Decay of Cytoplasmic Polyadenylated RNAs Labeled with [^{3}H]Orotate in Rat Liver and the Effect of the Re-utilization of Labeled Precursor on Such Decay†

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ABSTRACT: The loss of [^{3}H]orotic acid from cytoplasmic free polysomal and membrane-bound polysomal poly(A)-RNAs in liver was studied. Male Holtzmann rats were adapted to 8 + 40 h feeding cycles (8 h feeding at the start of the dark period followed by 40 h starvation with periodic controlled lighting) for 4 weeks prior to each experiment. RNA was labeled in vivo by the intraperitoneal administration of [^{3}H] orotate at the start of the feeding cycle. The decay in poly(A)-RNA specific activity was followed during the fasting portions of subsequent cycles when glycogen levels were low and free and membrane-bound polysomes could be effectively separated. Under these conditions, with no metabolic inhibitors administered, no difference was seen in the rate of radioactive decay of free polysomal and membrane-bound poly(A)-RNAs. Evidence for labeled precursor re-utilization is presented. The observed half-life of these poly(A)-RNAs, without correction for labeled precursor re-utilization, was 37 h. Administration of 25 μmol of unlabeled orotic acid in an attempt to prevent poly(A)-RNA-labeled precursor re-utilization did not significantly change the observed half-life. The observed 37-h half-life, when mathematically corrected for re-utilization of labeled precursor, was found to be 20 h. This represents the actual half-life of decay of the poly(A)-RNAs synthesized at the start of the experiment. Compared with this relatively stable class of poly(A)-RNAs, a second rapidly turning over class of poly(A)-RNAs with a half-life estimated to be less than 1 h was detected in both the free and membrane-bound polysomes. It can be concluded that both stable and unstable poly(A)-RNAs can coexist in the hepatic cell both free in the cytoplasm and attached to cellular membranes. Furthermore the free cytoplasmic and membrane-bound poly(A)-RNAs contained in polysomes are equally accessible to the factors that determine mRNA stability.

In eukaryotic cells a 3′-poly(A) segment averaging 50 to 200 bases in length is attached to the majority of the cytoplasmic mRNA molecules (Berger and Cooper, 1975; Puckett et al., 1975). The presence of poly(A) covalently attached to cytoplasmic mRNA has allowed the specific isolation of poly(A)-RNA[1] on oligothymidylatecellulose or polyuridylate-Sepharose columns (Lindberg et al., 1972; Aviv and Leder, 1972). The present studies were carried out in order to determine the half-lives of the poly(A)-RNAs present in liver in vivo under physiologically defined conditions.

There are two methods commonly employed to determine the half-lives of poly(A)-RNAs directly. The first technique is to follow the kinetics of synthesis of the mRNA as it approaches a steady state in the presence of a constant precursor specific activity (Greenberg, 1972; Wiegers et al., 1975; Kramer et al., 1973). This method has been most feasible in cell culture where a constant precursor specific activity can be maintained in the cell medium (Wiegers et al., 1975). In whole animals, however, it has not been possible to maintain a constant precursor specific activity during RNA synthesis (Podobed et al., 1974). The second method used to determine poly(A)-RNA half-lives is that of monitoring directly the decay of label in the RNA with time. Theoretically the decay curve is the inverse of the synthesis curve but, due to re-utilization of labeled precursors as poly(A)-RNAs decay, the half-lives so determined are overestimates of the half-lives obtained solely from a study of the kinetics of synthesis (Wiegers et al., 1975). A cold chase of label prior to monitoring the decay of mRNA has been an approach commonly used to prevent label re-utilization. Techniques are available for the efficient cold chase of labeled precursors in cell culture (Puckett et al., 1975; Murphy and Attardi, 1973) but unfortunately such techniques have not had uniform success in whole animal studies.

Many whole animal studies utilized to determine mRNA half-lives have relied on the administration of metabolic inhibitors to suppress RNA synthesis while subsequently monitoring the decay of previously labeled RNA, polysome profiles or protein synthesis (Wilson and Hoagland, 1967; Wilson et al., 1967; Revel and Hiatt, 1964; Murty and Sidransky, 1972). The reported effects of ethionine on polysome disaggregation (Endo et al., 1975) and of actinomycin D on protein synthesis initiation (Singer and Penman, 1972) indicate that studies utilizing metabolic inhibitors to determine mRNA half-lives are difficult to interpret. An approach designed to determine the half-life of mRNA in the whole animal in the absence of metabolic inhibitors is thus desirable. Such a system is described in this paper.

Materials and Methods

Animals. Male rats (Holtzman Laboratories, Madison, Wis.) weighing 150–200 g were maintained on an 8 + 16 h feeding cycle (8 h feeding at the start of the 12-h dark period

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[1] Abbreviations used: poly(A)-RNA, RNA which contains a sequence of poly(A) as defined by its ability to bind to poly(U)-Sepharose; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

followed by 16 h of starvation) for 1 week. Animals were then switched to an 8 + 40 h feeding cycle (8 h feeding at the start of the 12-h dark period followed by 40 h of starvation) for 4 weeks prior to each experiment. For both feeding cycles lights were on in the animal room from 2100 to 0900 and off from 0900 to 2100. The 8 + 16 h cycle was used to allow animals to adapt to their surroundings before being placed on the more strenuous 8 + 40 h cycle (Watanabe et al., 1968; Yager et al., 1974). The animals were fed a 60% protein diet during the feeding period. The composition of the diet has been described in detail elsewhere (Watanabe et al., 1968). Rats were given free access to water throughout the experiments.

Rat liver RNA was labeled with [5-^{3}H]orotic acid made to volume with 0.15 M NaCl, and given as an intraperitoneal injection. Specific activities and details are given in the individual experiments. When a cold orotic acid chase was used rats were each given 25 μmol (3.9 mg/rat) of unlabeled orotic acid in 1 mL of 0.15 M NaCl by intraperitoneal injection.

