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Measurement of Ribonucleotide Pool Specific Activities by an in Vivo Method: Comparison with an in Vitro Method†

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ABSTRACT: A biological assay is described for the specific activity of the nucleotides incorporated into nuclear ribonucleic acid (RNA). Under labeling conditions in which the pool specific activity reaches steady state by a process described by a continuous function, this method allows the determination of pool specific activity at all times of labeling. In *Drosophila* cultured cells incubated with [^{3}H]uridine, the incorporation of radioactivity into transfer RNA (tRNA) and the absolute rate of tRNA synthesis were used to obtain an absolute value for the average specific activities of the uridine 5′-triphosphate (UTP) and cytidine 5′-triphosphate (CTP) precursor pools. The average specific activity of the UTP and CTP pool obtained by this in vivo method was 2–3-fold higher than the specific activity of the whole-cell UTP and CTP pools as assayed by an in vitro method. This suggests that the ribonucleotide pools in *Drosophila* cultured cells are compartmentalized. Use of the in vivo assay of ribonucleotide pool specific activities described here should avoid underestimates or overestimates which may arise when absolute rates of RNA synthesis are calculated from measurements of whole-cell ribonucleotide pool specific activities. The rate of labeling of tRNA was used to determine the rate at which the combined UTP and CTP pools equilibrate. This rate was then used to determine that the rate of processing of pre-tRNA to mature tRNA is 0.21 min^{-1}; this corresponds to a half-life of 3.3 min.

An important problem in understanding the control of eukaryotic gene expression is the relative contribution of transcription, posttranscriptional processing, and turnover to the level of expression of a particular gene. In order to assess the relative transcriptional efficiencies of the promotors of different genes, it is necessary to measure the absolute rate of synthesis of particular RNA[1] species.

Absolute rates of synthesis are frequently determined by measuring the rate of labeling of RNA after the addition of radioactive precursor. Knowledge of the behavior of the specific activity of the whole-cell ribonucleotide triphosphate pool during the labeling period is then used to convert the rate of labeling to a rate of synthesis. An underlying assumption of this method is that the ribonucleotide precursor pool is not compartmentalized, i.e., that the specific activity of the total ribonucleotide triphosphate pool extracted from the cell is identical with the specific activity of the ribonucleotide pool used for nuclear RNA synthesis.

There is controversy in the literature as to whether the specific activity of the whole-cell ribonucleotide pool is identical with the specific activity of the ribonucleotide precursor pool utilized for nuclear RNA synthesis. In HeLa cells, the steady-state specific activity attained in mRNA was equal to that attained in the total UTP pool (Kramer et al., 1973; Wiegers et al., 1976); furthermore, the ratio of uridine to cytidine specific activity was the same in hnRNA, pre-rRNA, polio virus RNA (which is synthesized in the cytoplasm), and the total acid-soluble pools (Wu & Soiero, 1971; Soiero & Ehrenfeld; 1973; Puckett & Darnell, 1977). These various lines of evidence are consistent with the hypothesis that ribonucleotide pools are not compartmentalized.

Studies on a number of other cell lines, however, support the concept that ribonucleotide pools are compartmentalized. Rates of labeling of nuclear RNA and Mengo virus RNA (which is synthesized in the cytoplasm), in cells which had undergone prior pool expansion, were compared to their re-

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[1] Abbreviations used: RNA, ribonucleic acid; mRNA, messenger RNA; tRNA, transfer RNA; UTP, uridine 5′-triphosphate; hnRNA, heterogeneous nuclear RNA; rRNA, ribosomal RNA; MEM, minimum Eagle's medium; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; CTP, cytidine 5′-triphosphate; Cl_3CCOOH, trichloroacetic acid; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; BSA, bovine serum albumin; UMP, uridine 5′-monophosphate.

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spective rates of labeling in control cultures (no pool expansion). The rate of labeling of nuclear RNA was unaffected by prior pool expansion, but the rate of labeling of viral RNA was depressed by prior pool expansion, from which it was concluded that there is compartmentalization of ribonucleotide pools (Plagemann, 1971, 1972; Khym et al., 1978). The difference in the rate of equilibration of the nuclear and cytoplasmic pools was used to explain why compartmentalization of ribonucleotide pools was not detected in HeLa cells (Khym et al., 1978).

Because of the uncertainty as to whether total cell measurements of the ribonucleotide pool specific activity reflect the behavior of the ribonucleotide pool utilized for nuclear RNA synthesis, it is advantageous to use the rate of labeling of an RNA species synthesized at a constant rate as a biological assay of ribonucleotide pool specific activity. The incorporation of radioactivity into both rRNA and tRNA has been used to monitor the behavior of the ribonucleotide pool (Spradling, 1975; Levis & Penman, 1977). Unless the absolute rate of synthesis of the rRNA or tRNA is known, however, the rate of labeling of these molecules serves only as a relative assay of ribonucleotide pool behavior and not as a measure of the absolute specific activity of the ribonucleotide pool.

We show here that with a knowledge of the amount of tRNA per cell, the cell generation time, and the rate of turnover of tRNA, it is possible to use the rate of labeling of tRNA as an assay for ribonucleotide pool specific activity during the initial phase of labeling, when the pool specific activity is changing rapidly. Furthermore, the ribonucleotide precursor pool specific activity, determined by this biological assay, can be compared to the total cell ribonucleotide pool specific activity determined by an enzymatic method in vitro. Comparison of ribonucleotide pool specific activities obtained by these two methods shows that the specific activity of the ribonucleotides incorporated into tRNA is higher than the specific activity of the whole-cell ribonucleotide pool. This is consistent with the idea that ribonucleotide pools are compartmentalized in *Drosophila* cultured cells and that their specific activities can be more accurately assessed by in vivo rather than in vitro methods.

