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Cyclic Adenosine 3′,5′-Monophosphate Dependent Phosphorylation of Ribosomal Proteins from Bovine Anterior Pituitary Gland†

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ABSTRACT: Ribosomal proteins were labeled by incubation of pituitary slices with [^{32}P]orthophosphate or by self-phosphorylation of ribosomes with [^{32}P]ATP. Phosphorylation of ribosomal protein was stimulated by cyclic AMP in the cell-free system and by N^6,2′-*O*-dibutyryl cyclic AMP in intact cells. Ribosome-associated protein kinase activity is enhanced after exposure of ribosomes to Triton X-100. The properties of this ribosome-associated protein kinase are similar to those of a protein kinase isolated from the soluble fraction of bovine anterior pituitary gland. The ribosome-associated enzyme catalyzes transfer of the γ-phosphate group of ATP to ribosomal proteins, histones, and other substrates. The enzymatic activity is inhibited by Ca^{2+} and stimulated by cyclic AMP with an apparent K_m for this nucleotide of 1×10^{-7} M. Cyclic IMP and cyclic GMP stimulate protein kinase activity at respective apparent K_m values of 1×10^{-6} and 5×10^{-5} M. Both basal and cyclic AMP-stimulated activities are inhibited by Ca^{2+}. Treatment of unwashed ribosomes with high salt concentrations removed approximately 50% of the phosphorylated proteins, the remaining label being located mainly in one band after polyacrylamide gel electrophoresis of the proteins extracted by 4 M LiCl and 8 M urea. Radioactivity was located in phosphoserine and phosphothreonine residues of ribosomal proteins. The data suggest a possible mechanism by which cyclic AMP could enhance protein synthesis at the translational level in the anterior pituitary gland.

Cyclic AMP[1] has recently been shown to stimulate both total protein synthesis and the release of specific hormones (Labrie *et al.*, 1971a; Adiga *et al.*, 1971; Wilber *et al.*, 1969; Ratner, 1970; Jutisz and de la Llosa, 1970; Fleischer *et al.*, 1969) in the anterior pituitary gland. The stimulatory effect of cyclic AMP on protein synthesis is not blocked by actinomycin D, thus suggesting an effect of the cyclic nucleotide at the translational level (Labrie *et al.*, 1971a).

The presence of a cyclic AMP dependent protein kinase in many tissues in which cyclic AMP is presumably acting as second messenger has led to the proposal that the wide variety of effects elicited by cyclic AMP are mediated by activation of protein kinase (Kuo and Greengard, 1969). In fact, besides the well-known effect of phosphorylation on phosphorylase kinase (De Lange *et al.*, 1968; Walsh *et al.*, 1968) and on glycogen synthetase (Huijing and Larner, 1966; Schlender *et al.*, 1969; Soderling *et al.*, 1970), there is increasing evidence for a cyclic AMP induced phosphorylation of many other important enzymatic and nonenzymatic systems. These data pertain to the hormone-sensitive lipase

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[1] Abbreviations used are: cyclic AMP, cyclic adenosine 3′,5′-monophosphate; dibutyryl cyclic AMP, N^6,2′-*O*-dibutyryladenosine 3′,5′-monophosphate.

(Corbin *et al.*, 1970; Huttunen *et al.*, 1970), histones, protamines, and other nuclear proteins (Langen, 1969; Marzluff *et al.*, 1969; Ingles and Dixon, 1967), *Escherichia coli* RNA polymerase (Martello *et al.*, 1970), reticulocyte and liver ribosomes (Kabat, 1970; Loeb and Blat, 1970; Eil and Wool, 1971), membranes (Weller and Rodnight, 1970), microtubules (Goddman *et al.*, 1970), and adenohypophyseal secretory granules (Labrie *et al.*, 1971c).

Since cyclic AMP dependent protein kinase has recently been found in the anterior pituitary gland (Labrie *et al.*, 1971b; Lemaire *et al.*, 1971), and the possibility arises that phosphorylation of ribosomal proteins may be a mechanism for translational control of adenohypophyseal protein synthesis, we have investigated the phosphorylation of ribosomal proteins and some pertinent properties of the ribosome-associated protein kinase.

Materials and Methods

Chemicals. [γ-^{32}P]ATP (10–12 Ci/mmole) and [^{32}P]-orthophosphate (30–100 Ci/μg of P) were obtained from Amersham/Searle. [γ-^{32}P]ATP was stored in 0.05 M Tris-HCl (pH 7.0) at $-90°$ and used within 1 month of purchase. Cyclic AMP, dibutyryl cyclic AMP, cyclic IMP, and cyclic GMP were obtained from Boehringer Mannheim GmbH. ATP, *O*-phospho-DL-serine, *O*-phospho-DL-threonine, and calf thymus histones (type II-A) were supplied by Sigma.

