Acetylation of Ribosome-Associated Proteins in Vitro by an Acetyltransferase Bound to Rat Liver Ribosomes†

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ABSTRACT: Incubation of rat liver ribosomes with [1-^{14}C]acetyl-coenzyme A results in the incorporation of [^{14}C]acetyl into a material insoluble in cold trichloroacetic acid. The acetyltransferase involved in the self-acetylation of ribosomes can be released by high salt washing of the ribosomes; the activity of the solubilized enzyme can be assayed using histones as acetyl acceptors. Electrophoretic analysis of acetylated ribosomes or ribosomal proteins indicated that the acetyl radicals are associated with a group of relatively basic proteins, having molecular weights ranging from 10,000 to 45,000. Chromatographic analysis of the enzymatic hydrolysates of proteins extracted from acetylated ribosomes indicates that acetylation is mainly or exclusively NH_2 terminal. Almost 80% of the acetyl proteins are released from the ribosomes by high salt treatment. Most of the acetyl radicals not solubilized by the high salt treatment were found in the 60S subunit, associated with a protein(s) having an apparent molecular weight of 43,000. This acetyl protein(s) was released from the 60S subunit by EDTA treatment and was found in a ribonucleoprotein complex having a buoyant density of 1.56.

Modification of protein structure by enzymes is known to play an important role in the regulation of cell metabolism (Holtzer and Duntze, 1971). Acetylation of internal lysine residues within histones has been reported (DeLange et al., 1969). The acetylating activity probably involved in this process has been isolated from nuclei (Galwith, 1968). Amino terminal acetylation of histones and a number of biologically active proteins (Liew et al., 1970; Stegnik et al., 1971) have also been reported, although the enzymatic mechanisms of this process and its relationships to protein synthesis are not clear (Stegnik et al., 1971). Previous studies from this laboratory demonstrated the occurrence of an acetyltransferase in rat liver cytoplasm which is active with histone as substrate (Pestana et al., 1971). These studies also provided evidence for the presence of the enzymatic acetylation of amino-acylated tRNA (Pestana and Pitot, 1972) in the cytosol. During the course of a search for endogenous substrates of the cytoplasmic histone acetyltransferase, it was observed that microsomes and polysomes were acetylated on incubation with radioactive acetyl-coenzyme A, even when no acetyltransferase was added to the assay system. This preliminary result prompted a study of the acetylation in vitro of rat liver ribosomes in relation to the enzymatic mechanism of the process, the characterization of the reaction product, and the functional significance of the acetylation. In this report the occurrence of an acetyltransferase bound to rat liver ribosomes is demonstrated. The enzyme may be involved in the acetylation in vitro of several proteins associated with ribosomes. Some characteristics of the acetylated proteins, including their electrophoretic mobility and the identification of the acetylated amino acids, are described. A similar characterization of polyribosomes acetylated in vivo (Liew and Gornall, 1973) was reported while this manuscript was in preparation.

Materials and Methods

Polyribosomes and Ribosomes from Rat Liver. Male Holtzman rats weighing 200–300 g were used in all experiments described. The animals were fasted overnight before sacrifice. In certain experiments the RNA was labeled with [^{3}H]orotic acid (200 μCi/rat) given intraperitoneally 15–16 hr before sacrifice. Livers were homogenized in two volumes of 0.44 *M* sucrose in TKM[1] (0.1 *M* Tris (pH 7.4)–0.025 *M* KCl–0.001 *M* $MgCl_2$). Total polyribosomes were prepared by sodium deoxycholate treatment of the postmitochondrial supernatant (Shires et al., 1971). Ribosomes were prepared as 80S particles, free of mRNA, by a procedure patterned after that of Martin and Wool (1969). In brief, the polyribosomes were incubated with all the factors required for protein synthesis (Stahelin and Falvey, 1971) and puromycin. The incubation mixture contained per milliliter 1 μmol of ATP, 0.5 μmol of GTP, 0.5 μmol of puromycin, 10 μmol of phosphoenolpyruvate, 50μg of pyruvate kinase, 0.25 ml of polyribosomal suspension (10–20 A_{260} units), and 0.7 ml of liver postmicrosomal supernatant in 0.4 *M* sucrose–TKM containing 1 m*M* dithiothreitol. After incubation at 37° for 45 min, the incubation mixture was chilled and the ribosomes were collected by sedimentation through a cushion of 1.5 *M* sucrose in TKM. Centrifugation was for 12 hr at 39,000 rpm in a 50.1 Ti rotor (Beckman). The ribosomes prepared by this procedure showed ratios of A_{260}/A_{280} in excess of 1.7 and readily dissociate into subunits in high ionic strength medium.

In Vitro Acetylation of Ribosomes. Ribosomes (0.5–5 A_{260} units) were incubated at 37° with 0.02 μCi of [1-

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[1] Abbreviations used are: TKM, 0.1 *M* Tris (pH 7.4)–0.025 *M* KCl–0.001 *M* $MgCl_2$; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Table I: The in Vitro Acetylation of Ribosomes.[a]

Expt No.	Assay System	[^{14}C] Acetyl Incorporated (pmol)
I	Complete	1.1
	Complete + ribosomes	5.7
	Complete + ribosomes – acetyltransferase	5.8
II	Complete	1.2
	Complete + histones	7.4
	Complete + histones – acetyltransferase	0.4

[a] The complete assay system was contained in 0.2 ml, 1.25 μmol of Tris (pH 7.5), 20 μmol of KCl, and 15 μg of cytoplasmic acetyltransferase (40% ammonium sulfate precipitate from the postmicrosomal supernatant). Four OD_{260} units of ribosomes (experiment 1) or 200 μg of histone (experiment II) was added as shown. The incubation with [^{14}C]acetyl coenzyme A was for 10 min at 37°. The reaction was stopped with cold Cl_3CCOOH and the radioactivity in the precipitate measured as described under Materials and Methods.

