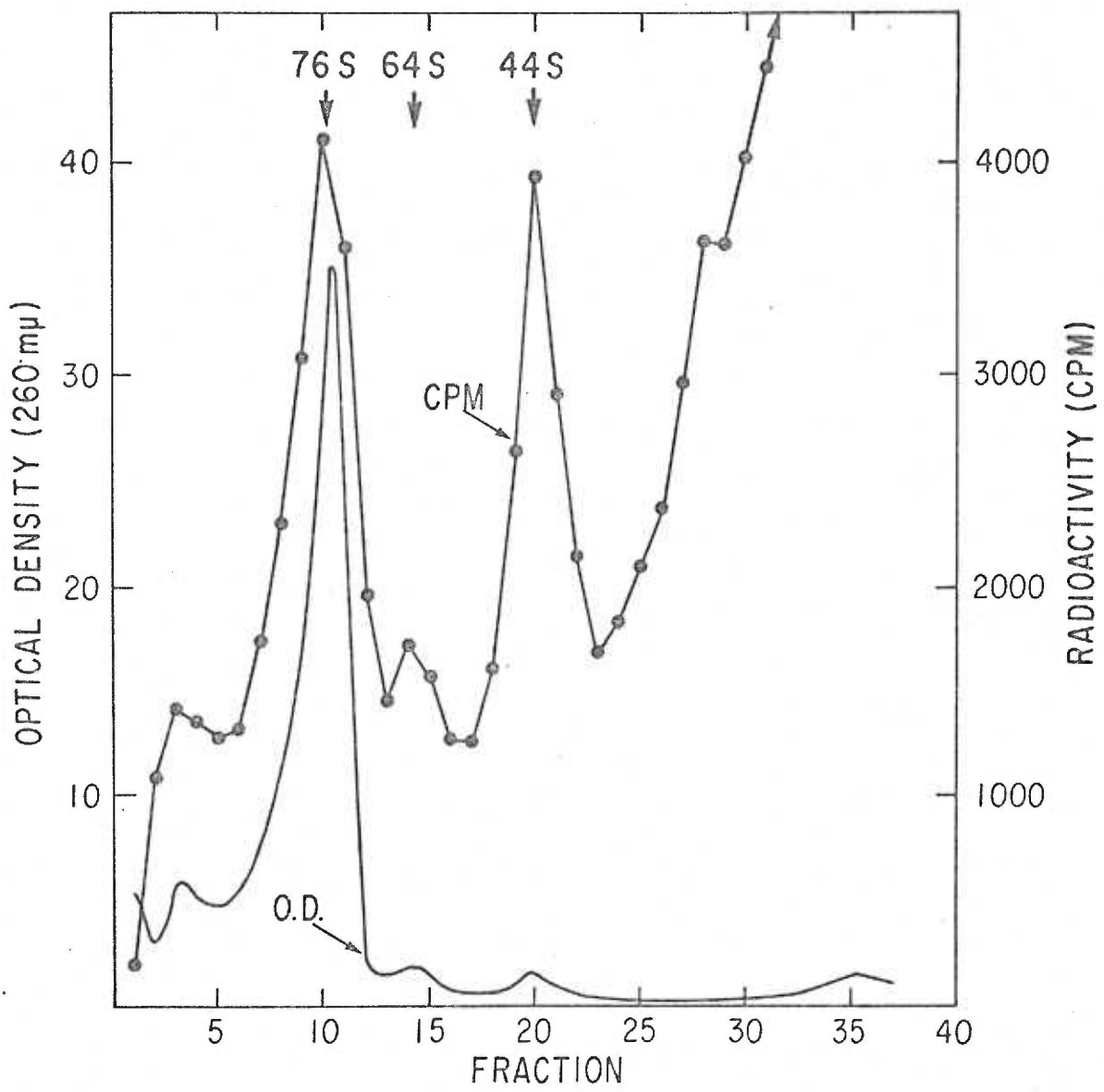
76S
64S
44S
40
30
20
10
4000
3000
2000
1000
CPM
O.D.
OPTICAL DENSITY (260 mμ)
RADIOACTIVITY (CPM)
5
10
15
20
25
30
35
40
FRACTION

Figure 2

Figure 3. Electrophoresis of ^{32}P-labeled proteins from sarcoma-180 polyribosomes in sodium dodecyl sulfate-polyacrylamide gels. Polyribosomes were pelleted at the bottom of the sucrose gradient analyzed in Fig. 2. This pellet of polyribosomes was dissolved in a small amount of electrophoresis buffer containing 0.6% sodium dodecyl sulfate and an aliquot was removed for digestion with pronase. The digested and undigested samples were then analyzed by electrophoresis at 5 ma per gel for 80 min. Radioactive regions of the gels are visualized by autoradiography. Electrophoresis is from right to left and was in 4% polyacrylamide gels.

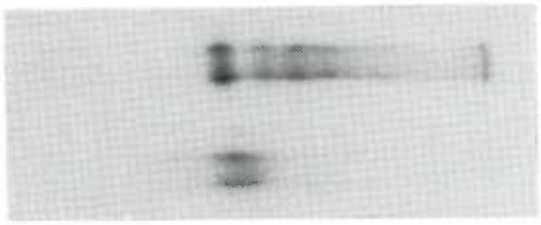

Figure 3

conditions, the electrophoretic mobility of proteins is a function of their molecular weights, the more rapidly migrating polypeptides having the lower molecular weights (44). It can be seen that incubation with pronase converts the radioactive components into more rapidly migrating materials. This supports the conclusion that the major radioactive components are phosphoproteins.

Preparations of ^{32}P-labeled polyribosomes, single ribosomes, and native subribosomal particles from S-180 cells were subjected to mild acid hydrolysis and subsequent paper electrophoresis (see Materials and Methods). All of the hydrolysates contained radioactive phosphoserine and phosphothreonine. As was true with reticulocytes (9) and rat liver (16), more radioactive phosphoserine than phosphothreonine was present in the hydrolysates.

3. Radioactive contamination of ribosomal phosphoproteins

One possible source of adsorbed radioactive contamination on ribosomal particles are ^{32}P-labeled phospholipids. Sarcoma-180 ribosomes containing ^{32}P-labeled phosphoproteins were extracted sequentially with a series of lipid solvents as described in Methods (Section II, B, 5). No appreciable loss of radioactivity from precipitated ribosomal phosphoproteins was observed upon extraction with the lipid solvents. Hence, contamination of ribosomal particles with ^{32}P-labeled phospholipids is not a significant factor in these incorporation values.

Non-ribosomal phosphoproteins adsorbed onto ribosomal

particles constitute another possible source of radioactive contamination. Phosphoprotein contamination in the ribosomal gradient profiles may be derived from ribonucleoprotein of nuclear origin (61), proteins bound to mRNA (62), or membrane proteins (56,57). To test these possibilities, I extracted S-180 ribosomes with 0.5% sodium deoxycholate prior to sedimentation through sucrose gradients as in Figure 1. I could detect no significant differences in the ^{32}P-radioactivity or in the absorbance profiles between deoxycholate extracted and non-extracted ribosomal preparations. Since 0.5% sodium deoxycholate removes all three types of contaminants (56,60,61), I conclude that ^{32}P incorporation into the ribosomal gradient profiles is not due to contaminating nonribosomal proteins of these origins. This consideration, however, does not exclude the possibility of contamination by other cytoplasmic phosphoproteins that may become adsorbed to the surface of ribosomal particles during their isolation from cell lysates.

The effects of different ribosomal isolation procedures on the quantities of radioactivity in preparations of ribosomal phosphoproteins are tabulated in Table I. Cyclic-AMP stimulation of the incorporation of [^{32}P]-orthophosphate into ribosomal phosphoproteins (Table I, experiment 2) will be discussed in a later section.

The principles implicit in the different isolation procedures used for the experiments summarized in Table I are: (1) washing ribosomes in different ionic strength buffers, (2) precipitation of ribosomes from solution by 50 mM $MgCl_2$, and (3) dissociation of

TABLE I

Effect of Isolation Procedures on Sarcoma-180 Ribosomes

Experiment	Isolation Procedure*	Specific Activity of Ribosome Preparations (cpm/µg protein)	Ratio Protein/RNA	Stimulation with Cyclic-AMP
1	A	564		
	D	507		
	B^+	267		
2	B	243	0.76	1.20
	C	147	0.68	1.18
	E	48	0.94	1.72
	F	468	1.18	1.07
	G	186	0.72	1.66
	Total Cytoplasmic Supernatant Fraction Specific Activity (cpm/µg protein)	= 371		1.85

*The labeling and isolation procedures are described in Methods (Section II, B, 2).

B^+ is procedure B plus treatment with 0.5% sodium deoxycholate.

ribosomes by incubation with puromycin followed by sedimentation in high ionic strength buffer C. The ionic strengths of buffers used for isolation procedures A, D, and B (experiment 1, Table I) are 0.02, 0.17, and 0.29, respectively. This experiment shows that a substantial portion of the ^{32}P-labeled phosphoprotein material is extracted from ribosomes by higher ionic strength buffers. Sarcoma-180 ribosomes are stable in the buffers used for isolation procedures A and D but are slightly unstable in buffer B which produces the greatest loss of radioactive material. Certain ^{32}-phosphoproteins remain associated with ribosomes regardless of the buffers employed for their preparation (Table I). This conclusion will be documented more fully later in this section.

Purification of ribosomes by magnesium precipitation (procedure F, Table I) produces ribosomes with the highest specific activity and also with the highest protein to RNA ratio. When this preparation was washed by sedimentation through medium salt buffer B (procedure G), a 60% lowering of specific activity was observed and the protein/RNA ratio was reduced to a level comparable with other KCl washed ribosomes (isolation procedures A-D).

The lowest specific radioactivity was obtained when ribosomes were dissociated in the presence of puromycin, followed by sedimentation through high salt buffer C (procedure E). However, the puromycin dissociation procedure involves incubation of relatively impure solutions of ribosomes at 37° for 10 min. This loss of radioactivity

may be caused by phosphoprotein phosphatase action during the incubation period. A similar enzyme contaminates impure preparations of reticulocyte ribosomes (15). Therefore, this data should not be interpreted as indicating a removal of phosphoproteins from ribosomes by isolation procedure E. Moreover, the unusually high protein to RNA ratio (0.94) suggests that there is no large loss of protein during dissociation. I cannot presently explain why the protein RNA ratio is higher for this procedure than for procedure C where the same high ionic strength buffer is used for washing the ribosomes.

^{32}P-labeled ribosomal phosphoproteins were fractionated on 8% polyacrylamide gels followed by autoradiographic analysis to identify specific ribosomal phosphoproteins (49). This procedure works well for ribosomal material from rabbit reticulocytes but I often observe a high background of radioactive contamination when analyzing ribosomal proteins from sarcoma-180 cells. Figure 4 shows a polyacrylamide gel analysis of phosphoproteins from sarcoma-180 ribosomes which were isolated by procedures B, G, and E. Magnesium precipitation of ribosomes prior to sedimentation through medium salt buffer B removes a large amount of background contamination. I must caution, however, that this procedure may also remove real ribosomal constituents. More specifically, protein band IV (found both on the large and small subunits) and other large molecular weight proteins found exclusively on the small subunits are absent from ribosomes following magnesium precipitation. This may be significant because native subunits do not

Figure 4. Electrophoresis of ^{32}P-labeled proteins from sarcoma-180 ribosomes prepared with Isolation Procedures B, G, and E, respectively (Section II, B, 2). Fractionation of proteins was accomplished by electrophoretic migration toward the bottom at 5 ma per gel for 125 min. in 8% polyacrylamide gels. Radioactive regions of the gels are visualized by autoradiography.

