Symp. Biol. 23, 1.
McCarthy, B. J., and McConaughy, B. L. (1968), *Biochem. Genet. 2*, 37.
McCormick, W., and Penman, S. (1969), *J. Mol. Biol. 39*, 315.
McEwen, C. R. (1967), *Anal. Biochem. 20*, 114.
Melli, M., Whitfield, C., Rao, K. V., Richardson, M., and Bishop, J. O. (1971), *Nature (London), New Biol. 231*, 8.
Moav, B., and Nemer, M. (1971), *Biochemistry 10*, 881.
Moore, R. C., Ed. (1966), Treatise on Invertebrate Paleontology, Lawrence, Kan., University of Kansas Press.
Nemer, M. (1962), *J. Biol. Chem. 237*, 143.
Nemer, M., and Lindsay, D. T. (1969), *Biochem. Biophys. Res. Commun. 35*, 156.
Ohno, S. (1972), *Brookhaven Symp. Biol. 23*, 366.
Oliver, S., and Chalkley, R. (1972), *Expt. Cell Res. 73*, 295.
Olsen, M. O. J., Jordan, J., and Busch, H. (1972), *Biochem. Biophys. Res. Commun. 46*, 50.
Panyim, S., Bilek, D., and Chalkley, R. (1971), *J. Biol. Chem. 246*, 4206.
Rall, S. C., and Cole, R. D. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 534.
Rinaldi, A. M., and Monroy, A. (1969), *Develop. Biol. 19*, 73.
Robertson, H. D., and Jeppeson, P. G. N. (1972), *J. Mol. Biol. 68*, 417.
Sanger, F. (1971), *Biochem. J. 124*, 833.
Schaap, T. (1971), *J. Theoret. Biol. 32*, 293.
Schilkraut, C., and Lifson, S. (1965), *Biopolymers 3*, 195.
Shearer, R. W., and McCarthy, B. J. (1967), *Biochemistry 6*, 283.
Slater, D. W., and Spiegelman, S. (1970), *Biochim. Biophys. Acta 213*, 194.
Smith, E. L., DeLange, R. J., and Bonner, J. (1970), *Physiol. Rev. 50*, 159.
Straus, N. A., and Bonner, T. I. (1972), *Biochim. Biophys. Acta 277*, 87.
Sutton, W. D., and McCallum, M. (1971), *Nature (London), New Biol. 232*, 83.
Suzuki, Y., and Brown, D. D. (1972), *J. Mol. Biol. 63*, 409.
Ullman, J. S., and McCarthy, B. J. (1973), *Biochim. Biophys. Acta 294*, 416.
Vaughan, M. H., Pawlowski, P. J., and Forchhammer, J. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2057.
Vincent, W. S., Halvorson, H. O., Chen, H. R., and Shen, D. (1969), *Expt. Cell Res. 57*, 240.
Weinberg, E. S., Birnstiel, M. L., Purdom, I. F., and Williamson, R. (1972), *Nature (London) 240*, 225.
White, H. B., Laux, B. E., and Dennis, D. (1972), *Science 175*, 1264.
Whiteley, A. H., McCarthy, B. J., and Whiteley, H. R. (1696), *Proc. Nat. Acad. Sci. U. S. 55*, 519.
Williamson, R., Morrison, M., Lanyon, G., Eason, R., and Paul, J. (1971), *Biochemistry 10*, 3019.

Messenger Ribonucleic Acid Metabolism in Mammalian Mitochondria. Origin of Ethidium Bromide Resistant Poly(adenylic acid) Containing Ribonucleic Acid in Ehrlich Ascites Mitochondria†

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ABSTRACT: The patterns of inhibition of actinomycin D and ethidium bromide on both *in vivo* and *in vitro* RNA synthesis in Ehrlich ascites mitochondria have been studied. Both the *in vivo* and the *in vitro* labeled mitochondria contain high molecular weight RNA migrating between 3 and 28 S. Only the *in vivo* RNA but not the *in vitro* RNA contains RNase resistant filter bindable poly(A) sequences. The *in vitro* RNA synthesis is extremely sensitive to ethidium bromide, while the *in vivo* synthesis of mtRNA is less sensitive to this drug (55–60%). Actinomycin D, on the other hand, affects the *in vivo* RNA synthesis by about 35%, and has no effect on the *in vitro* system. The ethidium bromide resistant mtRNA contains an increased amount of poly(A) as against actinomycin D resistant RNA which is extremely low in poly(A) content. The ethidium bromide resistant RNA has a half-life of 35–40 min, which coincides with the half-life of *in vivo* labeled poly(A) in mitochondria. Actinomycin D resistant RNA and the *in vitro* RNA, on the other hand, decay more rapidly with a half-life of 10 min. On the basis of these results and also kinetics of ethidium bromide resistance and appearance of poly(A), it has been proposed that the ethidium bromide resistance observed in the *in vivo* experiments is probably because of the effect of nRNA synthesis and that poly(A) containing RNA found in mitochondria are of nuclear origin. This hypothesis was further strengthened by DNA–RNA hybridization experiments.

Extensive research during the last decade has unequivocally shown the presence of specific systems for the synthesis of DNA, RNA, and protein in mitochondria (Ashwell and Work, 1970). Since mitochondrial systems are dissimilar to nucleocytoplasmic systems, many scientists consider these organelles as partly autonomous (for references, see Ashwell and Work, 1970). Nevertheless, it is now widely accepted that mitochondrial biogenesis is a complex event involving the expression of both mitochondrial and nuclear genomes (Woodward *et al.*, 1970; Borst, 1972). Most of the mito-

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chondrial proteins are known to be coded by nuclear genes (Ashwell and Work, 1970; Borst, 1972; Kuntzel, 1971). However, the exact mechanism of transfer of nuclear specified information into mitochondria is a matter of conflict (Borst, 1972).

Using specific inhibitors of cytoplasmic and mitochondrial protein synthesizing systems, it has been convincingly shown that almost all of the mitochondrial ribosomal proteins in both *Neurospora* and yeasts (Kuntzel, 1969; Kuntzel, 1971; Linnane and Haslam, 1970; Lizardi and Luck, 1971) are imported from the cytoplasm. More recently, using the selective inhibition technique, Barath and Kuntzel (1972a, 1972b) have demonstrated that enzymes like RNA polymerase and tRNA synthetases in *Neurospora* mitochondria are induced by ethidium bromide, an inhibitor of mitochondrial transcription and translation. On the basis of these results and also some DNA–RNA hybridization experiments in yeasts (Borst, 1972; Reijnders *et al.*, 1972), it has been postulated that the nuclear mRNAs coding for mitochondrial proteins are exclusively translated on the cytoplasmic ribosomes (Barath and Kuntzel, 1972a, 1972b).

