Isolation of Low Molecular Weight, Methylated Ribonucleic Acids from 10S to 30S Particles of Chinese Hamster Cell Fractions*

M. D. Enger† and R. A. Walters

ABSTRACT: Cultured Chinese hamster cells contain low molecular weight, methylated RNAs which are distinct from transfer or ribosomal species (LMM-RNAs). These RNAs are unique in their content of minor nucleotides. Three contain N^2,N^2-7-trimethylguanylic acid. In this paper we report on analyses of sedimentation properties of LMM-RNAs as they exist in fractions of cells mechanically or chemically disrupted in iso- or hypotonic buffers. Cell-fraction LMM-RNAs were found to exist in forms that sediment more rapidly than free RNAs. In fractions prepared and analyzed at low ionic strength, LMM-RNA electrophoresis species VII concentrated in the 20S to 30S region of a sucrose gradient subsequent to zone sedimentation. LMM-RNA electrophoretic species III, IV, and VI sedimented more rapidly and in a dispersed fashion. In 0.1 M salt, species III, IV, VI, and VII concentrated in the 10S to 30S region.

Protease treatment reduced the sedimentation rates of cytoplasmic forms to those of free RNAs. Addition of cytoplasm to phenol-extracted species prior to zone sedimentation did not increase their sedimentation rates. The sedimentation rates of cell-fraction LMM-RNAs and the amount of these species present in the cell predicted that postribosomal particles would be apparent in zone sedimentation analyses if soluble materials which obscure the 1S to 40S region were first removed. Such removal was effected using differential centrifugation. Thus, a number of postribosomal particles were made apparent; however, their number exceeded that predicted if all contained LMM-RNAs. In particular, a particle of approximately 10 S was found which has no LMM-RNA but contained 4S RNA as well as a more rapidly migrating (in electrophoresis), methylated species and two more slowly migrating, unmethylated species whose presence in previous preparations had been masked by the presence of larger amounts of LMM and ribosomal species of similar electrophoretic mobility. All postribosomal particles contain 4S RNA. All have A_{260}/A_{280} ratios of 0.9–1.0. None contain a detectable amount of DNA.

Cultured Chinese hamster cells (line CHO) contain at least four low molecular weight, methylated RNAs (LMM-RNAs) which are distinct from transfer or ribosomal species (Zapisek *et al.*, 1969). These RNAs, isolated and purified by preparative gel electrophoresis, are qualitatively and quantitatively unique in their content of methylated nucleotides. Three of these RNAs contain a newly discovered methylated nucleotide not present in other RNAs. This nucleotide has been identified with N^2,N^2-7-trimethylguanylic acid (Saponara and Enger, 1969).

RNAs possessing similar electrophoretic mobilities have been found in cells of mammalian, amphibian, and avian origin (Knight and Darnell, 1967; Hodnett and Busch, 1968; Dingman and Peacock, 1968; Larsen *et al.*, 1968; Weinberg and Penman, 1968; Moriyama *et al.*, 1969; Rein and Penman, 1969). Studies on the metabolic properties of such RNAs in HeLa cells have shown that they are relatively stable, are not synthesized coordinately with any of the other types of HeLa RNA, and are homogeneous in size (Weinberg and Penman, 1969). Of nuclear origin, they are only loosely associated with chromatin or other nucleoprotein complexes of the HeLa cell. In several instances, their molar concentration is high. Species D of Weinberg and Penman (species 5 of Hodnett and Busch or our species VI) comprises about 0.5% of the cell's RNA or about 10^6 molecules per cell. Hodnett and Busch (1968) have isolated the rat liver LMM-RNA cognates in milligram amounts. They find them to have no amino acid acceptor activity.

Most studies indicate that RNAs similar to our LMM-RNAs are localized in nuclear and nucleolar fractions. Our earlier studies on the location of these RNAs in the Chinese hamster cell appeared to be in accord with the above observations in that we found over 90% of them in our nucleolar fraction (Zapisek *et al.*, 1969). Subsequently, however, we determined that LMM-RNA localization in the nucleolar fraction (nuclear pellet) was due primarily to the fact that LMM-RNAs adsorb to bentonite which was present during fractionation and which sedimented with nucleoli (Walters *et al.*, 1970). When cell fractionation was performed in the absence of bentonite, over half of the LMM-RNAs were found in the cytoplasmic fraction.

We have observed that centrifugation of isolated cytoplasm for 3 hr at 100,000*g* sediments about half of the cytoplasmic content of LMM-RNAs along with ribosomes. This observation, taken with the fact that the LMM-RNAs of the fractionated cell exist in forms that absorb to negatively charged bentonite, suggested the possibility that they are complexed with sufficient positively charged material—presumably protein—to greatly increase their sedimentation rate and to change their net charge. In this paper we report on more detailed analysis of the sedimentation properties of LMM-RNAs as they exist in Chinese hamster cell fractions. These

* From the Biomedical Research Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544. *Received March 13, 1970.* This work was performed under the auspices of the U. S. Atomic Energy Commission.

† To whom correspondence should be addressed.

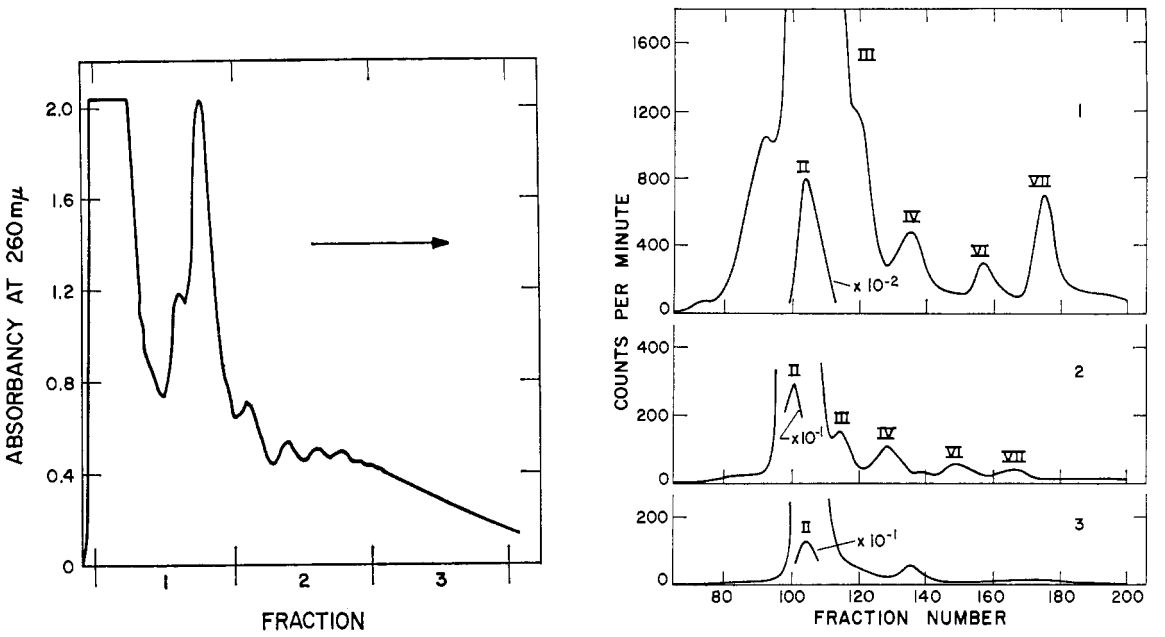


FIGURE 1: (A) Absorbancy profile of a 7.5–35% (57 ml) sucrose gradient after zone sedimentation of cytoplasm for 2.25 hr at 25,000 rpm in a Spinco 25.2 rotor. Chinese hamster cells had been exposed to 0.25 μCi/ml of L-[*methyl*-^{14}C]methionine during growth from 94,000 to 400,000 cells per ml (cell doubling time of 18 hr). Cytoplasm was prepared in the low ionic strength buffer RSB as described in the Experimental Section. Cytoplasm (2 ml) derived from 10^8 cells was layered over and sedimented through each of three gradients. The absorbancy profile of one of these is shown. Fractions were collected as designated in the figure, material in each fraction was precipitated with ethanol, and the ethanol precipitate was dissolved in sodium cacodylate buffer (0.05 M, pH 7.0, 0.5% in sodium dodecyl sulfate) and extracted with an equal volume of phenol in the cold. After ether extraction and ethanol precipitation, samples were redissolved and low molecular weight RNAs from each of the three fractions were isolated with the aid of zone sedimentation as previously described (Zapisek *et al.*, 1969). (B) The types and amounts of LMM-RNAs present were determined after resolution by gel electrophoresis. LMM-RNA content is expressed in terms of incorporated isotope from methyl-labeled methionine; LMM-RNA absorbancy is usually too low for detection, and labeling with nucleoside precursors results in relatively greater contamination from non-LMM-RNAs (such as 5S ribosomal RNA) having similar electrophoretic mobilities. (The specific activity of isolated RNAs is the same before and after treatment with 1 M hydroxylamine, pH 7.0, 37°, 1 hr.) Transfer RNA is found in peak II; peaks designated III, IV, VI, and VII are LMM-RNAs. Peaks IV, VI, and VII contain trimethylguanylic acid.

studies were performed at several ionic strengths, using both cytoplasmic and nuclear fractions. The sedimentation of phenol-extracted LMM-RNAs added to cell fractions was studied. These studies led to the resolution of a postribosomal particle of approximately 23 S from which one of the trimethylguanylic acid containing LMM-RNAs, electrophoresis peak VII, was extracted.