Experimental Procedures

Cell Culturing and Labeling. Exponentially growing suspension cultures of *Drosophila* line 2-L (Lengyel et al., 1975) adapted from Schneider line 2 (Schneider, 1972) were maintained in Dulbecco's modified MEM containing 2% GIBCO lactalbumin hydrolysate and 10% fetal calf serum (Lengyel et al., 1975). At 25 °C the cells have a generation time of 24 h. Cells were labeled during exponential growth (2×10^6–6×10^6/mL) with 5–50 μCi/mL [^{3}H]uridine (26–28 Ci/mmol).

Cell Fractionation and RNA Purification. Cells were fractionated by a modification of the method of Levis & Penman (1977). Cells were poured over an equal volume of crushed frozen wash buffer (0.01 M Tris, pH 7.4, 0.1 M NaCl, and 0.01 M magnesium acetate), collected by centrifugation at 1500 rpm in an IEC PR6000 centrifuge for 4 min at 4 °C, and washed once with wash buffer. Cells were resuspended at 1/20th of the original volume in lysis buffer (0.03 M Tris, pH 7.4, 0.03 M NaCl, and 0.01 M magnesium acetate) containing 0.5% diethyl pyrocarbonate and lysed by the addition of NP40 to 0.5%. Nuclei were removed by centrifugation over a 6-mL sucrose pad (0.75 M sucrose, 0.03 M Tris, pH 7.4, 0.03 M NaCl, and 0.01 M magnesium acetate) at 4500 rpm for 5 min at 4 °C. The cytoplasmic supernatant was removed from the top of the sucrose pad, added to an equal volume of 2 × $NaDodSO_4$ buffer (0.2 M NaCl, 0.02 M Tris, pH 7.4, 0.002 M EDTA, and 1% $NaDodSO_4$) containing 500 μg/mL proteinase K, and incubated at 37 °C for 3 h. The nucleic acid was precipitated by the addition of 2 volumes of absolute ethanol at −20 °C. The efficiency of extraction of RNA using this protocol is 99% (Falkenthal, 1980). Messenger RNA was removed from all cytoplasmic samples by two separate cycles of denaturation and chromatography on oligo(dT)–cellulose (T3, Collaborative Research) as described by Anderson & Lengyel (1979). Poly(A)$^-$ RNA therefore refers to RNA which consistently did not bind to oligo(dT)–cellulose.

Determination of UTP and CTP Pool Specific Activity. Pool specific activities were measured by an isotope dilution assay of whole-cell extracts using RNA polymerase (Maxson & Wu, 1976). This technique has advantages over chromatographic techniques in terms of sensitivity (as little as 1 ng of triphosphate can be detected), precision (with a [^{3}H]UTP standard, the value of the specific activity determined by this assay was within 2.5% of the manufacturer's stated specific activity), and time required (16 separate determinations can easily be carried out in 1 day).

Cl_3CCOOH-soluble extracts of whole cells which contained the labeled ribonucleotide pools were prepared as follows. Aliquots of washed cells were lysed by the addition of 2 volumes of 10% Cl_3CCOOH. After 30 min at 4 °C the Cl_3CCOOH-insoluble material was collected by centrifugation in a 1.5-mL polyallomer tube in a Beckman microfuge for 10 min at 4 °C. The supernatant was removed, extracted 5 times with 100% ether to remove the Cl_3CCOOH, and then stored at −20 °C.

To measure the specific activity of the UTP (or CTP) pool, we incubated labeled extract with 2 μCi of [^{14}C]ATP (or [^{14}C]GTP) in 0.08 M Tris, pH 8.0, 0.04 M $MgCl_2$, 0.02 M β-mercaptoethanol, 2 mg/mL BSA, 0.05 OD_{260} unit of poly[d(A–T)] (or poly[d(G–C)]), 1 unit of *E. coli* RNA polymerase, and increasing amounts of unlabeled ATP (or GTP) in a total reaction volume of 0.1 mL for 30 min at 37 °C. The reaction was terminated by the addition of 1.0 mL of 0.5 N $HClO_4$ and 0.1 M sodium pyrophosphate. After 30 min at 4 °C the precipitated material was collected on a nitrocellulose filter and washed with 1.0 N HCl and 0.5 M sodium pyrophosphate. The ^{3}H and ^{14}C acid-insoluble radioactivity was then determined by scintillation counting, and the ratio of the ^{3}H cpm/^{14}C cpm incorporated was plotted as a function of the amount of unlabeled ATP (or GTP) in the reaction mixture. The slope of this line was used to calculate the ^{3}H-labeled ribonucleotide pool specific activity (Maxson & Wu, 1976).

Determination of the Mass of tRNA, 5S RNA, and rRNA per Cell. In order to determine the steady-state amount of tRNA per cell, we electrophoresed aliquots of cytoplasmic RNA from a known number of cells and known amounts of yeast tRNA (used as a standard) on 10% polyacrylamide gels (Hirsch & Penman, 1973) at 5 mA/gel for 4 h. The amount of tRNA loaded onto the gel (5–50 μg) was determined from its absorbance at 260 nm, assuming that 43.5 μg/mL tRNA has an A_{260} of 1.0. The gels were scanned at 260 nm with a Beckman spectrophotometer equipped with a linear transport, and the areas under the 4S and the 5S peaks of the tracings were quantitated by cutting out and weighing the peaks. The weight of the yeast tRNA peak was used as a standard to convert the weight of the *Drosophila* tRNA peaks to a mass of tRNA.

For determination of the amount of rRNA per cell, aliquots of cytoplasmic RNA were centrifuged on 15–30% $NaDodSO_4$–sucrose gradients, the gradients were scanned with an

ISCO UV monitor at 254 nm, and the areas under the 19S rRNA peak, the 26S rRNA peak, and the 4S plus 5S peak were determined by cutting out and weighing the peaks. The ratio (weight of 19S plus 26S rRNA peaks)/(weight of the 4S plus 5S peak) was 5.2.

The mass of tRNA, 5S RNA, and rRNA per cell was determined in four separate experiments. On average there were 0.83 ± 0.08 pg of tRNA, 0.13 ± 0.01 pg of 5S RNA, and 4.9 ± 0.4 pg of rRNA per cell.