Based on the size of mtDNA,[1] from animal cells and the size of rRNAs and tRNAs it codes, Dawid (1972) has suggested that most of the mRNAs translated in the animal mitochondria may be of nuclear origin (Dawid, 1970, 1972). This hypothesis has been supported by experiments showing the ability of isolated mitochondria to incorporate synthetic polynucleotides (Swanson, 1971) and nuclear hnRNA (Kisselev and Gaitskhoki, 1972) which then serve as templates for protein synthesis in these organelles.

Thus, although it is certain that the majority of nuclear mRNAs coding for mitochondrial proteins are translated in the cytoplasm, it is not unambiguously clear whether nuclear mRNAs are normally transported to and translated in mitochondria.

Recently, we showed the existence of a class of RNA containing poly(adenylic acid) sequences (poly(A)) in Ehrlich ascites mitochondria (Avadhani *et al.*, 1973). In this paper, we present experimental evidence which indicates that the poly(A) containing RNA in Ehrlich ascites mitochondria are of nonmitochondrial and, presumably, of nuclear origin.

Materials and Methods

Cells. Hypotetraploid cells of Lettre Ehrlich ascites cells grown for 7 days in the peritoneal cavity of Swiss colony mice were used in these studies. Details of maintenance and growth have been described elsewhere (Chun *et al.*, 1969).

Pulse Labeling of Cells. Freshly harvested cells were diluted threefold with Locke Ringer medium to get a density of about 10^8 cells/ml. Incubation was carried out in a shaker water bath at 37°. After 15 min, one or more ^{3}H-labeled precursors were added as mentioned in the footnotes to respective experiments. ^{3}H-labeled cytidine (27.4 Ci/mmol) and adenosine (18.3 Ci/mmole) were purchased from Schwarz/Mann laboratories, and uridine (15 Ci/mmol) was a product of International Chemical and Nuclear Corp.

Preparation of Mitochondria. Unless otherwise mentioned, all the following steps were carried out at 0–4°. Cells were washed twice with 0.15 M NaCl and suspended in three volumes of homogenizing medium (10 mM Tris-HCl (pH 7.4)–1 mM $MgCl_2$–20 mM KCl–0.25 M sucrose). Homogenization was carried out in a glass homogenizer as described earlier (Avadhani *et al.*, 1973). The homogenate was centrifuged twice at 1200*g* for 10 min each in a refrigerated Beta Fuge (Lourdes Instrument Corp.) to eliminate whole cells and nuclei. The resultant supernatant was centrifuged at 7500*g* for 15 min to pellet mitochondria. The crude mitochondrial pellet was washed three times with Tes buffer (10 mM Tris-HCl (pH 7.4)–2 mM EDTA–0.25 M sucrose) and the resultant preparation was directly used for the *in vitro* RNA synthesis. In the case of *in vivo* labeling experiments, the mitochondria isolated as above were suspended in 6–7 volumes of 0.5% digitonin (Sigma) in Tes buffer, incubated on ice for 5 min. Mitochondria were then pelleted at 7500*g* for 15 min, washed once with Tes buffer, and used for isolating RNA. This additional treatment in the case of *in vivo* labeled mitochondria was necessary for minimizing cytoplasmic contamination (Dawid, 1970).

Extraction of RNA. RNA was prepared by a phenol–chloroform–sodium dodecyl sulfate extraction technique essentially as described earlier (Avadhani *et al.*, 1973), except that extraction was carried out at room temperature and the extraction buffer contained 50 mM Tris-HCl (pH 7.5) instead of sodium acetate buffer (pH 6.0) used in previous experiments. Extraction at elevated pH is known to improve the recovery of messenger-like RNAs (Lee *et al.*, 1971). The major disadvantage of extraction at alkaline pH, however, is the increased possibility of contamination of RNA fraction with DNA. The RNA preparation was, therefore, routinely treated with 10 μg/ml of DNase (RNase-free, Worthington Biochemical Corp.) in a buffer containing 10 mM Tris-HCl (pH 7.5)–1 mM $MgCl_2$–20 mM KCl at 30° for 20 min. DNase was then removed by extraction with phenol–chloroform and the RNA was precipitated with two volumes of 70% ethanol containing 3.0% potassium acetate. The RNA precipitate was washed three times with ethanol as described (Avadhani *et al.*, 1973) and stored at −20° under ethanol until used.

The concentration of RNA was determined on the basis of optical density readings at uv 260 nm (25 OD = 1 mg of RNA).

In Vitro Labeling of Mitochondria. Mitochondria were suspended in a buffer containing 10 mM Tris-HCl (pH 8.0), 7.0 mM $MgCl_2$, 20 mM KCl, 2 mM 2-mercaptoethanol (Sigma), 2 mM phosphoenolpyruvate (Sigma), and 1 μg/ml of pyruvate kinase (Sigma) at a concentration of 12–14 mg of mitochondrial protein/ml. ^{3}H-labeled CTP and/or ATP (both from Schwarz/Mann laboratories, specific activity 15 Ci/mmol) were then added as indicated in the respective experiments. Incubation was carried out at 37°, unless otherwise mentioned, for 30 min. Mitochondria were then washed twice at 20,000*g* for 10 min with Tes buffer and used for extracting the RNA.

Polyacrylamide Gel Electrophoresis. RNA samples were electrophoresed on 2.4% polyacrylamide gels according to the method of Bishop *et al.* (1967). The gels were sliced into 1-mm thick slices; each slice was taken in a scintillation vial, incubated at room temperature with 0.5 ml of 3 N protosol (New England Nuclear) for 3 hr, and counted with 10 ml of toluene based scintillation fluid.

Preparation of Nuclear and Mitochondrial DNA. The procedure of Wang (1967) was used for the preparation of pure nuclei. The final nuclear pellet was sedimented through 2.4 M sucrose also as described by Wang (1967) to eliminate whole cells, debris, and mitochondria. Mitochondria were prepared by digitonin washing as described above and digested with

[1] Abbreviations used are: hnRNA, heterogeneous RNA; nRNA, nuclear RNA; mtRNA, mitochondrial RNA; SSC, standard saline citrate.