Experimental Procedures

Isotope Incorporation. Chinese hamster cells (Tjio and Puck, 1958) were exposed to isotope during growth from approximately 80,000 to 450,000 cells per ml in Ham's F-10 medium containing one-half the usual complement of methionine. Adenosine (10^{-4} M) was usually added to reduce incorporation of methyl label into purine rings.

Zone Sedimentation. Samples were sedimented through exponential gradients prepared using RNase-free sucrose (Schwarz BioResearch, Inc.) solutions whose compositions are described in the appropriate figure legends. Gradients were analyzed and fractions collected with the aid of an ISCO model D fractionator, using a Beckman DB with a flow cell for an ultraviolet monitor. Most centrifugations utilized the 25.2 Beckman–Spinco rotor and tubes (1.25 × 3.5 in.). Apparent *s* values were assigned on the following bases. In the case of 7.5–35% gradients, values were estimated from postsedimentation positions relative to an assumed value of 60 S for the larger ribosomal subunit. In the case of 5–20% gradients, they were estimated relative to the rate of sedimentation of 18S and 28S ribosomal RNAs through 5–20% gradients (in the absence of Mg^{2+}). Values are uncorrected and approximate. They are assigned for purposes of reference and nomenclature.

Cell Fractionation. The procedure used for preparation of cytoplasmic and nuclear fractions has recently been described in detail (Walters *et al.*, 1970). It involves rupturing 0.25 M sucrose-washed cells with 1% Nonidet P-40 detergent (Borun *et al.*, 1967). Rough nuclei produced by 15-min NP-40 treatment are then stripped of visible cytoplasmic adhesions by addition of sodium deoxycholate to 0.5%, followed by several 20-sec periods of vigorous agitation, and at least 15-min standing at 1–3°. Apparently clean nuclei are produced more rapidly in low ionic strength buffer (RSB: 0.01 M NaCl–0.01 M Tris–1.5 mM Mg^{2+}) than when buffers of higher ionic strength are employed. Nuclei produced as described above are free of cytoplasm as judged by fluorescent microscopy of acridine orange stained preparations. They contain 99.7% of radioactive thymidine incorporated during growth of several generations. The cytoplasmic fraction contains 87% of total RNA and 90% of ribosomal RNA. Approximately 60% of LMM-RNAs are found in the cytoplasm subsequent to fractionation.

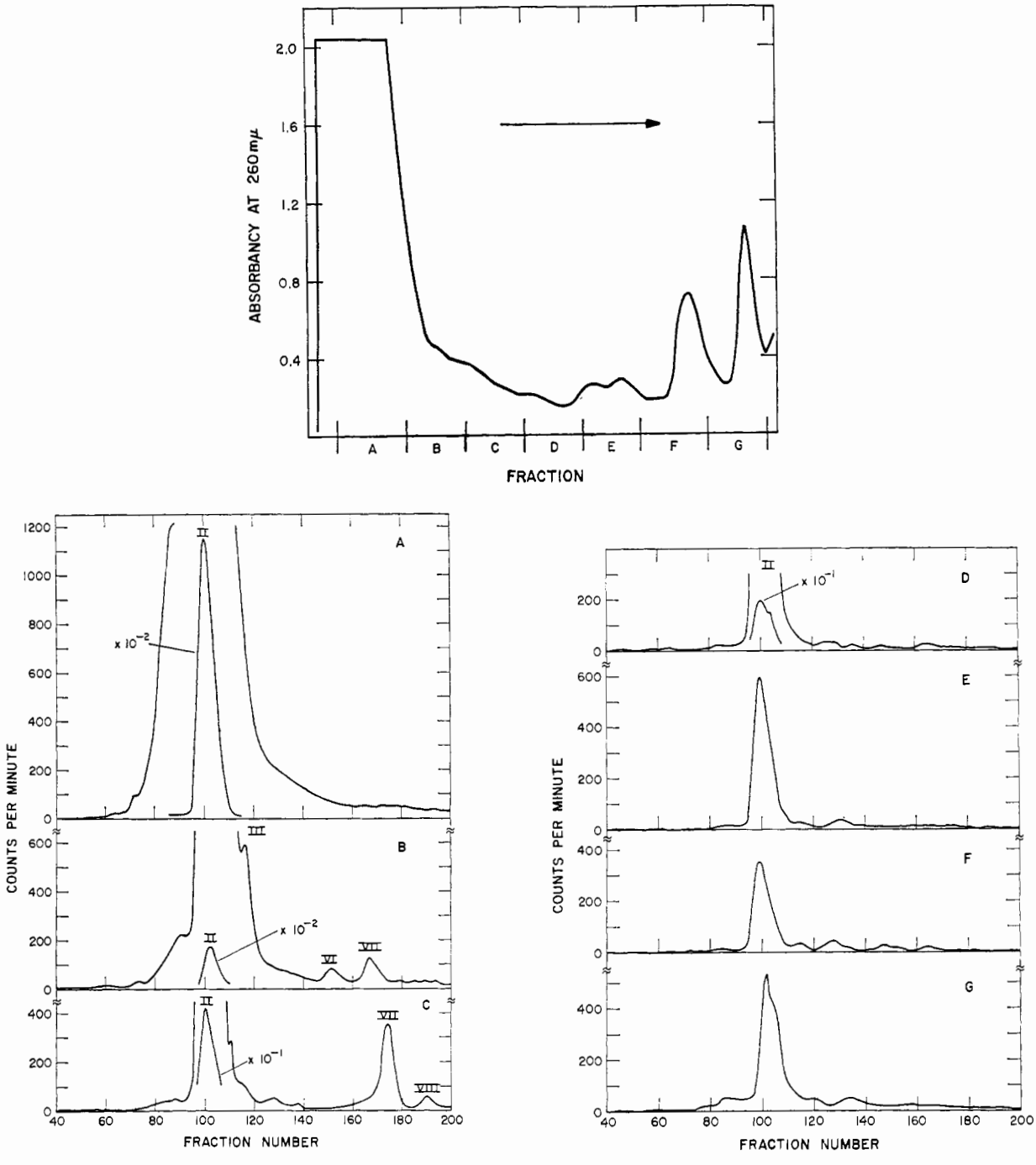


FIGURE 2: (A) An absorbancy pattern is shown for RSB cytoplasm from 10^8 [*methyl*-^{3}H]methionine-labeled (NEN, 1 mCi/1.1 mg) cells sedimented through a 7.5–35% sucrose gradient for 18 hr at 24,000 rpm, 2°. Fractions A–G from two such gradients were collected, and RNAs were concentrated, extracted, and resolved by preparative gel electrophoresis. The electrophoretic patterns are shown in B.

A nuclear brei suitable for sedimentation studies is produced by blending nuclei for 15 sec at 50,000 rpm in an Omnimixer micro blender. Whole cells are blended for 1 min. Both nuclei and cells (3×10^8) are blended in 3 ml of RSB or RSB made to 0.1 M or to 0.13 M in NaCl (0.1 RSB or 0.13 RSB).

Gel Electrophoresis. This method of resolving low molecular weight RNAs has been described by Zapisek *et al.* (1969).