Isolation of Polysomes and Nuclei. Adult liver contains a large amount of endogenous RNase activity. In order to isolate undegraded poly(A)-RNAs, it was necessary to employ RNase inhibitors during the initial liver homogenizations as well as in all isolations and analyses. The RNase inhibitors used were 50% (w/w) high-speed particle-free liver supernatant, glutathione, sodium dextran sulfate, and bentonite. Fifty percent (w/w) particle-free liver supernatant was prepared by the procedure of Venkatesan and Steele (1972). Livers were homogenized with an equal weight of homogenization buffer (0.25 M sucrose–50 mM Hepes (pH 7.6)–75 mM KCl–5 mM $Mg(C_2H_3O_2)_2$) and the supernatant was centrifuged at 208 000g for 170 min to remove all polysomes, organelles, and membranes. This supernatant contains endogenous RNase inhibitor(s) (Venkatesan and Steele, 1972; Blobel and Potter, 1966a) which are kept in an active reduced state by glutathione (Gribnau et al., 1969; Sirakov and Kochakian, 1969). Bentonite was purified by the method of Garrett et al. (1973) and utilized as an RNase inhibitor together with sodium dextran sulfate (Jacoli et al., 1973).

All glass and metal ware were acid washed, rinsed with distilled H_2O, and heated before use to 180 °F for at least 4 h. Metal caps for centrifugation were soaked in 30% H_2O_2 for at least 0.5 h before use, then rinsed, and dried. Disposable plastic gloves were worn at all times.

Liver homogenization was performed in 2 to 4 volumes of the homogenization buffer as detailed above containing 100 μg/mL sodium dextran sulfate and 3 mM glutathione. Cell breakage was achieved with 10 strokes of a motor-driven Teflon pestle (0.006–0.009-in. clearance) rotating at 1750 revolutions/min. To separate free from membrane-bound polysomes, the technique of Venkatesan and Steele (1972) was followed as modified to protect against RNase activity (vide supra). Thirteen-milliliter aliquots of the initial homogenates were centrifuged in a SW41 Beckman rotor first at 2000 rpm for 2 min and then at 28 000 rpm for 12 min (162×10^4 g min). The supernatant decanted from this centrifugation contained the free polysomes.

The pellet from the above spin was suspended by means of a spatula in 12 mL of 50% (w/w) particle-free liver supernatant (made up in homogenization buffer) containing 3 mM glutathione, 100 μg/mL sodium dextran sulfate, and 1 mg/mL bentonite. To this was added $\frac{1}{6}$ volume of freshly prepared 10% (w/w) Triton X-100 (made up in homogenization buffer) and the mix subjected to 3 complete vertical strokes with a pestle rotating at 1750 revolutions/min. To remove nuclei, the suspension was centrifuged at 1470g for 5 min in a Sorvall RC-2B centrifuge kept at 4 °C. The supernatant above the nuclear pellet was decanted and to it was added $\frac{1}{6}$ volume of freshly prepared 13% (w/w) sodium deoxycholate (made up in H_2O) to solubilize any remaining membrane fragments. The polysomes released from membranes were contained in this supernatant.

Five-milliliter aliquots of both supernatants, containing either free or membrane-bound polysomes, were applied to discontinuous sucrose gradients consisting of 3 mL of 1.38 M sucrose layered over 3 mL of 2.0 M sucrose both made up in homogenization buffer containing particle-free liver supernatant (2 mL of 50% (w/w) liver supernatant per 50 mL of homogenization buffer). The polysomes were pelleted through the 2 M sucrose layer by centrifuging the gradients at 145 000g for 18 to 24 h at 4 °C in a 50 Ti Beckman rotor. Polysome pellets were stored at −70 °C until use.

Nuclei were isolated by the procedure of Blobel and Potter (1966b). Nuclei appeared as white pellets below the 2.3 M sucrose layer. Microscopic examination of the nuclei showed them to be intact and free of cytoplasmic contamination.

RNA Extraction. All polysome pellets were extracted as previously described (Perry et al., 1972) with modifications incorporated to protect against RNase action (vide supra). Polysome pellets were resuspended at room temperature in RNA extraction buffer (10 mM Tris, pH 7.4, 0.1 M NaCl, 1 mM EDTA, and 0.5% $NaDodSO_4$) containing 100 μg/mL sodium dextran sulfate and 1 mg/mL bentonite. To this was added an equal volume of 1:1 chloroform–phenol (double-distilled and H_2O-saturated). Agitation was performed by hand shaking at room temperature (25 °C) for 5 min. The phases were separated by centrifugation at 10 000 rpm for 10 min in a Sorvall RC-2B at 4 °C and the aqueous layer was removed. The organic phase was shaken again with the extraction buffer and the combined aqueous phases extracted twice more with chloroform–phenol. The final aqueous phase was made to 0.1 M NaCl and precipitated overnight by the addition of 2 volumes of 95% ethanol. The RNA pellet was collected and washed by centrifugation at least once with 2 M LiCl to remove DNA, glycogen, and small RNA fragments (Parish, 1972) and subsequently washed with 95% ethanol. The drained pellets were dissolved in cold distilled H_2O and stored at −20 °C prior to poly(U)-agarose column chromatography.

Poly(U)-Agarose Column Chromatography. Poly(U)-agarose columns (1 mL of gel suspension (Pharmacia) per column) were washed prior to use with distilled H_2O and then with formamide-containing buffer (90% formamide, 10 mM Tris, pH 7.4, 10 mM $(Na)_2EDTA$, and 0.2% $NaDodSO_4$) to remove loosely bound poly(U). The columns were equilibrated by washing with 50 mL of binding buffer (10 mM Tris, pH 7.4, 0.1 M NaCl, 10 mM $(Na)_2EDTA$, and 0.2% $NaDodSO_4$) (Adesnik et al., 1972). To polysomal RNA in H_2O, an equal volume of twice concentrated binding buffer was added and the solution made to 50 μg/mL with sodium dextran sulfate. The RNA was applied to the column as quickly as possible under gravity flow and the column washed first with 30 mL of binding buffer and then with 20 mL of wash buffer (10 mM Tris, pH 7.4, and 0.1 M NaCl) (Eiden and Nichols, 1973). The last step was employed to remove $NaDodSO_4$ and EDTA from the column so that all A_{260} eluted from the column would be in poly(A)-RNA. The poly(A)-RNA was eluted from the column in 2 mL of distilled H_2O and used directly to determine its specific activity. The yields of poly(A)-RNA per mg of ribosomal RNA at each time point varied considerably (4 to 14 μg of poly(A)-RNA per mg ribosomal RNA). In spite of the

variability in yield, the poly(A)-RNA specific activities at each time point were very similar (see figures).