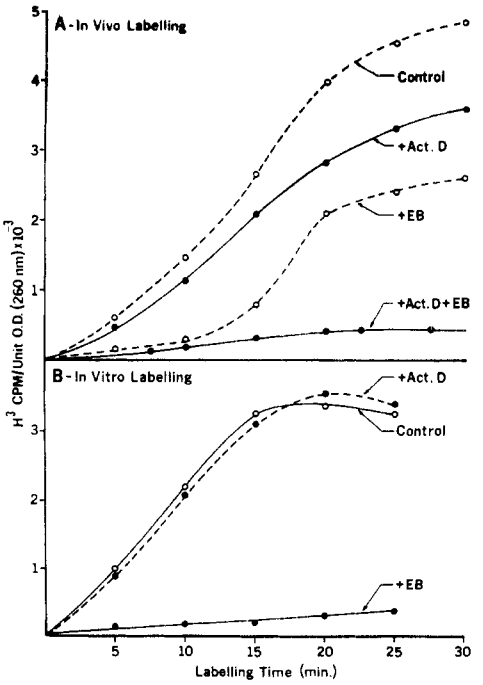

FIGURE 1: *In vivo* and *in vitro* labeling of mtRNA. (A) Cells were labeled with 5 μCi/ml of [^{3}H]cytidine with or without added drugs. After intervals of time, mitochondria were isolated, purified with digitonin treatment, and used for RNA isolation all as described in the Materials and Methods. (B) Mitochondria were isolated from fresh unincubated cells and labeled with 10 μCi/ml of [^{3}H]CTP as described in the Materials and Methods, and used for RNA preparation. Actinomycin D and ethidium bromide when added were at the final concentrations of 10 and 1 μg/ml, respectively, in both *in vivo* and *in vitro* experiments. In the former case, the drugs were added at the beginning of preincubation, while in the latter case, they were added at "zero" time of labeling.

100 μg of DNase (Worthington)/15 mg of mitochondrial protein in a buffer containing 10 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, and 0.25 M sucrose for 30 min at room temperature. DNA was isolated from purified nuclei and mitochondria essentially as described by Marmur (1961).

DNA–RNA Hybridization. The hybridization experiment was carried out essentially as described by Gillespie and Spiegelman (1965). Heat-denatured nDNA in 6 × SSC was filtered through presoaked membrane filters (Schleicher & Schuell, Type B-6), washed with 20–30 ml of 6 × SSC, and immobilized by incubating at 80° under vacuum for 18 hr. Using ^{3}H-labeled DNA, only 76–79% of the input sample was found to be retained on the filters. Hybridization was carried out in tightly closed scintillation vials at 72° for 18 hr. The final volume of reaction mixture was 2.0 ml.

After hybridization, the filters were washed twice with 10 ml each of 2 × SSC and then incubated in 5.0 ml of 2 × SSC containing 20 μg each of RNase A and T_1 (both from Worthington Biochemical Corp.) at room temperature for 45 min. Washing was carried out by incubating the filters in 25 ml of 2 × SSC for 15 min. After four such washings, the filters were dissolved in 10 ml of Cab-o-Sil scintillation fluid and counted in a Packard TriCarb spectrometer.

Because of the observed difficulties due to the nonspecific binding of mtRNA to membrane filters during DNA–RNA hybridization (Wood and Luck, 1969), appropriate controls were run. Every concentration of RNA to be tested for hybridization was also incubated with blank filters. The values with such blank filters were subtracted from respective experimental values. Hybridization values reported in this paper were the mean of at least three independent determinations.

Results

Labeling of mtRNA in Vivo. A typical pattern of [^{3}H]cytidine incorporation into mtRNA *in vivo* has been shown in Figure 1A. The incorporation level appears to saturate in a period of about 30 min. Addition of ethidium bromide, a specific inhibitor of mitochondrial transcription, inhibits the incorporation by 85–90% during the first 10 min. After this initial lag, there is a slow recovery in the rate of incorporation to about 52% of the control at 30 min (Figure 1A). Actinomycin D, an inhibitor of nRNA synthesis, also inhibits the mtRNA labeling by about 32%. The pattern of inhibition with actinomycin D, however, is different from the one seen with ethidium bromide. The actinomycin D inhibition and ethidium bromide resitance observed in the *in vivo* metabolism of mtRNA are not direct but only indirect effects because of the apparent involvement of nuclear synthesis (see Discussion). A combination of ethidium bromide and actinomycin D, however, results in a complete inhibition of mtRNA synthesis *in vivo* (Figure 1A).

For the sake of convenience, the RNAs labeled *in vivo* in the presence of ethidium bromide or actinomycin D will be referred to as ethidium bromide resistant and actinomycin D resistant RNA, respectively.

RNA Synthesis by Isolated Mitochondria. The efficiency of incorporation of labeled nucleosides and nucleotides by isolated mitochondria appears to be determined by the intactness of preparations (see Grant and Poulter, 1973). The isolation of mitochondria for the *in vitro* incorporation of RNA precursors was, therefore, carefully carried out without any digitonin treatment. These preparations are tightly coupled as tested by their ability to respire and phosphorylate (C. Ritter, N. G. Avadhani and R. J. Rutman; results not included in this paper). Even though in the present experiments ^{3}H-labeled CTP and ATP were used as precursors, this system can utilize labeled nucleosides as well with a high efficiency. Furthermore, the system does not require all the four nucleoside triphosphates and other conditions as reported for other systems (Fukamachi *et al.*, 1972; Aaij *et al.*, 1970). These latter results will be published elsewhere.

Incorporation of radioactivity by isolated mitochondria reaches a plateau in 15 min (Figure 1B) as in other systems (Saccone *et al.*, 1969; Gamble and McCluer, 1970). The labeling is greatly inhibited (85–90%) by ethidium bromide, but unaffected by actinomycin D (Figure 1B). The inability of the latter to inhibit RNA synthesis by isolated mitochondria may be due to impermeability (Dawid, 1970; Gamble and McCluer, 1970). In any case, the two drugs under study affect the *in vivo* and *in vitro* labeling of mitochondria differently. RNA synthesized by isolated mitochondria will be referred to hereafter as *in vitro* RNA.