Results

Zone Sedimentation of Cytoplasm. We first determined the distribution of LMM-RNAs among fractions of cytoplasm prepared in the low ionic strength buffer RSB and sedimented to produce polysomal and postribosomal (up to 100 S) sedimentation patterns. From the data shown in Figure 1, it can be seen that, although the majority of LMM-RNAs are found in the region of the gradient containing material

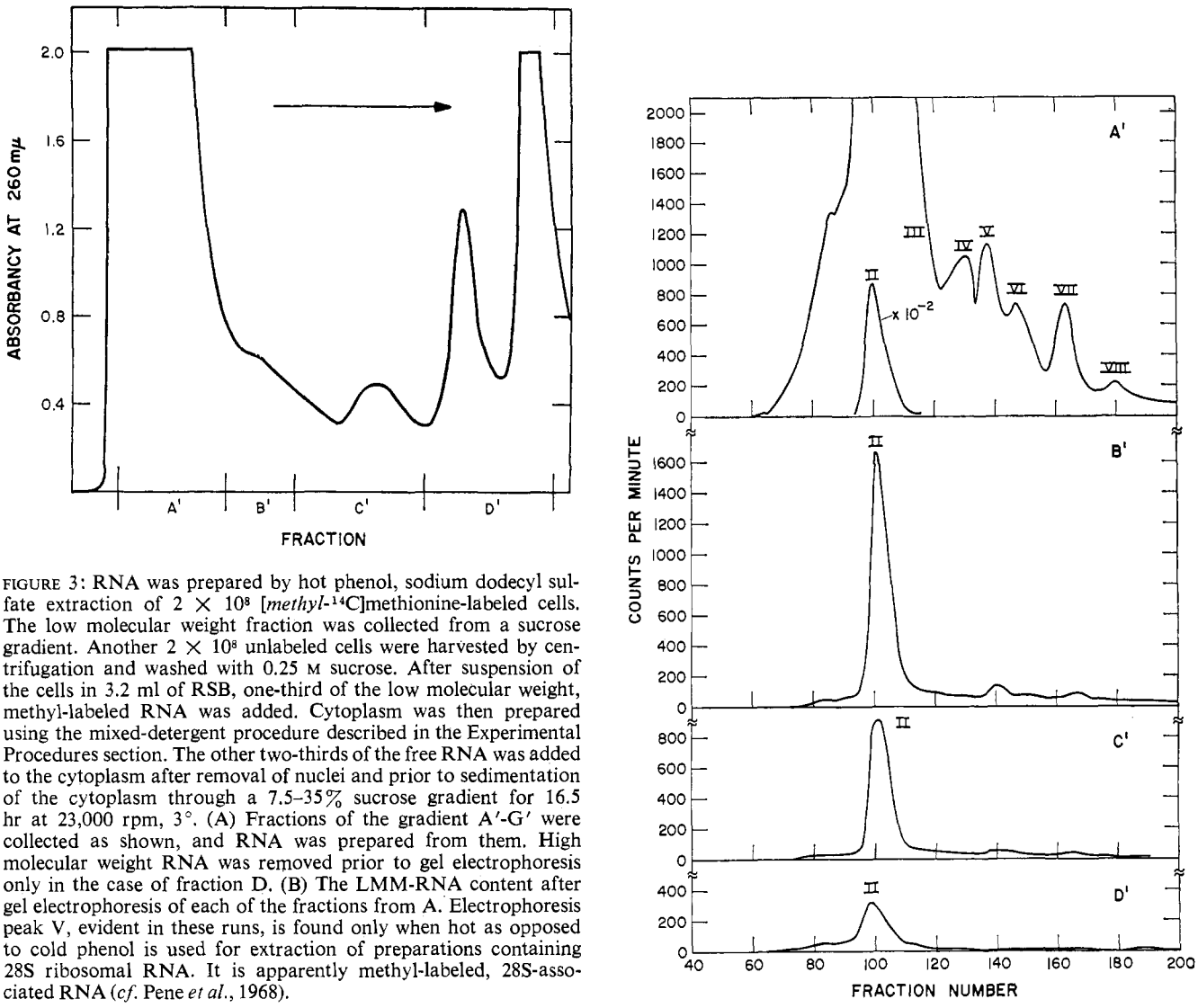
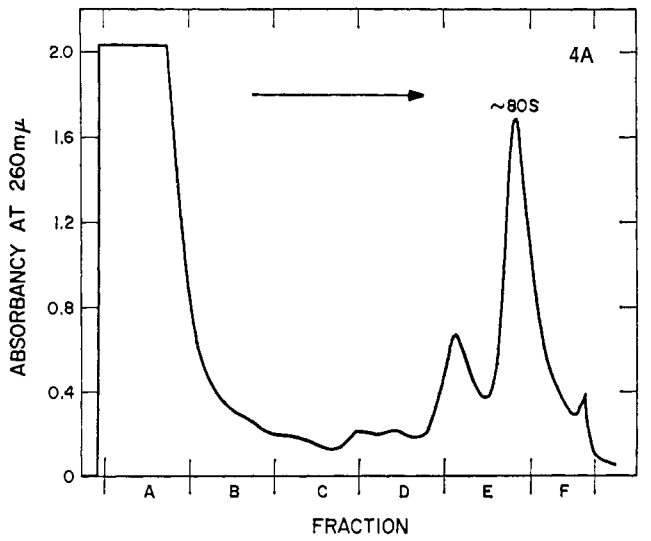


FIGURE 3: RNA was prepared by hot phenol, sodium dodecyl sulfate extraction of 2 × 10^8 [*methyl*-^{14}C]methionine-labeled cells. The low molecular weight fraction was collected from a sucrose gradient. Another 2 × 10^8 unlabeled cells were harvested by centrifugation and washed with 0.25 M sucrose. After suspension of the cells in 3.2 ml of RSB, one-third of the low molecular weight, methyl-labeled RNA was added. Cytoplasm was then prepared using the mixed-detergent procedure described in the Experimental Procedures section. The other two-thirds of the free RNA was added to the cytoplasm after removal of nuclei and prior to sedimentation of the cytoplasm through a 7.5–35% sucrose gradient for 16.5 hr at 23,000 rpm, 3°. (A) Fractions of the gradient A′-G′ were collected as shown, and RNA was prepared from them. High molecular weight RNA was removed prior to gel electrophoresis only in the case of fraction D. (B) The LMM-RNA content after gel electrophoresis of each of the fractions from A. Electrophoresis peak V, evident in these runs, is found only when hot as opposed to cold phenol is used for extraction of preparations containing 28S ribosomal RNA. It is apparently methyl-labeled, 28S-associated RNA (*cf.* Pene *et al.*, 1968).

sedimenting with or slower than the 80S ribosome, a significant portion is found in the region of small polysomes. That their presence in the polysome region does not quantitatively correlate with the amount of polysome material in the portion of the gradient extracted is seen by comparing the electrophoretically isolated LMM-RNA content of fractions 2 and 3 (Figure 1B). The peak at electrophoresis fraction 135 of fraction 3 is possibly 5S ribosomal RNA which has incorporated methyls from methionine into the purine bases *per se* (adenosine had not been added to the growth medium in this experiment).

Since the majority of LMM-RNAs sediment between 2 and 90 S, we next looked at their relative distribution in this region. Thus, cytoplasm was sedimented in such a fashion that polysomes completely sedimented (Figure 2A). The peak in cut G (Figure 2A) is due to the 80S ribosome. The 60S peak (cut F) contains 28S but not 18S RNA and is, therefore, the large ribosomal subunit. There are two peaks in the small subunit region (cut E) when, as is the case here, sedimentation is performed in low ionic strength buffer. The more rapidly sedimenting peak contains methylated 18S RNA and is on that basis a form of the smaller ribosomal subunit. The more slowly sedimenting peak contains 18S RNA as well as RNA which, after phenol extraction, sediments at approximately 12S. The amount of 18 S present is 0.1 that in the faster peak; it may be contaminant from that peak. Since the 12S RNA is unmethylated, it is not degraded ribosomal RNA but is more probably messenger. This post-ribosomal peak possibly represents a species of informosome (Spirin, 1969) liberated and/or resolved at low ionic strength. When we examined the RNAs extracted from cuts A–G (Figure 2B), we found that no LMM-RNAs were sedimenting as free RNA. That is, none was extracted from cut A. Further, we found that cut C contains LMM-RNA VII, some of VIII, but no corresponding amounts of III, IV, or VI. These species distribute among more rapidly sedimenting fractions. Their recovery is low in this instance because gradients were run prior to electrophoresis in the case of cuts E, F, and G in order to remove high molecular weight RNAs. Their endogenous distribution is shown more accurately below.