After the poly(A)-RNA was eluted, the remaining radioactivity (approximately 20% of the total dpm bound to the column) could be removed with formamide-containing buffer but not with further H_2O washings. Washings were continued until no additional dpm could be removed from the columns which were then stored at 4 °C in the presence of 0.02% sodium azide.

Analysis of RNA. Polyacrylamide gel electrophoresis of RNA was performed by the method of Weiss and Pitot (1974). Acid-soluble determinations of nucleotide pools and the Fleck-Munro assay were carried out as described by Blobel and Potter (1968). Charcoal adsorption and elution of nucleotides was performed according to the procedure of Fuhrman and Gill (1975). RNA determinations with orcinol were based on the original procedure (Ceriotti, 1955).

For the quantitative determination of radioactivity, Scintisol (Isolab) was added directly to aqueous samples. Gels after electrophoresis were sliced into 2-mm sections and incubated overnight at 75 °C in 0.5 mL of 30% H_2O_2. To each sample a 10 mL volume of Scintisol was added. All radioactivity was measured in Nuclear Chicago liquid scintillation counters.

Materials. [5-^{3}H]Orotic acid was obtained from the New England Nuclear Corp. Unlabeled orotic acid was purchased from Nutritional Biochemicals Corp. Poly(U)-agarose (type 6) was obtained from P-L Biochemicals. Activated charcoal, $NaDodSO_4$, and deoxycholate were obtained from Sigma, bentonite was from Fisher Scientific Co., and sodium dextran sulfate 500 (mol wt 5×10^5) was from Pharmacia. Formamide was purchased from Matheson Coleman and Bell and Triton X-100 from Calbiochemicals. All other chemicals were of analytical grade.

Results

Turnover of Poly(A)-RNAs Isolated From Free and Membrane-Bound Polysomes. The decay of labeled cytoplasmic poly(A)-RNAs was followed beginning 24 h after rats had been injected with [5-^{3}H]orotic acid. As seen in Figure 1, relatively stable classes of poly(A)-RNAs are labeled by this protocol. The labeled free polysomal and membrane-bound poly(A)-RNAs decayed with the same observed half-life of 37 h. Comparing the two decay curves it is further seen that the specific activities observed at corresponding time points are virtually identical, within the precision of the measurements. Poly(A)-RNA half-lives determined through a study of the kinetics of decay of the labeled RNA are known to be overestimates of the actual poly(A)-RNA half-lives due to the effect of label re-utilization. This has been observed in tissue culture and in rat liver after [5-^{3}H]orotate administration (Wiegers et al., 1975; Blobel and Potter, 1968) (see Results on nuclear RNA decay).

An attempt to study the decay of labeled free polysomal and membrane-bound poly(A)-RNA in the absence of labeled precursor re-utilization employed a cold chase technique basically as described by Wilson and Hoagland (1967). RNA was labeled at the start of the feeding period. An in vivo chase of intracellular labeled pyrimidine nucleotides was attempted by administering unlabeled orotic acid intraperitoneally (25 μmol/rat) at the end of the 8-h feeding period (data not shown). No difference in the uptake or turnover of label in poly(A)-RNAs could be detected under chase conditions relative to those of the nonchase experiment. The cold orotic acid chase experiment was carried out for a total of 6 days subsequent to exposure to [^{3}H]orotate. No tendency was seen for

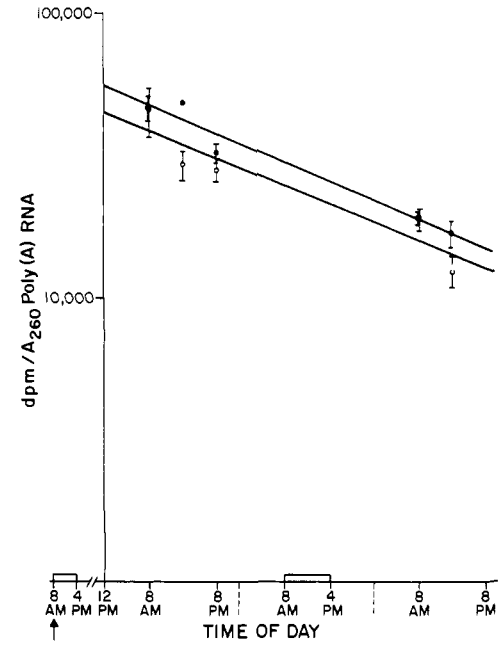

FIGURE 1: Turnover of poly(A)-RNAs during 8 + 40 h controlled feeding cycles. Rats were labeled with 200 μCi of [5-^{3}H]orotic acid, specific activity 17.8 Ci/mmol, at 8:00 a.m. of the feeding day (shown by arrow on abscissa). The broken lines connected to the abscissa represent the divisions between consecutive days. Rat weights at the start of the experiment were 272 ± 6.0 g. Each time point represents the average value from three rat livers processed separately ± the standard error of the mean; the straight lines for all figures were drawn based on linear regression analysis. The 8-h feeding period is represented by the open bar on the abscissa. (○—○) Free polysomal poly(A)-RNA decay; (●—●) membrane-bound poly(A)-RNA decay.

the poly(A)-RNA decay curves to plateau at these longer times after labeling as might be expected if more than one population of stable RNA were present as a large percentage of the total RNA.