[^{14}C]acetyl-coenzyme A (57 μCi/mmol), in the presence of 0.05 *M* Tris-HCl (pH 7.5) and 0.1 *M* KCl. Controls consisting of heat inactivated ribosomes (incubated 10 min at 70°) were always run in parallel. The assays were carried out in duplicate in a final volume of 0.2 ml. The reaction was stopped by the addition of 4 ml of 10% Cl_3CCOOH in the cold; the precipitates were collected on Millipore filters, washed, and counted as described (Pestana et al., 1971). For the large scale acetylation of ribosomes, the reaction mixture was enlarged 20 to 50 times and the reaction stopped by chilling to 0°. Histone acetyltransferase activity was assayed as described previously (Pestana et al., 1971).

Polyacrylamide Gel Electrophoresis. Electrophoresis in 0.1% sodium dodecyl sulfate–10% acrylamide gels was performed as described by Bickle and Traut (1971). Prior to electrophoresis, the samples were heated for 10 min at 60° in the presence of 1% sodium dodecyl sulfate–1% mercaptoethanol. After electrophoresis for 4 hr at 2.5 mA/tube the gels were soaked in 7.5% acetic acid for 2 hr and then stained wth 0.1% Coomassie Blue in 7.5% acetic acid–50% methanol and destained by diffusion in the same medium. Electrophoresis in 8 *M* urea was carried out as described by Traub et al. (1971). The gels were stained with 0.1% Amido Black in 7.5% acetic acid–20% ethanol and destained by diffusion. The absorbance at 600 nm of the stained gels was determined in the gel scanner attachment of a Gilford 2400 spectrophotometer. For radioactive measurements, the gels were fractionated by extrusion in a Gilson automatic gel fractionator. The minced gel fractions were incubated in counting vials with 0.5 *M* NaOH for 13–15 hr at room temperature. After acidification with acetic acid, 10 ml of Scintisol (Isolab) was added to each vial and the radioactivity measured in a Packard scintillation counter with an 80% efficiency for ^{14}C.

Analysis of the Enzymatic Hydrolysates of Ribosomal Proteins. Protein was extracted from acetylated ribosomes (250 A_{260} units) with acetic acid–$MgCl_2$ (Hardy et al., 1969), dialyzed extensively against 0.01 *M* acetic acid, and freeze-dried. The dried material (about 8 mg of protein) was resuspended in 10 ml of 0.1 *M* ammonium bicarbonate (pH 8.1) and digested through successive additions of 100 μg of trypsin, pronase, and carboxypeptidases A and B for 10–12 hr at room temperature. A drop of toluene was added with each enzyme addition in order to prevent bacterial growth. At the end of the incubation any cold Cl_3CCOOH insoluble material was removed by acid precipitation. The Cl_3CCOOH was extracted with ether and the water phase concentrated by evaporation under vacuum at 37°. The enzymatic digest was placed on a small Dowex 50 H^+ column (0.5 × 5 cm) and eluted with 30 ml of water followed by 30 ml of 0.5 *N* NH_4OH. The recovery of radioactivity was always higher than 70% at this stage. The eluates were dried under vacuum and analyzed by paper and column chromatography. Paper chromatography in Whatman 3MM paper was developed in the descending vertical direction with 1-butanol–acetic acid–water (12:3:5). For column chromatographic analysis a column (0.9 × 53 cm) of cation exchanger, Beckman UR-30, was used. Elution with 0.3 *M* lithium citrate (pH 2.8) was carried out at 40° at a flow rate of 1 ml/min. The outflow was split into two streams, one for the ninhydrin color development and the other was coupled to a fraction collector for radioactive measurements. Fractions were collected every 2 min and the radioactivity was measured in 0.5-ml aliquots after addition of 10 ml of Scintisol. Standard *N*-[^{3}H]acetyl amino acids were prepared by treatment of 1 mmol of the amino acid with 1 mmol of [^{3}H]acetic anhydride (5 Ci/mol) in boiling acetic acid (Greenstein, 1957).

Sucrose and CsCl Gradient Centrifugation. Isokinetic sucrose gradients ranging from 0.35 to 1.1 *M* sucrose in 0.002 *M* Tris-HCl (pH 7.5), 0.5 *M* KCl, and 0.005 *M* $MgCl_2$ were used to dissociate the ribosomes into subunits. The gradients were centrifuged for 4 hr at 18,000 rpm in an SW 41 rotor (Beckman). For the isopycnic sedimentation in CsCl, the samples to be analyzed were fixed for 3 hr at 0° with 2% formaldehyde in 10 m*M* potassium phosphate buffer (pH 7); after incubation, solid CsCl (0.6 g) was added to obtain a final density of 1.3 g/cm^3 in a volume of 2 ml. This solution was layered over 2 ml of CsCl dissolved in phosphate buffer at a density of 1.64 g/cm^3 and centrifuged at 20° for 30 hr at 49,000 rpm in an SW 50 rotor (Beckman). Fractions (6 drops) were collected and the density and radioactivity in 0.1-ml aliquots measured.