A number of investigators have noted that RNA sediments



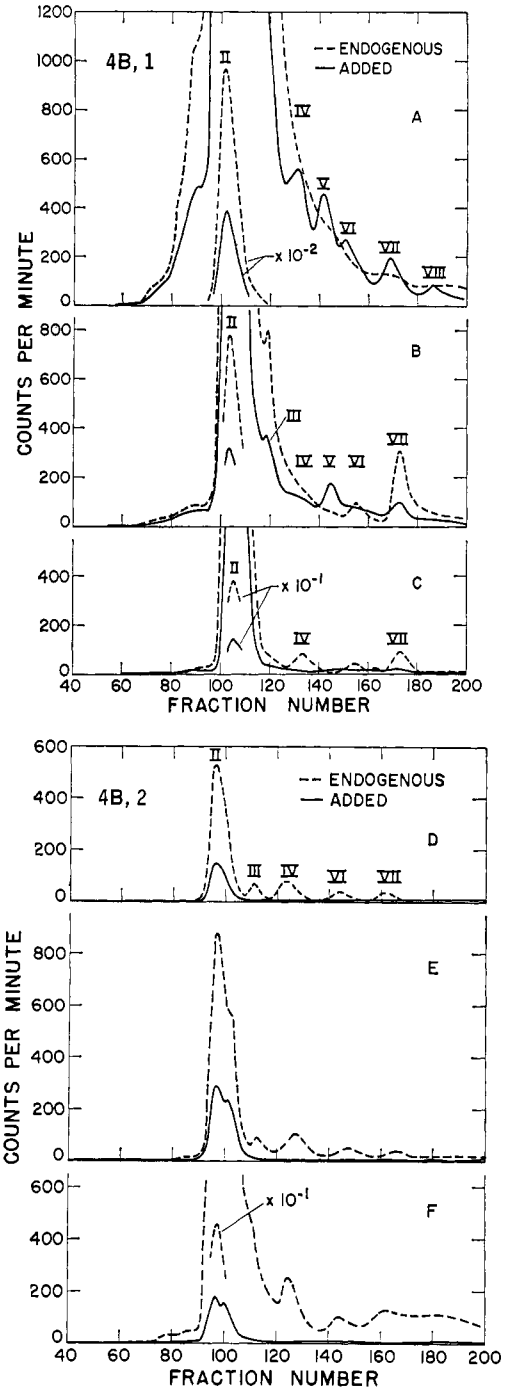


FIGURE 4: Low molecular weight, [*methyl*-^{14}C]methionine-labeled RNA was added to 2.7 × 10^8 [*methyl*-^{3}H]methionine-labeled cells suspended in 4 ml of RSB prior to disruption with Nonidet P-40. Cytoplasm was then prepared by the further addition of sodium deoxycholate, as described in the Experimental Procedures section, and was sedimented through three 7.5–35% sucrose gradients (made up in RSB) for 17 hr at 22,500 rpm, 2°, in the Spinco 25.2 rotor. Distribution of added and endogenous LMM-RNAs among the gradient fractions designated A–F in Figure 4A is shown by the gel electrophoretic patterns of B. Fraction F includes the pellet at the tube bottom. The RNA phenol extracted from each fraction was subjected to electrophoresis without removal of high molecular weight RNA *via* zone sedimentation.

rapidly when added to cytoplasm because of nonspecific association with cytoplasmic protein (Girard and Baltimore, 1966; Ovchinnikov *et al.*, 1968; Spirin, 1969). Therefore, we performed several experiments designed to determine if such association is the cause of the observed rapid sedimentation of LMM-RNAs. In the first experiment labeled, small, phenol-extracted RNA in an amount equal to the endogenous RNA was added to the cytoplasm prior to sedimentation. As seen in Figure 3, almost all *added* RNA was extracted from fraction A, which included material sedimenting slower than an apparent *s* of approximately 20. This indicates that the free RNA added to cytoplasm does not associate with cytoplasmic material to cause an increase in its apparent rate of sedimentation.

In the second experiment of this kind the added RNA, labeled with [^{14}C]methionine, was equal in mass to approximately 0.2 that of the endogenous [^{3}H]methionine-labeled, low molecular weight RNA. In cut A (Figure 4B), the radioactivity due to endogenous RNA is greater in the transfer RNA region than that of the added RNA, yet LMM-RNA peaks are seen in the case of added but not in the case of endogenous LMM-RNA. Again, peak VII concentrates

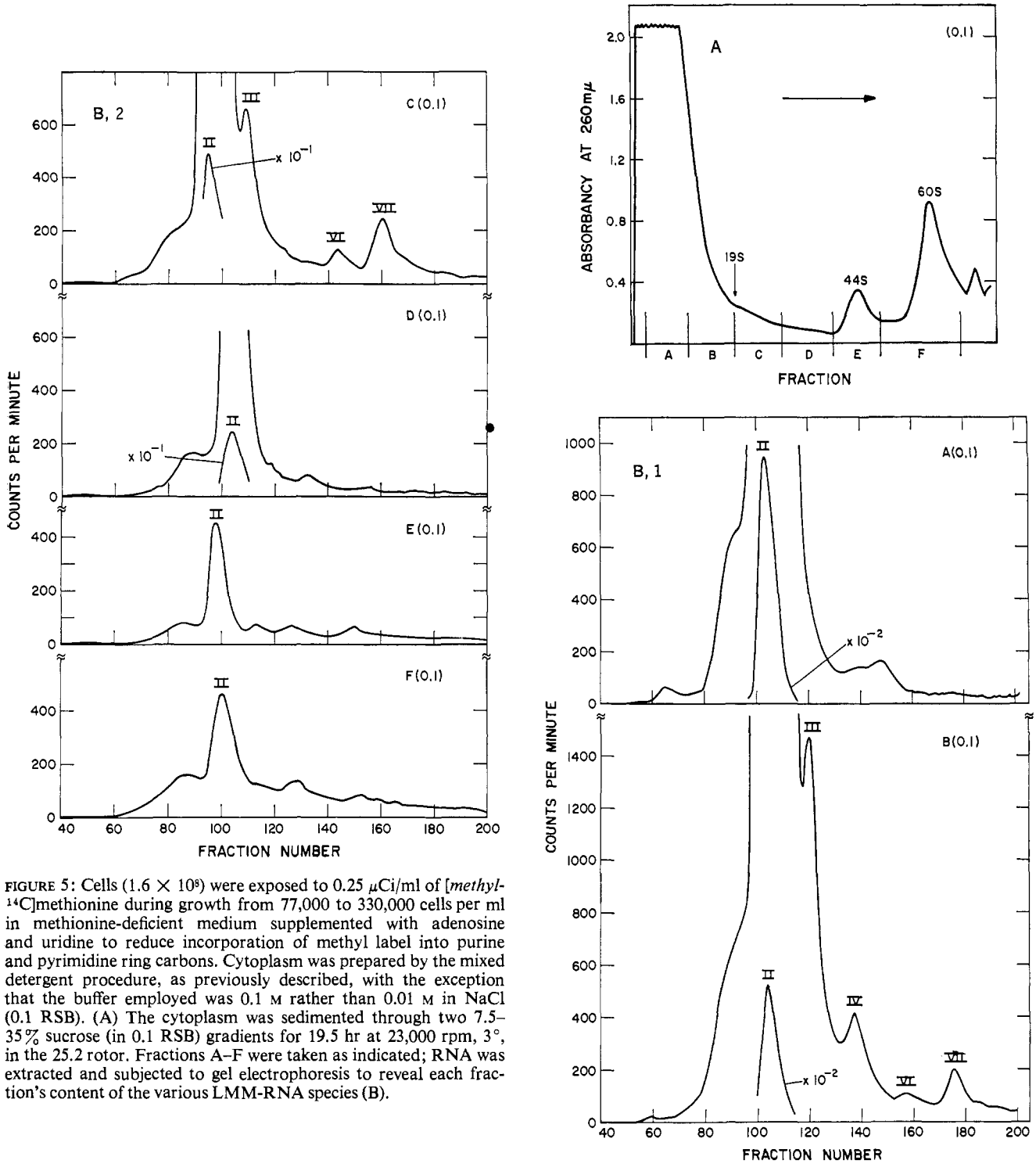


FIGURE 5: Cells (1.6×10^8) were exposed to 0.25 μCi/ml of [*methyl*-^{14}C]methionine during growth from 77,000 to 330,000 cells per ml in methionine-deficient medium supplemented with adenosine and uridine to reduce incorporation of methyl label into purine and pyrimidine ring carbons. Cytoplasm was prepared by the mixed detergent procedure, as previously described, with the exception that the buffer employed was 0.1 M rather than 0.01 M in NaCl (0.1 RSB). (A) The cytoplasm was sedimented through two 7.5–35% sucrose (in 0.1 RSB) gradients for 19.5 hr at 23,000 rpm, 3°, in the 25.2 rotor. Fractions A–F were taken as indicated; RNA was extracted and subjected to gel electrophoresis to reveal each fraction's content of the various LMM-RNA species (B).

in the 20S to 30S region, while LMM-RNAs III, IV, and VI are found in the more rapidly sedimenting regions. That their behavior may be due to aggregation at low ionic strength is indicated by LMM-RNA sedimentation distribution analysis of cytoplasm prepared in 0.1 RSB and sedimented through sucrose solutions made up in 0.1 RSB. A single peak is observed in the region of the small ribosomal subunit in this instance (Figure 5A). LMM-RNAs III, IV, and VI are now extracted from fraction B (~10 to 19 S). VII distributes among B and C so as to suggest an apparent S slightly greater than 20. Although III, IV, and VI sediment more slowly in 0.1 RSB, they still are not sedimenting as free RNAs—they are not concentrated in cut A.