An additional attempt as was made to correct poly(A)-RNA half-lives for the overestimation resulting from labeled precursor re-utilization. The poly(A)-RNA specific activity at each time point was divided by the 28S ribosomal RNA specific activity at that same time point. A plot (not shown) of the resulting ratios over time was employed to estimate a "corrected" poly(A)-RNA half-life. The measurement of the decay in specific activity of the stable rRNA species represented an attempt to control internally for poly(A)-RNA labeled precursor re-utilization. This protocol has appeared previously in the literature (Wiegers et al., 1975; Schultz, 1973). The attempted correction for the effect of poly(A)-RNA labeled precursor re-utilization was unsuccessful in yielding "corrected" poly(A)-RNA half-lives that were as long as or somewhat longer than the half-life observed in plotting the decline in specific activity of poly(A)-RNA alone over time (data not shown). The method employed in this report to correct poly(A)-RNA half-lives for the effect of labeled precursor re-utilization was to derive an equation that corrected the poly(A)-RNA specific activity at each time point on the basis of the rate at which radioactivity decayed from the acid soluble pool (see Appendix).

Polyacrylamide gel electrophoresis of the free polysomal and membrane-bound poly(A)-RNAs demonstrated that these molecules are not preferentially degraded during isolation and analysis (Figure 2). Only if the free polysomal and membrane-bound poly(A)-RNAs are approximately of the same size will a comparison of specific activities be valid. Some

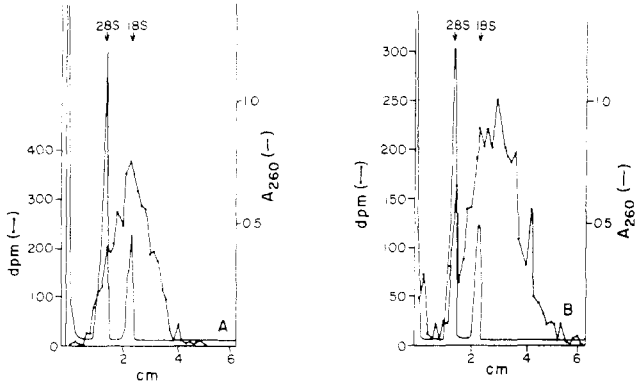

FIGURE 2: Polyacrylamide gel electrophoresis of poly(A)-RNAs. The poly(A)-RNAs analyzed represent samples obtained from the experiment presented in Figure 1. Electrophoresis was performed as described previously (Weiss and Pitot, 1974). The positions of the unlabeled 28S and 18S rRNA markers are shown. (A) Free polysomal poly(A)-RNA; (B) membrane-bound poly(A)-RNA.

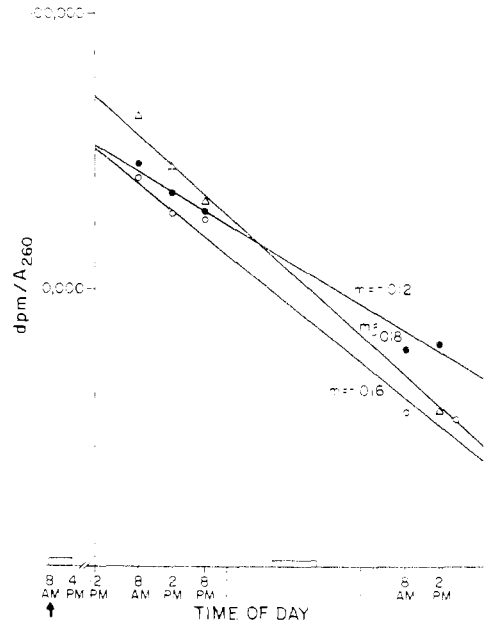

FIGURE 3: Decay of the acid-soluble RNA precursor pool from the nonchase experiment during 8 + 40 h cycles as determined with different techniques. The radioactive decay of the acid-soluble pool based on perchloric acid solubility is redrawn from Figure 4; the same homogenates used for perchloric acid extraction were used to follow the radioactive decay of the precursor pool based on charcoal adsorption and elution (Fuhrman and Gill, 1975) and based on Dowex chromatography (Hurlbert et al., 1954). Each time point based on acid solubility is the average value obtained from three separate determinations. Each time point based on charcoal adsorption and elution is the average value obtained after combining equal volumes of three homogenates prior to analysis. This is also the case for the time points based on Dowex chromatography. Specific activity based on: (●—●) acid solubility; (○—○) charcoal adsorption and elution; and (△—△) isolation of UMP on Dowex columns.

variability in size was occasionally seen in these studies with free polysomal poly(A)-RNAs being somewhat larger than membrane-bound poly(A)-RNAs. However, the size differences are not sufficiently large so as to affect any comparison of specific activities. The small differences seen in comparing poly(A)-RNA specific activity values at the same time point may be attributed to RNase activity. During the initial separation of free from membrane-bound polysomes, the nuclei, lysosomes, and mitochondria pellet with the membrane fraction. These organelles are known to contain a large proportion of the cellular RNase activity (Sirakov and Kochakian, 1969).

Radioactive Decay of Acid-Soluble Pools under Chase and Nonchase Conditions. The half-lives for the radioactive decay of the acid-soluble pools are quite similar in the presence or the absence of a chase with unlabeled orotate (data not shown). The specific activities of the pools at corresponding time points are essentially identical. In the cold chase experiment where time points were taken over a 6-day period, the acid-soluble pool decay plateaus and little decay of radioactivity from the pool is seen after 8 a.m. of the third day.

In this and related studies, it is important that the metabolic precursor pool for RNA synthesis be measured (Hauschka, 1973). Measurement of the acid-soluble pool specific activity is complicated by the fact that radioactive derivatives other than nucleotides are acid soluble. In addition the presence of unlabeled acid-soluble components, such as aromatic amino acids, might contribute to the A_{260} used in specific activity calculations. In such cases the measured acid-soluble pool specific activities would not represent the true precursor pool for RNA synthesis. In order to determine the significance of such possibilities, the specific activity of the cytoplasmic acid-soluble pool for the nonchase experiment was determined by three different methods. Nucleotides were analyzed by acid solubility, charcoal adsorption and elution and Dowex-1 column chromatography (Figure 3). Under the conditions of analysis shown in Figure 3, uridine triphosphates are broken down to the diphosphate and monophosphate derivatives (Hauschka, 1973). Previous work has demonstrated that, 24 h subsequent to injection of [5-^{3}H]orotate into the rat, there is an equilibration of the specific activities of the uridine mono-, di-, and triphosphates in the liver (Hurlbert and Potter, 1954). These investigators proposed that the measured acid-soluble pool in liver served both as a direct precursor pool for nuclear RNA synthesis and as a major, though possibly indirect, precursor pool for cytoplasmic RNA synthesis. We have assumed that the specific activity of the measured UMP and UDP pool is a close approximation to that of the immediate precursor pool employed for poly(A)-RNA synthesis. The kinetics of decay in specific activity of the nucleotide pool was virtually the same with the three methods of analysis we have employed. Using Dowex chromatography it was further observed that the eluted material gave an $A_{275/260}$ reading of 0.66 which is characteristic of uridylate (Hurlbert et al., 1954).