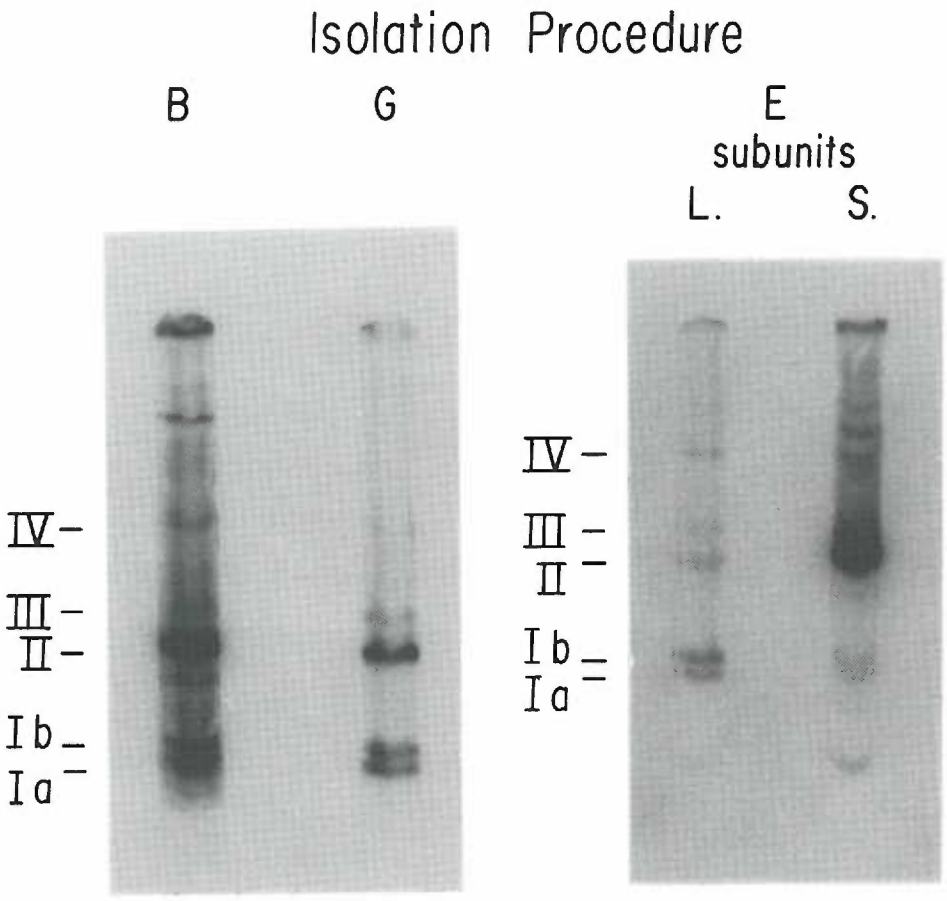
Isolation Procedure
B
G
E
subunits
L.
S.
IV
III
II
Ib
Ia
IV
III
II
Ib
Ia

Figure 4

precipitate as efficiently in the presence of 50 mM magnesium ions as do polyribosomes and single ribosomes and the phosphorylation pattern of phosphoproteins associated with native 44S subunits has not yet been determined.

Results of polyacrylamide gel analysis of phosphoproteins from sarcoma-180 ribosomes (Fig. 4) strongly indicates the occurrence of at least 4 and most likely 5 distinct ribosomal phosphoproteins since they are observed regardless of the isolation procedure employed. Conclusive proof that these are real ribosomal constituents rather than adsorbed contaminants will be presented in a later section where a comparison of sarcoma-180 ribosomal phosphoproteins to rabbit reticulocyte ribosomal phosphoproteins is presented.

4. Kinetics of [^{32}P]-orthophosphate incorporation into ribosomal phosphoproteins

The kinetics of [^{32}P]-orthophosphate incorporation into the ribosomal protein fraction of S-180 cells is shown in Figure 5. The ribosomal proteins were separated from ribosomal RNA by a standard method (64) and were then treated with 10% trichloroacetic acid at 90° for 20 min before they were filtered onto membranes. The combination of these two purification methods suffices for removal of all contaminating radioactive RNA from the labeled proteins, even when the ^{32}P incorporation was done in the absence of actinomycin D. These data show that the ribosomal phosphoprotein metabolism is not significantly influenced by the latter drug or by the drug-induced inhibition of RNA

Figure 5. Kinetics of [^{32}P]orthophosphate incorporation into sarcoma-180 ribosomal proteins in the presence or absence of actinomycin D. Two flasks containing sarcoma-180 cells were incubated with [^{32}P]orthophosphate as described under Materials and Methods. The incubations were identical except that only one flask contained actinomycin D (10 µg per ml). At various times during the incorporation, total ribosomes were prepared (Isolation procedure B. Section II, B, 2). RNA was then removed from the ribosomal proteins by the method of Leboy et al. (64). The proteins were then precipitated with 10% trichloroacetic acid, were incubated at 90° for 20 min to hydrolyze any contaminating nucleic acids, and were filtered onto membranes for assay of radioactivity. Protein content was measured by the method of Lowry et al. (63).

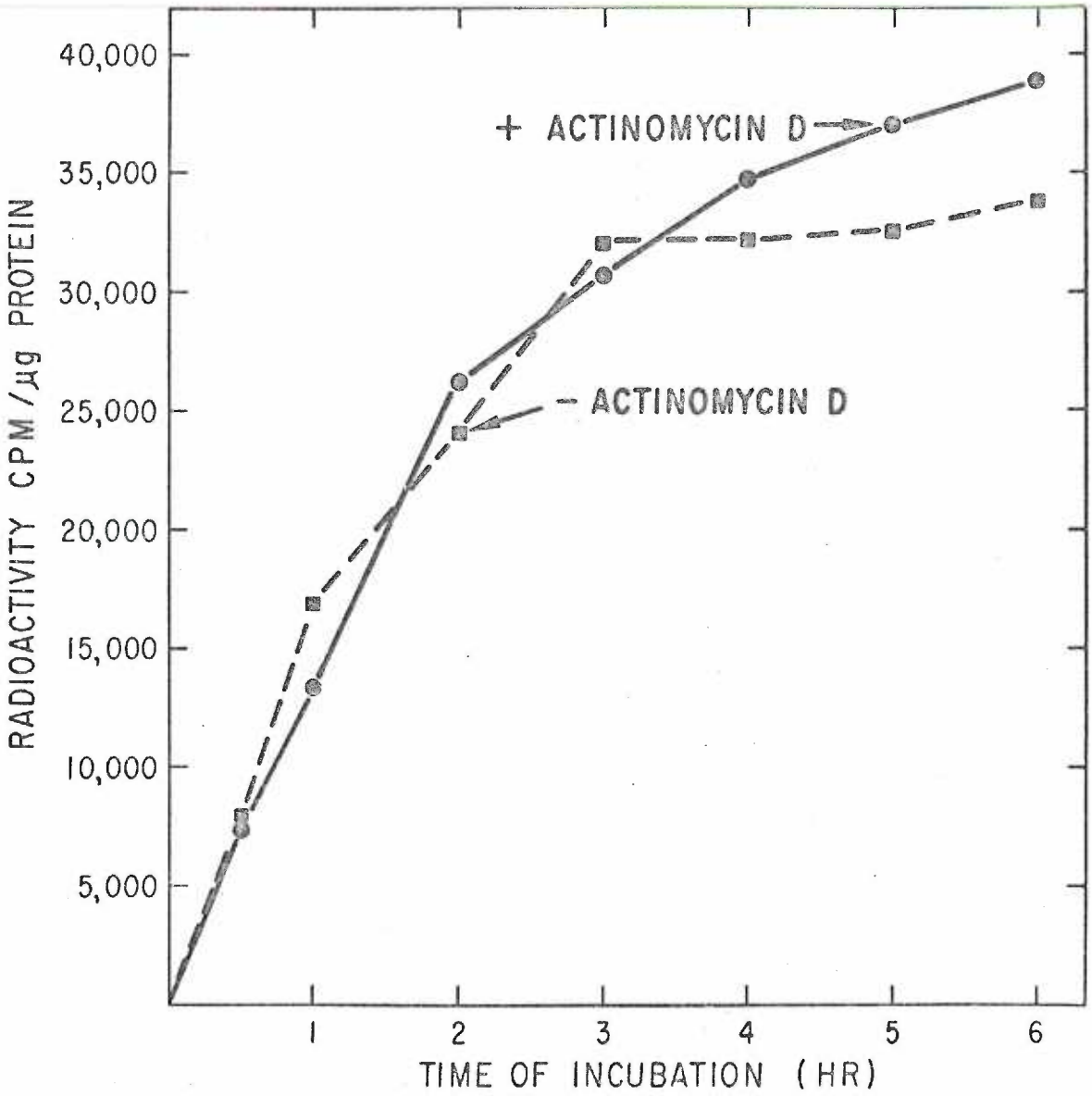
40,000
35,000
30,000
25,000
20,000
15,000
10,000
5,000
RADIOACTIVITY CPM / µg PROTEIN
+ ACTINOMYCIN D
- ACTINOMYCIN D
1
2
3
4
5
6
TIME OF INCUBATION (HR)

Figure 5

synthesis.

B. Comparison of Ribosomal Phosphoproteins from Mouse Sarcoma-180 Cells with Those from Rabbit Reticulocytes

1. Polyribosomes and single ribosomes

The above studies establish that sarcoma-180 ribosomes are phosphorylated on several polypeptide chains. My initial studies of these polypeptides suggested that they were similar to the phosphoproteins of reticulocyte ribosomes. Accordingly, I have made a more detailed comparison of the phosphoproteins from these sources.

A comparative electrophoretic analysis of the ^{32}P-labeled constituents of sarcoma-180 and rabbit reticulocyte polyribosomes and single ribosomes is shown in Figure 6. As in Figure 3, separation was on 4% polyacrylamide gels containing sodium dodecyl sulfate and fractionation is according to molecular weight. Radioactive components are visualized by autoradiography. Previously reported evidence suggests that the most rapidly migrating material, labeled "P", is not a protein and that it is soluble in 10% trichloroacetic acid (9). Furthermore, P occurs in variable yields in polysome preparations from S-180 cells and from reticulocytes, and it is extracted from polysomes when they are washed with 0.5% sodium deoxycholate. Although it is not hydrolyzed by alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 2.1.3.3), its chemical structure remains unknown. All of the other ^{32}P-labeled components resolved in the gels are phosphoproteins since they are degraded by pronase. Furthermore, these

Figure 6. Comparative electrophoresis of ^{32}P-labeled ribosomal proteins from rabbit reticulocytes and from mouse sarcoma-180 cells. The electrophoresis is in 4% polyacrylamide gels containing 0.6% sodium dodecyl sulfate (see Materials and Methods). The radioactive bands are visualized by autoradiography. Ribosomes from both cell types were precipitated at pH 5.1 and were then fractionated in the SW 27 rotor in isokinetic 15 to 40% sucrose gradients made with Buffer B. Polyribosomes were recovered from a gradient centrifuged for 3 hours at 25,000 rpm, while single ribosomes were recovered from an identical gradient centrifuged for 17 hours at 25,000 rpm. S-SIN and S-POLY are S-180 single ribosomes and polyribosomes, respectively. R-SIN and R-POLY are reticulocyte single ribosomes and polyribosomes, respectively. R+S-SIN is a mixture of reticulocyte and of S-180 single ribosomes. R+S-POLY is a mixture of reticulocyte and S-180 polyribosomes.