We assumed that LMM-RNAs sediment more rapidly in these cytoplasmic fractions than they do when phenol-extracted because they are associated with protein. To explore this possibility, we pelleted the LMM-RNAs and then resedimented them through a gradient after treating

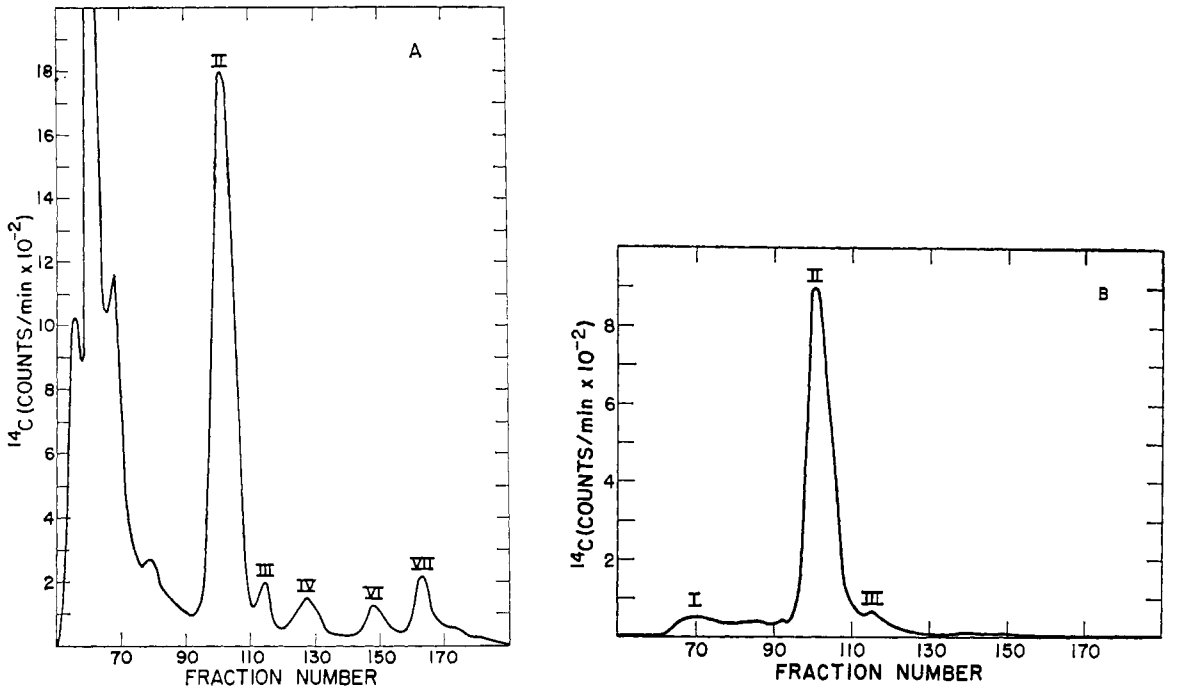


FIGURE 6: Cells were exposed to 0.25 μCi/ml of [*methyl*-^{14}C]methionine during growth from 100,000 to 500,000 cells per ml. They were fractionated as described in the Experimental Section. The cytoplasmic fraction was diluted, divided, underlaid in each instance with a 1-ml pad of 45% sucrose, and centrifuged 9 hr at 30,000 rpm in a Spinco 30 rotor. One pellet was dissolved in buffer (0.05 M sodium cacodylate, pH 7.0, 1.5 mM $MgCl_2$). The other was dissolved in 0.5 ml of buffer to which 0.05 ml of a fresh solution containing 1 mg/ml of protease (Pronase, Calbiochem, B grade, 45,000 PUK/g; 4 mg/ml of protease was predigested at least 30 min at 37° before use) was added. After 1 hr at 3°, another 0.05 ml of protease was added, and the mixture was held at 37° for 5 min and returned to 3° for 1 hr. The protease-treated and control suspensions were layered over 5–20% sucrose (1.5 mM in Mg^{2+}) in a 25.2 rotor tube and centrifuged 11 hr at 25,000 rpm, 3°. Ethanol precipitation of the 1S to 10S fractions was followed by phenol extraction of the ethanol precipitate and gel electrophoresis of the extracted RNA: (A) protease treated, and (B) untreated control.

one sample with protease. Figure 6 shows that protease treatment causes these RNAs to sediment similarly to phenol-extracted species.

As previously noted, most investigators find LMM-type RNAs to be nuclear. If they are exiting from the nucleus during isolation and accumulating associated protein during the process, artifactual particles could be produced which would not be formed when "free" RNA is added to cytoplasm. Therefore, we blended nuclei as described in the experimental section (in RSB) and determined if the LMM-RNA forms released behave as do cytoplasmic species. Since the data are qualitatively the same as in the case of cytoplasmic preparations (Figure 1), the data are not shown. As with cytoplasm, no LMM-RNA was found in the 1S to 10S region of nuclear material subsequent to blending in RSB: LMM-RNA VII again sedimented in the 20S to 30S region. Analysis of LMM-RNA distribution among polysome fractions (as in Figure 1) showed the majority of LMM-RNAs to sediment in the 1S to 90S region. Those found in the polysome region did not distribute in proportion to polysome mass. As is seen in Figure 7, this was also true in the case of nuclei blended in 0.1 RSB. In the absence of electron microscope facilities, we cannot state with certainty that the polysomes obtained by blending nuclei are nuclear and not derived from the outer nuclear membrane, although nuclear polysomes have been reported in mammalian cells (Sadowski and Howden, 1968). In any event, LMM-RNA forms released by blending nuclei *do not* sediment as free RNAs. As will be documented in the next section, LMM-RNA forms released by blending whole cells also behave as if particulate.

Resolution of Electrophoresis Peak VII Containing Particle. From the data presented in the previous section, we concluded that a particle containing LMM-RNA VII sediments in the 20S to 30S range and that, in low ionic strength buffer, none of the other LMM-RNA species would sediment in this region. Further, the amount of electrophoretic species VII contained in the cell (approximately 0.5% of total RNA) indicated that this particle should be apparent in an ultraviolet scan of a gradient subsequent to zone sedimentation if the large amount of soluble cytoplasmic material such as transfer RNA and proteins were removed. Accordingly, we subjected RSB-cytoplasm to a preliminary preparative centrifugation under conditions designed to pellet most of a 20S particle. This pellet was then suspended in buffer, and the suspension was sedimented through a sucrose gradient to resolve postribosomal particles. Unexpectedly, a number of particles were revealed which have apparent *s* values of 10, 18, 23, and 32 (Figure 8A). That VII concentrates in the 23S region is seen in Figure 8B. The amount of VII recovered in fraction 3 is about one-half the amount to be found in the cytoplasm of the number of cells processed. Note that cuts 2, 3, and 4 contain RNAs which have electrophoretic mobilities similar to transfer RNA. Such 4S material was present even when preparations similar to cut 3 were resedimented prior to phenol extraction and gel electrophoresis. As will be evident from the data presented below, sodium dodecyl