Radioactive Decay of Homogenate and Nuclear RNAs. The kinetics of radioactive decay for the homogenate and nuclear RNAs in the nonchase experiment are shown in Figure 4. The homogenate RNA specific activity falls only slightly over the time course of the experiment. The homogenate RNA was shown to consist predominantly of rRNA which decayed with the identical kinetics shown for the homogenate RNA (data not shown). The nuclear RNA specific activity decays with almost the same half-life as that seen for the decay of the acid-soluble pool. Previous work has demonstrated that in the mammalian cell nuclear RNA decays with a uniform half-life of 3 to 23 min (Brandhorst and McConkey, 1974; Soeiro et al., 1968). The data presented in this paper imply that a relatively stable nuclear RNA is present with a half-life of several hours. It should, however, be kept in mind that, when an RNA decays much faster than its precursor pool, it will be expected to follow the decay kinetics of the precursor pool. In the case of nuclear RNA, the precursor pool is assumed to be the cytoplasmic acid-soluble pool (see Appendix and Hurlbert and Potter, 1954). It is possible that a very unstable nuclear RNA is present which, due to the constant re-utilization of label from

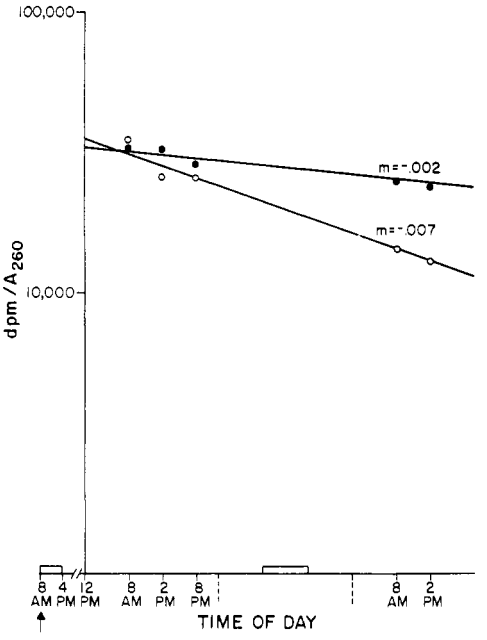

FIGURE 4: Decay of homogenate RNA and nuclear RNA during 8 + 40 h cycles. Homogenates from the rat livers used in Figure 1 were employed in this experiment. Nuclei were isolated as described in Materials and Methods. RNA was determined by alkaline hydrolysis of acid-insoluble RNA (Blobel and Potter, 1968). Each time point represents the average value from three rat liver homogenates, each processed separately. (●—●) Homogenate RNA decay; (○—○) nuclear RNA decay.

the acid-soluble pool, appears to be stable. We feel that the detection of a stable nuclear RNA supports the hypothesis that label re-utilization is occurring during the synthesis of rat liver RNAs. The same conclusion has been reached in a separate experiment utilizing rat liver RNAs labeled with [5-^{3}H]orotate (Blobel and Potter, 1968). In the latter work animals fed ad libitum were employed and the nuclear RNA and acid pools were found to decay in parallel exhibiting half-lives of 108 and 104 h, respectively.

Pulse Synthesis of Poly(A)-RNAs. Extrapolating from the work of Kafatos and Gelinas (1974), the half-life value determined in such a study most likely represents an average turnover for many messenger RNA species decaying with similar but not identical kinetics. It is of course possible that the observed half-life value instead represents an average decay for classes of poly(A)-RNAs that individually turn over at very different rates. A poly(A)-RNA that turned over much faster or slower than the class of poly(A)-RNAs with an observed 37-h half-life and which was a small percentage of the total poly(A)-RNA present would not be detected in the experiment described in Figure 1.

In a further study of free polysomal and membrane-bound cytoplasmic poly(A)-RNAs, the incorporation of [5-^{3}H]orotic acid at early times after administration of label was examined. Murty and Sidransky (1972) found evidence for a difference in free polysomal and membrane-bound mRNA turnover in rat liver using either actinomycin D to suppress further RNA synthesis or RNase to degrade selectively the mRNA in polysomes. An experiment was performed utilizing their basic protocol. Rats adapted to the cyclic regimen (see Materials and Methods) were used in this study. Since glycogen levels must be low in order to separate free from membrane-bound polysomes, the animals were labeled in the fasting portion of the 8 + 40 h cycle. Animals were sacrificed and killed at short time intervals (30 to 120 min) thereafter (data not shown).

The free polysomal poly(A)-RNAs at early times after giving label reach a higher specific activity than the membrane-bound poly(A)-RNAs. The fact that no difference in specific activity between free and membrane-bound poly(A)-RNAs was seen at the earliest time point examined is attributed to small amounts of nuclear RNA and acid-soluble contamination eluting with the isolated poly(A)-RNAs at this time point. The nuclei originally pellet with the membrane fraction and any nuclear RNA contamination is largely in this fraction. At 30 min after administration of the label, most of the radioactivity is in nuclei and the acid-soluble pool. A very small amount of nuclear RNA and acid-soluble components contaminate the cytoplasm and bind to poly(U) columns at any time point. The amount that does bind at a time when little label has entered cytoplasmic poly(A)-RNAs is sufficient to cause the free polysomal and membrane-bound poly(A)-RNA specific activities to appear identical (data not shown). After this 30-min time point, the free polysomal poly(A)-RNA specific activity is clearly greater than the membrane-bound poly(A)-RNA specific activity. The acid-soluble pool shows a substantial fluctuation of specific activity during this time course making an exact determination of poly(A)-RNA half-life unfeasible (data not shown).