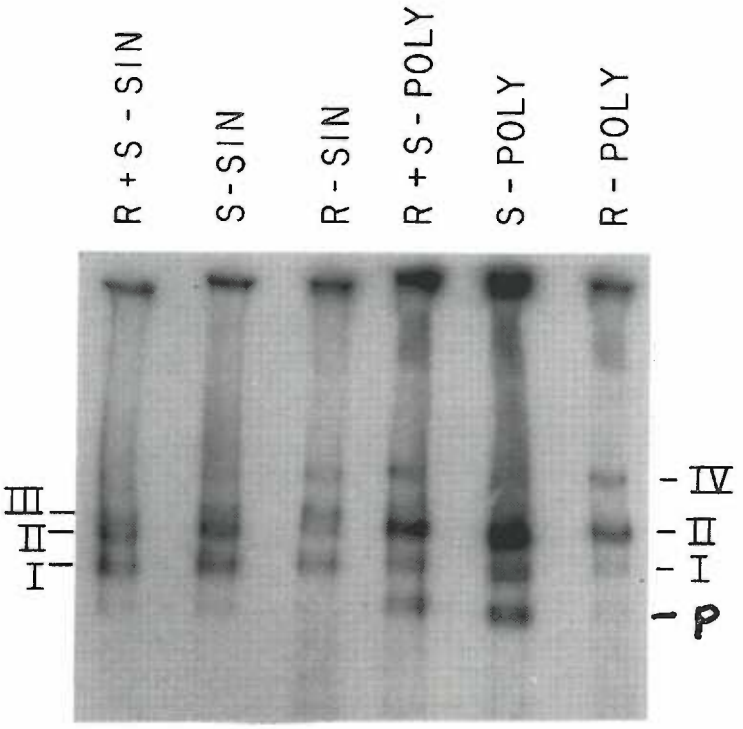

Figure 6

phosphoproteins from S-180 and from reticulocyte ribosomes are strikingly similar. No components occur only in S-180 or only in reticulocyte ribosomes since a mixture of ribosomes from these two sources exhibit the same number of electrophoretic bands as do the unmixed ribosomes. This suggests that the ribosomal phosphoproteins from these two cells have the same molecular weights, as would be expected if they were homologous proteins related by evolution.

Polyribosomes and single ribosomes have distinctive phosphorylation patterns in both cells (see Fig. 6). For example, Band II is more heavily phosphorylated in polysomes than in single ribosomes. Moreover, Band III seems to be reproducibly more heavily labeled in single ribosomes than in polysomes. However, differences between the two cells also exist. For example, Band I is often phosphorylated as highly in S-180 polysomes as in single ribosomes, whereas it is always labeled relatively more heavily in the single ribosomes of reticulocytes (see also references 9 and 12). In spite of such minor differences, however, the phosphorylation patterns in Figure 6 are typical of the results obtained and illustrate the distinctiveness of the polysome- and single ribosome-banding patterns.

2. Subunit localizations of ribosomal phosphoproteins

Figure 7 shows an electrophoretic analysis of ^{32}P-labeled phosphoproteins found associated with ribosomal subunits from mouse sarcoma-180 cells and from rabbit reticulocytes. Ribosomes were isolated from cells and separated into subribosomal particles with isolation

Figure 7. Comparative electrophoresis of ^{32}P-labeled ribosomal proteins from subribosomal particles of rabbit reticulocytes and mouse sarcoma-180 cells. Subribosomal particles were prepared from ^{32}P-labeled ribosomes by Isolation Procedure E, Section II, B, 2. L. and S. are 60 S and 40 S subunits, respectively. Electrophoresis was in 8% polyacrylamide gels at 5 ma per gel for 125 min with migration toward the bottom. Radioactive regions of the gels were visualized by autoradiography.

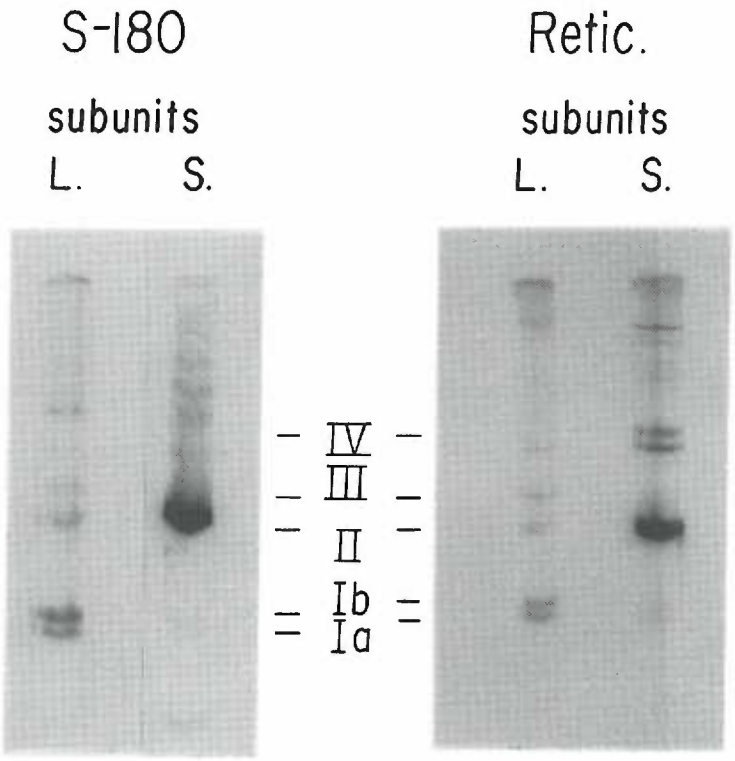

Figure 7

procedure E (Methods, II, B, 2). Hence, the subribosomal particles analyzed are a mixture of native subunits and of subunits derived from the dissociation of polysomes and single ribosomes. Radioactive components were separated according to their molecular weights by migration in an electrophoretic field through 8% polyacrylamide gels. Eight percent polyacrylamide gels provide a more sensitive fractionation of radioactive material than the 4% gels used for the experiment shown in Figure 6. The major advantage of 8% gels is that Band I resolves into two bands (Ia and Ib), and that larger molecular weight components (> protein Band IV) are better resolved in the higher density polyacrylamide gel.

This electrophoretic analysis of ribosomal phosphoproteins from sarcoma-180 cells and from reticulocytes shows that the previously described similarity in ribosomal phosphoproteins from these two different sources (Figure 6) extends to the subunit localization of the specific radioactive bands. Ribosomal phosphoproteins Ia, Ib, and III are found associated with the large subunit and phosphoprotein II is found associated with the smaller subunit. Additional slow moving radioactive bands not given identification numbers are also associated with the small subunit. Phosphoproteins having a similar mobility to component IV are found on both types of subunits. These ribosomal phosphoproteins have the same molecular weights from both sources. I regard this striking similarity between sarcoma-180 ribosomes and reticulocyte ribosomes as conclusive proof that I am observing

real ribosomal constituents and not merely contaminating phosphoproteins associated only loosely with ribosomes.

C. Shifting of Single Ribosomes into Polysomes in Sarcoma-180 Cells

The addition of glucose and amino acids to a depleted Eagles medium in which mouse Ehrlich tumor cells were being cultured causes a slow shift of single ribosomes into polysomes in the tumor cells (65). This shift was complete to 95% polysomes and 5% single ribosomes in 4 hrs. A more rapid movement of single ribosomes into polysomes has been observed in Landschutz ascites cells (66). This ribosomal shift was in response to the addition of a mixture of amino acids to cells being cultured both in vivo and in vitro. The in vivo study produced a polysome/single ribosome ratio of 3:1 in 90 min. However, Lee and Brawerman (33) reported a complete shift of single ribosomes into polysomes in mouse sarcoma-180 tumor cells within 10 min of adding amino acids and glucose to an in vitro culture of those cells.

This report of a rapid shift of single ribosomes into polysomes prompted my selection of mouse sarcoma-180 tumor cells for the study of eukaryotic ribosomal phosphoproteins. I reasoned that a prerequisite for a complete shift of single ribosomes into polyribosomes was the activation of the previously inactive single ribosomal particles (34,35). Moreover, the kinetics of the shift in sarcoma-180 cells was likely to be rapid enough to allow a study of

ribosomal phosphoproteins as they shifted from single ribosomes into polyribosomes. Hence, I had hoped that a study of ribosomal protein phosphorylation patterns could be correlated to ribosomal involvement in protein synthesis.

The procedure used for inducing the rapid ribosomal shift in sarcoma-180 cells was to starve cells freshly harvested from the peritoneum of mice by incubation in non-supplemented Krebs buffer for 20 min at 37°. Glucose and amino acids were added to the culture medium following starvation and the incubation was continued at 37°. This addition of nutrients induced a shift of single ribosomes into polysomes that was reported to be complete within 10 min.

My attempts to reproduce this work of Lee and Brawerman (33) were unsuccessful. The starvation incubation for 20 min did cause all polysomes to shift into the single ribosome fraction; however, the addition of nutrients did not cause the reverse shift to go to completion. In different experiments the polysome to single ribosome ratio after 15 min incubation with nutrients varied from 0.3 to 1.2. Hence, the shift of single ribosomes into polysomes was far from complete.

The cells used for these experiments were freshly removed from animals. I therefore questioned whether a rapid ribosomal shift to polysomes could be dependent upon some factor only available to the sarcoma-180 cells in vivo. To determine the effect of nutrients on tumor cell ribosome shifts in vivo, I injected solutions of amino acids,

glucose, and amino acids plus glucose into the peritoneum of different mice carrying tumors. Samples of peritoneal fluid containing ascites tumor cells were removed at different times after injection of nutrients and the ratio of polysomes to single ribosomes in these cells was determined. The mice remained alive throughout this procedure.

The results of these experiments are shown in Figure 8. It is immediately apparent from these results that nutrient induced ribosomal shifts in vivo are relatively slow in sarcoma-180 cells. The maximal rate being comparable to the previously mentioned rate observed in mouse Ehrlich cells cultured in vitro (66). Also, a more rapid shift is observed with amino acids than with glucose. Sarcoma-180 cells harvested from different mice contain ribosome populations with widely varying polysome to single ribosome ratios. Experiments were selected for Figure 8 where the polysome/single ribosome ratios were comparable at time 0.

I next returned to in vitro incubations of sarcoma-180 cells to determine the effect of the starvation incubation on the rate of nutrient induced shifting of single ribosomes into polysomes. Results from a typical experiment are shown in Figure 9. The starvation incubation (Fig. 9a to 9c) causes polysomes to enter into the single ribosome peak. However, this shift does not greatly elevate the maximal height of the single's peak; rather the ribosomes appear to shift into a shoulder on the peak in the location of ribosomal dimers. Upon addition of nutrients and continuation of the incubation for 15

Figure 8. The in vivo kinetics of the shifting of single ribosomes into polysomes of sarcoma-180 cells upon injecting concentrated solutions of amino acids, glucose, or amino acids plus glucose into the peritoneum of host mice. The volume of the peritonel fluid was assumed to be 10 ml when determining the quantity of supplements for injection. The final intraperitonel concentration of nutrients were those recommended by Lee and Brawerman for in vitro incubations (33).

Ascites sarcoma-180 tumor cells were removed from the animals with a hypodermic syringe at the times indicated and ribosomes were prepared for sedimentation in sucrose density gradients. Ribosomes precipitated at pH 5.1 (Section II, A, 6) were dissolved in Buffer D (Section II, A, 1) and 40 $O.D._{260}$ units of material was layered on a sucrose gradient made with the same buffer. See figure 1 for a description of sucrose gradients and sedimentation conditions.

The quantities of single ribosomes and polysomes in the preparations were determined by measuring the respective areas under the $O.D._{260}$ profile curves, as in figure 1.