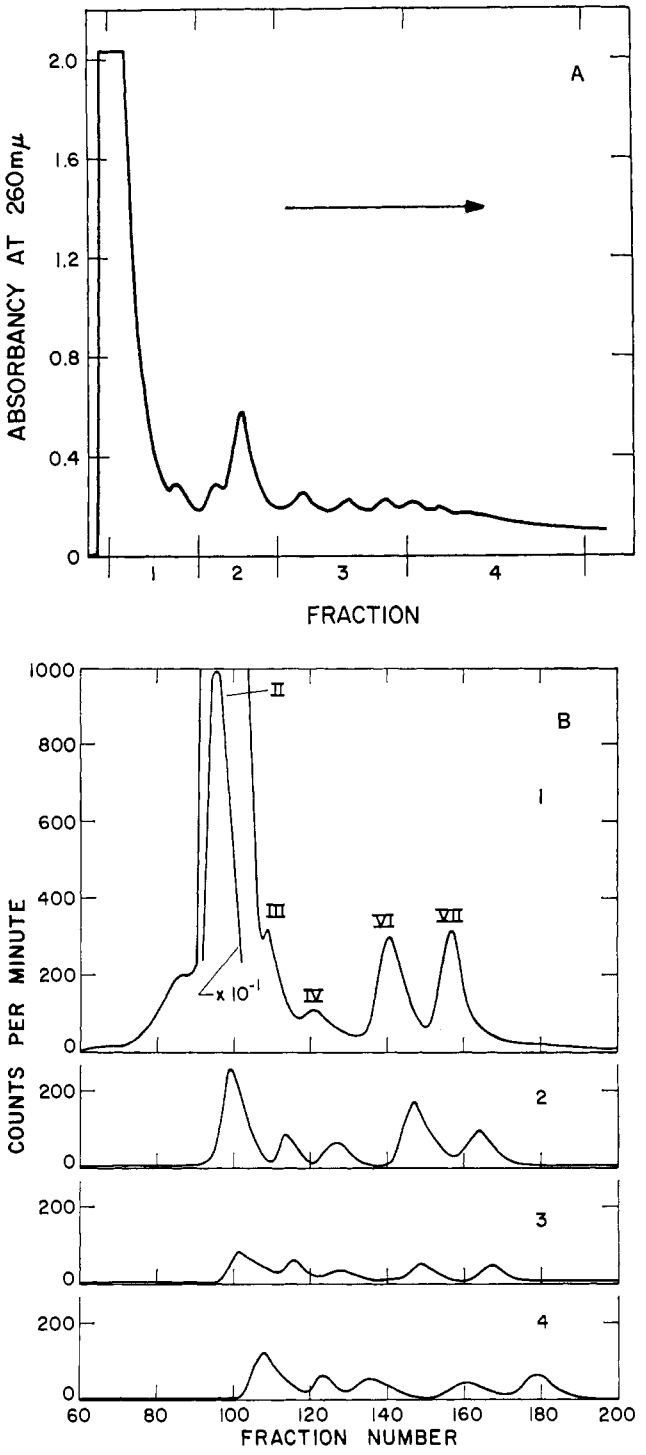


FIGURE 7: Nuclei from 8 × 10^8 cells, labeled and fractionated as described in the legend to Figure 1, were blended in 0.1 RSB buffer. Chromatin was removed by low-speed centrifugation, and the resultant supernatant was layered over two 7.5–35% sucrose (in 0.1 RSB) gradients and centrifuged 2.5 hr at 22,500 rpm, 2°, in the 25.2 Spinco rotor. (A) RNA was extracted from the fractions designated 1–4 and subjected to gel electrophoresis (B).

sulfate–phenol extraction of the 10S (fraction 1) region also produces a large amount of 4S material, as well as several other species of differing mobility; however, no VII is found in this region. Since a particle of ~10 S was observed in the above experiment and since such a particle would not be completely sedimented in 20 hr at 100,000*g*, we subjected our next preparation of this type ([^{3}H]uridine, [^{14}C]methionine labeled) to greater force × time during the pelleting step. As seen in Figure 9A, recovery of material in the 10S region is now considerably greater. The phenol–sodium dodecyl sulfate-extractable RNAs of this region are whown in Figure 9B. Two methyl- and uridine-labeled species are present. One migrates more rapidly than transfer RNA, and the other has an electrophoretic mobility similar to that of transfer RNA. There are also two unmethylated uridine-labeled peaks which represent species previously obscured by other uridine- and methyl-labeled RNAs. The more slowly moving peak (fraction 140) is not the 28S-associated RNA which migrates in this region, since (a) 28S-associated RNA is not released with the cold phenol–sodium dodecyl sulfate extraction used in this preparation, and (b) 28S-associated RNA is methylated. Thus, the 10S region contains a number of previously undescribed RNAs. It may also contain a correspondingly large number of particles as well as large proteins unassociated with RNA.

We used a portion of sedimentation fraction 2, 3, and 4 (Figure 9) to determine if phenol-extractable, high molecular weight RNA was present. None was found. Since DNA-containing 16S particles have been found in an animal cell system (Bell, 1969), we looked for DNA. As can be seen from Table I, no significant amount of radioactivity from

TABLE I: Relative RNA and DNA Contents of Postribosomal Particles as Determined from Amounts of [6-^{3}H]Uridine Incorporated.[a]

Fraction	RNA (cpm [^{3}H])	DNA (cpm [^{3}H])
1	21,776	38
2	10,670	5
3	10,442	23
4	6,266	25

[a] Aliquots (0.5 ml) of fractions 1–4 of Figure 9A were mixed with carrier RNA and DNA and worked up as follows: 5 ml of 10% trichloroacetic acid was added to each. After 30 min at 0°, the resultant precipitate was collected by centrifugation, washed with 10 ml of 2% potassium acetate in ethanol, and then digested with 1 ml of 0.3 M KOH for 18 hr at 37°. The KOH digest was neutralized and then brought to 0.2 M in perchloric acid. After standing at 0° for 30 min, the precipitate was deposited by centrifugation and washed with 5 ml of 0.2 M perchloric acid. The 0.2 M supernatants were made to 10 ml, and 0.5 ml was taken for counting to determine incorporation into RNA. The 0.2 M perchloric precipitate was suspended in 5 ml of 0.5 M perchloric acid, heated to 70° for 30 min, and cooled. The precipitate was then spun out and washed with another 5 ml of 0.5 M perchloric acid. An 0.5-ml aliquot of the 0.5-M perchloric supernatants was counted for determination of incorporation into DNA. The efficiency of counting was essentially the same for the two supernatants in the system used (Insta-gel Emulsifier, Packard Instrument Co., Inc.).

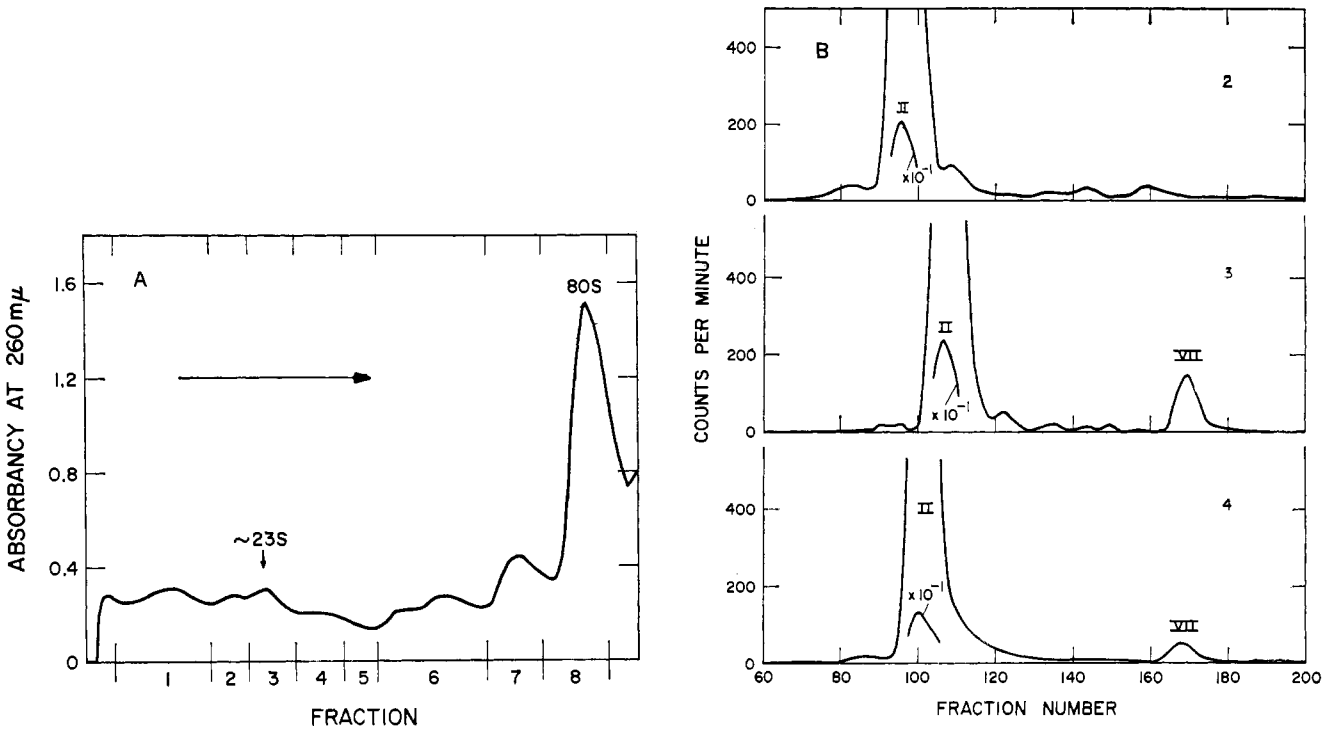


FIGURE 8: Cells (4 l.) were exposed to 2 mCi of [*methyl*-^{3}H]methionine during growth from 100,000 to 400,000 cells per ml. Cytoplasm was prepared using 25.6 ml of RSB and 3.2 ml each of 10% Nonidet P-40 and 5% sodium deoxycholate. After removal of nuclei (15 min at 1000 *g*), cytoplasm was divided into two aliquots, each of which was layered over 2 ml of 30% sucrose in RSB in IEC A211 centrifuge tubes. After 20 hr at 100,000*g* average, 3°, the resultant pellets were suspended in 2 ml of RSB each by gentle agitation with a magnetic stir bar for 6 hr at 4°. The resultant solutions were rinsed from the tubes with another 1 ml of RSB, and the total was layered over three 7.5–35% sucrose in RSB gradients. After 18 hr at 22,500 rpm, 3°, in the 25.2 tubes, gradients were analyzed and fractions 1–8 taken as designated (A). The gel electrophoresis patterns of low molecular weight, methylated RNAs of fractions 2, 3, and 4 are shown in (B).

6-[^{3}H]uridine was found in material resistant to KOH but released by hot perchloric acid.