Chemicals and Radiochemicals. Ribonuclease A and Pronase, B grade, were obtained from Calbiochem; trypsin and carboxypeptidases A and B were products of Worthington. Pyruvate kinase, ribonuclease A, phosphoenolpyruvate (tricyclohexylamine salt), and histone II A were obtained from Sigma. Dowex 50 H^+ (200–400 mesh) AG grade, acrylamide and methylene-*N,N'*-bisacrylamide were purchased from Bio-Rad. N^ϵ-Acetyllysine was obtained from Cyclochemicals. All other chemicals used were of the highest purity available. [^{3}H]Orotic acid (18 Ci/mol) and [^{3}H]acetic anhydride (500 Ci/mol) were purchased from the Radiochemical Centre. [1-^{14}C]Acetyl-coenzyme A (50 Ci/mol) and the ^{3}H-labeled amino acid mixture were products of the New England Nuclear Company.

Results

In Vitro Acetylation of Ribosomes. Incubation of rat liver ribosomes with [^{14}C]acetyl-coenzyme A results in the uptake of [^{14}C]acetyl into a cold Cl_3CCOOH insoluble form. The presence of added cytoplasmic acetyltransferase (Pestana et al., 1971) is not required for this acetylation to occur (experiment I in Table I), although the enzyme preparation is active with histone as substrate (experiment II in Table I). This lack of effect of the cytoplasmic acetyltrans-

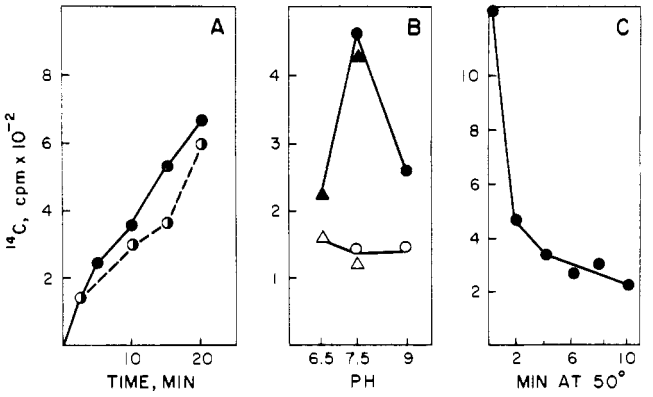

FIGURE 1: Properties of the in vitro acetylation of ribosomes. (A) Time course. Ribosomes (4 OD_{260} units) were incubated as described in the legend to Table I, either alone (○) or in the presence of the cytoplasmic acetyltransferase preparation (◑). The cold Cl_3CCOOH insoluble radioactivity found at the indicated time periods was corrected for the small amount of radioactivity incorporated into heated ribosomes and into the cytoplasmic acetyltransferase preparation. (B) Effect of pH. Incubation for 15 min at 37° was performed as described under Materials and Methods, except for the indicated changes in pH and the substitution of Tris buffer (circles) for Hepes buffer (triangles). The open symbols represent the values obtained with heat-inactivated ribosomes; the solid symbols represent the values for native ribosomes. (C) Heat inactivation. Ribosomes (4 OD_{260} units) suspended in the assay mixture described under Materials and Methods, minus [^{14}C]acetyl-CoA, were incubated at 50° for the indicated time period and chilled in an ice bath. Radioactive acetyl-coenzyme A was added to these heat-treated ribosomes and the extent of acetylation was measured after an additional incubation for 20 min at 37°.

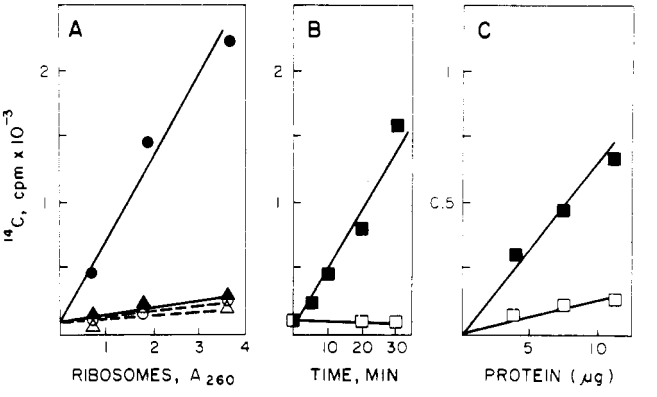

FIGURE 2: Acetylating activity of potassium chloride washed ribosomes and of the ribosomal supernatant. Ribosomes resuspended in 50 m*M* Tris-HCl (pH 7.5) were adjusted to 0.5 *M* KCl and 0.005 *M* $MgCl_2$ and then collected by centrifugation for 30 min at 120,000*g*. The resulting KCl washed ribosomes and the KCl ribosomal supernatant were assayed for acetylating activity as described under Materials and Methods. In A is a comparison of the acetylating activity of intact (circles) and KCl washed ribosomes (triangles). Open symbols are values for controls employing heat-treated ribosomes. B and C show the effects of the time of incubation and the amount of protein, respectively, on the acetylating activity of the KCl supernatant assayed with (closed symbols) or without (open symbols) the addition of 200 μg of histones. In B, 7μg of supernatant protein was present at each time point. In C, the incubation at 37° was for 20 min.

ferase on the acetylation of ribosomes is also demonstrated in Figure 1A, in which the time course of acetylation of ribosomes is shown to be essentially unaffected by the presence of the cytoplasmic enzyme during the incubation. These results differ from those of Liew and Gornall (1973) who observed a 60% increase in the acetylation of ribosomes when incubated in the presence of whole cell sap as the source of cytoplasmic acetyltransferase. The presence of a high concentration of Mg^{2+} in their incubation mixture may account for the differences seen in the results from the two laboratories, since this ion strongly inhibits the endogenous acetylation of ribosomes (vide infra) without affecting the activity of the cytoplasmic acetyltransferase (Pestana and Pitot, 1975).