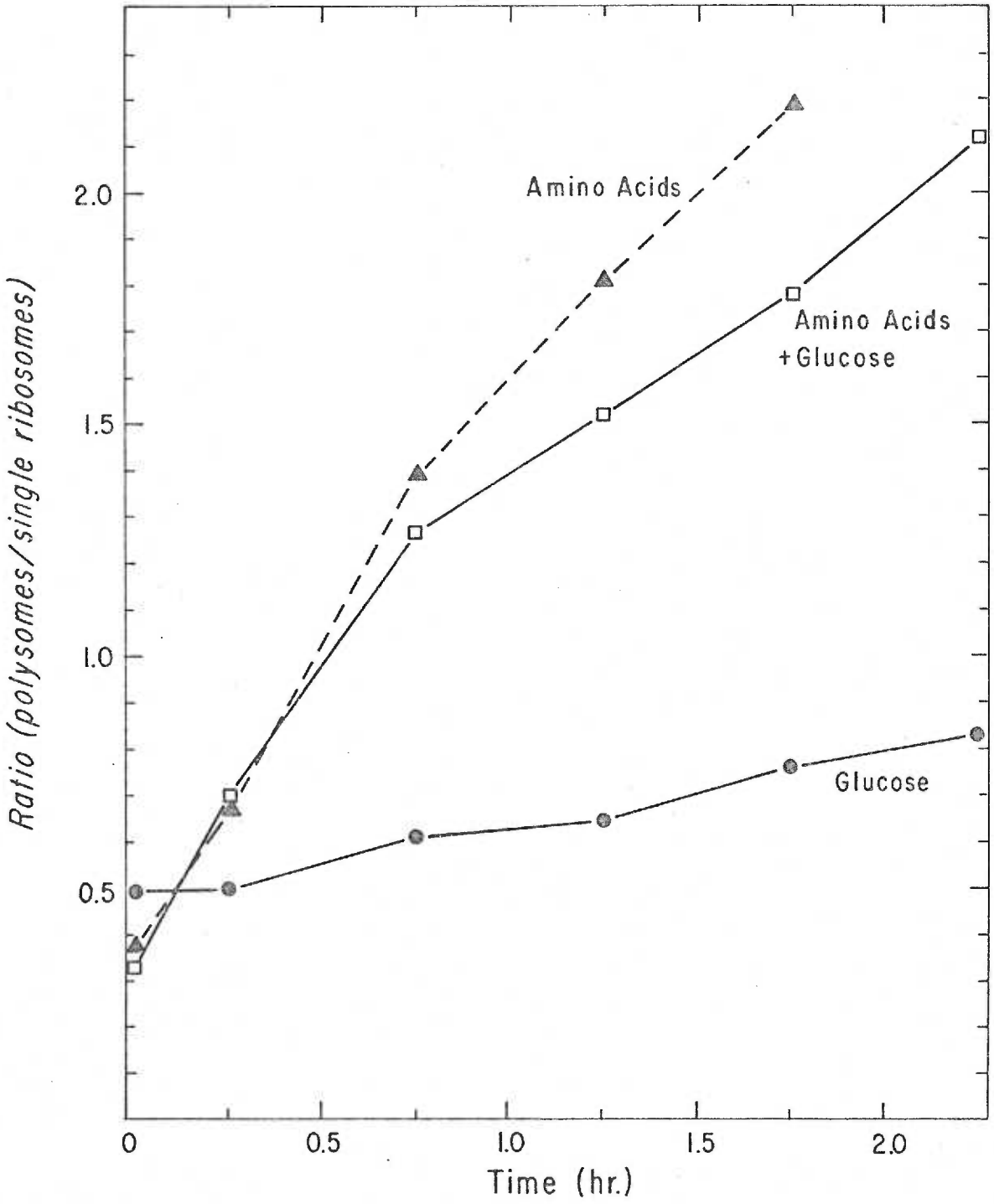
Ratio (polysomes/single ribosomes)
2.0
1.5
1.0
0.5
0
0.5
1.0
1.5
2.0
Time (hr.)
Amino Acids
Amino Acids
+Glucose
Glucose

Figure 8

Figure 9. Sucrose gradient sedimentation of ribosomes from sarcoma-180 tumor cells following incubation in vitro. Cells were removed from animals, washed, and suspended in incubation media as described in Methods (Section II, A, 6). Ribosomes were prepared and fractionated as described in figure 8.

The $O.D._{260}$ profiles show ribosomes from cells after subjection to the following in vitro incubation conditions; 9a, Sarcoma-180 cells as they were when removed from animals prior to in vitro incubation. 9b, Cells incubated with full nutrients (33) at 37° for 15 min. 9c, Cells incubated at 37° for 20 min under starvation conditions. 9d, nutrients were added to cells following starvation (i.e., 9c) and the incubation was continued at 37° for 15 min. 9e, The same as for 9d except that the incubation following addition of nutrients was continued for 45 min at 37°.

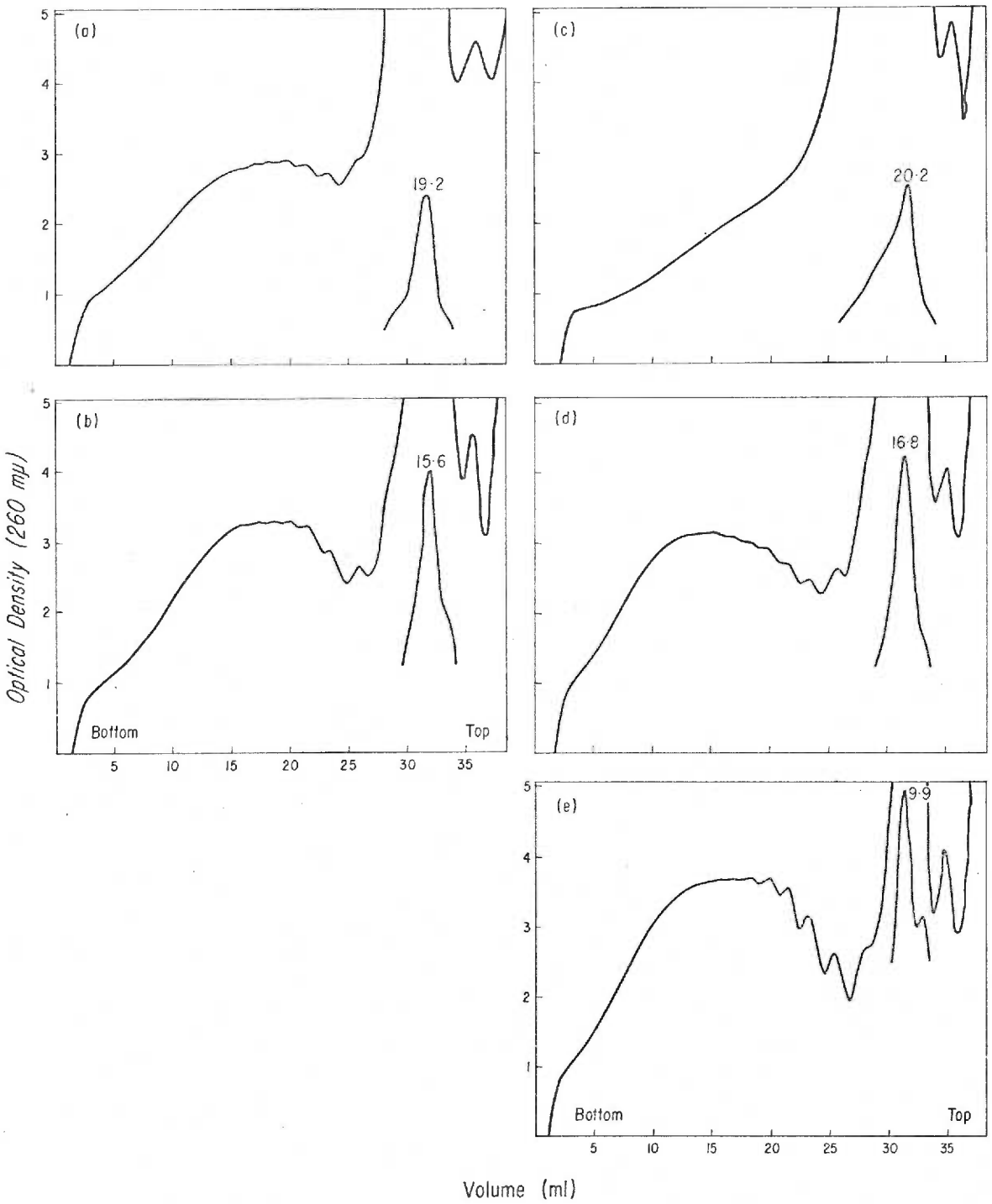
(a)
19·2
(c)
20·2
(b)
15·6
(d)
16·8
(e)
9·9
Bottom
Top
Optical Density (260 mμ)
Volume (ml)

Figure 9

min (Fig. 9d), there occurs a rapid increase in the number of polysomes. Continuation of this incubation for an additional 45 min results in single ribosomes entering polysomes but this increase in the amount of polysomes appeared to be at a much slower rate. Obviously, the starvation incubation had little or no effect on the quantity of ribosomes in polysomes following the 15 min nutrient incubation (compare Fig. 9b go 9d). Subsequent experiments where this procedure was repeated using sarcoma-180 cells from different mice confirmed this observation. Although the ratio of polysomes to single ribosomes varied widely when tumor cells were removed from animals, an incubation with nutrients for 15 min following starvation (e.g., Fig. 9d) always produced ribosome distribution patterns similar to control experiments (e.g., Fig. 9b).

The slower shifts shown in Figure 9 (9a to 9b and 9d to 9e) appear to be at similar rates to in vivo shifts (Fig. 8). Therefore, I have concluded that an accelerated shift of single ribosomes into polysomes upon nutrient addition to previously starved cells was restricted in magnitude to that quantity of single ribosomes produced by the starvation incubation.

This conclusion suggests that the ribosomes constituting that material fractionated on sucrose gradients and identified as single ribosomes were heterogeneous with respect to their ability to enter into polysomes. A special group of single ribosomes that had not

become part of the inactive pool of monosomes could have accounted for all of the rapid single ribosome to polysome shift post starvation. Hence, a study of the phosphorylation patterns of ribosomal phosphoproteins before and after this shift could not be rigorously correlated with a change in ribosome activity. I therefore did not pursue this line of investigation further.

D. Effects of Cyclic-AMP on the Phosphorylation of Ribosomal Proteins in Sarcoma-180 Tumor Cells

1. Phosphoproteins in purified ribosomes

My goal in this study was to analyze the effects of cyclic-AMP on the incorporation of [^{32}P]-orthophosphate into ribosomal proteins. However, phosphoprotein contaminants have been reported to occur in ribosome preparations from various tissues (56,57,60,61); and it is conceivable that the phosphorylation of such contaminants might be very strongly influenced by cyclic-AMP. Accordingly, I felt that it was essential at the onset to obtain some information concerning the sources and specific activities of the common ribosomal contaminants which may be present in ribosomes purified by the procedures described earlier (Section II, B, 2).

When suspensions of sarcoma-180 cells are incubated with [^{32}P]-orthophosphate there occurs a rapid incorporation of radioactivity into the ribosomal proteins and also into the cytoplasmic non-ribosomal supernatant proteins (see Methods II, A, 6 and I, B, 2 for a description of these fractions). Figure 10 shows the kinetics of incorporation

Figure 10. Kinetics of [^{32}P]-orthophosphate incorporation into sarcoma-180 cytoplasmic supernatant proteins (see Section II, A, 6) and into ribosomal proteins. Measurements were made on samples before and after extraction of preparations with lipid solvents (see Section II, B, 5). Ribosomes were prepared by Isolation Procedure F (Section II, B, 2). Protein content was measured by the method of Lowry et al. (63) and radioactivity was assayed as described in figure 5.