Solutions. Buffer A (20 mM Tris-HCl, pH 7.6, 100 mM KCl, 40 mM NaCl, 10 mM magnesium acetate, and 6 mM 2-mercaptoethanol) was used for the isolation of adenohypophyseal ribosomes. Buffer B is 50 mM sodium acetate–10 mM magnesium acetate, pH 6.5. Krebs-Ringer bicarbonate buffer containing 0.01 M glucose (KRBG) was prepared and used without addition of unlabeled phosphate (Cohen, 1957).

Preparation of Total Ribosomes. Bovine anterior pituitary glands from adult male and female animals were collected in local slaughterhouses, immediately dissected free from connective tissue and posterior and intermediary lobes, and immersed in ice-cold KRBG. Anterior pituitaries were homogenized in 3 vol of buffer A containing 0.25 M sucrose and the homogenate was filtered through cheesecloth before centrifugation at 10,000*g* for 10 min. Triton X-100 was added to the 10,000*g* supernatant at a final concentration of 1% and ribosomes were sedimented through a layer of 1.0 M sucrose in buffer A at 150,000*g* for 2.5 hr. These ribosomes were used for studies of *in vitro* phosphorylation or assay of protein kinase activity either before or after salt washing. Salt-washed ribosomes were prepared by resuspending unwashed ribosomes in buffer A containing the appropriate high salt concentrations and resedimenting through a layer of buffered 1.0 M sucrose.

Isolation of Free, Membrane-Bound, and Detergent-Released Ribosomes. The 10,000*g* supernatant (6 ml) was layered over 3 ml of 0.5 M sucrose in medium A, which was itself layered over 3 ml of buffered 2.0 M sucrose. After centrifugation at 150,000*g* for 3 hr, free ribosomes were recovered in the pellet and microsomes were found at the 0.5–2.0 M interface. Microsomes were further fractionated into smooth and rough fractions by centrifugation on a linear 1.0–2.0 M sucrose gradient at 95,000*g* for 12 hr and collected by centrifugation of the appropriate bands diluted 1:1 (v/v) with medium A. Detergent-released ribosomes were prepared by treating rough microsomes with 1% Triton X-100 and sedimenting through 1.0 M sucrose.

Incubation of Pituitary Slices. Pituitary slices (0.8 mm thickness) were incubated under O_2–CO_2 (95:5) in several changes of KRBG without phosphate for 30 min at 37° in order to deplete the intracellular phosphate pool. [^{32}P]-Orthophosphate (1.0 mCi/ml) and, where indicated, dibutyryl cyclic AMP[1] (5 mM) were added and incubation was continued for a further 3 hr. After washing the slices in several changes of ice-cold KRBG, ribosomes were prepared and treated as described above.

Assay of Protein Kinase Activity and of Cell-Free Ribosome Phosphorylation. Incubations were carried out according to slight modifications of the procedure of Kuo and Greengard (1970). For assay of ribosome-associated protein kinase activity, ribosomes (40–50 μg) were incubated in 0.2 ml of buffer B containing 50 μg of histones, 1×10^{-5} M [γ-^{32}P]ATP, and, where indicated, given concentratons of a nucleoside cyclic monophosphate. Incubations were carried out for 5 min at 30° and were terminated by the addition of ice-cold 10% trichloroacetic acid. Ribosome phosphorylation was measured by incubating in the absence of histones and, where indicated, with the addition of 10 μg of a purified pituitary protein kinase fraction prepared as described (Labrie *et al.*, 1971b). ^{32}P incorporation was determined after addition of 250 μg of bovine serum albumin as carried protein, centrifugation at 2500*g* for 10 min, and incubation of the precipitate in 10% trichloroacetic acid at 90° for 30 min. The tubes were chilled and the protein was resedimented, suspended in 0.3 ml of 1 M NaOH, and reprecipitated with cold trichloroacetic acid. This procedure was repeated once and the final pellet was resuspended in 0.2 ml of 1 M NaOH.

Hydrolysis of Ribosomal Proteins. Ribosomal RNA was hydrolyzed by incubating in 10% trichloroacetic acid at 90° for 1 hr. The residue was spun down, washed with cold trichloroacetic acid, and hydrolyzed in 6 M HCl at 105° for 4 hr in tubes sealed under nitrogen. The hydrolysate was electrophoresed on Whatman No. 3MM paper for 1 hr at 2.5×10^3 V and at pH 1.85 (2.5% formic acid–7.8% acetic acid). [^{32}P]Orthophosphate, phosphoserine, and phosphothreonine were run as markers. Amino acids were visualized with a ninhydrin spray and radioactivity was located by autoradiography. Electrophoretic analyses were left in contact with Kodak RPX-omat (RP-14) films for periods of 3–30 days before processing with a Pakorol-XU automatic developer.