Optimization of the Conditions for the Acetylation of Ribosomes. The requirements for the self-acetylation of ribosomes are relatively simple. [^{14}C]Acetyl incorporation can be studied in ribosomal suspensions in a neutral medium with radioactive acetyl coenzyme A as the donor of acetyl radicals. Although there are no known requirements for monovalent cations in the reaction, 0.1 *M* KCl was always present in the assay mixture in order to minimize the possibility of nonenzymatic acetylation (A. Pestana, unpublished results). Under these conditions, the acetylation of ribosomes was found to be a thermolabile process (Figure 1C) with an optimal pH near 7.5 (Figure 1B). At pH 7.5 and with heat-inactivated ribosomes (10 min at 70°) as a control for nonenzymatic acetylation, the acetylation of ribosomes was linear with time for at least 20 min (Figure 1A). Addition of GTP, ATP, ITP, or CTP at 1 m*M* final concentration caused a 70–90% inhibition of the self-acetylation of ribosomes. Mg^{2+} ions were also found to affect the acetylation of ribosomes; concentrations as low as 0.1 m*M* of added $MgCl_2$ resulted in a 50% inhibition of acetylation. For this reason, $MgCl_2$ was never added to the incubation mixture, although this omission might impair the stability of the ribosomal structure, resulting in the release of some proteins (Blobel, 1971).

Solubilization of an Acetyltransferase from the Ribosomes. In order to study the mechanism of the acetylation of ribosomes, attempts were made to solubilize the enzymatic activity involved in this process. As a result of these studies it was observed that no acetylation takes place when ribosomes washed with 0.5 *M* KCl were used in place of the unwashed preparation (Figure 2A). This appeared to be an effect of the high salt treatment, since no decrease in the self-acetylation of ribosomes was observed when the particles were washed at lower KCl concentrations (data not shown). On the other hand, the supernatant from high salt-washed ribosomes was found to be very active in the acetylation of histones in a time-dependent process (Figure 2B) which was proportional to the amount of supernatant protein in the assay (Figure 2C). Attempts to acetylate salt-washed ribosomes with the supernatant preparation were unsuccessful.

Nature of the Ribosomal Constituent Acetylated. In preliminary attempts to characterize the chemical nature of the product of the acetylation of ribosomes, the distribution of [^{14}C]acetyl between RNA and protein was studied. A suspension of ribosomes with their RNA tritiated in vivo (see Materials and Methods) was acetylated in vitro with [^{14}C]acetyl-CoA. The ribosomes were fractioned into RNA and protein by treatment with acetic acid in the presence of 0.1 *M* $MgCl_2$, followed by precipitation of the RNA by low-speed centrifugation (Hardy et al., 1969). The distribution of ^{3}H and ^{14}C indicated the protein nature of the acetylated ribosomal constituent, since virtually all the [^{14}C]acetyl was found in the supernate, essentially free of [^{3}H]RNA. Also significant in this respect is the fact that an identical distribution of [^{14}C]acetyl radioactivity was observed in sodium dodecyl sulfate electrophoresis of whole acetylated ribosomes and the acetyl proteins extracted by

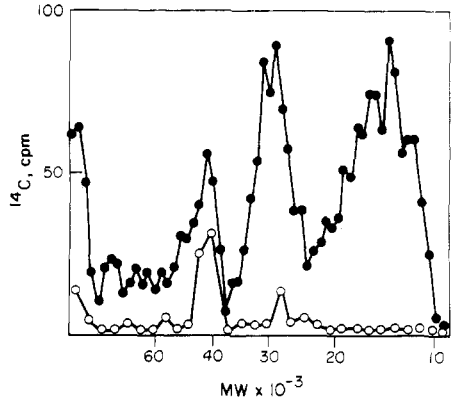

FIGURE 3: Sodium dodecyl sulfate-acrylamide electrophoresis of acetylated ribosomes. Acetylated ribosomes (4 A_{260} units) and large ribosomal subunits (3 A_{260} units) were analyzed in 10% acrylamide gels in the presence of 0.1% sodium dodecyl sulfate as described under Materials and Methods. Marker proteins were also run in separate gels to calibrate mobility with molecular weight. The gels were fractionated and counted as described under Materials and Methods. The solid symbols denote the radioactivity from complete ribosomes and the open symbols correspond to the radioactivity from large ribosomal subunits.

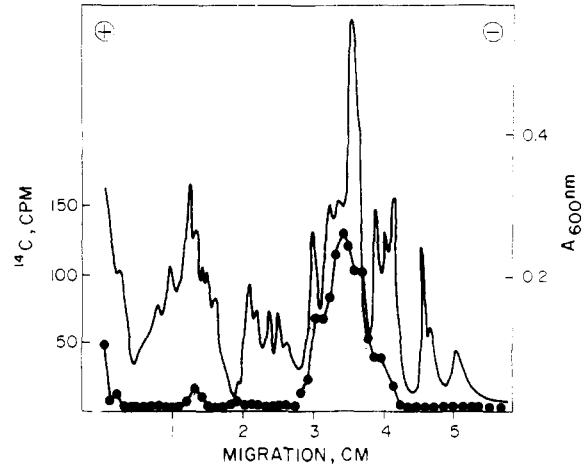

FIGURE 4: Urea-acrylamide electrophoresis of acetylated ribosomes. Acetylated ribosomes (4 A_{260} units) were treated with ribonucleases A and T_1 before electrophoresis in 8 N urea-10% acrylamide gels. The stained gels were scanned and counted as described under Materials and Methods. The solid circles correspond to the radioactivity in the gel fractions while the continuous line without symbols represents the optical density.

the procedure outlined above (A. Pestana, unpublished observations).