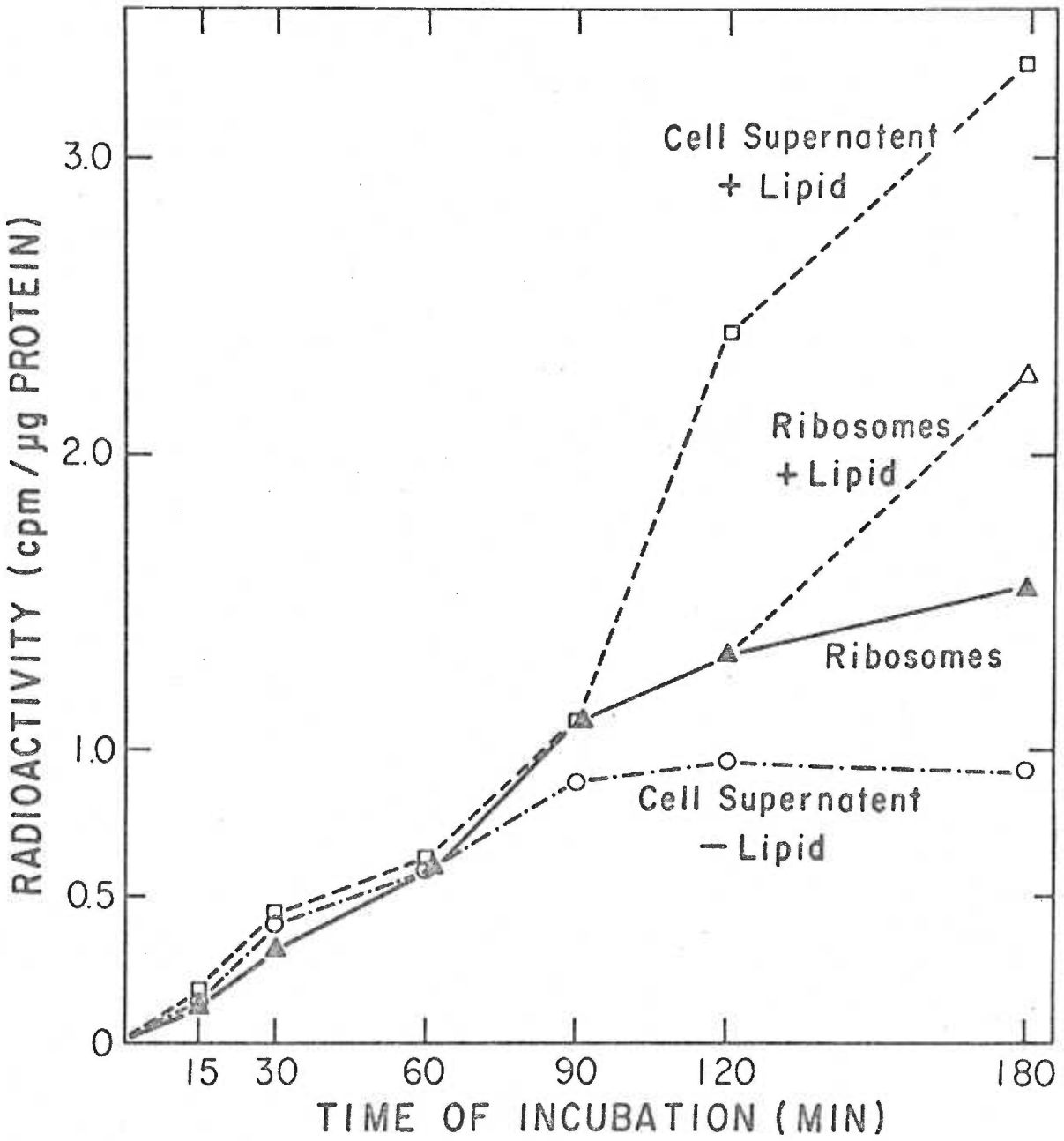
RADIOACTIVITY (cpm / µg PROTEIN)
3.0
2.0
1.0
0.5
0
15
30
60
90
120
180
TIME OF INCUBATION (MIN)
Cell Supernatent
+ Lipid
Ribosomes
+ Lipid
Ribosomes
Cell Supernatent
— Lipid

Figure 10

into these subcellular fractions. Although these fractions do contain some radioactive material which is extractable with nonaqueous solvents, such material becomes labeled to a significant extent only after 60 min of incorporation (Fig. 10).

The results of this experiment also revealed that [^{32}P]-orthophosphate incorporation into ribosomal proteins represents only a small percentage (2-4.5%) of the total incorporation into cytoplasmic proteins. Accordingly, a rather small extent of contamination with supernatant proteins could lead to an appreciable error in analysis of radioactivity incorporated into ribosomes. In addition, these data provide some information concerning the magnitude of phosphoprotein metabolism in sarcoma-180 cells. Since we know that approximately 5 out of the 76 different ribosomal proteins are phosphoproteins (9, 12), I infer that roughly this same fraction of all sarcoma-180 cytoplasmic proteins may also be phosphorylated. Obviously, this estimate would be in error if the specific activity of the cytoplasmic phosphoproteins were very different than that of the ribosomal phosphoproteins. However, this is an interesting estimate, nonetheless, because it raises the possibility that phosphorylation of proteins may be much more widespread than has been previously suspected. Whether such active phosphoprotein metabolism occurs in all cells or is unique to tumor cells likewise remains unknown.

2. Stimulation by cyclic-AMP of [^{32}P]-orthophosphate incorporation into ribosomal phosphoproteins

The addition of cyclic-AMP and theophylline to a suspension of S-180 cells can have a dramatic effect on the incorporation of [^{32}P]-orthophosphate into ribosomal phosphoproteins, as shown in Figure 11. Theophylline alone produced no response but served to enhance the response of the cells to cyclic-AMP when both were added together. However, I have observed a high degree of variability in the response of S-180 cells to cyclic-AMP. S-180 cells isolated from different animals and assayed in parallel experiments have produced results as dissimilar as no stimulation of incorporation in one experiment and a nine-fold stimulation of incorporation into ribosomal phosphoproteins in the other. Factors which influence the degree of stimulation will be discussed in a later section.

That the stimulation of incorporation of [^{32}P]-orthophosphate into ribosomes shown in Figure 11 represents increased incorporation into real ribosomal phosphoproteins was indicated by data presented earlier but not discussed at that time (Table I, Section III, A, 3, experiment 2). Cyclic-AMP stimulated the incorporation of [^{32}P]-orthophosphate into all ribosomal preparations. Moreover, the greatest stimulation was observed when ribosomes of the highest purity were assayed (isolation procedures E and G, Table I). The level of stimulation for these procedures approaches that of the total cytoplasmic phosphoproteins (Table I).

Figure 12 shows the ^{32}P-radioactivity analysis of transectionally sliced polyacrylamide gels in which sarcoma-180 ribosomal

Figure 11. Stimulation of [^{32}P]-orthophosphate incorporation into ribosomes by addition of cyclic AMP (3 mM) and theophylline (3 mM) to an in vitro incubation of sarcoma-180 cells. Cells were incubated at 37° with full nutrients as described previously (Section II, A, 6). [^{32}P]-orthophosphate was added at time 0 and samples were removed at the times indicated. After 60 min of incubation, 1/2 of the incubation was transferred into a flask containing cyclic AMP and theophylline (+c-AMP) and the incubations were continued at 37°. Theophylline was added with cyclic-AMP to inhibit cyclic-AMP phosphodiesterase.

Sarcoma-180 ribosomes were purified by Isolation Procedure E (Section II, B, 2). Protein content was calculated from O.D.$_{260}$ measurements and radioactivity was assayed as described for figure 5.

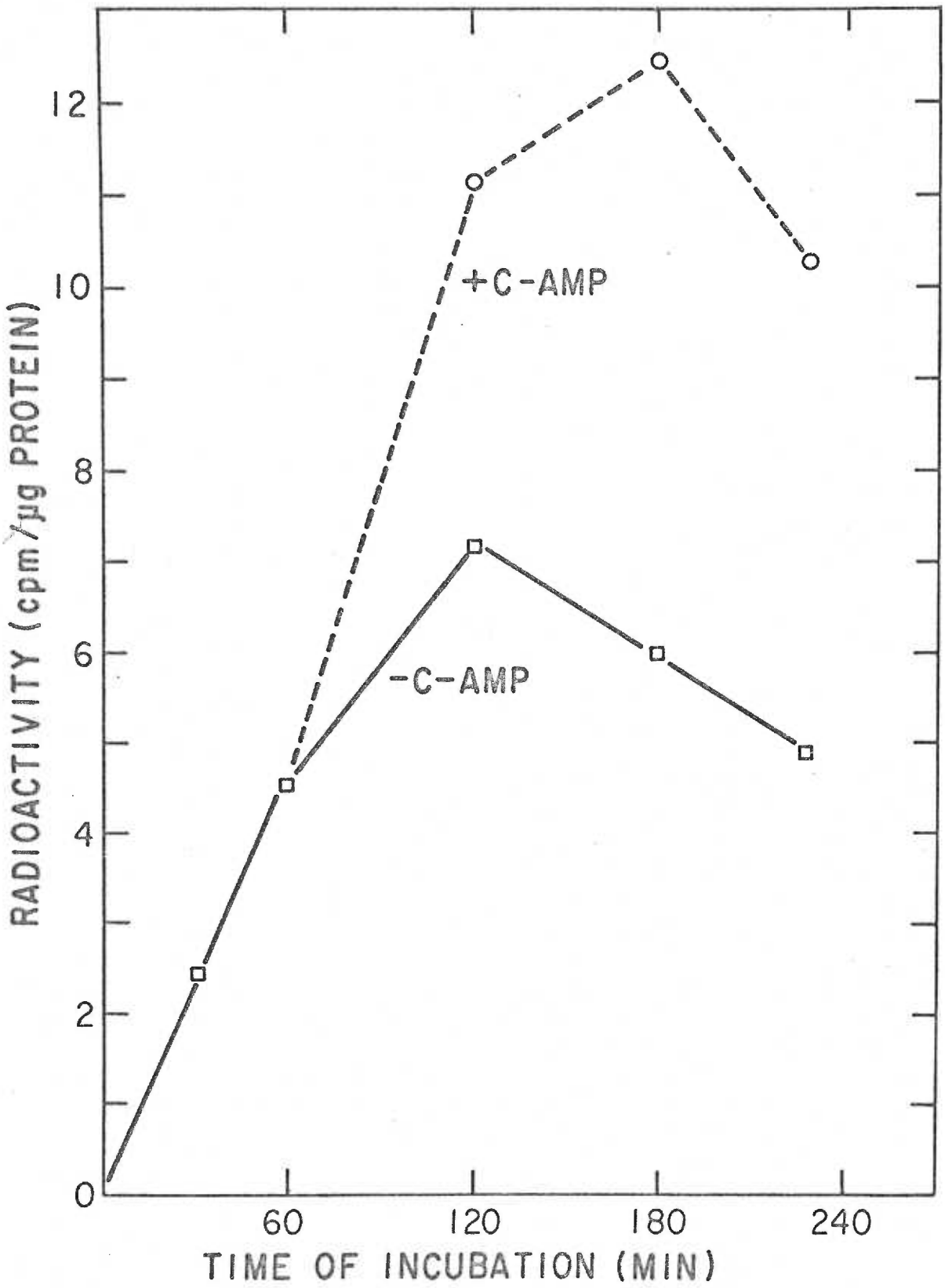
RADIOACTIVITY (cpm/µg PROTEIN)
0
2
4
6
8
10
12
+C-AMP
-C-AMP
60
120
180
240
TIME OF INCUBATION (MIN)

Figure 11

Figure 12. Electrophoresis of ^{32}P-labeled ribosomal constituents from sarcoma-180 ribosomal subunits in 8% polyacrylamide gels (i.e., as in figures 4 and 7). Cells were incubated with [^{32}P]-orthophosphate as described in Methods (Section II, A, 6), 1/2 of the cells were incubated in the presence of cyclic AMP (3 mM) and theophylline (3 mM).

Migration is toward higher fraction numbers. The gels were cross-sectioned and the sections were counted in a scintillation counter, see Methods (Section II, C, 7).