Electrophoretic Analysis of the in Vivo and the in Vitro RNAs. In order to verify if the radioactive precursors are incorporated into high molecular weight components, both the *in vivo* and the *in vitro* mtRNA samples were electrophoresed on 2.4% polyacrylamide gels. It was essential to know the size distribution of these labeled RNAs especially because very few reports have shown the incorporation of label into high molecular components in an *in vitro* system (Fukamachi *et al.*, 1972; Grant and Poulter, 1973). It is seen from Figure 2 that both the *in vivo* and the *in vitro* RNAs contain components ranging from 3 to 25 S. Actinomycin D and ethidium

TABLE I: Poly(A) Content of mtRNA.[a]

Description of RNA	% of Total Counts: Binding to Filters (*a*)	% of Total Counts: Resistant to RNase (*b*)	*b*/*a* × 100
In vivo RNA	39.5	4.91	12.3
Ethidium bromide resistant RNA	57.9	6.78	11.8
Actinomycin D resistant RNA	2.5	0.25	10.0
In vitro RNA	Negligible	Negligible	

[a] *In vivo* RNAs used in this experiment were obtained essentially as described in Figure 1A, excepting that labeling was carried out for 25 min with 5 μCi/ml each of [^{3}H]cytidine and [^{3}H]adenosine. *In vitro* labeling was carried out for 15 min with 10 μCi/ml of [^{3}H]ATP as described in Figure 1B. Various RNA samples were checked for their ability to bind to Millipore filters as described earlier (Avadhani *et al.*, 1973). Ribonuclease-resistant counts were also determined by a procedure (Avadhani *et al.*, 1973) adopted from Lee *et al.* (1971) and Perry *et al.* (1972).

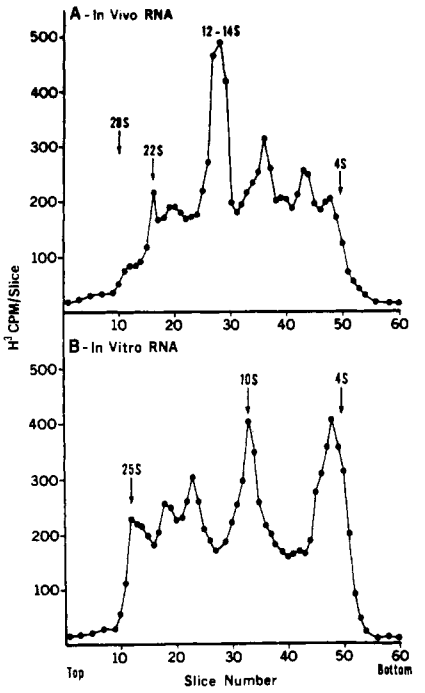

FIGURE 2: Polyacrylamide gel electrophoresis of RNA. Both *in vivo* (A) and *in vitro* (B) RNAs were prepared as described in Figure 1. RNA was dissolved in 5 mM Tris-HCl (pH 7.4), and aliquots of 0.03–0.04 ml representing 35,000–40,000 ^{3}H cpm were electrophoresed on 2.4% polyacrylamide gels of 6.0-cm long. Details of electrophoresis and analyses of the gels were as described in the Materials and Methods.

bromide resistant fractions also show a similar heterogeneity in the distribution of label (results not included here). Though there appears to be a significant difference between the *in vivo* and *in vitro* RNA patterns, it is not the purpose of our present studies to investigate these differences and their significance.

Poly(A) Content of mtRNA. Previously, we have demonstrated the presence of poly(A) sequences in mtRNA using two different techniques (Avadhani *et al.*, 1973). In the present work, the Millipore filter binding technique of Lee *et al.* (1971) was used to estimate the poly(A) containing RNA populations (Perry *et al.*, 1972) in various mtRNA fractions. About 39% of the *in vivo* RNA labeled with [^{3}H]adenosine is retained by the filter (Table I). Similarly labeled ethidium bromide resistant RNA binds to the filters to the extent of 58%. Actinomycin D resistant RNA, however, appears to contain a very low percentage of filter bindable counts. All three of the species of RNA contain RNase resistant fractions characteristic of poly(A) sequences (Lee *et al.*, 1971; Perry *et al.*, 1972). The RNase resistant material migrates as a 4–5-S peak on 6% polyacrylamide gels (Avadhani *et al.*, 1973) indicating an average size of 100–150 nucleotides for poly(A) sequences. The ratio of percentage filter binding/RNase resistance for all three fractions was nearly identical (Table I). This excludes the possibility of nonspecific binding of mtRNA to Millipore filters as noted for *Neurospora* mtRNA under different conditions (Wood and Luck, 1969).

Further, the *in vitro* mtRNA in the present system fails to bind to the filters (Table I). Consistent with the binding data, this RNA fraction also lacks the RNase resistant fraction. These results conclusively show that only the *in vivo*, but not the *in vitro*, RNA contains poly(A). Further, ethidium bromide, increases the poly(A) containing RNA population in mitochondria; whereas, actinomycin D which has hardly any effect on mitochondrial transcription in our system (Figure 1B) drastically reduces mitochondrial poly(A).

Extreme sensitivity of mitochondrial poly(A) to an inhibitor of nRNA, but not mtRNA, synthesis suggests two possibilities. (1) Poly(A) containing RNA species are synthesized outside mitochondria, presumably in the nucleus. (2) The poly(A) fragments isolated with mtRNA are contaminants from cytoplasmic polysomes. The latter possibility is unlikely since treatment of mitochondria with RNases and extensive purification with digitonin does not alter the poly(A) content of mtRNA (Avadhani *et al.*, 1973). Further, ribosomes and rRNA isolated from such preparations fail to show the presence of cytoplasmic specific components (*e.g.*, 18- or 28S RNA, results not included in this paper) indicating the purity of the mitochondria used for the *in vivo* RNA preparations. Absence of cytoplasmic specific rRNAs and mtRNAs in these mitochondrial preparations is further substantiated by the fact that cytoplasmic polysomal RNA does not compete with any of the mtRNA fractions in DNA–RNA hybridization experiments described later in this paper.