Electrophoresis of Acetylated Proteins. Acetylated ribosomes and the acetic acid-$MgCl_2$ extracted proteins were analyzed in sodium dodecyl sulfate-acrylamide gel electrophoresis as described under Materials and Methods. Three main radioactive peaks were seen to correspond to groups of proteins with apparent molecular weights of about 40,000, 30,000, and 10,000-20,000 (Figure 3). The acetyl proteins were also analyzed in an urea-acrylamide electrophoretic system at pH 4.5. Whole acetylated ribosomes were used in this analysis as the source of acetyl proteins. The proteins were released from the ribonucleoprotein complexes by a short incubation with ribonucleases A and T_1 (Traub et al., 1971) prior to electrophoresis. Most of the [^{14}C]acetyl was found associated with 4 protein bands having a relatively high mobility (Figure 4). In other electrophoretic analysis

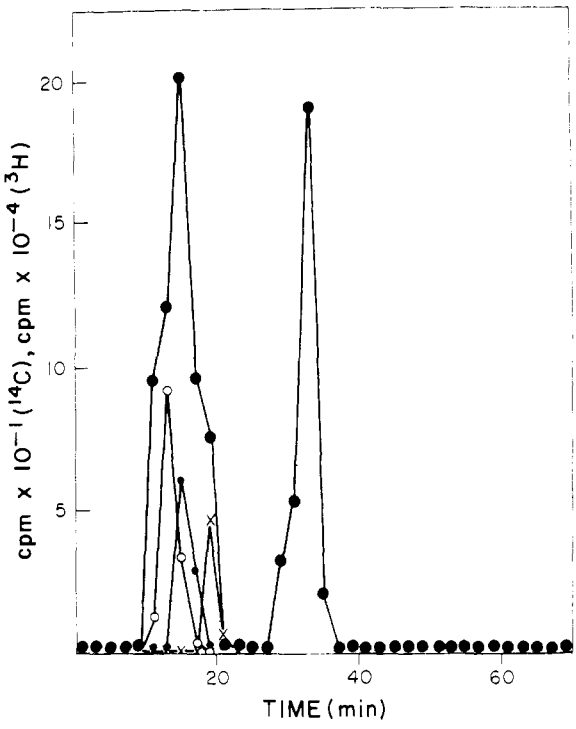

FIGURE 5: Column chromatography of acetyl amino acids. An aliquot from the Dowex 50 H^+ water eluate (see text) was analyzed on an ion exchange column as described under Materials and Methods. Fractions were collected every 2 min and the radioactivity found in 0.5 ml is shown in large solid circles. The figure also shows the elution pattern of tritiated standards: N-acetylglcyine (○), N-acetylserine (●), and N-acetylalanine (×).

runs for shorter times, [^{14}C]acetyl was also found associated with a fast moving band which migrated ahead of the tracking dye.

Analysis of the Enzymatic Hydrolysates of Acetyl Proteins. To characterize further the acetylated moieties, the proteins extracted from acetylated ribosomes were digested with trypsin, Pronase, and carboxypeptidases A and B as described under Materials and Methods. The resulting hydrolysate containing acetyl amino acids and peptides was placed on a small Dowex 50 H^+ (0.5 × 5 cm) column which was then washed with 30 ml of water followed by 30 ml of 0.5 M NH_4OH to obtain, respectively, the "acidic" (NH_2-terminal blocked amino acids and peptides) and "basic" (free NH_2-terminal) fractions (Marchis-Mouren and Lipmann, 1965), each of which were dried under vacuum. The water eluate was found to contain 90% of the total radioactivity eluted and gave no color when assayed with ninhydrin. Column chromatographic analysis of this fraction (Figure 5) revealed the presence of two radioactive peaks. The first peak, which represented about 75% of the [^{14}C]acetyl in the "acidic" fraction, was eluted between 14 and 20 min; this is the time at which the standards [^{3}H]acetylglycine, -serine, and -alanine were found. No positive identification of the second peak has been obtained thus far. The "acidic" fraction was also analyzed in descending paper chromatography in 1-butanol, acetic acid, and water. The results (Figure 6) indicate the presence of N-[^{14}C]acetylalanine, which is well resolved in this system, and also suggest the presence of N-[^{14}C]acetylserine and acetylglycine in the enzymatic hydrolysates of ribosomal acetylated proteins. Column chromatographic analysis of the "basic" fraction (data not shown) revealed the presence of a broad radioac-

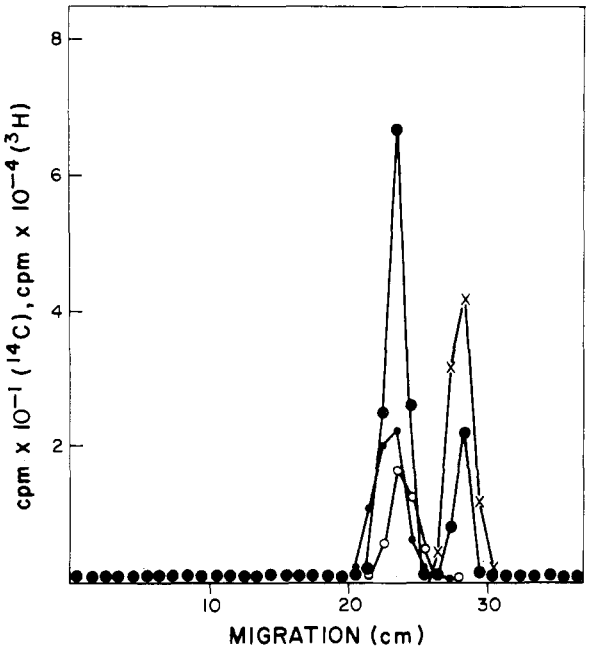

FIGURE 6: Paper chromatography of acetyl amino acids. An aliquot of the water eluate from the Dowex 50 H^+ column was analyzed by paper chromatography as described under Materials and Methods. Standard ^3H-labeled acetylated amino acids were also included. The dried chromatograms were cut into 1-cm strips and the radioactivity was measured by liquid scintillation counting. Symbols are identical with those in Figure 5.