A comparison of this study of poly(A)-RNA synthesis with the previous poly(A)-RNA decay experiments indicates that both unstable and stable poly(A)-RNA populations are present in rat liver. Over the course of the reported experiments, the mg of RNA/g of liver determinations were constant so that steady-state kinetics can be assumed (data not shown). Using a corrected half-life of 20 h from the decay curve for poly(A)-RNAs, the decay constant is 3.46% h (see Appendix). Since the decay curve is theoretically the inverse of the synthesis curve (Wiegers et al., 1975), 3.46%/h can also be assumed to be the steady-state constant of synthesis. During the 120-min labeling period of the pulse experiment, 6.80% of the poly(A)-RNA would be calculated to be synthesized if only stable poly(A)-RNA with a 20-h half-life were present. If less than 10% of the poly(A)-RNA were labeled, the specific activities seen in the pulse experiment would be substantially lower than the specific activities seen in the decay experiments. This was not found to be the case. The free polysomal poly(A)-RNA specific activity after 30-min exposure to the labeled precursor was of the same magnitude as the poly(A)-RNA specific activities seen in the decay experiments. This result indicates that a very unstable poly(A)-RNA fraction is present in the liver and that this fraction makes a large contribution to the uptake of label at early times after administration of the labeled precursor. When the poly(A)-RNA labeling data from the synthesis studies is plotted as the inverse of the synthesis curve on semilogarithmic graph paper, a poly(A)-RNA half-life of less than 1 h can be shown for both the free polysomal and membrane-bound poly(A)-RNAs (data not shown). This latter calculation assumes steady-state kinetics in the cells and a first-order decay for messenger RNA (Schultz, 1973).

Discussion

In comparing the half-lives for the decay of free polysomal and membrane-bound poly(A)-RNAs, no difference in turnover was found under the conditions of the experiments described in this study. The observed half-life of 37 h could be corrected on the basis of the decay in specific activity of the uridine phosphate(s) pool to a half-life of 20 h in each case (see Appendix). It is thus concluded that the majority of the steady-state poly(A)-RNAs are relatively stable and decay

with the same half-life whether present in free cytoplasmic polysomes or in polysomes bound to membranes.

An attempted cold orotic acid chase of labeled nucleotides had no effect on the half-lives observed or on the specific activities observed in either the poly(A)-RNAs or the acid-soluble pools. The reason for this is not understood at present since a similarly designed chase experiment in regenerating rat livers gave different results. The specific activities of poly(A)-RNAs after an in vivo chase in 24 h regenerating rat liver were significantly lower than those seen under nonchase conditions (manuscript in preparation). This indicates that large amounts of unlabeled orotic acid can be taken up by the liver resulting in a dilution of the specific activity of the acid-soluble pool. The conclusion to be drawn from our studies is that a cold chase of label in adult rat liver is not able to prevent poly(A)-RNA labeled precursor re-utilization. The observed poly(A)-RNA half-lives determined directly from decay curves are thus overestimates of the actual poly(A)-RNA half-lives. The detection of a stable nuclear RNA that decays in parallel with the measured acid-soluble pool supports this contention (Blobel and Potter, 1968).

The equation presented in the Appendix to this paper corrects poly(A)-RNA specific activities at each time point for labeled precursor re-utilization. The correction is based on the rate of decay of label from the acid-soluble pool. In this regard the behavior of the acid-soluble pool in the cold chase experiment has important implications. In the cold chase experiment, time points were analyzed out to later times than in the nonchase experiment. After 8 a.m. of the third day studied in the chase experiment the amount of radioactivity remaining in the acid-soluble pool plateaued and did not decay at the times examined thereafter (data not shown). The reasons for the stabilization of the amount of radioactivity present in the acid-soluble pool at the late times analyzed under chase conditions are not known. Possible explanations include (1) an equilibrium stage existing wherein the amount of radioactivity entering the acid-soluble pool equals the amount of radioactivity decaying from the acid-soluble pool, (2) a sequestration of the label remaining in the acid-soluble pool into a nondecaying pool separate from the pool for RNA synthesis, or (3) the complex behavior of the acid-soluble pool being related in some manner to the 8 + 40 h controlled feeding cycles to which animals are adapted. Compartmentalization of nucleotide pools has been demonstrated in Novikoff hepatoma cells growing in suspension culture using rapid kinetic labeling studies of the order of minutes (Plagemann, 1971a,b). However, as later studies by Plagemann in this system have shown (Plagemann, 1972), apparent equilibration of these pools probably occurs within a few hours. In the studies reported in this paper which cover several days and in which the decay is not measured until 24 h after the initial administration of the label, we feel that it is unlikely that differential labeling of precursor pools due to pool compartmentalization plays a significant role in differences or similarities seen in poly(A)-RNA turnover as reported in this study. A more detailed discussion of the assumptions involved in correcting poly(A)-RNA decay for label re-utilization may be seen in the Appendix.

In these studies both rapidly turning over the stable poly(A)-RNA populations were detected in both the free and membrane-bound polysomes. Fast and slow decaying mRNAs have also been found in the same cell type in other systems. In HeLa cells Puckett et al. (1975) demonstrated that 35 to 50% of the pulse-labeled mRNA decayed with a half-life of 1 to 2 h. In addition two classes of stable mRNA with half-lives of 7 and 24 h were observed. In investigations of resting human lymphocytes, Berger and Cooper (1975) studied poly(A)-RNAs and detected one population with a half-life of less than 17 min and another population that was stable and did not decay during 24 h of observation. The decay of poly(A)-RNA and ribosomal RNA of mouse kidney after the administration of [5-^{3}H]orotate was reported by Ouellette and Malt (1976). In this investigation beginning 12 h after the administration of the label, a short-lived component of poly(A)-RNA with an apparent half-life of 6 h was shown to exist as well as a second class consisting of slightly less than half of the labeled poly(A)-RNA having a half-life of 24 h. In these experiments while the authors indicated that re-utilization of labeled precursor may exist, no attempt was made to correct for such re-utilization.