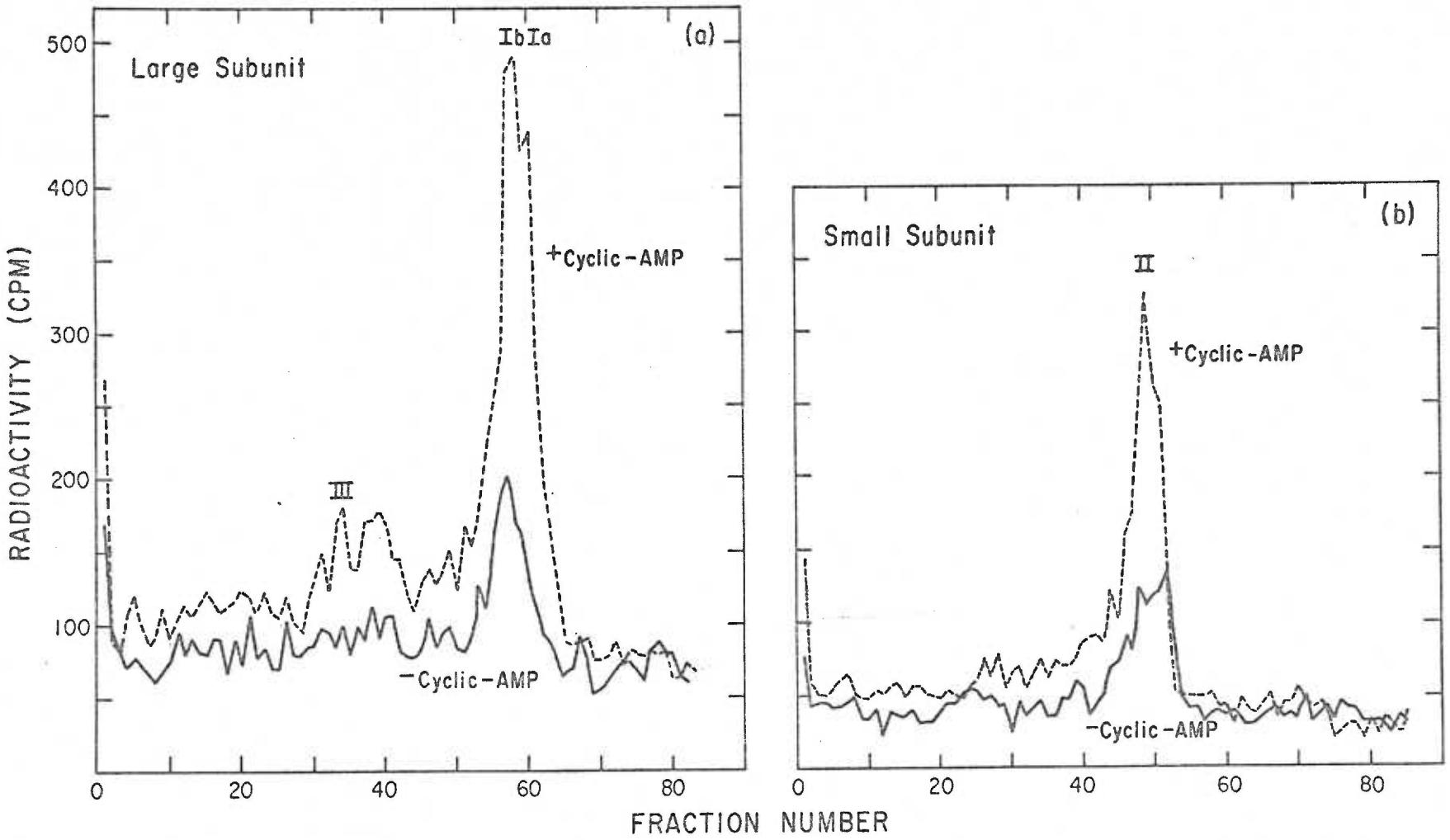
Large Subunit
(a)
IbIa
III
+Cyclic-AMP
−Cyclic-AMP
Small Subunit
(b)
II
+Cyclic-AMP
−Cyclic-AMP
RADIOACTIVITY (CPM)
500
400
300
200
100
0
20
40
60
80
FRACTION NUMBER

Figure 12

phosphoproteins from isolated subunits were fractionated. Cyclic-AMP stimulation was induced under the standard conditions mentioned previously and produced an overall increase of ^{32}P-incorporation into ribosomal proteins of 2·4 when ribosomes were purified with isolation method B. The remaining ribosomes were dissociated into subunits by isolation method E. The cyclic-AMP induced stimulation of incorporation for the small and large ribosomal subunits were by factors of 2·2 and 3·1, respectively. Cyclic AMP enhanced the labeling of all ribosomal phosphoproteins in the experiment shown in Figure 12 and in other experiments where ribosomes were purified using a magnesium precipitation step (isolation procedure G). Band II, which is located in the small subunit (Fig. 7) was stimulated by about 2-fold (calculation from experiment shown in Fig. 12), whereas the phosphoproteins located in the large subunits (bands Ia, Ib, III, and IV) were also stimulated to a similar extent. These experiments showed that cyclic-AMP does stimulate the rate of incorporation of [^{32}P]-orthophosphate into real ribosomal phosphoproteins. Moreover, this stimulated incorporation does not appear to have any specificity with respect to the phosphoprotein affected.

3. Factors influencing the effect of cyclic-AMP

Factors which influence the degree of stimulation of incorporation of [^{32}P]-orthophosphate into ribosomal proteins are: (1) the time of cell preincubation prior to adding [^{32}P]-orthophosphate and cyclic-AMP, (2) the glucose concentration in the incubation medium,

(3) the time of incubation with [^{32}P]-orthophosphate prior to assay (Fig. 11), and (4) any time lapse between adding cyclic-AMP and [^{32}P]-orthophosphate to a cell suspension. Since cyclic-AMP has been implicated as playing a role in control of tumor cell growth (67), and since phosphoprotein metabolism may be central to cyclic-AMP actions in animal cells (27,28), I have examined these effects in greater detail.

The effect of pre-incubating cells in Krebs medium on cyclic-AMP stimulation of ribosomal protein phosphorylation is shown in Figure 13. The cells used for these experiments displayed little or no response to cyclic-AMP just after they were harvested from animals. The sarcoma-180 cells in control incubations (-cyclic-AMP) progressivly lost their ability to incorporate [^{32}P]-orthophosphate into ribosomal proteins, regardless of the levels of glucose and amino acids in the medium. At the same time, however, the addition of cyclic-AMP to these preincubated cells produced a stimulation of phosphorylation to higher levels than for the same cells prior to the preincubation.

The effectiveness of preincubation in enhancing the response of the cells to cyclic-AMP was not dependent on the nutrient content of the medium. However, glucose was required by the cells to maintain their viability and to sustain high levels of [^{32}P]-orthophosphate incorporation upon stimulation.

The effect of allowing time to lapse between adding cyclic-AMP

Figure 13. The effects of preincubating sarcoma-180 cells in Krebs medium on the cyclic AMP induced stimulation of [^{32}P]-orthophosphate incorporation into ribosomal phosphoproteins. Cells were incubated at 37° and, where indicated, the incubations were supplemented with nutrients as described in Methods (Section II, A, 6).

Incubation samples were transferred at times indicated into flasks containing [^{32}P]-orthophosphate and the incubation was continued at 37° for 30 min. Some of these flasks also contained 3 mM cyclic AMP and 3 mM theophylline (+ c-AMP).

Ribosomes were isolated with procedure B (Section II, B, 2), radioactivity was assayed as described for figure 5, and protein content was calculated from measurements of O.D.$_{260}$.

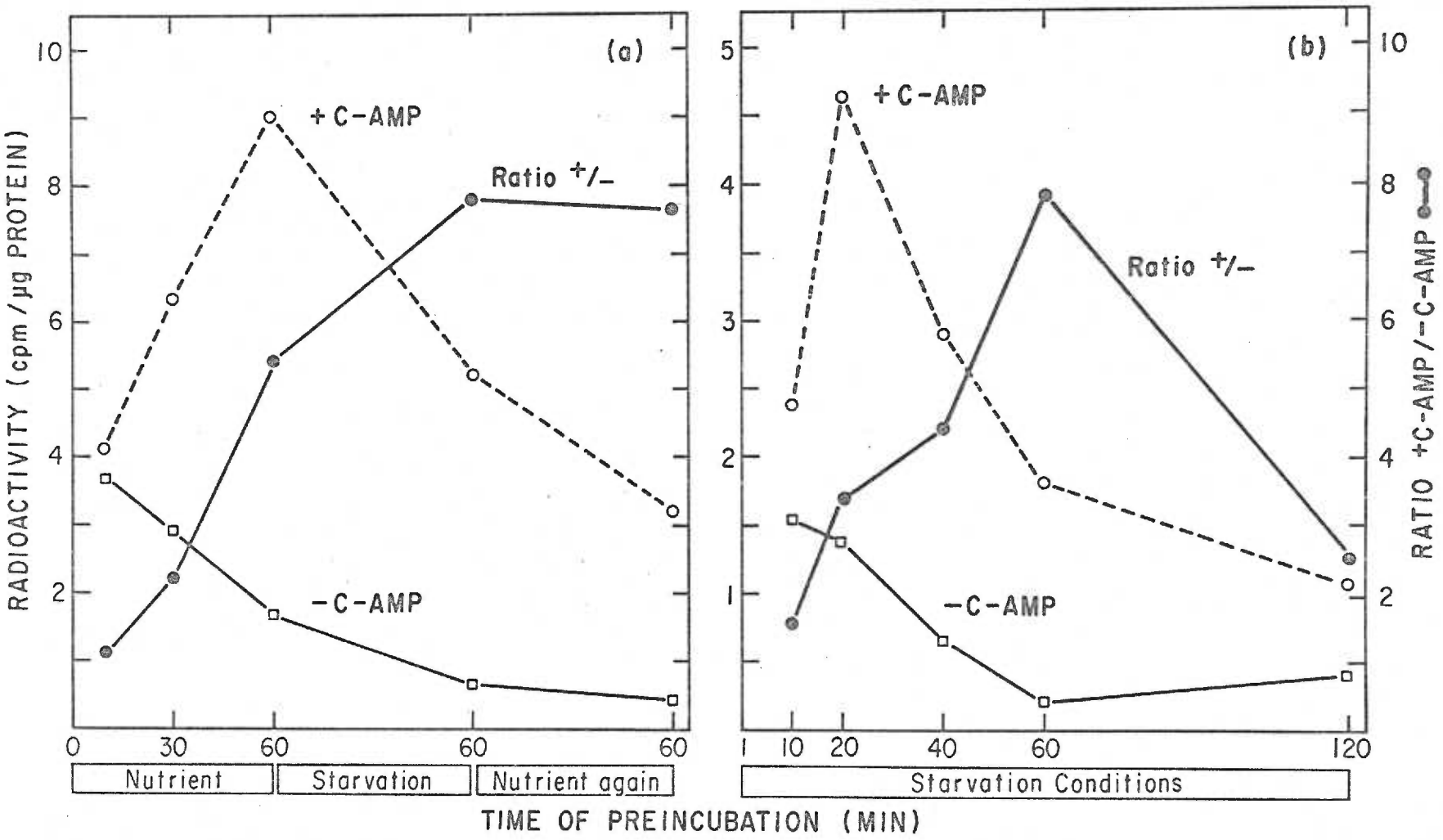
(a)
(b)
+C-AMP
-C-AMP
Ratio +/-
RADIOACTIVITY (cpm/µg PROTEIN)
RATIO +C-AMP/-C-AMP
0
30
60
60
60
1
10
20
40
60
120
Nutrient
Starvation
Nutrient again
Starvation Conditions
TIME OF PREINCUBATION (MIN)

Figure 13

and [^{32}P]-orthophosphate to a cell suspension is displayed in Figure 14. Cyclic-AMP loses its effectiveness shortly after being introduced into the medium, even in the presence of theophylline. Simultaneous addition of the cyclic nucleotide and [^{32}P]-orthophosphate produced a stimulation of 1.8 which diminished to essentially no effect within 20 min. The reintroduction of additional cyclic-AMP and theophylline (3 mM each) at the time of radioactive orthophosphate addition (asterick data at 30 min) produced a return of cyclic nucleotide dependent stimulation. The magnitude of [^{32}P]-orthophosphate incorporation upon restimulation is far less than at the zero time. However, the stimulation was again by a factor of 1.8. My interpretation of this data is that cyclic-AMP is degraded rapidly after being added to the medium and that the same factor which was limiting for cyclic-AMP stimulation at time zero was also limiting at 30 min.