Decay Patterns of Various RNA Species. If the poly(A) containing RNA population is of extramitochondrial origin, it is reasonable to suspect that it has a different rate of turnover than the RNA synthesized inside mitochondria. It was, therefore, decided to study the decay patterns of various RNA species. In spite of the degradative effect of ethidium bromide on mtRNA (Zylber *et al.*, 1969), incubation of labeled mitochondria with this drug prevented any further incorporation of endogenous label and enabled one to look at the patterns of degradation of RNAs labeled under different conditions. A uniform addition of this drug to all the samples is, therefore, expected to give only a qualitative picture on the rates of degradation of various RNAs in these organelles. As seen from Figure 3, *in vitro* RNA and actinomycin D resistant RNA fractions decay with a $T_{1/2}$ of 12–14 min. The ethidium bromide resistant RNA, on the other hand, appears to be much longer lived since it has a $T_{1/2}$ of 35–40 min. It is clear from these data that RNA species which either lack or contain insignificant poly(A) are turned over more rapidly than poly(A) containing species. The decay rates also suggest an apparent similarity between

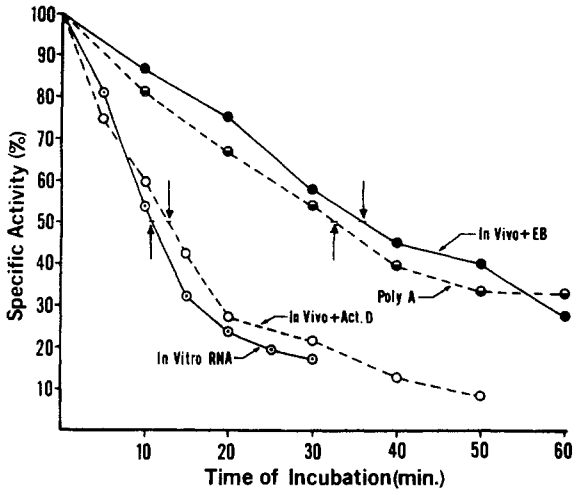

FIGURE 3: Decay patterns of various RNAs. Cells were labeled with 5 μCi/ml each of [^{3}H]cytidine and [^{3}H]adenosine with or without ethidium bromide or actinomycin D as described in Figure 1. After labeling for 25 min mitochondria were isolated and suspended in five volumes of incubation buffer (10 mM Tris-HCl, (pH 7.8)-5 mM $MgCl_2$-40 mM KCl-0.25 M sucrose). Ethidium bromide was added to a final concentration of 2.0 μg/ml to prevent incorporation of endogenous radioactivity. Incubation was carried out at 37°. After intervals of time, samples were withdrawn, RNA extracted, and specific activity determined. *In vivo* RNA labeled in the absence of any drug was used to determine the decay pattern of poly(A). The poly(A) content of the RNA was estimated by digestion with RNases and subsequent binding with Millipore filters as described in Table I. For determining the decay pattern of the *in vitro* RNA, isolated mitochondria were labeled as described in Figure 1B, suspended in the incubation medium containing ethidium bromide, and incubated as described above. The specific activities of various RNA samples (^{3}H cpm/unit OD 260 RNA or RNase-resistant filter bindable ^{3}H cpm/unit OD 260 RNA in the case of poly(A)) at "zero" incubation time were considered to be 100%.

ethidium bromide resistant fraction and poly(A) containing population of the *in vivo* RNA in mitochondria.

Kinetics of Ethidium Bromide Resistance and Appearance of Poly(A) RNA. The ethidium bromide resistant RNA and poly(A) containing RNA have similar physicochemical properties, since both contain poly(A) and decay at a similar rate. The question arises if the two species are the same. As shown in Figure 4, the patterns of ethidium bromide resistance and appearance of poly(A) containing RNA as a function of time have great resemblance. Ethidium bromide resistance in mitochondria decreases with a lag of 10–15 min which coincides with the appearance of poly(A) containing RNA. One possible explanation of these results is that poly(A) containing RNAs are synthesized outside and brought into mitochondria which then contributes to an apparent decrease in ethidium bromide resistance. Further, the lag probably represents the time taken for the transportation of poly(A) containing RNA.

DNA–RNA Hybridization. One of the ways to verify the nuclear origin of poly(A) containing ethidium bromide resistant actinomycin D sensitive RNA is to verify the complementarity between these RNA species and the nDNA. As shown in Figure 5, the *in vivo* mtRNA hybridizes with nDNA to the extent of 0.35%. The rate of hybridization is not significantly affected when 20-fold concentrations of cytoplasmic polysomal RNA are included. Similar concentrations of nuclear hnRNA, however, causes 85–90% reduction in hybridization. These results clearly demonstrate that mtRNA

TABLE II: Hybridization of Ethidium Bromide Resistant and Actinomycin D Sensitive RNAs with nDNA.[a]

Type of RNA	Competed With	% Hybridization
Actinomycin D resistant		0.046
Ethidium bromide resistant		0.32
	Cytoplasmic rRNA	0.34
	Nuclear hnRNA	0.002
	mtRNA	0.009

[a] Actinomycin D resistant and ethidium bromide resistant RNAs were prepared as described in Figure 5. The amounts of DNA and RNA used in this experiment were 10 and 250 μg, respectively. Competition was carried out with 20-fold excess of unlabeled RNA. Cytoplasmic rRNA and nuclear hnRNA were prepared as described in Figure 5. mtRNA was prepared from digitonin-washed mitochondria as described in the Materials and Methods. The counts for nonspecific adsorption of RNA on filters containing no DNA were 185 and 203 for ethidium bromide resistant and actinomycin D resistant RNAs, respectively.

contains species specific for nDNA and that the hybridization observed is not due to a contamination from cytoplasmic ribosomes and messengers as in a previously reported case (Reijnders *et al.*, 1972). The specificity of a portion of mtRNA for nDNA is further confirmed by competing the hybridization with nuclear hnRNA (Figure 5).

The nuclear origin of poly(A) containing mtRNA was also evident from experiments reported in Tables II and III. Actinomycin D resistant RNA which lacks poly(A) hybridizes insignificantly (0.046%) with nDNA, while the ethidium bromide resistant fraction which is rich in poly(A) hybridizes significantly (0.32%). Further, hybridization of the ethidium bromide resistant fraction with nDNA is not competed by cytoplasmic polysomal RNA, but only nuclear hnRNA and mtRNA (Table II). Consistent with these results, both the *in vitro* RNA and actinomycin D resistant RNA hybridize specifically with mtDNA (5.62–6.95%). Ethidium bromide resistant RNA, on the other hand, hybridizes with mtDNA only to the extent of 0.037% (Table III).

The observed hybridization between mtRNA and nDNA in these experiments is probably not because of the cross contamination of the nuclear fraction with organelle DNA, since nuclei were purified by a procedure that does not permit the cross-contamination (Wang, 1967). Nevertheless, it is not possible to determine the purity of nuclear and mitochondrial specific DNAs in Ehrlich ascites system using the conventional buoyant density gradient technique since both the DNAs have nearly similar density characteristics (F. S. Lewis, R. J. Rutman, and N. G. Avadhani, unpublished results; Borst *et al.* (1968)). The purity of DNA preparations used in these experiments, however, is evident from the specificity of hybridization, *i.e.*, actinomycin D resistant RNA with mtDNA and ethidium bromide resistant RNA with nDNA (Tables II and III).