Results

Mathematical Description of the Relationship between Ribonucleotide Pools and RNA Synthesis. The rate of synthesis of RNA is usually estimated by measuring the incorporation of radioactivity from exogenously added ^{3}H-labeled nucleosides into RNA. In order to obtain an absolute rate of synthesis from this incorporation of radioactivity with time, it is necessary to know the behavior of the specific activity of the ribonucleotide precursor pool during the time of the labeling.

A simple two-compartment model which describes the relationship between the specific activity of the ribonucleotide precursor pool and the amount of radioactivity in an RNA species is

$$S \xrightarrow{k_s} A \xrightarrow{k_d}$$

where A = the amount of radicactivity in the RNA, k_s = the absolute rate of synthesis of the RNA (picomoles of ribonucleotide per minute), S = the specific activity of the ribonucleotide percursor pool (dpm per picomole of ribonucleotide), and k_d = the rate constant for the decay of A (per minute). The rate of accumulation of radioactivity into the RNA species is then

$$dA/dt = k_sS - k_dA \quad (1)$$

where t = time. During labeling with exogenous ribonucleosides, the ribonucleotide pool specific activity, S, is usually not constant, particularly at early time points. In order to solve eq 1 for A, therefore, it is necessary to be able to describe S as a continuous function of t. The derivation of such a function, and its fit to pool specific activity data obtained during labeling of *Drosophila* cultured cells with [^{3}H]uridine, is presented below.

Previous studies of uridine metabolism in cultured cells have shown that UTP arises from both de novo synthesis and the phosphorylation of UMP derived from the degradation of RNA molecules within the cell [reviewed by Hauschka (1973)]. Various metabolic demands such as catabolism, metabolic interconversions, and RNA synthesis drain the UTP pool. A simple one-compartment model which describes the increase in the specific activity of the UTP pool after the addition of exogenous [^{3}H]uridine is

$$\xrightarrow{k_1} S \xrightarrow{k_2}$$

where k_1 is an empirical zero-order rate constant for the rate-limiting step of entry of exogenously added [^{3}H]uridine into the UTP pool of the cell (dpm per picomole per minute) and k_2 is the empirical rate constant for the turnover of the ribonucleotide pool (per minute).

On the basis of the one-compartment model shown above, the rate of labeling of the ribonucleotide precursor pool is

$$dS/dt = k_1 - k_2S \quad (2)$$

Equation 2 can be integrated to obtain

$$S = (k_1/k_2)(1 - e^{-k_2t}) \quad (3)$$

Since $dS/dt = 0$ once the specific activity of the ribonucleotide precursor pool has reached steady-state labeling, the steady-state specific activity of the ribonucleotide precursor pool, S_∞, is

$$S_\infty = k_1/k_2 \quad (4)$$

In experimental terms, k_2 is the rate constant at which the pool equilibrates (reaches steady-state labeling); the half-life for this equilibration is $\ln 2/k_2$.

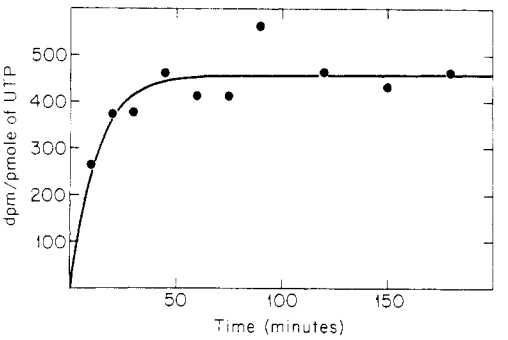

FIGURE 1: Specific activity of UTP pool during labeling with [^{3}H]uridine. Cells (3×10^6/mL) were labeled with [^{3}H]uridine (5 μCi/mL). At various times after the initiation of labeling, cells were harvested and lysed with 10% Cl_3CCOOH. The Cl_3CCOOH-soluble fraction was assayed for the specific activity of the UTP pool (see Experimental Procedures). The curve is a computer-generated least-squares fit of the data to eq 3. The values of k_1 (36 ± 1.3 dpm pmol^{-1} min^{-1}) and k_2 (0.079 ± 0.015 min^{-1}) were determined by an APL (A Programming Language, IBM) least-squares nonlinear regression routine.

Determination of Pool Specific Activity. During incubation with [^{3}H]uridine, radioactivity flows into both the UTP and CTP pools, since UTP is the direct metabolic precursor to CTP (Plagemann & Roth, 1969). The specific activity of these pools as a function of time was determined by two different methods. One of these measures the specific activity of the whole-cell UTP or CTP pool by an enzymatic isotope dilution method (see Experimental Procedures). We refer to this as the in vitro method for assaying pool specific activity. The other method is a biological assay. We show here that the rate of labeling of tRNA and a knowledge of the absolute rate of tRNA synthesis and turnover can be used to calculate average specific activities of the UTP and CTP precursor pools. We refer to this as the in vivo method for the determination of the pool specific activity.

(*a*) *In Vitro Method.* At various times during the labeling period, equal aliquots of cells were taken, and the Cl_3CCOOH-soluble fraction of whole-cell lysates was analyzed. Figure 1 shows the specific activity of the UTP pool (dpm per picomole of UTP) as a function of time after the initiation of labeling. The curve is a computer-generated least-squares fit of the data to eq 3; the mean square error of the fit shown in Figure 1 was 5.1%, indicating that eq 3 provides an accurate empirical description of the behavior of the pool specific activity as a function of time. In the experiment shown in Figure 1 the UTP pool attained steady-state labeling (460 dpm/pmol) approximately 30–40 min after the addition of exogenous [^{3}H]uridine; the half-life with which the pool achieved constant specific activity was 8.8 min.