The above analyses indicated that LMM-RNA VII concentrated in the 23S region of a sucrose gradient. We used the uridine- and methyl-labeled preparation to check the susceptibility of the material in the 23S region (cut 3, Figure 9A) to protease and to high salt. As is seen from the data presented in Figure 10, protease converts 23S material, both uridine- and methionine methyl-labeled, into more slowly sedimenting forms. High salt causes the uridine-labeled material to sediment more slowly. Methionine-labeled species, however, continue to sediment in the 20S to 30S region after high salt.

Postribosomal Particles in Whole Cell Blends. We were concerned that the detergents NP-40 and deoxycholate, used in all the above preparations, may have caused formation of particles which were not present *in vivo* and which, thus, might be absent in cell fractions prepared without the detergents. Therefore, we mechanically disrupted cells in isotonic buffer with a high-speed blender and looked for particles. As seen in Figure 11A, postribosomal particles are again detected. That electrophoretic species VI and VII sediment faster than 10 S is seen from the data of Figure 11B, where the RNA content of fractions representing regions of approximately 3 to 15 S (1) and 15 to 30 S (2) is shown. Again, a large amount of 4S RNA is found in relatively rapidly sedimenting forms. LMM-RNA particles were also found in whole cell blends prepared in the low ionic strength buffer RSB.

Discussion

We have shown that LMM-RNAs exist in Chinese hamster cell fractions in forms that sediment more rapidly than 10 S before but not after protease treatment. They are presumably associated with protein. There are a number of reasons for believing that this association is not artifactual. First, when phenol-extracted LMM-RNAs are added to cells prior to their disruption, the added RNAs sediment at less than (but the endogenous RNAs greater than) 10 S. This was the case whether the added RNA was equal in amount to the endogenous or 0.2 the cell LMM-RNA content. Second, a very specific interaction of one of the LMM-RNAs (electrophoretic peak VII) with protein occurs such that the particle formed is the only LMM-RNA containing one to be found sedimenting at approximately 23 S in low ionic strength buffer. Third, the particles are very stable; they do not dissociate during several days of sedimentation through sucrose solution, resuspension, and resedimentation. Finally, the particles are found in both nuclear and cytoplasmic fractions and are present after mixed detergent or mechanical disruption of Chinese hamster cells in 0.01 M Tris (pH 7.4, 1.5 mM Mg^{2+}) buffers that are 0.01, 0.1, or 0.13 M in NaCl.

Although a number of groups have studied RNAs similar to LMM-RNAs, none has reported them to be in more rapidly sedimenting particulates. Weinberg and Penman (1969) looked for HeLa forms sedimenting more rapidly than the free RNAs. They found none. In this instance,

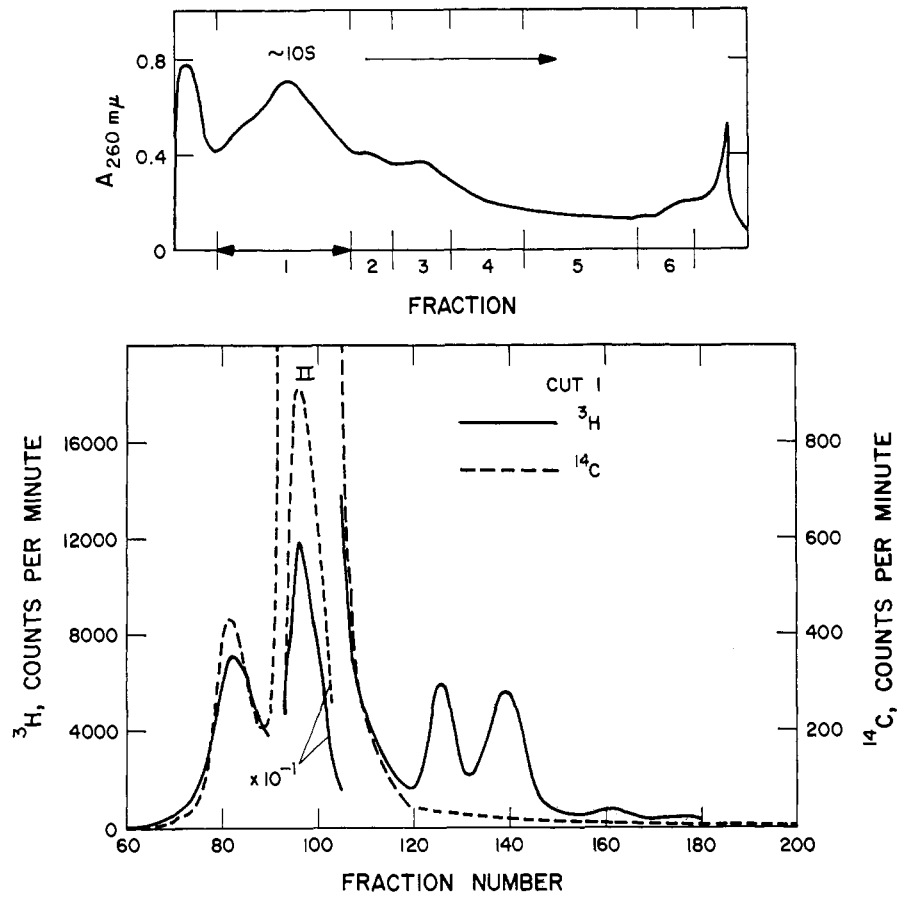


FIGURE 9: A 4-l. culture of Chinese hamster cells was exposed to a total of 2 mCi of [6-^{3}H]uridine (NEN, 5 mCi in 0.118 mg) and 500 μCi of [*methyl*-^{14}C]methionine (Schwarz BioResearch, 51 mCi/mmole) during growth from 100,000 to 355,000 cells per ml. Cytoplasm was prepared in RSB using mixed detergents. After a preliminary spin of 10 min at 20,000*g* average, the supernatant cytoplasm was layered over 2 ml of 30% sucrose (in RSB) in each of two IEC A211 tubes and centrifuged 20 hr at 170,000*g* average, 3°. The resultant pellets were suspended in 2 ml of RSB each, layered over three 5–20% sucrose (in RSB) gradients, and sedimented 18 hr at 24,000 rpm, 3°, in Spinco 25.2 tubes. The absorbancy (A_{260}) profile after centrifugation is shown in A. An aliquot from fraction 1 was used for determination of low molecular weight RNA content of the 10S region after gel electrophoresis (B). Aliquots of fractions 2–6 were phenol–sodium dodecyl sulfate extracted and analyzed for uridine- or methionine-labeled, high molecular weight RNA as previously described (Saponara and Enger, 1966). Aliquots (0.5 ml) of cuts 1–4 were analyzed for relative [6-^{3}H]uridine incorporation into RNA and DNA (Table I). The A_{260}/A_{280} ratios were monitored. The ratio throughout the region of fractions 1–4 is between 1.0 and 0.9. In the region of ribosomes, this ratio approximates 2.0.

there is a probable explanation for the absence of particulate forms. That is, the HeLa species were liberated from nuclei with high salt buffer (HSB) or from chromatin with 0.2 M salt prior to observation of sedimentation properties at reduced ionic strength. Treatment of the Chinese hamster cell particulate containing LMM-RNA species VII with HSB prior to sedimentation through a gradient of low ionic strength caused release of the RNA to the extent that its sedimentation rate was reduced to that of protease-released RNA. Perhaps, then, particulates containing LMM-RNA cognates are to be found only if suitably low ionic strength buffers (iso- or hypotonic) are used.

The function of LMM-RNAs is not known. The fact that they are not usually found in cytoplasmic fractions makes their direct involvement in translation processes unlikely. Weinberg and Penman (1969) found LMM-type RNAs to be but loosely attached to chromatin. Similarly, we find Chinese hamster cell chromatin, mechanically prepared using isotonic saline, to be free of detectable amounts of LMM-RNAs. These observations limit the possibilities for a role of LMM-RNAs in transcription processes.

We believe that a determination of the nature of the proteins and nucleic acids found in LMM-RNA containing particulates is of value in delineating possible functions for LMM-RNAs. Accordingly, we are engaged in isolation and characterization of the proteins involved. We are also attempting to determine whether any of these proteins are in cosedimenting but unassociated particles. Also, these particles contain methylated RNA which is similar to transfer RNA in electrophoretic mobility. Again, we hope to determine whether this 4S RNA is actually associated with the LMM-RNA-containing particles. We are collaborating with A. G. Saponara and A. E. Hampel in studies of the minor nucleotide content, GTψC sequence content, and amino acid acceptor activity of these RNAs. Hopefully, these studies will allow us to consider more meaningfully the possibility that LMM-RNA particles are involved in transfer RNA synthesis or maturation.