Discussion

Both *in vivo* and *in vitro* incorporation of radioactivity into RNA is affected by ethidium bromide, though to a varied ex-

TABLE III: Hybridization of Various RNAs with mtDNA.[a]

Type of RNA	Competed With	% Hybridization
In Vitro		5.62
In Vitro	Cytoplasmic rRNA	5.41
In Vitro	Nuclear hnRNA	5.67
Actinomycin D resistant		6.95
Actinomycin D resistant	Nuclear hnRNA	6.73
Actinomycin D resistant	Cytoplasmic rRNA	6.71
Ethidium bromide resistant		0.037

[a] The *in vitro* RNA was prepared by labeling mitochondria with 25 μCi/ml each of ^{3}H-ATP and ^{3}H-CTP as described in the Materials and Methods. The resultant RNA preparation had a specific activity of 15–17,000 cpm/μg. All the other details were as described in Figure 5 and Table II except that the mtDNA was denatured by alkali treatment as described by Wood and Luck (1969). At the concentration of RNA used (250 μg/2 ml), the extent of nonspecific adsorption of the *in vitro* RNA and the actinomycin D resistant RNA were 225 and 210 cpm, respectively. The hybridization observed with various RNAs such as 5.62% for the *in vitro* RNA, 6.95% for the actinomycin D resistant RNA, and 0.037% for the ethidium bromide resistant RNA represent 9441, 11,206 and 97 cpm, respectively.

tent. In the *in vitro* system it is inhibitory to the extent of 90%, while in the *in vivo* system its effect is only to about 52%. In HeLa cells, Zylber *et al.* (1969) observed that even low concentrations (0.2%) of ethidium bromide completely inhibited mitochondrial rRNAs and tRNAs. The total *in vivo* incorporation, however, was inhibited only up to about 60–65% in their system. These ethidium bromide resistant RNA fractions, though noted in several systems, have not received any attention or clarification. We feel that the ethidium bromide resistant fraction observed in the present work is of extramitochondrial origin.

RNA synthesis by isolated mitochondria is completely insensitive to actinomycin D in our system. As correctly pointed out by Grant and Poulter (1973) and Gamble and McCluer (1970), there appears to be an increasing inconsistency in the effects of various inhibitors of RNA synthesis on isolated mitochondria in various systems. Nevertheless, either solubilized or purified mitochondrial enzymes from a wide variety of sources are sensitive to these inhibitors (Kuntzel and Schafer, 1971; Scragg, 1971). Thus, the insensitivity to actinomycin D observed in the present experiments may be a problem of permeability (Dawid, 1970; Gamble and McCluer, 1970). Indirectly, this might reflect on the integrity of our mitochondrial preparations, since either swollen or damaged mitochondrial preparations appear to be inhibited by actinomycin D (Fukamachi *et al.*, 1972; Gamble and McCluer, 1970). This argument is further supported by the ability of our mitochondrial preparations to synthesize high molecular weight RNA without an external supply of nucleoside triphosphates (except the labeled nucleotides). It is thus apparent that tightly coupled mitochondria used for the *in vitro* experiments contain pools of precursors sufficient for the synthesis of RNA (Heldt and Klingenberg, 1965). Similar "self-sufficient" *in vitro* systems have been reported before (Gamble and McCluer, 1970; Grant and Poulter, 1973).

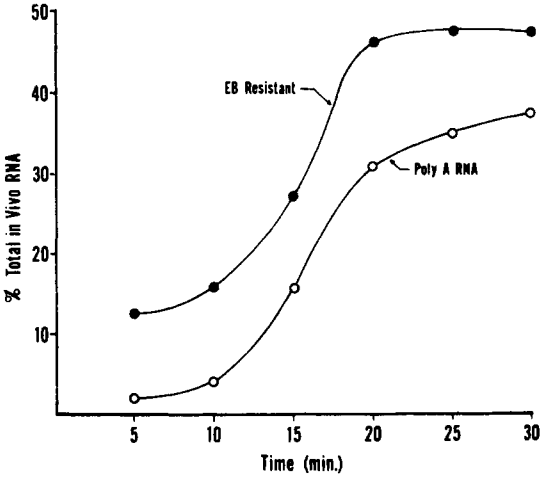

FIGURE 4: Kinetics of ethidium bromide resistance and poly(A) containing RNA population in mitochondria. mtRNA was labeled *in vivo* with 5 μCi/ml each of [^{3}H]cytidine and [^{3}H]adenosine for intervals of time between 5 and 30 min. Percentage poly(A) containing RNA population was determined on the basis of % ^{3}H cpm bound to Millipore filters. The percentage of ethidium bromide resistant RNA was calculated from data presented in Figure 1A.

The RNA synthesized by isolated mitochondria contain sequences ranging from 3 to about 25 S. An earlier paper showed the synthesis of such large components by liver mitochondria (Fukamachi *et al.*, 1972). The time of incubation in this latter case, however, was 60 min and the product contained ribosomal and preribosomal components. Recent studies on RNA synthesis by *Physarum polycephalum* mitochondria show the synthesis of the 13–15-S RNA component having a half-life of less than 5 min (Grant and Poulter, 1973).

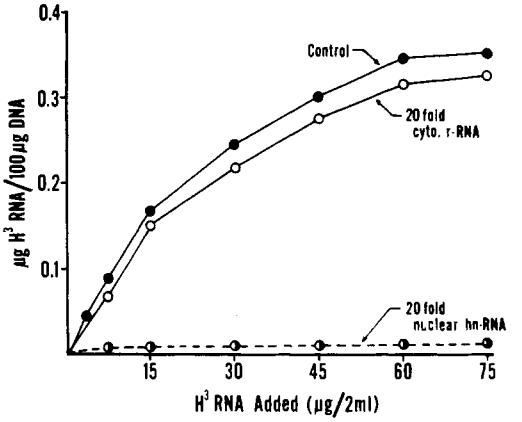

FIGURE 5: Hybridization of mtRNA with nuclear DNA. Heat-denatured DNA (10 μg) was immobilized on the filters. mtRNA was labeled *in vivo* for 25 min with 20 μCi/ml each of [^{3}H]cytidine, [^{3}H]adenosine, and [^{3}H]uridine. Resultant RNA had a specific activity of 20–25,000 cpm/μg. Nuclear hnRNA was prepared by hot phenol–sodium dodecyl sulfate extraction (reference sited in Adesnik *et al.*, 1972). The ribosomal–polysomal fraction was prepared by centrifuging the 20,000*g* supernatant fraction of the cell-homogenate over 1.0 M sucrose (10 ml of sucrose 19 ml of homogenate) for 14 hr at 75,800*g* in an SW-25.1 rotor of Beckman L-2 ultracentrifuge at 3–5°. The resultant pellet was extracted with phenol–chloroform–sodium dodecyl sulfate as described in the Materials and Methods. Nonspecific adsorption of labeled mtRNA in these experiments ranged from 32 cpm for the lowest concentration of RNA to 162 cpm for the highest concentration of RNA used. These values, however, were subtracted from the experimental values. The maximum % hybridization observed (0.356) represents 1120 cpm.