Polyacrylamide Gel Electrophoresis of Ribosomal Proteins. Ribosomal proteins were extracted by resuspending in 8 M urea–4 M LiCl at 0° for 24 hr. After sedimentation of RNA by centrifugation, ribosomal proteins were dialyzed against several changes of 0.03 M HCl containing 2.5 M urea. Polyacrylamide gels (7.5%) containing 1.1% of bisacrylamide were prepared according to the method of Panyim and Chalkley (1969) and were prerun for 3 hr before sample application.

Measurement of Radioactivity. Ribosomes and ribosomal proteins dissolved in NaOH were counted in Bray's scintillation fluid (Bray, 1960) using a Packard Model 3375 spectrometer. Gel slices were dried onto aluminum planchets and counted using a low background Nuclear Chicago gas-flow counter.

Determination of RNA and Protein. Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard and RNA according to Fleck and Munro (1962).

Results

Ribosome-Associated Protein Kinase. Incubation of adenohypophyseal ribosomes with [γ-^{32}P]ATP results in self-phosphorylation of ribosomal protein components. This

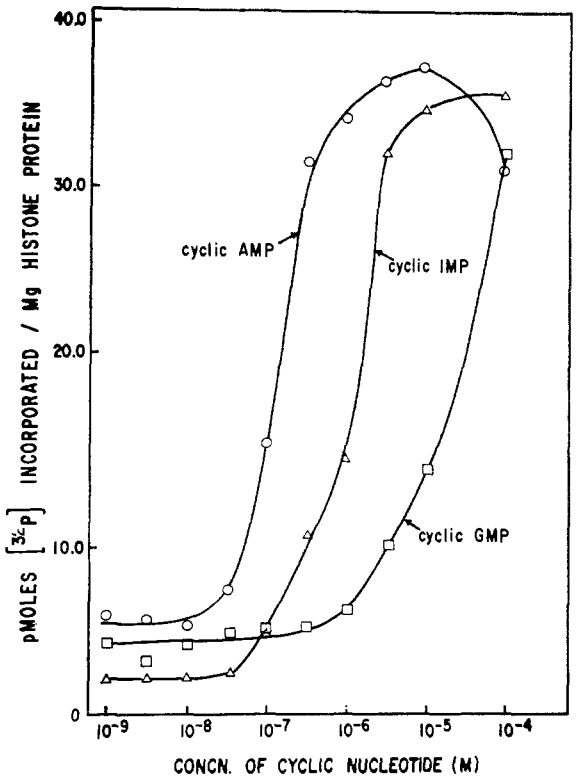

FIGURE 1: Effect of nucleoside cyclic monophosphates on ribosome-associated protein kinase activity. NH_4Cl-washed ribosomes (44 μg) obtained after Triton X-100 treatment were incubated with histones (50 μg) and [γ-^{32}P]ATP (1×10^{-5} M) in 0.2 ml of buffer (sodium acetate, 50 mM; magnesium acetate, 10 mM; pH 6.5) for 5 min at 30°. ^{32}P incorporated was measured as described under Materials and Methods except for the indicated concentrations of the various cyclic nucleotides: (○) cyclic AMP; (△) cyclic IMP; (□) cyclic GMP.

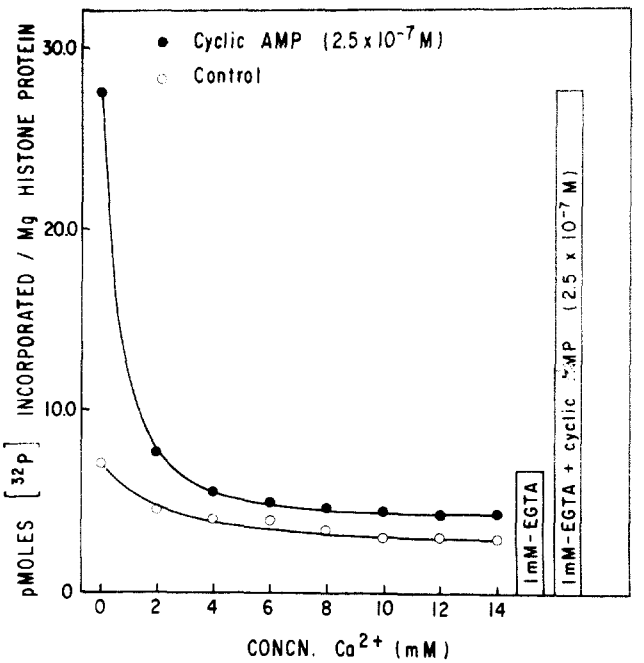

FIGURE 2: Inhibition of ribosome-associated protein kinase by Ca^{2+}. Detergent-released and 0.5 M NH_4Cl washed ribosomes (40 μg) were incubated with histones (50 μg) and [γ-^{32}P]ATP (1×10^{-5} M) in 0.2 ml of buffer B for 5 min at 30°. ^{32}P incorporated was measured as described under Materials and Methods except for the indicated concentration of Ca^{2+} or EGTA. The effects of Ca^{2+} and EGTA were determined on both basal (○) and cyclic AMP dependent protein kinase activities (●).

ribosome-associated protein kinase exhibits a broad substrate specificity and measurement of calf thymus histone phosphorylation has been used as a test of maximal enzymatic activity. Under optimal concentrations of ATP and histones, the ribosome-associated protein kinase exhibits maximal activity at pH 6.5 and optimal Mg^{2+} concentrations of 10–15 mM. These conditions are close to those found for the protein kinase isolated from bovine anterior pituitary tissue (Labrie *et al.*, 1971b).