The specific activity of the CTP pool as a function of time after the addition of [^{3}H]uridine was also analyzed by the isotope dilution method. Even though the rate of entry of label into the CTP pool is clearly not constant (because of the increasing specific activity of the UTP pool), eq 3 also provides a satisfactory empirical description of CTP pool specific activity as a function of time (Figure 2). During labeling with 5–50 μCi/mL (0.19–1.9 μM) exogenous [^{3}H]uridine, the

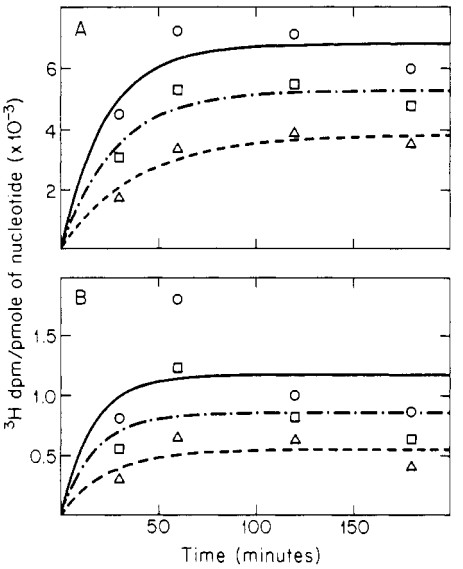

FIGURE 2: Specific activity of UTP and CTP pools during labeling with [^{3}H]uridine. Cells (3×10^6/mL) were labeled with [^{3}H]uridine. At various times after the initiation of labeling, the cells were harvested and lysed with 10% Cl_3CCOOH. The Cl_3CCOOH-soluble fraction was assayed for the specific activity of the UTP and CTP pools (see Experimental Procedures). The curves are computer-generated least-squares fits of the data to eq 3 as described in the legend to Figure 1. (A) Cells labeled with 50 μCi/mL [^{3}H]uridine. (B) Cells labeled with 5 μCi/mL [^{3}H]uridine. (○) Specific activity of UTP; (△) specific activity of CTP; (□) average of UTP and CTP specific activities, i.e., (sp act. of UTP + sp act. of CTP)/2. The values of the computer-determined rate constants and their root mean square errors are as follows: (A) (○) $k_1 = 300 \pm 23$ dpm pmol^{-1} min^{-1}, $k_2 = 0.044 \pm 0.004$ min^{-1}; (△) $k_1 = 100 \pm 7$ dpm pmol^{-1} min^{-1}, $k_2 = 0.026 \pm 0.002$ min^{-1}; (□) $k_1 = 195 \pm 14$ dpm pmol^{-1} min^{-1}, $k_2 = 0.037 \pm 0.003$ min^{-1}. (B) (○) $k_1 = 72 \pm 17$ dpm pmol^{-1} min^{-1}, $k_2 = 0.060 \pm 0.010$ min^{-1}; (△) $k_1 = 23 \pm 4$ dpm pmol^{-1} min^{-1}, $k_2 = 0.042 \pm 0.008$ min^{-1}; (□) $k_1 = 49 \pm 10$ dpm pmol^{-1} min^{-1}, $k_2 = 0.057 \pm 0.013$ min^{-1}.

steady-state specific activity attained by the CTP pool is half of that of the UTP pool specific activity. The half-life for the equilibration of the CTP pool is ~1.6 times longer than that of the UTP pool. This delay reflects both the increasing specific activity of the UTP pool and the rate of conversion of UTP to CTP.

The average specific activity of the UTP and CTP pools as a function of time is also shown in Figure 2. The rate at which this average pool equilibrates is 1.1–1.2 times slower than the rate of turnover of the UTP pool alone.

(*b*) *In Vivo Method.* The enzymatic isotope dilution method measures the specific activity of individual nucleotide triphosphates of the whole cell. In order to characterize the specific activities of the UTP and CTP pools used for nuclear RNA synthesis, we analyzed the rate of incorporation of radioactivity into tRNA. The two methods of determining pool specific activities are then compared below.

Because tRNA is synthesized at a constant rate per cell in asynchronous, logarithmically growing cells (Spradling, 1975) and is found in the cytoplasmic fraction immediately after its synthesis (Zieve & Penman, 1976; Levis, 1977), incorporation of radioactivity into tRNA can be used as a biological assessment of the precursor ribonucleotide pool specific activity during labeling. Cells were labeled for various periods of time, cytoplasmic RNA was extracted and separated from mRNA, and low molecular weight RNAs were separated by electrophoresis in 10% polyacrylamide gels (see Experimental Procedures). An example of such a measurement of radioactivity in tRNA is shown in Figure 3.

The accumulation of radioactivity in tRNA can be described as a function of its rate of synthesis and turnover and the

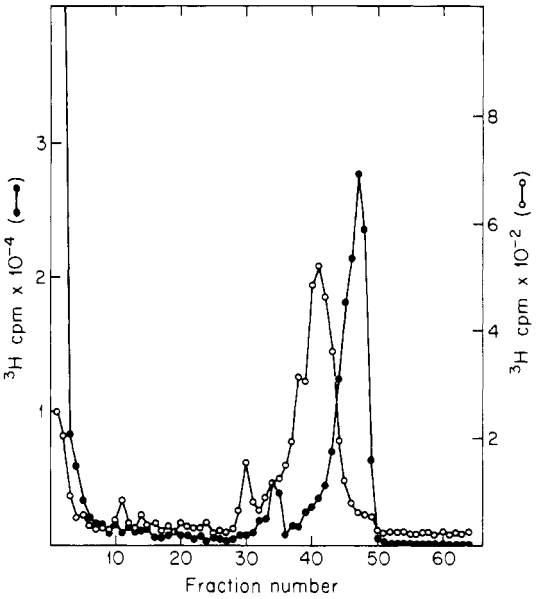

FIGURE 3: Size distribution of low molecular weight cytoplasmic poly(A)$^-$ RNAs during a pulse label. Cytoplasmic poly(A)$^-$ RNA was isolated from the culture described in Figure 1 as described under Experimental Procedures at (○) 5 min and (●) 120 min after the addition of [^{3}H]uridine and was electrophoresed on 10% polyacrylamide gels (Hirsch & Penman, 1973) at 5 mA/gel for 4 h.