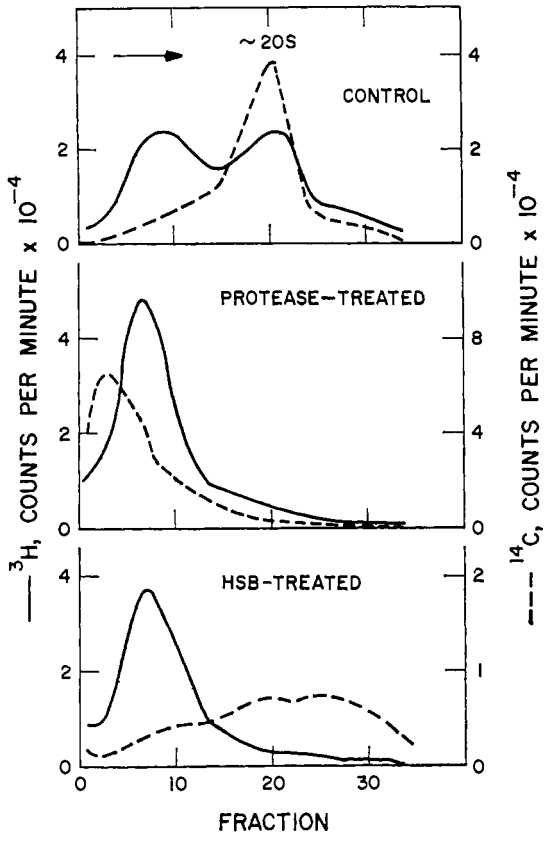


FIGURE 10: Two-thirds of fraction 3 (Figure 9A) was sedimented for 22 hr at 50,000 rpm in the IEC A321 rotor, 3°. After suspension in 1 ml of RSB, three aliquots were taken: 0.33 ml was mixed with 0.55 ml of HSB × 1.6 (HSB is 0.5 M NaCl, 0.05 M Mg^{2+}, 0.01 M Tris, pH 7.4; 1.6 × HSB is 0.8 M in NaCl) and allowed to stand 1 hr at 4°; to another 0.33-ml aliquot was added 0.1 ml of 5 mg/ml of predigested protease; and the third aliquot was held in RSB as a control. After 1-hr digestion the protease-treated, high salt buffer-treated, and control aliquots were layered over 5–20% gradients made up in RSB and sedimented 18 hr at 22,500 rpm. The gradients were fractionated into 0.75-ml aliquots for determination of isotope content as previously described (Saponara and Enger, 1966). The distribution of label among the fractions of the top half of the gradients is shown in this figure.

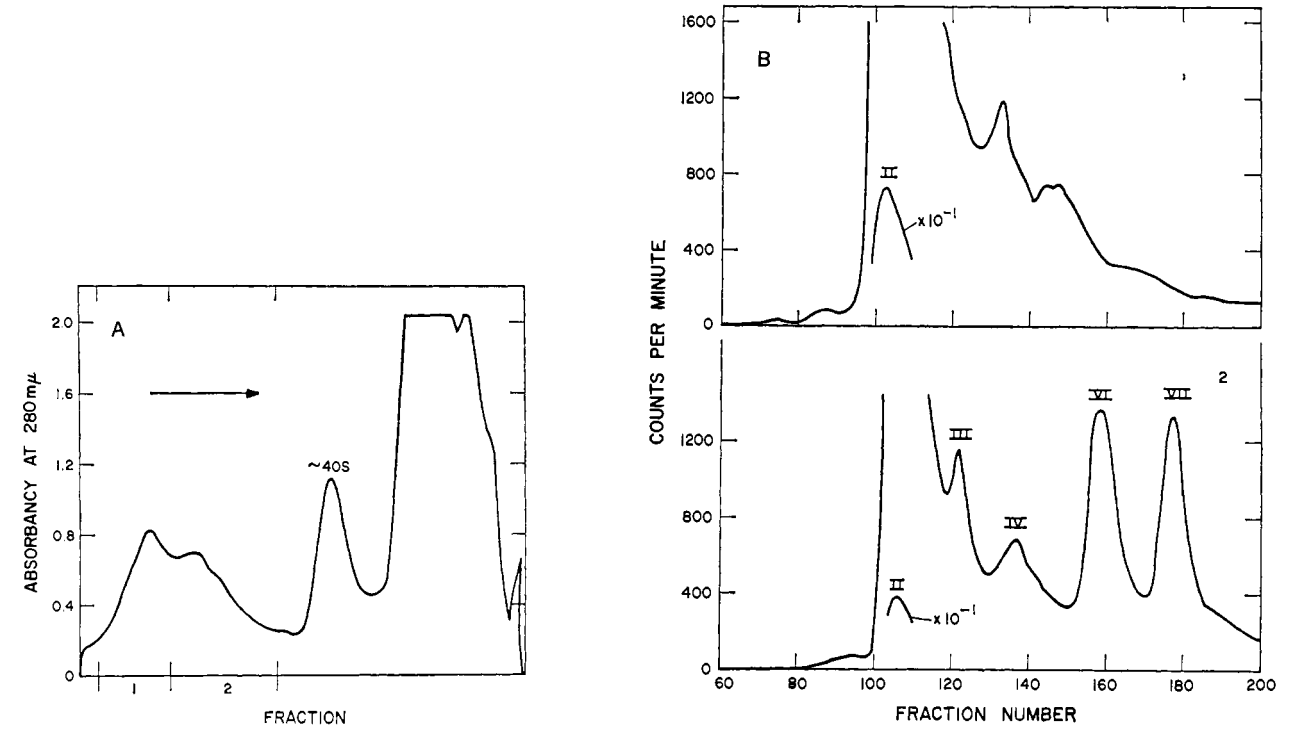


FIGURE 11: Cells (1.8 l.) were exposed to 500 μCi of [5-³H]uridine (Schwarz BioResearch, Inc., 4 Ci/mmole) during growth from 80,000 to 420,000 cells per ml in regular F-10 medium. After harvest, they were washed by deposition from 0.25 M sucrose, suspended in 0.13 RSB (0.13 M NaCl, 0.01 M Tris, pH 7.4, 1.5 mM Mg^{2+}), and blended with a high-speed blender in batches; the total volume of the blend was 11 ml. The blend was diluted to 30 ml and chromatin removed by centrifugation at 20,000*g* average for 15 min. The supernatant was centrifuged through 2 ml of 30% sucrose in 0.13 RSB for 20 hr at 140,000*g* average. The resultant pellet was suspended in 1.5 ml of 0.13 RSB, layered over a 7.5–35% sucrose (in 0.13 RSB) gradient, and centrifuged 20 hr at 22,500 rpm in the 25.2 Spinco rotor. The resultant absorbancy profile is shown in A. The gel electrophoresis patterns obtained upon analysis of RNA extracted from fractions 1 and 2 are shown in B.

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Thermal Perturbation Difference Spectra of Proteins Containing Tryptophyl Residues*

Jake Bello

ABSTRACT: The thermal perturbation difference spectral method was applied to the study of the exposed tryptophyls of chicken lysozyme and α-chymotrypsinogen. In 6 M guanidinium chloride both proteins as well as their derivatives having reduced disulfides gave spectral results indicating substantially complete exposure of tryptophyls. In aqueous buffer the method indicates 3.5–4 exposed tryptophyls in lysozyme compared with about 0.9 reactive toward Koshland's reagent (2-hydroxy-5-nitrobenzyl bromide). The thermal perturbation spectrum of α-chymotrypsinogen contains an additional extremum (303 nm), not found for lysozyme or for the model, *N*-acetyltyrosinamide. The spectra of the reduced proteins are markedly different from those of the native proteins.

The spectrum of α-chymotrypsinogen is dependent on the manner in which temperature changes are carried out. The spectrum of the model compound is strongly dependent on the solvent.

Thermal perturbation difference spectra are generated when solutions of identical composition, but at different temperatures are examined spectrophotometrically. We have reported earlier on the use of the thermal perturbation method for the estimation of exposed tyrosines of RNase (Bello, 1969a,b), for the study of water structure in mixed solvents (Pittz and Bello, 1970), and for qualitative indication of preferential hydration of tyrosine in a mixed solvent (Pittz and Bello, 1969). Cane (1969) has also presented an independent development of this technique. We now present data on *N*-AcTrp-NH_2[1] and on the tryptophyl-containing proteins, chicken egg lysozyme, bovine α-chymotrypsinogen, and, in passing, β-lactoglobulin. We shall see that tryptophan gives results that are much more complex than those of tyrosine.

* From the Department of Biophysics, Roswell Park Memorial Institute, Buffalo, New York 14203. *Received March 2, 1970.* This work was supported by Grant GM-13485 from the Institute of General Medical Sciences, National Institutes of Health and Grant GB 7523 from the National Science Foundation.

[1] Abbreviations used are: *N*-AcTrp-NH_2, *N*-acetyltryptophanamide; *N*-AcTyr-NH_2, *N*-acetyltyrosinamide; RCAM, disulfides reduced and alkylated with carboxamidomethyl groups; Gu·HCl, guanidinium chloride.