It thus seemed important to investigate whether this protein kinase was a contaminant of the ribosome preparation or was a specific component of the ribosomes. Many proteins found in association with ribosomes are loosely bound and may be removed by washing with solutions of high ionic strength. The results shown in Table I indicate that although some of the ribosome-associated protein kinase is removed by salt washes, even quite drastic treatment fails to remove more than 50% of the activity originally associated with the ribosomes. Cyclic AMP dependent activity is removed to a proportionately greater extent than is the cyclic AMP independent activity. Assay of ribosome washes for protein kinase activity showed that the enzyme was in fact removed by high salt concentrations and not simply inactivated.

TABLE I: Effect of Salt Washes on Ribosome-Associated Protein Kinase Activity.[a]

Salt Solution	Ribosome-Associated Protein Kinase Act., % Unwashed −cAMP	+cAMP
0.5 M KCl	94.8	80.0
0.5 M NH_4Cl	70.4	51.8
0.75 M NH_4Cl	71.8	40.4
1.0 M NH_4Cl	64.4	39.2

Detergent-released ribosomes were prepared as described under Materials and Methods, resuspended in the appropriate high salt concentration, and resedimented through a layer of buffered 1 M sucrose. Maximal ribosome-associated protein kinase activity was assayed in the presence of excess histones, as described in the text. Protein kinase activity is expressed as a percentage of that associated with unwashed ribosomes. Cyclic AMP, when present, was at a concentration of 2.5×10^{-7} M.

Relative Efficiencies of Nucleoside 3',5'-Monophosphates on the Activation of Ribosome-Associated Protein Kinase. Of all the nucleoside cyclic monophosphates tested, cyclic AMP was the most efficient at stimulating the ribosome-associated protein kinase (Figure 1). Half-maximum stimulation of the protein kinase by cyclic IMP and cyclic GMP required, respectively, concentrations of 10- and 500-fold that of cyclic AMP (Figure 1). In the presence of exogenous substrate, the apparent K_m of the ribosome-associated protein kinase for cyclic AMP was measured at 1×10^{-7} M using both unwashed and 0.5 M NH_4Cl washed ribosomes.

Inhibition by Calcium. Calcium ion is intimately involved in anterior pituitary gland function, this ion being required for the release of adenohypophyseal hormones under the influence of cyclic AMP, hypothalamic releasing factors, and

TABLE II: Protein Kinase Activity of Rough Microsomes and Ribosomes and Detergent-Released Ribosomes.[a]

Fraction	pmol of ^{32}P incorp./mg of Histone Protein
Free ribosomes	26.4
Free ribosomes + cyclic AMP	29.0
Rough microsomes	45.6
Rough microsomes + cyclic AMP	48.6
Detergent-treated ribosomes	30.6
Detergent-treated ribosomes + cyclic AMP	93.0

[a] Free and detergent-released ribosomes and rough microsomes were prepared as described under Materials and Methods. Samples of each (20 μg of RNA) were incubated in buffer B with histone (50 μg), [γ-^{32}P]ATP (1×10^{-5} M), and, where indicated, cyclic AMP (2.5×10^{-7} M). ^{32}P incorporated into histone was measured as described in the text.

high concentrations of potassium (Wakaba *et al.*, 1968; Samli and Geschwind, 1968; Vale *et al.*, 1967; Zimmerman and Fleischer, 1970). The effect of Ca^{2+} on the activity of the ribosome-associated protein kinase is shown in Figure 2. Ca^{2+}, at all concentrations used, inhibits both the basal and the cyclic AMP induced activities of the ribosome-associated protein kinase. At 2 mM Ca^{2+}, the cyclic AMP dependent activity is inhibited to 25% of the control rate. In the presence of 10 mM magnesium acetate, 1 mM EGTA, a chelating agent specific for Ca^{2+} (Raaflaub, 1956), has no effect on either the basal or the cyclic AMP dependent protein kinase activity.

Relative Protein Kinase Activity of Rough Microsomes, Free Ribosomes, and Detergent-Released Ribosomes. Both free and detergent-released ribosomes exibit endogenous protein kinase activity (Table II). Rough microsomes exhibit greater basal endogenous protein kinase activity per milligram of ribosomal RNA than do either free or detergent-treated ribosomes, the difference resulting probably from activity associated with components of microsomal membranes. However, cyclic AMP (2.5×10^{-7} M) resulted in little stimulation of the protein kinase activity associated with either the free ribosomes or rough microsomes, but caused a threefold stimulation of the protein kinase associated with Triton X-100 released ribosomes.