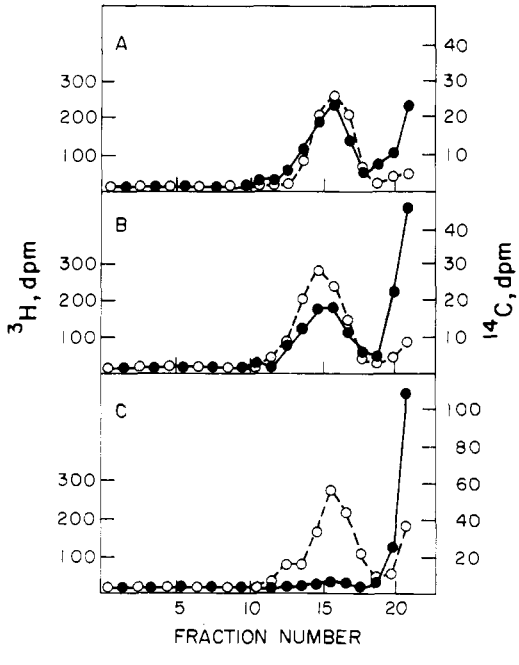

FIGURE 7: Release of acetylated proteins from ribosomes by KCl treatment. Ribosomes with their RNA tritiated in vivo were resuspended in 50 mM Tris-HCl (pH 7.5) (60 A_{260} units/ml) and incubated with [^{14}C]acetyl-CoA as indicated in Materials and Methods. After incubation, 50-μl aliquots (3 A_{260} units) were used for centrifugation in 15–35% linear sucrose gradients. In A, the ribosomes were diluted with 50 μl of 50 mM Tris buffer (pH 7.5) and centrifuged in a sucrose gradient (15–35%) containing the same buffer. In B and C, the ribosomes were adjusted to 0.1 M and 0.5 M KCl, respectively, and centrifuged in sucrose gradients containing 0.1 M and 0.5 M KCl in Tris buffer. Centrifugation was for 60 min at 49,000 rpm in an SW 51 rotor (Beckman). Fractions (5 drops) were collected and assayed for radioactivity as described under Materials and Methods. The open symbols represent [^{3}H]RNA and the closed symbols are the [^{14}C] acetyl label.

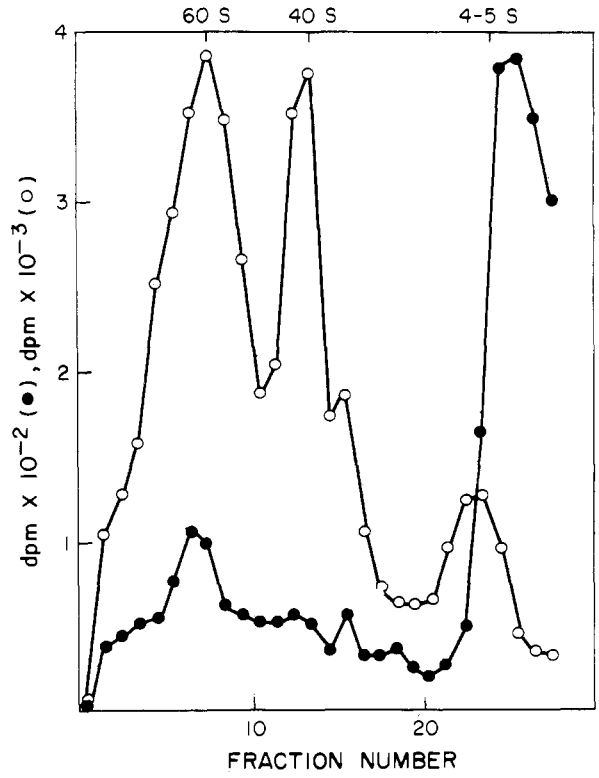

FIGURE 8: Analysis of acetylated ribosomes dissociated into subunits in high salt-containing sucrose gradients. Acetylated ribosomes (30 A_{260} units) were suspended in 0.5 M KCl and 5 mM $MgCl_2$ and centrifuged in isokinetic sucrose gradients as described in Materials and Methods. Fractions (10 drops) were collected through a puncture in the bottom of the tube and analyzed for cold Cl_3CCOOH insoluble radioactivity. The open symbols represent [^{3}H]RNA and the closed symbols are the [^{14}C]acetyl label.

tive peak, which is eluted between 140 and 160 min, well before the elution of the standards N^{α}- or N^{ϵ}-acetyllysine (elution times of 220 and 360-min, respectively). The exact molecular species in this peak have not yet been identified.