This series of experiments enabled me to better define conditions under which cyclic-AMP produces an enhancement of [^{32}P]-orthophosphate incorporation into sarcoma-180 ribosomal proteins. Hence, I adopted a standard procedure of 30 min preincubation of cells in Krebs medium supplemented with glucose and amino acids prior to testing for cyclic-AMP induced stimulation of phosphorylation. Fetal calf serum commonly employed in such medium was excluded because it was found to have no noticeable effect on the phosphorylation reactions being observed; accordingly, it would unnecessarily complicate the interpretation of results. Two important considerations at this

Figure 14. The effect of preincubating sarcoma-180 cells in the presence of cyclic AMP on the stimulation of incorporation of radioactivity into ribosomal phosphoproteins. The experimental procedure was as described for figure 13 with full nutrients except that the cells were divided at time 0 and cyclic AMP plus theophylline (3 mM each) was added to one incubation.

Samples from these preincubations were added to flasks containing [^{32}P]-orthophosphate at times indicated and the incubations were continued at 37° for 30 min. The asterix data point at 30 min preincubation is for a sample of cells from the + c-AMP preincubation to which additional cyclic AMP and theophylline were added at original levels together with [^{32}P]-orthophosphate. Ribosomes were prepared and analysed as described for figure 13.

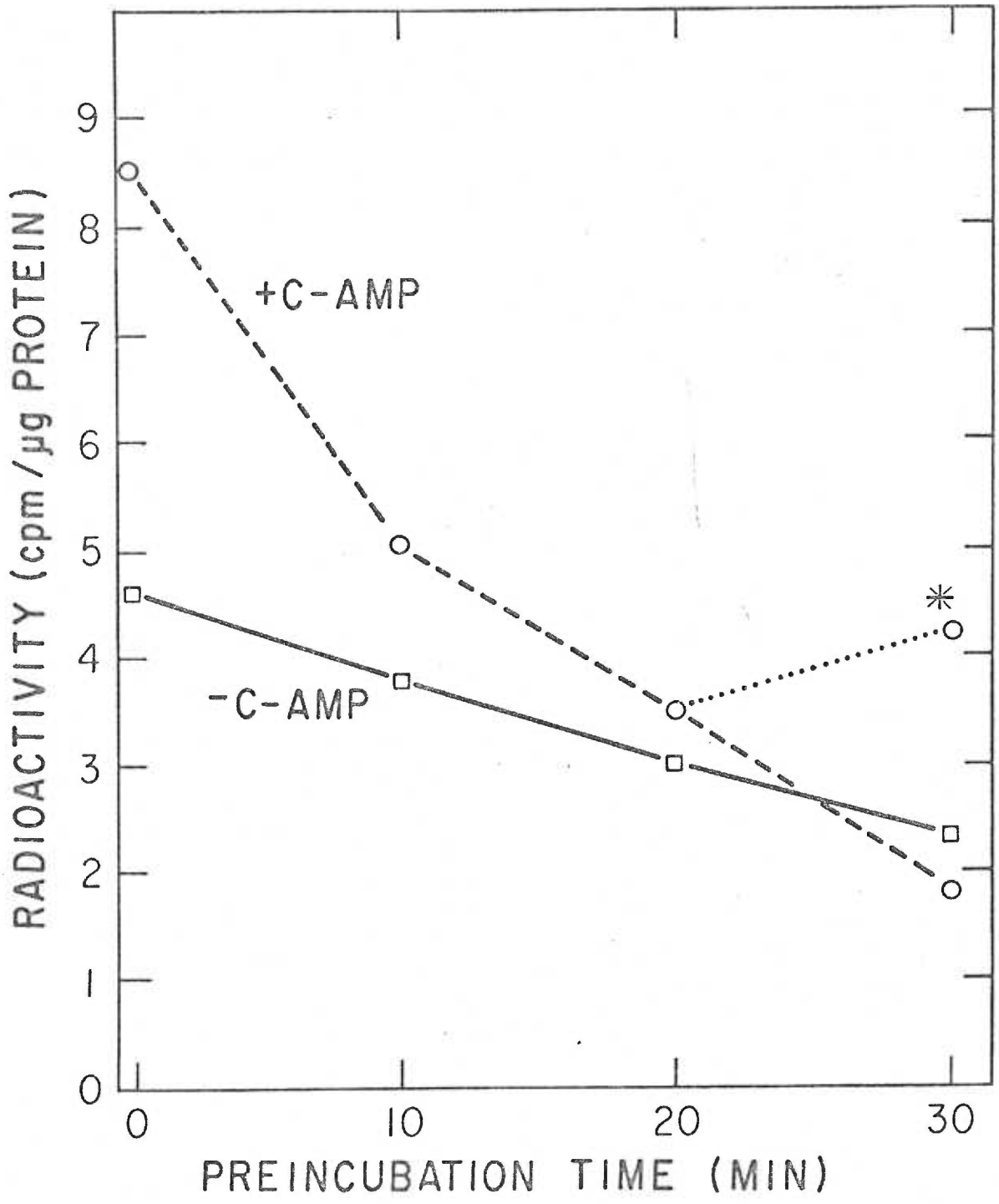
RADIOACTIVITY (cpm / µg PROTEIN)
0
1
2
3
4
5
6
7
8
9
0
10
20
30
PREINCUBATION TIME (MIN)
+C-AMP
-C-AMP
*

Figure 14

stage of the investigation were whether the cyclic-AMP effect influenced the specific activity of ATP and whether any correlation existed between the stimulation of [^{32}P]-orthophosphate incorporation into ribosomes and the rate of protein synthesis in sarcoma-180 cells.

4. The effects of cyclic-AMP and of sodium fluoride on ATP specific activity

Changes in the specific activity of ATP could obviously influence the labeling data I have described; the γ-phosphate of ATP is the immediate source of ribosomal protein phosphate (15,16,18, 21). Moreover, I have previously shown that the cyclic-AMP induced stimulation of radioactivity into ribosomes in sarcoma-180 cells is non-specific with respect to individual phosphoproteins (Fig. 12). A cyclic-AMP induced increase in the specific activity of ATP could produce this result. I therefore analyzed the effects of cyclic-AMP on the incorporation of [^{32}P]-orthophosphate into ATP in sarcoma-180 cells. Since sodium fluoride activates adenylate cyclase (68) and enhances ribosomal protein phosphorylation in rabbit reticulocytes (69), I also assayed its influence on the labeling of ATP in sarcoma-180 cells.

The data in Table 2 demonstrates that sodium fluoride lowers the intracellular concentration of ATP and drastically inhibits the incorporation of [^{32}P]-orthophosphate into ATP in sarcoma-180 cells. However, cyclic-AMP reproducibly elevates both the intracellular concentration and the specific activity of ATP in the same cells. The

TABLE II

Effect of Cyclic-AMP and Sodium Fluoride on ATP Intracellular Concentration and Specific Activity

Experiment	Incubation Addition	ATP (μ moles/ml cells)	Stimulation (Inhibition)	ATP S.A. (cpm/μ mole x 10^7)	Stimulation (Inhibition)	Ribosomal Proteins S.A. (cpm/μ mole x 10^6)	Stimulation (Inhibition)
1	None	1.72		11.6		6.48	
	c-AMP	2.12	1.23	13.4	1.15	7.88	1.21
2	None	1.52		73.8		55.2	
	c-AMP	1.70	1.12	83.2	1.12	76.8	1.39
	NaF	0.203	(0.134)	5.2	(0.063)	2.88	(0.052)
3	None	1.84		66.1		21.9	
	c-AMP	2.52	1.38	82.1	1.24	33.8	1.55
	NaF	0.330	(0.179)	5.1	(0.078)	4.64	(0.21)

Ribosomes were prepared with isolation procedure B and were assayed as described for Table I (Section III, A, 3). ATP content and specific activity were determined as described in Methods (Section II, D, 3).

degree to which cyclic-AMP raised the specific activity of ATP in all experiments was less than the relative stimulation of incorporation into ribosomal proteins. However, such differences are difficult to interpret rigorously (see Discussion). The important point to be derived from these experiments is that cyclic-AMP does indeed enhance the incorporation of [^{32}P]-orthophosphate into ATP in sarcoma-180 cells. Such a change in ATP specific activity presumably contributes to some extent to the cyclic-AMP induced stimulation of incorporation of radioactivity into ribosomal phosphoproteins. My observation that cyclic-AMP increases the incorporation of [^{32}P]-orthophosphate into sarcoma-180 cells is in agreement with data obtained in liver and heart (29-31), and in reticulocytes (69).

5. Cyclic-AMP stimulation of the rate of protein synthesis in sarcoma-180 cells

The effects of cyclic-AMP on amino acid incorporation into proteins are complex; both positive and negative effects have been observed in different eukaryote cells (27). In sarcoma-180 cells, cyclic-AMP causes a stimulation of incorporation of ^{14}C-labeled amino acids into proteins. This stimulation varied in magnitude in different experiments from 1·1 to 2·8. Parallel incubations were undertaken for this series of experiments in which the cyclic-AMP induced stimulation of [^{32}P]-orthophosphate incorporation into ribosomal phosphoproteins was assayed. Although cyclic-AMP was stimulatory for both types of radioisotope incorporations, no numerical correlation between

the two stimulations was observed. Both stimulatory effects varied in different experiments in what appeared to be an unrelated manner.

The effects of cyclic-AMP on cellular metabolism are numerous and complex; and it is clear that the cellular rate of protein synthesis can be limited by many factors other than ribosome activity *per se*. Accordingly, it is unclear whether the stimulation of protein synthesis is in any way related to ribosomal phosphoprotein metabolism.

IV. DISCUSSION

A. Phosphorylation of Ribosomal Proteins in Sarcoma-180 Cells

Much of my initial work with sarcoma-180 tumor cells closely paralleled earlier studies with rabbit reticulocytes (9). Ribosomal particles were fractionated by sedimentation in sucrose gradients and cosedimentation of radioactivity with the optical density at 260 nm was used to show the incorporation of [^{32}P]-orthophosphate into polysomes, single ribosomes, and ribosomal subunits (Figures 1 and 2). That this incorporation of radioactivity was not into RNA was shown by several lines of evidence (Section III, A, 2) and phospholipids were also excluded as being a major contributor to the radioactive assay (Section III, A, 3). Proof that [^{32}P]-orthophosphate was incorporated into phosphoproteins was obtained from electrophoretic analysis of ribosomal preparations following pronase treatment (Figure 3) and by isolation of radioactive phosphoserine and phosphothreonine from mild acid hydrolysates of ^{32}P-labeled ribosomal preparations.

In some respects, however, procedures for sarcoma-180 cells differed from procedures used for reticulocytes. Cells were lysed with the use of a detergent (Triton X-100) to avoid disruption of nuclei (59). Removal of intact nuclei from cell lysates was desirable to avoid possible contamination of ribosomal particles with phosphorylated histones and with other nuclear phosphoproteins (70-72). The ionic strength of the cell lysis medium was raised to 140 mM monovalent cations for sarcoma-180 cells to limit the release of membranous

proteins into the lysate (56,57). This was done to avoid artifactual adsorption of polysomes onto modified membranes and to limit possible contamination of ribosomes by released membrane phosphoproteins (see Section III, A, 1).

In spite of the above mentioned precautions, radioactive contamination of ribosomal particles was a major problem in the characterization of ribosomal phosphoproteins from sarcoma-180 cells. Ribosome isolation procedures which were adequate for preparing phosphoproteins from reticulocytes were inadequate for studies with sarcoma-180 cells. Tumor cell ribosomes prepared by three reticulocyte methods often showed a high background in polyacrylamide gels (Figure 4); and more drastic purification procedures were therefore employed. Furthermore, it was necessary to evaluate the effects of different ribosomal isolation procedures on the quantity of phosphoprotein radioactivity remaining in those preparations (Table I). Radioactive phosphoproteins were found in all ribosomal preparations regardless of the procedure used. Subsequent electrophoretic fractionation and analysis strongly indicated the occurrence of at least 4 and most likely 5 distinct ribosomal phosphoproteins. One explanation for the difficulties encountered with these cells may be that ribosomal phosphoproteins constitute only a small proportion (2 to 4.5%) of the total phosphoprotein radioactivity found in the cell cytoplasmic supernatant fraction from which ribosomes are prepared. This provides a large source of potential contamination by adsorbed non-ribosomal phosphoproteins.