Self-Phosphorylation of Ribosomes in the Cell-Free System. The ribosome-associated protein kinase catalyzes quite efficiently the self-phosphorylation of ribosomal components. When phosphorylation of the added kinase fraction itself has been subtracted, addition of a purified adenohypophyseal protein kinase results in no increase of the phosphorylation of unwashed ribosomes, and only slightly increased phosphorylation of 0.5 M NH_4Cl washed ribosomes (Table III). A large proportion of the components of unwashed ribosomes which becomes phosphorylated can be removed by high salt washes. Cyclic AMP (2.5×10^{-7} M) resulted in almost 100% stimulation of self-phosphorylation of 0.5 M NH_4Cl washed ribosomal components (Table III).

Phosphorylation of Ribosomes in Intact Cells. Salt-washed ribosomes isolated from pituitary slices previously incubated in the presence of [^{32}P]orthophosphate were also phosphorylated. A large proportion of the label was incorporated into RNA but ribosomal proteins and, to a lesser extent, lipids were also labeled. This is in contrast to phosphorylation in a cell-free system, where phosphate is predominantly transferred

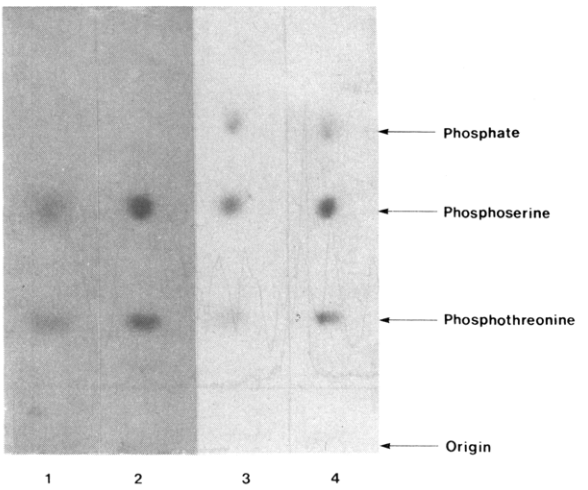

FIGURE 3: Autoradiograph of hydrolysate of ^{32}P-labeled ribosomal proteins separated by high voltage paper electrophoresis at pH 1.85. Ribosomes were phosphorylated *in vitro* using [γ-^{32}P]ATP or by pituitary slice incubation with [^{32}P]orthophosphate as described under Materials and Methods. After removal of RNA by 10% trichloroacetic acid at 90° for 30 min, the residue was hydrolyzed in 6 N HCl at 105° for 4 hr. Samples shown are as follows: (1) ribosomes phosphorylated with [γ-^{32}P]ATP in a cell-free system; (2) ribosomes phosphorylated with [γ-^{32}P]ATP in a cell-free system in the presence of cyclic AMP; (3) ribosomes isolated from pituitary slices incubated with [^{32}P]orthophosphate; (4) ribosomes isolated from pituitary slices incubated with [^{32}P]orthophosphate in the presence of dibutyryl cyclic AMP. Equal amounts of these pairs of ribosome preparations were taken for hydrolysis and identical volumes of hydrolysate electrophoresed. Phosphoserine and phosphothreonine were coelectrophoresed as markers and visualized with ninhydrin.

TABLE III: Self-Phosphorylation of Ribosomal Proteins by Incubation with [γ-^{32}P]ATP.[a]

Additions	pmol of ^{32}P incorp./mg of Ribosomes (Mean ± SEM)
Unwashed ribosomes	24.8 ± 0.4
Unwashed ribosomes + cyclic AMP	31.8 ± 0.2
Unwashed ribosomes + protein kinase	23.8 ± 0.2
Unwashed ribosomes + protein kinase + cyclic AMP	30.6 ± 0.4
Washed ribosomes	4.8 ± 0.2
Washed ribosomes + cyclic AMP	8.6 ± 0.4
Washed ribosomes + protein kinase	5.8 ± 1.0
Washed ribosomes + protein kinase + cyclic AMP	9.4 ± 1.0

[a] Unwashed or 0.5 M NH_4Cl washed ribosomes (50 μg) obtained by detergent treatment were incubated for 5 min at 30° in 0.2 ml of buffer B with [γ-^{32}P]ATP (1×10^{-5} M) and, where indicated, cyclic AMP (2.5×10^{-7} M) or a purified adenohypophyseal soluble cyclic AMP dependent protein kinase (10 μg). ^{32}P incorporated into ribosomal proteins was measured as described under Materials and Methods. Where necessary, the background phosphorylation of added protein kinase was subtracted.