Analysis of Acetylated Ribosomes in Sucrose Gradients. Having characterized the acetylated ribosomal proteins in terms of size, charge, and the chemical nature of most of the acetyl groups, we next studied the structural relationships of acetyl proteins and ribosomal subunits. In the experiment described in Figure 7, it is shown that increasing the salt concentration results in the solubilization of acetyl proteins. Almost 35% of the acetyl proteins were found in the supernatant when no KCl was added. With 0.1 M KCl in the gradient the amount of acetylated protein solubilized was 49% of the total, and in KCl concentrations of 0.5 M the solubilization of acetylated proteins was near complete. Since these experiments were performed in the absence of Mg^{2+} ions the effect of high salt treatment was also studied in the presence of added Mg^{2+} ions. A suspension of ribosomes acetylated in vitro was adjusted to 0.5 M KCl and 5 mM $MgCl_2$ and centrifuged in an isokinetic sucrose gradient as described in Figure 8. Under these conditions a measurable amount of acetyl proteins representing nearly 20% of the total [^{14}C]acetyl insoluble in cold Cl_3CCOOH remained with the ribosomal particles, most of it bound to the 60S subunit. The ribosomal supernatant (fractions 20–30) contained 80% of the acetylated proteins and a small amount of RNA. Electrophoretic analysis of this fraction (Figure 9) indicates that most of the RNA has the mobility of 4S RNA. In addition, two bands of acetylated proteins

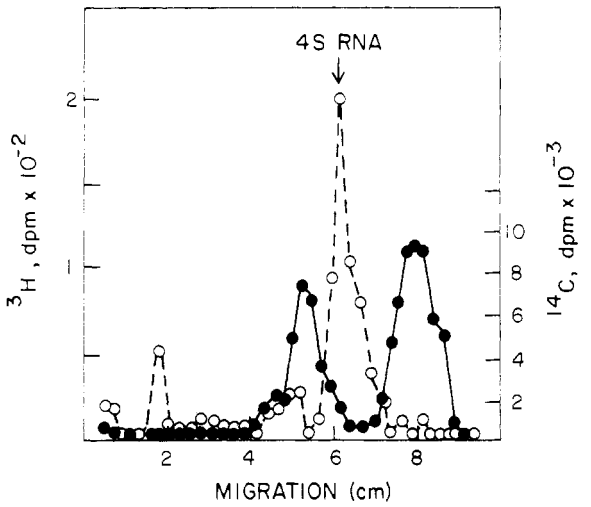

FIGURE 9: Electrophoretic analysis of macromolecules from the high salt ribosomal supernatant. The supernatant from the 0.5 *M* KCl treatment of acetylated ribosomes (pooled fraction 20–28 from Figure 8) was dialyzed, freeze-dried, and resuspended in a small volume of 0.3% sodium dodecyl sulfate in 50 mM Tris-HCl (pH 7.5). Electrophoresis of this solution in the presence of 0.1% sodium dodecyl sulfate was carried out as described under Materials and Methods, except that the acrylamide concentration was 7.5%. The gel was cut into 2-mm slices, the slices were transferred to scintillation vials, and 0.5 ml of 30% H_2O_2 containing 0.5% NH_4OH was added to each vial. After incubation overnight at 37°, 10 ml of Scintisol was added and the radioactivity measured in a Packard scintillation counter with 10% efficiency for ^{3}H and 60% efficiency for ^{14}C. The symbols are as in Figure 7.

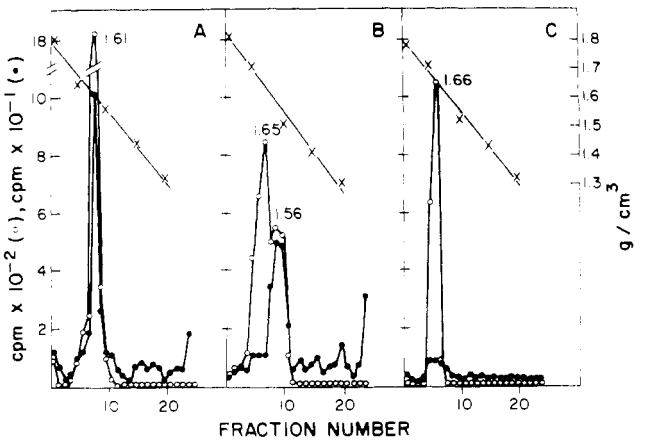

FIGURE 10: Banding pattern of acetylated 60S subunits and of its EDTA derivatives in CsCl after formaldehyde fixation. Acetylated large subunits with their RNA tritiated in vivo were employed in this experiment. In A, the particles were fixed (see Materials and Methods) and centrifuged in CsCl without EDTA treatment. In C, the particles were treated with EDTA as described by Blobel (1971) and then were reisolated by differential centrifugation before fixation. In B, the subunits were treated with EDTA as above and fixed directly, without reisolation. Four A_{260} units were used on each experiment. Fixation, centrifugation, and assay of fractions were done as described under Materials and Methods. The symbols are as shown in Figure 8.

with apparent molecular weights of 30,000 and 10,000–20,000 were also observed in this fraction.

Characterization of the Acetylated Proteins in the 60S Subunit. Acetylated 60S ribosomal subunits were isolated from sucrose gradients as described above and were precipitated in the cold (−20°) with 10 volumes of absolute ethanol in the presence of 10 m*M* $MgCl_2$. Electrophoretic analysis in 2.3% acrylamide–agarose gels (Wilkinson et al., 1971) in the presence of sodium dodecyl sulfate indicated that these preparations contained 28S and 5S RNA and only a trace of 18S RNA, suggesting a contamination by the 40S ribosomal subunit of less than 5%. The proteins in the acetylated subunit were analyzed by sodium dodecyl sulfate–acrylamide electrophoresis as described above. Radioactive measurement of the gel fractions showed (see Figure 3) that most of the [^{14}C]acetyl is associated with a protein band having an approximate molecular weight of 40,000.