The kinetics of incorporation of [^{32}P]-orthophosphate into ribosomal proteins in sarcoma-180 cells (Figure 5) appears to be somewhat slower than for reticulocytes (14). This may indicate a slower turnover rate of phosphoryl groups in tumor ribosomal phosphoproteins; however, slower transport of [^{32}P]-orthophosphate into the cells or a slower equilibration of radioactivity with ATP could produce the same results.

B. Comparison of Ribosomal Phosphoproteins from Mouse Sarcoma-180 Cells with those from Rabbit Reticulocytes

These studies show that similar phosphoproteins occur in ribosome preparations from mouse sarcoma-180 cells and from rabbit reticulocytes. That phosphoproteins from these sources have the same molecular weights is suggested by their coelectrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (Figures 6 and 7). Furthermore, the subunit localizations of these phosphoproteins are the same from both sources (Figure 7). In addition, the specificity of ribosome phosphorylation in these cells is similar; polysomes and single ribosomes have distinctive phosphorylation patterns which appear to be common for both cells.

I believe that these similarities between S-180 cells and reticulocytes show conclusively that the phosphoproteins are true ribosomal constituents rather than contaminants of ribosome preparations. It is extremely unlikely that these two very different cells lysed under different conditions would produce similar ribosomal

contaminants.

Ribosomes play a central role in biological information transfer and are complex organelles which contain many constituent proteins (1,7,8). The proteins of eukaryote ribosomes are generally isolated after denaturation and cannot be assayed since they lack demonstrable biological activities. Consequently, it has not previously been possible to show homology between ribosomal proteins from different mammalian species. My studies suggest that homologous proteins occur in the ribosomes of mice and of rabbits. Moreover, the homologous ribosomal phosphoproteins are well suited for further evolutionary studies because they can be readily identified and assayed (e.g., as in Figures 6 and 7). This will certainly facilitate their purification. Hopefully, studies of the amino acid sequences of these purified phosphoproteins would help us to understand the evolution and functions of ribosomes.

Although the functions of ribosomal protein phosphorylations remain unknown, presumably they are important since this metabolism has been retained during mammalian evolution. Protein phosphorylation is a post translational event which requires a primary sequence containing either a serine or threonine residue accessible to a kinase enzyme and to ATP. Moreover, phosphorylation of these residues is a significant event since it alters the net charge of the protein. If such a drastic change in the protein were without functional effect, then very likely other amino acid substitutions at this site would

have been tolerated during evolution. However, such substitutions have apparently not occurred; and it may therefore be inferred that the phosphorylations perform a functional role. The phosphorylations occur in non-nucleated nondividing reticulocytes synthesizing predominantly hemoglobin as well as in a rapidly proliferating tumor. This also implies that their functions are not related specifically to cellular differentiation or to neoplasia.

The phosphorylation differences between polysomes and single ribosomes (Figure 6) are consistent with other evidence that these classes of ribosomes are not rapidly equilibrating in higher organisms (34,35,73,74). Single ribosomes are not participating in the ribosomal subunit-polysome cycle of protein synthesis and are believed to be in an inactive state.

C. Shifting of Single Ribosomes into Polysomes in Sarcoma-180 Tumor Cells

I had hoped to study ribosomal protein phosphorylation patterns during rapid shifts of single ribosomes into polysomes. This study was proposed in order to learn whether any obligatory coupling existed between the phosphorylation of ribosomes and changes in ribosomal activity. Changes in ribosome activity (75), or alternatively, the availability of mRNA to ribosomes (33), have been proposed as regulatory mechanisms for the control of protein synthesis in mammalian cells. Accordingly, elucidation of the effects of ribosomal protein phosphorylations on ribosomal activity could have contributed to our

understanding of the regulation of this important biological function.

I was unable to reproduce experiments reported previously that showed a complete shift of single ribosomes into polysomes in mouse sarcoma-180 tumor cells within 10 min (33). I did observe some shift of single ribosomes into polysomes (Figure 9) that was more rapid than similar shifts in vivo (Figure 8); however, the quantity of ribosomes capable of rapidly shifting into polysomes appeared to be limited to that number of ribosomes that moved out of polysomes as a result of previous starvation. Subsequent to my investigation of this subject, other researchers have published similar findings with Hela cells (76) and Ehrlich ascites tumor cells (77,78). These results suggest that sucrose gradient fractionation of ribosomal material may produce a peak containing single ribosomes which are functionally inhomogeneous (Figure 9c) with respect to their ability to initiate protein synthesis.

D. Effects of Cyclic-AMP on the Phosphorylation of Ribosomal Proteins in Sarcoma-180 Tumor Cells

Cyclic-AMP dependent protein phosphorylation reactions have been increasingly implicated in the control of metabolism and growth in mammalian cells (27). Blat and Leob (11) have reported that glucagon injection into rats stimulates the incorporation of [^{32}P]-orthophosphate into liver ribosomal proteins. Presumably, the mechanism of this stimulation was by activation (via cyclic-AMP) of the intracellular ribosomal kinase. However, their studies were not

conclusive because an increased incorporation of [^{32}P]-orthophosphate into ATP could have produced the same results.

The addition of cyclic-AMP to in vitro cultures of sarcoma-180 cells clearly stimulates the incorporation of [^{32}P]-orthophosphate into ribosomal particles (Figure 11). That this stimulation was into specific ribosomal phosphoproteins was shown in Figure 12. This stimulation of incorporation of radioactivity was non-selective with respect to the different ribosomal phosphoproteins. Recent experiments with rabbit reticulocytes have given results similar to those described here (69). In that case, however, cyclic-AMP caused a strong relative increase in the labeling of phosphoprotein II (see Figure 7); and it was concluded that this selective stimulation was consistent with a mechanism including stimulation of phosphorylation by action of a cyclic-AMP dependent protein kinase. Such a conclusion cannot be drawn from these studies with sarcoma-180 tumor cells due to the non-selective nature of the observed stimulation by cyclic AMP.

Cyclic-AMP has been shown to increase inorganic phosphate uptake into liver and heart tissues (29-31) and into reticulocytes (69). Such increased transport would be expected to result in an initial stimulation in the specific activity of intracellular ATP; this would then cause a more rapid labeling of phosphoproteins. The addition of cyclic AMP to cultures of sarcoma-180 cells causes stimulation of both the specific activity and intracellular concentration of ATP commensurate with the stimulation of incorporation of

radioactivity into ribosomal phosphoproteins (Table II). Although such data cannot exclude the possibility that cyclic-AMP can cause some increase in the phosphorylation of ribosomes, it seems unlikely from these experiments that such an effect is either large or highly reproducible.

This interpretation may have to be re-evaluated if the observed increase in ATP specific activity results from increased [^{32}P]-orthophosphate incorporation into the α phosphoryl group of ATP. Such an effect would result from increased synthesis of new adenosine nucleotides since the α phosphate of ADP and ATP are not in rapid equilibrium with extracellular [^{32}P]-orthophosphate (79,80). However, it is likely that the observed increase in ATP concentration results from a shift in ATP/ADP ratios since these ratios change very rapidly in response to alterations in the physiological environment of cells (81).

Distribution of [^{32}P]-orthophosphate between the β and γ phosphoryl groups in ATP normally reaches a rapid equilibrium in mammalian cells (80,82,83). However, a recent report has indicated that extracellular [^{32}P]-orthophosphate is incorporated into the γ phosphate more rapidly than into the β phosphate in Ehrlich tumor cells (81). If sarcoma-180 tumor cells respond in a similar fashion, the increase in ATP specific activity reported in Table II could easily account for all of the increased incorporation of radioactivity into ribosomal phosphoproteins.

Sodium fluoride drastically inhibits both the generation of ATP and the incorporation of [^{32}P]-orthophosphate into that ATP which is recovered after 30 min of incubation. Reduced transport of [^{32}P]-orthophosphate into the tumor cells could account for the reduction of ATP specific activity, but would not explain the dramatic inhibition of ATP synthesis. Most ATP in tumor cells is generated from glycolytic reactions rather than from mitochondria, even though the mitochondria appear to be functional (84). The low production of ATP by mitochondria has been explained by the hypothesis that they are starved for substrates in tumor cells. Sodium fluoride is a known inhibitor of phosphopyruvate hydratase (85) and it is presumably this inhibition which causes the lowering of ATP generation from glycolysis in sarcoma-180 cells.

The studies (Section III, D, 3) concerned with the effects of physiological variables such as nutrient supply or preincubation of cells prior to adding [^{32}P]-orthophosphate with or without cyclic-AMP, are interesting but difficult to interpret. They do, however, illustrate the complexity of the stimulation reaction. Cells showing no initial response to cyclic AMP upon removal from animals can be induced to respond by a period of in vitro preincubation (Figure 13); however, I have not been able to culture cells in such a way as to predict the magnitude of that response.

Regardless of the conditions of incubation, the ability of sarcoma-180 cells to incorporate radioactivity into ribosomal phosphoproteins diminishes with increased incubation time. This effect

may be simply explained by a reduced ability of the cells to transport phosphate across membranes or by some more complex mechanism such as the degeneration of an obligatory constituent for ribosomal protein phosphorylation resulting from in vitro culturing of the cells. The addition of cyclic-AMP to preincubated cells produced a stimulation of radioactivity incorporation that was to levels higher than for the same cells prior to preincubation (Figure 13a). If one assumes that the effect of preincubation on phosphate transport is negative (see above), this latter observation may be explained by the decay of an inhibitor of protein phosphorylation or by changes in some factor that is directly involved in the stimulation of ATP synthesis by cyclic-AMP.

Regardless of what mechanisms are involved in the response of sarcoma-180 cells to cyclic-AMP, it is apparent that I have been observing essentially short term effects. The results shown in Figure 14 indicate that cyclic-AMP is rapidly destroyed upon addition to cultures of sarcoma-180 cells and the effects of the addition rapidly disappear.

My attempts to correlate the cyclic AMP stimulation of incorporation of radioactivity into ribosomal phosphoproteins with the rate of protein synthesis, were equally inconclusive. Both reactions were stimulated in what appeared to be an unrelated manner. Obviously, the stimulation of [^{32}P]-orthophosphate incorporation into phosphoproteins by cyclic AMP can be a complex matter. Many variables must

be considered before final conclusions are reached.

The functions of ribosomal phosphoproteins in eukaryotic cells remain unknown. The previously discussed differences between sarcoma-180 cells and reticulocytes in their response to cyclic-AMP may ultimately be related to the different functions of these cells. However, such an assertion would be purely speculative at this time.

V. SUMMARY

[^{32}P]-orthophosphate is actively incorporated into phosphoserine and phosphothreonine residues of specific ribosomal proteins in mouse sarcoma-180 tumor cells. These phosphoproteins constitute only a small proportion (2 to 4.5%) of the total phosphoprotein radioactivity found in the cell cytoplasmic fraction from which the ribosomes are fractionated. However, comparison of these ribosomal phosphoproteins with those from rabbit reticulocytes show that they are true ribosomal constituents. Moreover, the retention of this metabolism during mammalian evolution indicates that they have important functions which are not related specifically to cellular differentiation or to neoplasia.

The failure of sarcoma-180 cells to respond readily to attempts to control their rate of protein synthesis suggests that they may not be a suitable choice for further investigations into the function of ribosomal phosphoproteins. Ascites sarcoma-180 cells are a heterogeneous mixture of cells in different stages of mitotic division. This heterogeneity may be responsible for some of the difficulties encountered in controling their biological activities. This may also account for the high degree of variability observed in their response to cyclic-AMP.

The experiments concerned with the effects of changes in physiological variables on cellular responses to cyclic AMP show that these responses are indeed complex. Accordingly, great care must be

exercised in making conclusions about the mechanisms by which cyclic AMP causes a stimulation of incorporation of radioactive orthophosphate into cellular macromolecules.

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