The half-life of rat liver mRNA has been estimated to be between 5 and 80 h (Wilson and Hoagland, 1967; Tominaga et al., 1971). In comparing the half-life values found in this study with those found in other studies, several reasons for the differences in results are apparent. Since poly(A)-RNAs were examined in this study, mRNAs lacking poly(A) in rat liver may turn over with a half-life different than that observed for the poly(A)(+)-mRNAs. There has been one other study reported that investigated the decay of poly(A)-RNA in rat liver (Tweedie and Pitot, 1974). In this report a half-life of 11.6 h was observed for the total cytoplasmic polysomal poly(A)-RNAs. The corrected 20-h half-life for poly(A)-RNAs reported in this study is quite similar to the previous estimate. The present investigation differs from the previous report in that cycled animals were employed, [5-^{3}H]orotate was utilized to label RNA, and both stable and fast-decaying poly(A)-RNAs free and attached to membranes were examined. Tweedie and Pitot (1974) plotted the specific activity of pH 9.0 RNA divided by the specific activity of pH 7.6 RNA as a function of time. The resultant plot was employed to obtain the half-life of the poly(A)-RNAs. In our study the decay in specific activity of poly(A)-RNA was used directly to obtain the half-life value which was then corrected for the effect of labeled precursor re-utilization.

Previous studies have utilized rats adapted to 12 + 36 h controlled feeding cycles (Gebert, 1967). The total cell RNA (mg/g liver) during one 12 + 36 h cycle varied by as much as 30% in the absolute amounts of RNA per liver within the cycle. RNA accumulated during feeding and the first 12 h of fasting and then fell to prefeeding values during the latter part of the fasting phase of the cycle. In comparison rats fed ad libitum did approximate a steady-state condition in which the total amount of RNA per liver remained relatively constant (Gebert, 1967). In the studies reported herein, determinations were always performed at the same time intervals in the fasting portions of the 8 + 40 h cycles in order to ensure that a reproducible steady-state relationship existed. Meaningful comparisons between animals and experiments could be accomplished in this manner.

A further unique aspect of this study of messenger RNA turnover in vivo was the fact that no metabolic inhibitors were utilized in the estimation of poly(A)-RNA turnover. Previous similar studies (Endo et al., 1975; Singer and Penman, 1972) have demonstrated that several metabolic inhibitors affecting RNA synthesis, administered in vivo or in vitro, can alter the stability and turnover of messenger RNA. These systems when compared with investigations in which the inhibitors were not used resulted in abnormally delayed or shortened half-lives of cytoplasmic mRNA.

From the findings reported in this study, one can conclude

that, when messenger RNA turnover is studied in liver in vivo without the use of metabolic inhibitors and with appropriate correction for re-utilization of the labeled precursor employed, the poly(A)-RNA of membrane-bound and free polysomes decays at essentially equivalent rates.

Appendix

In this paper the decay of the labeled poly(A)-RNAs was corrected for re-utilization of labeled nucleotides. The basic assumptions made in this correction were (1) the measured acid-soluble pool is the precursor pool for RNA synthesis, (2) the specific activity of the pool decays exponentially, (3) steady-state kinetics exist during the fasting portions of the cycles over which time points were taken, and (4) poly(A)-RNA decay is first order. In the reported work the experimental data are consistent with either a zero- or a first-order decay of poly(A)-RNA. Previous workers have shown that mRNA decay fits a first-order kinetics while the histone mRNA appears to decay with zero-order kinetics (Perry and Kelley, 1973). When estrogen is withdrawn from the estrogen-stimulated chick oviduct, the ovalbumin mRNA decays with a complicated kinetics that is neither zero order nor first order (Palmiter and Carey, 1974).

The derivation of the equation to correct for poly(A)-RNA labeled precursor re-utilization is based on the dpm contributed by the precursor both into and out of the poly(A)-RNA pool. To follow the flow of label, both the poly(A)-RNA and the acid-soluble pool were studied over the same time courses and are mathematically related as outlined below.

Let S = the incorporation of precursors (mg/h), k_m = the decay constant for poly(A)-RNA (%/h), and B = the steady-state level of incorporated precursor (mg).

At steady state

$$S = k_m(B)$$

Let A = the specific activity of the precursor pool (dpm/mg):

$$A \times S = \text{dpm incorporated/h}$$

With respect to the poly(A)-RNA pool, let M = specific activity of the precursor incorporated into poly(A)-RNA (dpm/mg):

$$M \times B = \text{total dpm in poly(A)-RNA}$$

$$k_m \times M \times B = \text{dpm lost/h from the poly(A)-RNA pool}$$

Thus

$$d(MB)/dt = S(A) - k_m(M)(B)$$

and dividing through by B

$$dM/dt = (S(A)/B) - k_m(M)$$

Since at steady state $S = k_mB$ then $dM/dt = k_m(A - M)$.

The equation is now in the form where a change in the specific activity of the labeled precursor in poly(A)-RNA as a function of time is related to both the input and output of label. Let A_0 = the specific activity of the precursor pool at $t = 0$ (dpm/mg) and k_i = the decay constant of the measured acid-soluble pool (%/h). When the pool decays exponentially, $A = A_0e^{-k_it}$.

Since the specific activity of the labeled precursor in poly(A)-RNA is not measured directly, we substitute m, the specific activity of the poly(A)-RNA.

The previous equation can now be rearranged to give

$$dm/dt + k_m(m) = k_mA_0e^{-k_it}$$

One solution of this equation follows:

$$m(t) = \frac{k_mA_0}{(k_m - k_i)}(e^{-k_it} - e^{-k_mt} + m_0e^{-k_mt})$$

$m(t)$ is the poly(A)-RNA specific activity, dpm/A_{260}, at time t, and m_0 is the specific activity at $t = 0$. A_0 is the acid-soluble pool specific activity, dpm/A_{260}, at time 0. k_i and k_m are the decay constants (% h) of the acid-soluble pool and the poly(A)-RNA pool, respectively.

m_0 was obtained by extrapolation of the experimental poly(A)-RNA decay curve to $t = 0$ which in all experiments was taken to be midnight of the day preceding that in which time points were first analyzed. A_0 was obtained in the same manner. k_i was obtained from the slope of the straight line representing the acid-soluble pool decay. Different values of k_m were chosen and then substituted into the above equation until the line that best fit the observed experimental poly(A)-RNA decay points was found. A corrected half-life can then be calculated from the k_m value that yielded the best fit to the experimental data. An observed half-life of poly(A)-RNA of 37 h when corrected for re-utilization of labeled precursors was estimated as 20 h.