For the characterization of the structural relationship of the acetyl protein(s) found in the 60S particle, acetylated large subunits with their RNA tritiated (see above) were treated with EDTA under the same conditions used by Blobel (1971) to release a 5S RNA–protein complex. The EDTA derivative of the large subunit (L_{3E}) was then reisolated by differential centrifugation, fixed in formaldehyde, and centrifuged in a CsCl gradient as described under Materials and Methods. Controls, consisting of large subunits fixed directly without the EDTA step, were also run in parallel (Figure 10A). As shown in Figure 10C, the EDTA treatment resulted in the loss of the acetylated protein and in an increase in the buoyant density (compare with Figure 10A). In order to visualize all the split products an aliquot containing acetylated 60S subunits was treated with EDTA as above, but then was directly fixed and centrifuged in CsCl without reisolation. As shown in Figure 10B, in addition to the L_E derivative (the large band with a buoyant density of 1.65), the gradient contains a smaller band with much of the [^{3}H]RNA and almost all of the [^{14}C]acetyl released from the large subunit. The buoyant density of this band indicates an RNA content of about 50%. Since the molecular weights of 5S RNA and the acetyl protein in the large subunit are close to 40,000, the data suggest that this acetylated protein is the protein moiety of the 5S RNA–protein complex described by Blobel (1971) and Peterman et al. (1972).

Discussion

The results described in this paper demonstrate the presence of an acetyl-coenzyme A dependent acetylation of rat liver ribosomes that can be studied in vitro without any requirement for an exogenous source of acetyltransferase (Table I and Figure 1A). The enzymatic nature of this process is suggested by the optimal pH near 7.5 (Figure 1B) and the thermolability (Figure 1C) of the acetylation, which contrasts with the alkaline requirements and thermostability described for the spontaneous acetylation of proteins (Paik et al., 1970). The acetyltransferase activity that is released from the ribosomes by 0.5 *M* KCl treatment (Figure 2) is probably the enzyme involved in the acetylation of ribosomes. However, no direct proof of this thesis is yet available, since attempts to reconstitute the system with washed ribosomes and its supernatant have thus far failed. Since the cytoplasm of the rat liver has an acetyltransferase also active with histone as substrate (Pestana et al., 1971), the question arises as to whether these two activities (cytoplasmic and ribosomal) correspond to different proteins or to one single protein that can exist free in the cytoplasm and bound to ribosomes. The former alternative is suggested by experiments described in an accompanying publication (Pestana and Pitot, 1975).

The product of the "self-acetylation" of ribosomes has been characterized as protein in nature and consists of several classes of proteins with molecular weights ranging from 10,000 to 45,000 (Figures 3 and 4). Extensive enzymatic

Table II: Distribution of Nascent Chains Labeled in Vitro between Ribosomes and the Ribosomal Supernatant.[a]

	Total Radioactivity	
	^{3}H (cpm)	%
Ribosomal supernatant	660,000	93
Ribosomes	48,000	7

[a] Rat liver polysomes (300 OD) were incubated with all the factors required for protein synthesis, puromycin and 100 μCi of a mixture of ^{3}H-labeled amino acids as described under Materials and Methods. At the end of the incubation, the ribosomes were centrifuged through 1.5 *M* sucrose and the radioactivity insoluble in hot Cl_3CCOOH was measured in triplicate from aliquots of the supernatant and the pellet.

hydrolysis of acetylated ribosomal proteins followed by chromatographic analysis indicates that the acetylation takes place predominantly in the amino terminal group of serine, glycine, alanine, and other unidentified amino acids (Figures 5 and 6). In contrast with the results of Liew and Gornall (1973) with polysomes acetylated in vivo, our data indicate that little or no acetylation of lysine (either at the α or ϵ amino groups) takes place during ribosomal acetylation in vitro. The reason for this discrepancy is not known. Our studies with polysomes acetylated in vivo indicate that 60% of the label is introduced into glutamic and aspartic acids and most of the remainder into *N*-acetylserine and the amino terminal group of several different amino acids as well as small amounts into the N^{ϵ}-acetyl amino group of lysine (Pestana and Pitot, 1975).

The release of acetyl proteins by high salt treatment of ribosomes (Figures 7 and 8) should be considered in light of the question as to whether these are ribosomal or contaminating proteins. Because the relationship of acetylated proteins to the function and structure of ribosomes is unknown, the term "ribosome associated protein" is used here to refer to proteins which are part of the ribosomes under the preparative procedure followed in this study. Walton et al. (1971) used a similar term in referring to the proteins phosphorylated in ribosomes by an exogenous protein kinase. It should be remembered in this context that prior to high salt treatment and sedimentation, the ribosomes have been incubated for 20 min at 37° in the absence of Mg^{2+} ions. Such drastic treatment will probably result in the release of some ribosomal proteins (Blobel, 1971). The acetylation of nascent peptides should also be considered. This possibility arises from the fact that our ribosomal preparation still contains nascent peptides (Table II). Other studies from this laboratory have shown N-terminal acetylation of nascent peptides on incubation of polyribosomes with acetyl-Co A (Pestana and Pitot, 1975).

One of the proteins acetylated in vitro fulfills the requirements for a protein inherent to the ribosomal structure. This is the acetyl protein with a molecular weight of 40,000 that can be extracted from the 60S subunit in the form of an RNA–protein complex (Figures 8 and 10). The parameters of this ribonucleoprotein are quite similar to those reported for a 5S RNA–protein complex extracted from rat liver ribosomes by formamide (Peterman et al., 1972). To our knowledge this is the first such characterization of an acetylated protein in eucaryotic ribosomes. In *Escherichia coli*, the L_7 ribosomal protein is known to have an N terminal *N*-acetylserine (Terhorst et al., 1972). The importance of 5S RNA in the protein-synthetic capacity of ribosomes raises the question of the functional relevance of the acetylation of a protein probably closely associated with 5S RNA as suggested by this study.

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