Such rapidly turned over RNA species were also observed in other *in vitro* systems (Gamble and McCluer, 1970); Wintersberger, 1966). Since the rRNAs have been reported to have a half-life of about 3 hr (Zylber *et al.*, 1971) and also since no radioactivity was seen in the ribosomal particles by 25 min in our system, the *in vitro* RNA in the present experiment does not seem to contain rRNAs. It is possible, however, that the *in vitro* RNA contains preribosomal as well as premessenger RNAs. Further investigations are in progress to characterize these components.

Poly(A) sequences were reported to be present in HeLa cell mitochondria (Perlman *et al.*, 1973) and Ehrlich ascites mitochondria (Avadhani *et al.*, 1973). Perlman *et al.* (1973) have ascribed a mitochondrial origin for the poly(A) containing RNA in these organelles. In the present studies, however, despite using a wide range of *in vitro* incubation conditions, it was not possible to detect any poly(A) in the *in vitro* RNA. In view of a recent finding that rat liver mitochondria contain enzymes for synthesizing poly(A) (Jacob and Schindler, 1972), it is possible that our *in vitro* system is not efficient enough to synthesize poly(A). In any case, the significance of poly(A) synthesizing enzyme in mitochondria is yet to be established. Further, Perlman *et al.* (1973) have proposed that the poly(A) containing RNA is exclusively mitochondrial in origin because it was not inhibited by Camptothecin, a plant alkaloid which is a specific inhibitor of nuclear RNA synthesis in many systems (see Kessel, 1971). However, this compound has been reported to be a poor inhibitor of RNA and DNA synthesizing systems in HeLa cells (Horwitz *et al.*, 1970); and also the nuclear hnRNA in HeLa cells is only partially inhibited by this compound (Abelson and Penman, 1972). In any event, because of the difference in the experimental conditions employed, our data cannot be directly compared with the results of Perlman *et al.* (1973).

In eukaryotic systems, poly(A) has been proposed to possess several functions, which include maturation and processing of nuclear hnRNA, packaging of mRNA for transportation, and some process in translation (Darnell *et al.*, 1971; Johnston and Bose, 1972; Adesnik *et al.*, 1972; Perry *et al.*, 1972). On the other hand, bacterial messengers lack poly(A). Since there are several similarities between bacterial and mtRNA and protein synthesizing systems (see Ashwell and Work, 1970; Borst and Grivell, 1971), it is quite possible that mRNA synthesized on mtDNA do not contain poly(A).

Since ethidium bromide treatment has been reported to enhance the degradation of mtRNA (Zylber *et al.*, 1969, 1971), the decay rates reported here might not directly reflect the *in vivo* situation. The inclusion of ethidium bromide, however, was essential to prevent any endogenous incorporation. The fact that ethidium bromide resistant RNA which contains poly(A) decays at a rate similar to poly(A) containing species from *in vivo* RNA also suggests a similarity between them. The *in vitro* labeled RNA, on the other hand, decays similarly to the actinomycin D resistant fraction lacking poly(A). These facts are compatible with an extra mitochondrial, presumably nuclear origin for these RNAs. Thus, the observed inhibition of the *in vivo* mtRNA by actinomycin D is not a direct effect since this drug was ineffective on the *in vitro* system, but an indirect effect by way of inhibiting the population of mtRNA which is synthesized in the nucleus. The elegant experiments of Adesnik *et al.* (1972) and La Torre (1972) have shown that the eukaryotic messengers are processed in less than 15 min in the nucleus and appear in the cytoplasm at about 15–20 min. Our kinetic data on the appearance of poly(A) containing RNA in mitochondria is in agreement with these reports and provides a logical explanation for the observed extent and rate of decrease of ethidium bromide resistance.

Further confirmation of the presence of RNA of nuclear origin in mitochondria comes from hybridization experiments. Earlier reports have shown the presence of a small per cent (0.2%) of RNA complimentary to nDNA in mitochondria (Wood and Luck, 1969; De Kloet *et al.*, 1971). Our results show a hybridization of 0.35% at saturating levels (Figure 5). Considering the size and complexity of eukaryotic genome, the extent of hybridization obtained in our experiments and also in other reports (Wood and Luck, 1969) is probably quite significant. The probability of contamination from cytoplasmic fractions is further reduced by being able to compete the hybridization specifically with nuclear hnRNA, but not cytoplasmic polysomal RNA. The hybridization results also prove that only ethidium bromide resistant RNA, but not actinomycin D resistant RNA has complementarity with nuclear genome.

As a whole, our results show the presence of nRNA, having the properties of messenger in mitochondria. This view supports the early experiments designed to show the possible transportation of nuclear mRNAs into mitochondria for translation (Dawid, 1972; Swanson, 1971; Kisselev and Gaitskhoki, 1972). On the other hand, our results do not argue against the synthesis of some messengers on mitochondrial templates as proposed by Grant and Poulter (1973). Nevertheless, it appears that such messengers might be lacking in poly(A) as are most bacterial messengers.

These data cannot be used to quantitate the exact amounts of n- and mtRNA in these organelles at any given time. Further details on the precursor-pool size, rates of polynucleotide synthesis, and rates of turnover in both mitochondria and nucleus are needed to arrive at a quantitative picture.

Acknowledgment

Excellent technical help of M. Kuan is greatly acknowledged. We are also thankful to F. S. Lewis for isolating n- and mtDNA.

References

Aaij, C., Saccone, C., Borst, P., and Gadaleta, M. N. (1970), *Biochim. Biophys. Acta 199*, 373.

Abelson, H. T., and Penman, S. (1972), *Nature (London), New Biol. 237*, 144.

Adesnik, M., Salditt, M., Thomas, W., and Darnell, J. E. (1972), *J. Mol. Biol. 71*, 21.

Ashwell, M. A., and Work, T. S. (1970), *Annu. Rev. Biochem. 39*, 251.