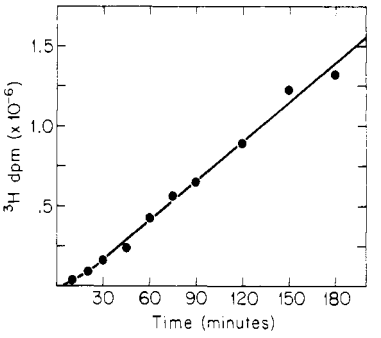

FIGURE 4: Rate of accumulation of radioactivity into tRNA. At various times after the initiation of labeling of the culture described in Figure 1, cells were harvested, and the poly(A)$^-$ RNA was prepared and electrophoresed on 10% polyacrylamide gels as described in the legend to Figure 3. The cpm in the pre-4S and the 4S region of the 10% polyacrylamide gels (as shown in Figure 3) were quantitated and corrected for counting efficiency. The dpm in the 4S region of the gel were then corrected for loss of nucleotides during the processing of the pre-4S RNA (Levis, 1977) and added to the dpm in the pre-4S region of the gel. The curve is a computer-generated least-squares fit of the data to eq A4 (see Appendix) in which k_b was fixed at 0.00022 min^{-1} and in which it was determined that $P(1) = 1.1 \times 10^4 \pm 0.02 \times 10^4$ dpm/min and $k_2 = 0.069 \pm 0.009$ min^{-1}.

ribonucleotide pool specific activity (see Appendix, eq A4). The curve in Figure 4 is a computer-generated least-squares fit of the radioactivity in tRNA as a function of time to eq A4. The excellent fit of this equation to the data (mean square error = 3.1%) indicates that eq 3 accurately describes the behavior of the specific activity of the UTP and CTP precursor pools. The half-life for the equilibration of the combined UTP and CTP pools obtained from this analysis was 10 min. This was 1.2 times greater than the half-life for the equilibration of the UTP pool determined by the in vitro method in the same experiment (Figure 1) and is within the range of the half-lives for the equilibration of the UTP and CTP pools determined by the in vitro method in other experiments (Figure 2). Thus, the average rate of equilibration of the UTP and CTP pools is similar whether measured by the in vivo or the in vitro method.

Table I: Comparison of Ribonucleotide Pool Specific Activity Obtained from Rates of tRNA Labeling and from Assay of Whole-Cell Ribonucleotide Pools[a]

(1) expt	(2) concn of exogenous uridine, μCi/mL	(2) concn of exogenous uridine, μM	(3) rate of tRNA labeling/cell, k_1k_a/k_2N[b] (dpm cell^{-1} min^{-1})	(4) calcd in vivo precursor pool sp act., k_1/k_2[c] (dpm/pmol of UTP + CTP in tRNA)	(5) measured whole-cell UTP pool sp act., k_1/k_2[d] (dpm/pmol of UTP)	(6) calcd whole-cell UTP + CTP pool sp act. in tRNA, k_1/k_2[e] (dpm/pmol of UTP + CTP)	(7) column 4/ column 6
1	5	0.19	1.17×10^{-3}	1100	470	400	2.8
2	20	0.76	4.24×10^{-3}	3900	2450	1900	2.1
3	50	1.9	7.43×10^{-3}	6800	5300	4100	1.7

[a] The rate of tRNA labeling (k_1k_a/k_2) and the various pool specific activities (k_1/k_2) are the values for steady state. [b] Parameter $P(1)$, obtained by a computer-generated fit of the accumulation of radioactivity in tRNA as a function of time (see legend to Figure 4 and Computer Analysis of Data under Analysis of Rate of Labeling of tRNA), divided by N, the number of cells per aliquot. [c] Value obtained by dividing the numbers in column 3 by the absolute rate of synthesis of tRNA per cell (see text). [d] Obtained by the isotope dilution method as described under Experimental Procedures. [e] Calculated as $N_uS_u + N_cS_c/N_u + N_c$ where N_u is the number of uridine residues in tRNA, S_u is the UTP pool specific activity measured by the isotope dilution assay, N_c is the number of cytidine residues in tRNA, and S_c is the CTP pool specific activity. The CTP pool specific activity at steady state is half of the UTP steady-state pool specific activity (see Figure 2).

The average specific activity of the UTP and CTP precursor pools can now be compared to the average specific activity of the whole-cell UTP and CTP pools as assayed by the in vitro method. The average specific activity of the UTP and CTP precursor pools, k_1/k_2, can be calculated by dividing k_1k_a/k_2N, the rate of labeling of pre-tRNA at steady-state pool specific activity per cell, by k_a/N, the absolute rate of synthesis of pre-tRNA per cell. The term k_1k_a/k_2 is obtained from the computer-generated least-squares fit to the data shown in Figure 4. N is the total number of cells per aliquot. The absolute rate of synthesis of tRNA per cell (picomoles per nucleotide per cell per minute) is $(M/0.75)(k_d + k_g)$, where M is the steady-state mass of tRNA per cell, k_g is the rate constant for cell doubling, and k_d is the rate constant for the decay of tRNA. The factor of 0.75 is used since the mature tRNA is 75% of the length of pre-tRNA (Burdon, 1975; Garber & Gage, 1979). The steady-state mass of mature tRNA per cell is 0.83 ± 0.07 pg (see Experimental Procedures). The rate constants for the decay of tRNA and the growth of the cells are 2.2×10^{-4} min^{-1} (Lengyel & Penman, 1977) and 4.8×10^{-4} min^{-1} (Falkenthal, 1980), respectively. The absolute rate of synthesis of pre-tRNA is thus 2.3×10^{-6} pmol of nucleotide cell^{-1} min^{-1} or 1.1×10^{-6} pmol of UTP plus CTP cell^{-1} min^{-1} [based on a precursor tRNA base composition of 46% U plus C (Hagenbüchle et al., 1979; Knapp et al., 1978; Ogden et al., 1979; Silverman et al., 1979a,b)].