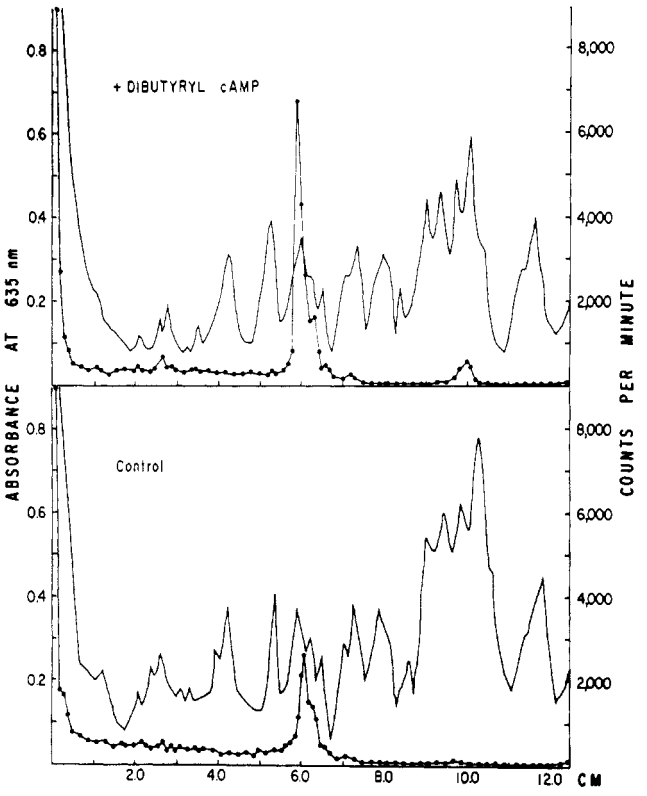

FIGURE 4: Polyacrylamide gel electrophoresis of ribosomal proteins. Pituitary slices were incubated for 3 hr with [^{32}P]orthophosphate (1 mCi/ml) either with or without dibutyryl cyclic AMP (5 mM). Proteins were extracted from 0.5 M KCl washed ribosomes and separated on polyacrylamide gels as described under Materials and Methods. After staining with Amido Black and scanning at 635 nM, 1-mm slices were cut for determination of radioactive content: (●) counts per minute; (—) absorbance at 635 nm.

to ribosomal protein (Table IV). Incubation of pituitary slices with [^{32}P]orthophosphate in the presence of dibutyryl cyclic AMP had no effect on the distribution of label between cold trichloroacetic acid, hot trichloroacetic acid, and organic solvent soluble materials and hence must stimulate the incorporation of phosphate into other materials besides ribosomal proteins. In different experiments, dibutyryl cyclic AMP stimulated the phosphorylation of the residue left after hot trichloroacetic acid and organic solvent extraction by, respectively, 12.5, 14.0, 25.0, 11.6, and 11.0% above that of controls.

Characterization of Phosphorylation Products. Ribosomes labeled with [^{32}P]phosphate by transfer from ATP in a cell-free system or after pituitary slice incubation with [^{32}P]-orthophosphate were hydrolyzed with 6 N HCl after removal of RNA. Figure 3 shows that radioactive material coelectrophoresed with the markers phosphoserine and, to a lesser extent, with phosphothreonine. The presence of cyclic AMP in the [γ-^{32}P]orthophosphate slice incubation resulted in increased specific activities of phosphoserine and phosphothreonine as detected by autoradiography (Figure 3).

Ribosomal proteins were extracted from detergent-released and 0.5 M KCl washed ribosomes after pituitary slice incubation with [^{32}P]orthophosphate. Separation on polyacrylamide gels (Figure 4) showed label to be present in one major band. Dibutyryl cyclic AMP stimulated the phosphorylation of this major band and also, but to a much lesser degree, caused the phosphorylation of a further band, which was unlabeled in the control preparation.

TABLE IV: Solubility Characteristics of Phosphorylated Ribosomal Components.[a]

Treatment	% ^{32}P Label Remaining Bound
A. Phosphorylation by [γ-^{32}P]ATP in cell-free system	
1. Cold 10% trichloroacetic acid	100.0
2. 10% trichloroacetic acid at 90° for 30 min	90.6
3. 10% trichloroacetic acid at 90° for 30 min + organic solvent	88.2
B. Phosphorylation by [γ-^{32}P]ATP in the presence of cyclic AMP	
1. Cold 10% trichloroacetic acid	100.0
2. 10% trichloroacetic acid at 90° for 30 min	90.0
3. 10% trichloroacetic acid at 90° for 30 min + organic solvent	87.2
C. Phosphorylation by [^{32}P]orthophosphate	
1. Cold trichloroacetic acid	100.0
2. 10% trichloroacetic acid at 90° for 30 min	31.7
3. 10% trichloroacetic acid at 90° for 30 min + organic solvent	27.1
D. Phosphorylation by [^{32}P]orthophosphate in the presence of dibutyryl cyclic AMP	
1. Cold 10% trichloroacetic acid	100.0
2. 10% trichloroacetic acid at 90° for 30 min	32.0
3. 10% trichloroacetic acid at 90° for 30 min + organic solvent	28.5