Avadhani, N. G., Kuan, M., Van Der Lijn, P., and Rutman, R. J. (1973), *Biochem. Biophys. Res. Commun. 51*, 1090.

Barath, Z., and Kuntzel, H. (1972a), *Proc. Nat. Acad. Sci. U. S. 69*, 1371.

Barath, Z., and Kuntzel, H. (1972b), *Nature (London), New Biol. 240*, 195.

Bishop, D. H. L., Claybrook, J. R., and Spiegelman, S. (1967), *J. Mol. Biol. 26*, 373.

Borst, P. (1972), *Annu. Rev. Biochem. 41*, 333.

Borst, P., and Grivell, L. A. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13*, 73.

Borst, P., Van Bruggen, E. F. J., and Ruttenberg, G. J. C. M. (1968), *in* Biochemical Aspects of the Biogenesis of Mito-

chondria, Slater, E. C., Tager, J. J., Papa, S., and Quagliariello, E., Ed., Bari, Italy, Adriatica Editrice, p 51.
Chun, E. H. L., Gonzales, L., Lewis, F. S., Jones, J., and Rutman, R. J. (1969), *Cancer Res. 29*, 1184.
Darnell, J. E., Philipson, L., Wall, R., and Adesnik, M. (1971), *Science 174*, 507.
Dawid, I. B. (1970), *Symp. Soc. Exp. Biol. 24* 227.
Dawid, I. B. (1972), *J. Mol. Biol. 63*, 201.
De Kloet, S., Andrean, B. A. G., and Mayo, V. S. (1971), *Arch. Biochem. Biophys. 143*, 175.
Fukamachi, S., Bartoov, B., and Freeman, K. B. (1972), *Biochem. J. 128*, 299.
Gamble, J. G., and McCluer, R. H. (1970), *J. Mol. Biol. 53*, 557.
Gillespie, D., and Spiegelman, S. (1965), *J. Mol. Biol. 12*, 829.
Grant, W. D., and Poulter, R. T. M. (1973), *J. Mol. Biol. 73*, 439.
Heldt, H. W., and Klingenberg, M. (1965), *Biochem. Z. 343*, 433.
Horwitz, S. B., Chang, C., and Grollman, A. P. (1970), *Pharmacologist 12*, 283.
Jacob, S. T., and Schindler, D. G. (1972), *Biochem. Biophys. Res. Commun. 48*, 126.
Johnston, R. E., and Bose, H. R. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1514.
Kessel, D. (1971), *Biochim. Biophys. Acta 246*, 225.
Kisselev, O. I., and Gaitskhoki, V. S. (1972), *Biokhimiya 37*, 1224.
Kuntzel, H. (1969), *Nature (London) 222*, 142.
Kuntzel, H. (1971), *Curr. Top. Microbiol. Immunol. 54*, 94.
Kuntzel, H., and Schafer, K. P. (1971), *Nature (London), New Biol. 231*, 265.
La Torre, J. (1972), *J. Cell Biol. 55*, 148a.
Lee, S. Y., Mendecki, J. R., and Brawerman, G. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1331.
Linnane, A. W., and Haslam, J. M. (1970), *Curr. Top. Cell. Regul. 2*, 101.
Lizardi, P. M., and Luck, D. J. (1971), *11th Annu. Meeting Amer. Soc. Cell Biol.*, p 170a.
Marmur, J. (1961), *J. Mol. Biol. 3*, 208.
Perry, R. P., La Torre, J., Kelley, D. E., and Greenberg, J. R. (1972), *Biochim. Biophys. Acta 262*, 220.
Perlman, A., Abelson, H. T., and Penman, S. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 350.
Reijnders, L., Kleison, C. M., Grivell, L. A., and Borst, P. (1972), *Biochim. Biophys. Acta 272*, 396.
Saccone, G., Gadaleta, M. N., and Gallerani, R. (1969), *Eur. J. Biochem. 10*, 61.
Scragg, A. H. (1971), *Biochem. Biophys. Res. Commun. 45*, 701.
Swanson, R. F. (1971), *Nature (London) 231*, 31.
Wang, T. Y. (1967), *Methods Enzymol. 12*, 417.
Wintersberger, E. (1966), *in* Regulation of Metabolic Processes in Mitochondria, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Amsterdam, Elsevier, p 439.
Wood, D. D., and Luck, D. J. L. (1969), *J. Mol. Biol. 41*, 211.
Woodward, D. O., Edwards, D. L., and Flavell, R. B. (1970), *Symp. Soc. Exp. Biol. 24*, 55.
Zylber, E. A., Perlman, S., and Penman, S. (1971), *Biochim. Biophys. Acta 240*, 588.
Zylber, E. A., Vesco, C., and Penman, S. (1969), *J. Mol. Biol. 44*, 195.

Protein Biosynthesis in the Spleen. V. Increase in Poly(uridylic acid) Binding Factor Following Primary Immunization†

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ABSTRACT: Ribosomes extracted from the spleens of immunized rats are more active in an *in vitro* protein synthesizing system than similar extracts from spleens of unimmunized controls. Part of this enhanced activity can be explained by increased amounts of the elongation factor EF-1 and by decreased amounts of ribosomal-bound ribonuclease in the immunized preparations. This communication describes the partial purification of a poly(uridylic acid) binding factor from a KCl extract of rat spleen microsomes that is present in greater amounts in immunized spleens than in controls. The major poly(U) binding protein of spleen does not appear to be identical with the major poly(U) binding protein of liver. Reconstitution of "stripped" ribosomes with microsomal KCl washes restores some of the poly(U) binding activity, but has no apparent effect on the polymerization ability of the ribosomes.

The initial step in the process of protein biosynthesis is the binding of messenger RNA to the small ribosomal subunit, yet little is known about the attachment process at the molecular level. In bacteria, the binding of natural messenger RNA containing the initiator codon AUG at 4 mM Mg^{2+} to the native 30S ribosomal subunit is catalyzed by initiation factor IF-3. The binding of *N*-formylmethionyl-tRNA is promoted by IF-2. The 50S subunit is joined to the tRNA–messenger–30S complex for the initiation of protein synthesis (Pestka and Nirenberg, 1966). The importance of initiation factors in this process is well documented, for ribosomes lacking initiation factors are incapable of binding natural mRNA (Brown and Doty, 1968; Brawerman *et al.*, 1969).

Despite the fact that the synthetic polyribonucleotide, poly(uridylic acid), contains no initiating codon, its reaction

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