If the ribonucleotide pool used for nuclear RNA synthesis is the same as the whole-cell ribonucleotide pool, then the specific activity of the nucleotides in newly synthesized tRNA should be the same whether calculated from the rate of labeling of tRNA at steady-state pool specific activity (the in vivo method) or from the steady-state specific activity of the whole-cell UTP and CTP pools (the in vitro method). A comparison of the calculated average specific activity of UMP and CMP in tRNA as determined by these two methods (Table I) reveals that the specific activities of the UTP and CTP pools used for nuclear RNA synthesis (column 4) are higher than the specific activities of the UTP and CTP pools of the whole cell (column 6). This suggests that the UTP and CTP pools of *Drosophila* cultured cells are compartmentalized.

If there are intracellular pools which are metabolically separate (i.e., compartmentalized), due to differences in the sizes and metabolic demands upon them, then these pools might turn over at different rates. This cannot be determined by approach to steady-state labeling of the pools such as shown in Figure 1, because this type of analysis does not allow resolution of multiple kinetic components when one component is <10% of the total. A more sensitive approach, that of pulse–chase labeling, was therefore used to determine if there are multiple rates of turnover for the UTP pool. Cells were pulse labeled for 5 min with [^{3}H]uridine and chased with an excess of unlabeled uridine and cytidine. At various times after the initiation of the chase, equal aliquots of cells were taken, and the Cl_3CCOOH-soluble fraction of whole-cell lysates was analyzed. Figure 5 shows the specific activity of the UTP pool (dpm per picomole of UTP) as a function of time after the initiation of the chase. The decay of the specific activity of the UTP pool shows two kinetic components: one which decays with a half-life of 9.8 ± 0.4 min and one which decays with a half-life of 103 ± 4 min. Because the relative sizes of the two pools are unknown, the specific activity of either pool cannot be determined.

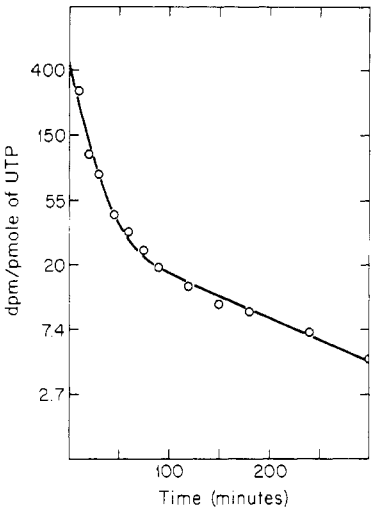

FIGURE 5: Specific activity of UTP pool during a uridine–cytidine chase. Cells (2.4×10^6/mL) were labeled with [^{3}H]uridine (10 μCi/mL). After 5 min of labeling, a chase was initiated by the addition of unlabeled uridine and cytidine to 5 mM each. At various times after the initiation of the chase, equal aliquots of cells were harvested and lysed with 10% Cl_3CCOOH. The Cl_3CCOOH-soluble fraction was assayed for the specific activity of the UTP pool (see Experimental Procedures). The curve is a computer-generated least-squares fit of the data to a linear transformation of the equation $Y = A_0e^{-k_ct} + B_0e^{-k_dt}$, where Y is the specific activity of the UTP pool, A_0 and B_0 are the dpm per picomole at $t = 0$ in components 1 and 2, respectively, k_c is the rate constant for the decay of component 1 (determined to be 0.072 ± 0.003 min^{-1}), and k_d is the rate constant for the decay of component 2 (determined to be 0.0067 ± 0.003 min^{-1}).

The direct demonstration by pulse–chase experiments of two kinetic components for the decay of the UTP pool supports

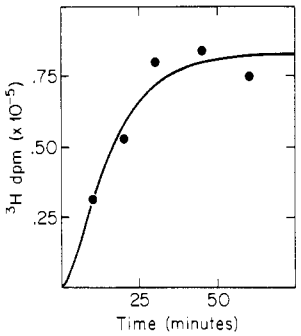

FIGURE 6: Rate of accumulation of radioactivity into pre-tRNA. Cells were labeled and poly(A)$^-$ cytoplasmic RNA was prepared and electrophoresed on 10% polyacrylamide gels as described in the legend to Figures 1 and 3. The cpm in the pre-4S region of the gel (an example of which is shown in Figure 3) were quantitated during the labeling period and corrected for the counting efficiency. The curve is a computer-generated least-squares fit of the data to eq A6 in which k_2 was fixed at 0.069 min^{-1} and in which it was determined that $P(1) = 1.4 \times 10^4 \pm 0.3 \times 10^4$ dpm/min and $k_p = 0.21 \pm 0.01$ min^{-1}.

the hypothesis that there are metabolically separate pools. Since the slowly decaying pool contributes <10% to the total specific activity of the whole-cell UTP pool (Figure 5), it is not observed in approach to steady-state analysis (Figure 1). The value of the specific activity at $t = 0$, the initiation of the chase, must therefore be a minimum value for the specific activity of the more rapidly turning over ribonucleotide pool.

Processing of tRNA Precursor. The experiment described in Figures 3 and 4 also allows the determination of the rate at which the precursor tRNA is processed to mature tRNA. The dpm in the precursor tRNA region of the polyacrylamide gels were quantitated at various times during the labeling. The curve in Figure 6 is a computer-generated least-squares fit to the data obtained by using the equation which describes the approach to steady-state labeling of pre-tRNA (see Appendix). As can be seen in Figure 6, the precursor tRNA reached steady-state labeling by 30 min. The rate constant for approach to steady-state labeling of the precursor tRNA, taking into account the changing specific activity of the ribonucleotide pool, was 0.21 min^{-1}. If it is assumed that the precursor tRNA does not decay and that it is converted to mature 4S tRNA with an efficiency of 100%, then the rate constant for its processing to mature tRNA is also 0.21 min^{-1}, corresponding to a half-life of 3.3 min.