References

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

Aviv, H., and Leder, P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1408.

Berger, S. L., and Cooper, H. L. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3873.

Blobel, G., and Potter, V. R. (1966a), *Proc. Natl. Acad. Sci. U.S.A. 55,* 1283.

Blobel, G., and Potter, V. R. (1966b), *Science 154,* 1662.

Blobel, G., and Potter, V. R. (1968), *Biochim. Biophys. Acta 166,* 48.

Brandhorst, B. P., and McConkey, E. H. (1974), *J. Mol. Biol. 85,* 451.

Ceriotti, G. (1955), *J. Biol. Chem. 214,* 59.

Eiden, J. J., and Nichols, J. L. (1973), *Biochemistry 12,* 3951.

Endo, Y., Tominaga, H., and Natori, Y. (1975), *Biochim. Biophys. Acta 383,* 305.

Faiferman, I., and Pogo, A. O. (1975), *Biochemistry 14,* 3808.

Fuhrman, S. A., and Gill, G. N. (1975), *Biochemistry 14,* 2925.

Garrett, C. T., Wilkinson, D. S., and Pitot, H. C. (1973), *Anal. Biochem. 52,* 342.

Gebert, R. A. (1967), Doctoral Dissertation, University of Wisconsin, Madison, Wisconsin.

Greenberg, J. R. (1972), *Nature (London) 240,* 102.

Gribnau, A. A. M., Schoenmakers, J. G. G., and Bloemendal, H. (1969), *Arch. Biochem. Biophys. 130,* 48.

Hauschka, P. V. (1973), *Methods Cell Biol. 6,* 361-462.

Hurlbert, R. B., and Potter, V. R. (1954), *J. Biol. Chem. 209,* 1.

Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R. (1954), *J. Biol. Chem. 209,* 23.

Jacoli, G. G., Ronald, W. P., and Lavkulich, L. (1973), *Can. J. Biochem. 51,* 1558.

Kafatos, F. C., and Gelinas, R. (1974), *MTP Int. Rev. Sci.: Biochem., Ser. One 9,* 223.

Kramer, G., Wiegers, U., and Hilz, H. (1973), *Biochem. Biophys. Res. Commun. 55,* 273.

Lindberg, U., Persson, T., and Philipson, L. (1972), *J. Virol. 10,* 909.
Murphy, W., and Attardi, G. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 115.
Murthy, M. R. V. (1972), *J. Biol. Chem. 247,* 1944.
Murty, C. N., and Sidransky, H. (1972), *Biochim. Biophys. Acta 281,* 69.
Ouellette, A. J., and Malt, R. A. (1976), *Biochemistry 15,* 3358.
Palmiter, R. D., and Carey, N. H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2357.
Parish, J. H. (1972), Principles and Practice of Experiments with Nucleic Acids, London, Longman Group Limited.
Perry, R. P., and Kelley, D. E. (1973), *J. Mol. Biol. 79,* 681.
Perry, R. P., LaTorre, J., Kelley, D. E., and Greenberg, J. R. (1972), *Biochim. Biophys. Acta 262,* 220.
Plagemann, P. G. W. (1971a), *J. Cell Physiol. 77,* 213.
Plagemann, P. G. W. (1971b), *J. Cell Physiol. 77,* 241.
Plagemann, P. G. W. (1972), *J. Cell Biol. 52,* 131.
Podobed, O. V., Brykina, E. V., Abakumova, O. Y., Chernovskaya, T. V., and Lerman, M. I. (1974), *Mol. Biol. (Moscow) 8,* 936.
Puckett, L., Chambers, S., and Darnell, J. E. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 389.
Revel, M., and Hiatt, H. H. (1964), *Proc. Natl. Acad. Sci. U.S.A. 51,* 810.
Schultz, G. A. (1973), *Can. J. Biochem. 51,* 1515.
Shiokawa, K., and Pogo, A. O. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2658.
Singer, R. H., and Penman, S. (1972), *Nature (London) 240,* 100.
Sirakov, L. M., and Kochakian, C. D. (1969), *Biochim. Biophys. Acta 195,* 569.
Soeiro, R., Vaughan, M. Y., Warner, J. R., and Darnell, J. E. (1968), *J. Cell Biol. 39,* 112.
Spirin, A. S. (1969), *Eur. J. Biochem. 10,* 20.
Tanaka, T., and Ogata, K. (1972), *Biochem. Biophys. Res. Commun. 49,* 1060.
Tominaga, H., Aki, J., and Natori, Y. (1971), *Biochim. Biophys. Acta 228,* 183.
Tweedie, J. W., and Pitot, H. C. (1974), *Cancer Res. 34,* 109.
Venkatesan, N., and Steele, W. J. (1972), *Biochim. Biophys. Acta 287,* 526.
Watanabe, M., Potter, V. R., and Pitot, H. C. (1968), *J. Nutr. 95,* 207.
Weiss, J. W., and Pitot, H. C. (1974), *Arch. Biochem. Biophys. 106,* 119.
Wiegers, U., Kramer, G., Klapproth, K., Rehpenning, W., and Hilz, H. (1975), *Eur. J. Biochem. 50,* 557.
Wilson, S. H., Hill, H. Z., and Hoagland, M. B. (1967), *Biochem. J. 103,* 567.
Wilson, S. H., and Hoagland, M. B. (1967), *Biochem. J. 103,* 556.
Yager, J. D., Jr., Lichtenstein, M. J., Bonney, R. J., Hopkins, H. A., Walker, P. R., Dorn, C. G., and Potter, V. R. (1974), *J. Nutr. 104,* 273.