[a] Detergent-released ribosomes were prepared from anterior pituitary glands either before or after incubation of pituitary slices with [^{32}P]orthophosphate. Phosphorylation using [γ-^{32}P]ATP was performed as described under Materials and Methods. Phosphorylated ribosomes were salt washed with 0.5 M NH_4Cl and then washed several times in cold 10% trichloroacetic acid. The amount of radioactivity associated with the cold trichloroacetic precipitable components and with the material left after extraction with 10% trichloroacetic acid at 90° for 30 min, or after further extraction of the residue with organic solvent (ethanol–ether–chloroform, 2:2:1), was determinated after dissolving the final residues in 1 M NaOH and counting in Bray's solution.

In contrast, phosphorylation of ribosomes *in vitro*, using [^{32}P]ATP, resulted in the labeling of several bands separated by polyacrylamide gel electrophoresis (Figure 5). The addition of cyclic AMP increased the phosphorylation of all these bands, especially those close to the gel top, but did not result in the phosphorylation of any additional material. In the presence of exogenous pituitary gland protein kinase the level of phosphorylation of all bands was increased both in the basal and cyclic AMP stimulated states.

Discussion

It is clear from these data that adenohypophyseal ribosomes are subject to modification by phosphorylation by an endogenous protein kinase and that the enzymatic activity can be stimulated by cyclic AMP. In fact, protein kinase activity was found in association with all types of ribosomal particles studied, namely the free, membrane-bound, and detergent-

released ribosomes. Phosphorylation was observed both during incubation of the intact cell and in the cell-free system. No more than 50% of the protein kinase activity associated with unwashed adenohypophyseal ribsomes could be removed by washing with 1 M NH_4Cl. This is in contrast to the protein kinase associated with reticulocyte ribosomes which may be completely removed by washing in a solution of high ionic strength (Kabat, 1970). It was noticed that high salt washes did not proportionately remove cyclic AMP independent and cyclic AMP dependent activities, the latter being eluted to a greater extent than the former. This may result from a greater affinity of the ribosome for the catalytic subunit of the protein kinase than for the receptor–catalytic complex. Alternatively, only the catalytic subunit may be in true association with ribosomal particles, while the cyclic AMP receptor–protein kinase complex may result from cytosol contamination (Lemaire *et al.*, 1971).

Treatment of ribosomal particles with Triton X-100 resulted in a stimulation of protein kinase activity. This unmasking of protein kinase has been noticed previously in both rough and smooth microsomes (Lemaire *et al.*, 1971). Unmasking of protein kinase (Maeno *et al.*, 1971) and phosphodiesterase (Ingles and Dixon, 1967) activities has been observed by similar treatment of rat brain microsomes.

Although Ca^{2+} is essential for the secretion of adenohypophyseal hormones, the relevancy of this ion to protein synthesis is uncertain. It is of note that the protein kinase associated with adenohypophyseal ribosomes, in common with the protein kinases found in the soluble fraction (Labrie *et al.*, 1971b), is inhibited by Ca^{2+}, but the physiological significance of this fact, if any, remains to be elucidated.

As evidenced by electrophoresis of the acid hydrolysate of ribosomal components, the incorporation of ^{32}P from [γ-^{32}P]- or [^{32}P]orthophosphate is into *O*-phosphoserine and *O*-phosphothreonine residues of the polypeptide chains. Phosphorylation of proteins associated with ribosomes has also been demonstrated in rabbit reticulocytes (Kabat, 1970), rat liver (Loeb and Blat, 1970; Eil and Wool, 1971), and rat adrenal cortex (Walton *et al.*, 1971). In all of these systems, with the exception of rabbit reticulocytes, phosphorylation has been shown to be stimulated by cyclic AMP. However, the exent of phosphorylation reported varies considerably. In rat liver, at least 12 ribosomal proteins are reported to be phosphorylated (Eil and Wool, 1971) while in rat adrenal cortex but one is modified (Walton *et al.*, 1971). Our findings indicate that one major band, as separated on polyacrylamide gels, is phosphorylated in ribosomes extracted after incubation of pituitary slices and that incorporation of ^{32}P is increased by the presence of dibutyryl cyclic AMP during slice incubation. It is, however, possible that one band on polyacrylamide gels of ribosomal proteins may contain more than one protein and that not all ribosomal proteins are extracted by 4 M LiCl and 8 M urea. A separate band is also phosphorylated in the presence of dibutyryl cyclic AMP but not in its absence. However, the level of phosphorylation of this second band is considerably lower than the major band. In contrast to the phosphorylation of ribosomal proteins following incubation of pituitary slices, when ribosomes are incubated *in vitro* with [^{32}P]ATP, at least six different bands become labeled and the phosphorylation of the major band seen after slice incubation is decreased in importance. It is possible that the protein kinase *in vitro* lacks the substrate specificity that it exhibits *in vivo*, or that isolation of ribosomes prior to phosphorylation exposes additional sites to which the protein kinase may gain access.