Discussion

Most measurements of ribonucleotide pool specific activities are obtained from analyses of whole-cell ribonucleotide pools or hydrolyzed steady-state labeled RNA. Controversies have arisen in the literature as to the correct method for ascertaining the specific activity of the ribonucleotide pool which serves as precursor for RNA synthesis (Wu & Soiero, 1971; Plagemann, 1971, 1972; Davidson, 1976; Puckett & Darnell, 1977).

We describe here a biological method for the measurement of the ribonucleotide precursor pool specific activity, even at early times in the labeling period when pool specific activities are rapidly changing. The rate of incorporation of radioactivity into tRNA combined with a knowledge of the absolute rate of tRNA synthesis was used to calculate the average specific activity of the UTP and CTP pools. The average specific activity determined in this way was compared to the average specific activity of the whole-cell UTP and CTP pools determined by an in vitro assay. Although the rate of equilibration of the ribonucleotide pools determined by the two methods was similar, the value for the average UTP and CTP pool specific activity obtained by the rate of labeling of tRNA was higher than that obtained by the assay of the whole-cell pools.

These data suggest that ribonucleotide pools may be compartmentalized in *Drosophila* cultured cells. Additional support for this conclusion is the biphasic decay of the specific activity of the UTP pool during a chase (Figure 5). A biphasic decay in the specific activity of the UTP pool during a chase experiment has also been found in myeloma cells (Cowan & Milstein, 1974). Assuming that there is compartmentalization, we conclude that the component which turns over more rapidly ($\tau_{1/2} = 10$ min) during the chase is the nuclear pool, since the half-life of the ribonucleotide precursor pool, obtained by the analysis of tRNA labeling (Figure 4), was also 10 min.

As the cells were incubated with higher concentrations of exogenous uridine, the steady-state specific activity of the total cellular pool approached the steady-state specific activity of the pool used for nuclear RNA synthesis (Table I, column 7). This can be explained by a model in which there is a small nuclear pool and a large cytoplasmic pool of ribonucleotide triphosphates (Plagemann, 1971, 1972). When cells are incubated in the presence of low concentrations (0.19 μM) of labeled uridine, the specific activity attained by the nuclear UTP and CTP pools is higher than that attained by the cytoplasmic pools. As the concentration of exogenous uridine is increased (to 1.9 μM), more labeled uridine is incorporated into the cytoplasmic pools; thus, the specific activity of the whole-cell UTP and CTP pools approaches the specific activity of the UTP and CTP pools used for nuclear RNA synthesis. This suggests that it should be possible to obtain whole-cell pool specific activities which are the same as RNA precursor pool specific activities by incubating cells with higher concentrations of exogenous labeled uridine. A possible drawback of this approach, however, is that the pool expansion caused by such a procedure might affect RNA metabolism.

Because the conclusion that there is compartmentalization of ribonucleotide pools rests on the value of the specific activity of the UTP and CTP precursor pools, as calculated from the rate of labeling of tRNA, being higher than the specific activity of the total cellular UTP and CTP pools, as assayed by the in vitro method, it is important to evaluate the accuracy of the values used to calculate pool specific activities from tRNA labeling. The main potential source of error is the value used for the absolute rate of synthesis of tRNA, since this was used to calculate the steady-state specific activity of the ribonucleotides in tRNA. The absolute rate of tRNA synthesis was calculated from the steady-state mass and the half-life of tRNA (eq 5). The half-life of tRNA used in this calculation was 53 h (Lengyel & Penman, 1977); this is similar to the 60-h half-life of tRNA in 3T3 cells (Abelson et al., 1974). A half-life for tRNA of 13 h, 4-fold less than the previously measured half-life (Lengyel & Penman, 1977), would be required in order to increase the calculated value for the absolute rate of tRNA synthesis by a factor of 2 (which would abolish the differences in steady-state specific activity of the ribonucleotides in tRNA, as calculated by the two methods, Table I).

The value used for the steady-state mass of tRNA per cell also affects the calculated value of the absolute rate of tRNA synthesis. The value used for the steady-state mass of tRNA per cell would also have to be increased by a factor of 2, in order to increase the absolute rate of tRNA synthesis by a factor of 2, thereby decreasing the steady-state specific activity of the nucleotides in tRNA by a factor of 2. The value for the amount of rRNA per cell (upon which the value for the

amount of tRNA per cell rests) is already 36% higher than the previously reported value for this cell line (Lengyel et al., 1975); it is therefore extremely unlikely that the value for the mass of tRNA per cell used here is an underestimate.

In conclusion, the specific activity of the total cellular ribonucleotide pool is not the same as that used for RNA synthesis in vivo in *Drosophila* cultured cells. There is evidence in the literature that this is also the case in other cell lines (Plagemann, 1971, 1972; Khym et al., 1978). We have shown, however, that the rate of labeling of tRNA can be used as a biological method to measure the specific activity of the ribonucleotide pool used for nuclear RNA synthesis, even at early times of labeling before the ribonucleotide pool has reached constant specific activity. In cultured cells which have a constant doubling time and thus a constant rate of tRNA synthesis, this approach should be useful for obtaining accurate measurements of the rates of transcription of specific genes.

Appendix

Analysis of Rate of Labeling of tRNA. (a) Theory. The rate of accumulation of radioactivity in tRNA in an aliquot of cultured cells, over a period of time which is short relative to the cell generation time, is described by

$$dT/dt = k_a S - k_b T \quad \text{(A1)}$$

where k_a = the absolute rate of synthesis of tRNA (picomoles of nucleotide per aliquot per minute), k_b = the first-order rate constant for the cytoplasmic decay of tRNA (per minute), S = the specific activity of the ribonucleotide precursor pool (dpm per picomole), and T = the dpm in tRNA per aliquot (precursor tRNA and tRNA) at time t. Rearranging eq A1 gives

$$dT/dt + k_b T = k_a S \quad \text{(A2)}$$

Substituting eq 3 which describes the pool specific activity into eq A2 and integrating with respect to t gives.

$$T = \frac{k_1 k_a}{k_2 k_b}(1 - e^{-k_b t}) + \frac{k_1 k_a (e^{-k_b t} - e^{-k_2 t})}{k_2 (k_b - k_2)} \quad \text{(A3)}$$

The first term of eq A3 describes the accumulation of label into tRNA if the pool specific activity were constant. The second term corrects for the changing pool specific activity; at large t (after the nucleotide pool has reached constant specific activity) this correction term goes to 0.