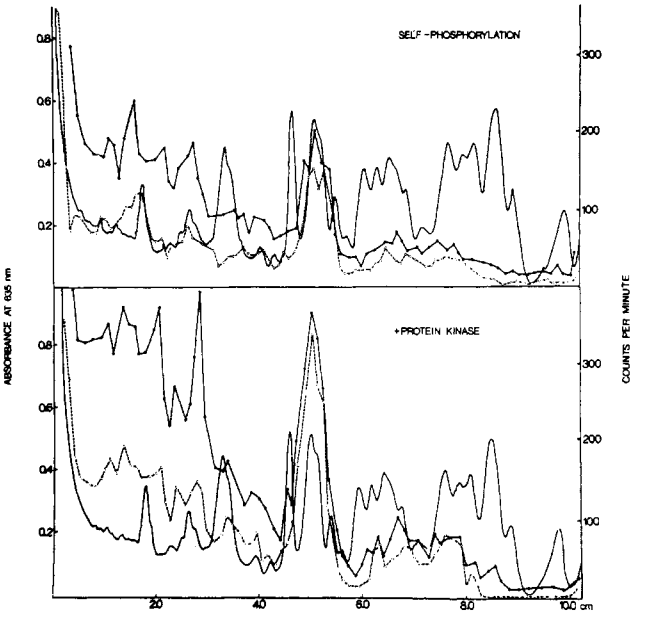

FIGURE 5: Polyacrylamide gel electrophoresis of ribosomal proteins. Ribosomes in buffer B were incubated with [^{32}P]ATP (5×10^{-6} M) for 15 min at 30°. Cyclic AMP (1×10^{-6} M) and exogenous protein kinase (50 μg/ml) were present where indicated. Following incubation, ribosomes were 0.5 M KCl washed and reisolated. Ribosomal proteins were extracted and separated as described in the legend to Figure 4: (O---O) counts per minute; (●—●) counts per minute in the presence of cyclic AMP; (—) absorbance at 635 nm.

The cyclic AMP induced stimulation of phosphorylation was much greater in a cell-free system using [γ-^{32}P]orthophosphate. The significance of this apparent discrepancy will have to be evaluated in relation with the time course of ribosomal protein phosphorylation and the possible role of phosphatase during the 3-hr incubation period with dibutyryl cyclic AMP.

The present data show that protein components of both free and membrane-bound ribosomes are phosphorylated by an endogenous protein kinase and that this process can be stimulated by cyclic AMP. It remains to be seen whether or not the functional activity of ribosomes is changed by ribosomal protein phosphorylation and if this could be the basis of a mechanism which could explain our previous data (Labrie *et al.*, 1971a) of a stimulation of adenohypophyseal protein synthesis at the translational level.

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Stability and Homogeneity of Preparations of Ribosomal Particles from *Escherichia coli*†

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ABSTRACT: Techniques have been developed for preparing ribosomal particles from *Escherichia coli* (MRE 600) so that the particles are unusually stable against autodegradation when kept unfrozen for long periods. Criteria for stability were constancy of RNA and RNP migration on polyacrylamide gels, and preservation of activity in poly(U)-directed incorporation of amino acid. Stability could be maintained for 1 week at 25° or 4 weeks at 4°. Gel migration was also used to assess the homogeneity of both monosome and subunit preparations. Evidence for multiple conformations was obtained, and some progress was made toward improving homogeneity.

Our aim in starting this work was to stabilize ribosomal particles from *Escherichia coli* for *in vitro* crystallization trials. For the same purpose we also set out to assess any lack of homogeneity in our preparations, not just for cross-contamination between different particles but for the existence of more than one conformation of each particle type. We believe that improved stability and homogeneity are prerequisites for any serious attempt to crystallize ribosomes; the work is also expected to be of some interest to other users of cell-free systems.

The part of a ribosome most sensitive to damage is its rRNA. Extracts of ribosomes always contain some endonucleases which attack the exposed regions of rRNA; such enzymes are more dangerous than exonucleases, since the latter can only attack one end of the molecule which may in any case be protected. Nucleases may in some cases form part of the ribosomal "split" protein fraction (Spahr, 1964; Szer, 1969). *E. coli* MRE 600, used in this work, is a mutant which contains only low levels of the endonuclease RNase I, but RNase II (an exonuclease) is present in usual amounts (Singer and Tolbert, 1965) and endonuclease IV has been found (Spahr and Gesteland, 1968). We have endeavored to reduce the activity of endogenous nucleases, and to distinguish their effects from those of nucleases introduced accidentally from outside during the preparations.

The stability requirements for crystallization are probably

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