(b) Computer Analysis of Data. In order to fit the data for the accumulation of label into tRNA by computer, we replaced the group of parameters $k_1 k_a / k_2$ in eq A3 by the single parameter $P(1)$. Equation A3 therefore becomes

$$T = \frac{P(1)}{k_b}(1 - e^{-k_b t}) + \frac{P(1)(e^{-k_b t} - e^{-k_2 t})}{(k_b - k_2)} \quad \text{(A4)}$$

Parameters $P(1)$ and k_2 were allowed to vary during the fit. Parameter k_b, the rate constant for the cytoplasmic decay of tRNA, was set to the value 0.00022 min^{-1} (Lengyel & Penman, 1977). The time course of the experiment was not long enough to attain steady-state labeling of the tRNA; it was thus not possible to make an independent measurement of the half-life of tRNA in these experiments. The fact that $P(1)$ was set equal to $k_1 k_a / k_2$ did not affect the computer fit in terms of the values obtained and their respective root mean square errors.

Analysis of Rate of Labeling of tRNA Precursor. (a) Theory. The rate of accumulation of radioactivity in the tRNA precursor can be described by

$$dT'/dt = k_s S - k_p T' \quad \text{(A5)}$$

where T' is the dpm per aliquot in the tRNA precursor at time t, k_s is the absolute rate of synthesis of the tRNA precursor (picomoles of nucleotide per aliquot per minute) (which is the same as the rate of synthesis of total tRNA, k_a), S is the specific activity of the nucleotide precursor pool (dpm per picomole), and k_p is the first-order rate constant for the processing of the tRNA precursor. Rearranging eq A5, substituting eq 3 for S, and integrating with respect to t gives

$$T' = \frac{k_1 k_s}{k_2 k_p}(1 - e^{-k_p t}) + \frac{k_1 k_s (e^{-k_p t} - e^{-k_2 t})}{k_2 (k_p - k_2)} \quad \text{(A6)}$$

(b) Computer Analysis of Data. In order to fit the data, we set the group of parameters $k_1 k_s / k_2$ equal to $P(1)$. This reduces eq A6 to the same form as eq A4. When fitting the data, k_2, the rate constant for the equilibration of the pool, was fixed at the value determined by the analysis of the rate of labeling of total tRNA (see Computer Analysis of Data under Analysis of Rate of Labeling of tRNA).

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Purification and Characterization of the Intestinal Promoter of Iron(3+)–Transferrin Formation†

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ABSTRACT: The nonceruloplasmin enzyme located in the intestinal mucosa which promotes the incorporation of iron into transferrin has been resolved into a small, heat-stable component and a heat-labile protein component. The small, heat-stable component was purified from the high-speed supernatant of intestinal mucosal homogenates by ion-exchange chromatography and gel filtration and identified as xanthine. The heat labile protein component was purified from the high-speed supernatant of intestinal mucosal homogenates by heat treatment, gel filtration, and ion-exchange chromatography. The physical, spectral, and kinetic properties of the heat-labile protein component strongly suggest that it is xanthine oxidase. By promotion of the oxidation and incorporation of iron into transferrin, intestinal xanthine oxidase could perform a similar function in iron absorption as ceruloplasmin serves in the mobilization of iron from liver stores.

Strong evidence from several laboratories (Osaki & Johnson, 1969; Ragan et al., 1969; Osaki et al., 1970, 1971; Roeser et al., 1970; Evans & Abraham, 1973; Williams et al., 1974) has established that ceruloplasmin facilitates the mobilization of iron from liver stores. Considerable evidence suggests that ceruloplasmin facilitates iron mobilization by promoting the oxidation and incorporation of iron into transferrin (Osaki et al., 1966, 1970, 1971; Osaki, 1966; Osaki & Johnson, 1969). Although a physiological role for ceruloplasmin has been demonostrated in iron mobilization, Brittin & Chee (1969) found no relationship between ceruloplasmin and the intestinal absorption of iron. The dietary iron that traverses the intestinal mucosal cell must be eventually incorporated into transferrin for transport in the blood. The identification of a nonceruloplasmin enzyme in the intestinal mucosa that promotes the incorporation of iron into transferrin has been described in a preliminary report (Topham, 1978), and it has been postulated that this intestinal enzyme could serve a similar function in iron absorption as ceruloplasmin serves in iron mobilization. The present paper describes the purification and characterization of this enzyme system from rabbit intestinal mucosa. Data are presented which strongly suggest that this intestinal enzymatic promoter of Fe^{3+}–transferrin formation is xanthine oxidase.

Experimental Procedures

Materials

Rabbit Intestinal Mucosa. Frozen intestinal mucosa from the duodenum of young rabbits was purchased from Pel-Freez Biologicals, Inc., Rogers, AR.

Apotransferrin. A 2.0% (w/v) solution of iron-free transferrin (apotransferrin; Calbiochem-Behring, La Jolla, CA) was prepared in deionized, glass-distilled water and extensively dialyzed as previously recommended (Johnson et al., 1970). This apotransferrin was 98–99% pure as determined by polyacrylamide gel electrophoresis.

Homogenizing Media. High purity, crystalline sucrose was dissolved in deionized, glass-distilled water to yield a concentration of 0.25 M. Sodium azide, 0.02% (w/v), was added to the sucrose solution to prevent microbial growth. A previous report (Topham, 1978) indicated that the intestinal enzyme was insensitive to azide. The pH of this solution was adjusted to 7.4 with dilute NH_4OH. This homogenizing medium was used to prepare the intestinal extracts used for preliminary studies of the intestinal enzyme system and for the isolation and identification of the small, heat-stable component of the intestinal enzyme system.

The homogenizing medium used to prepare intestinal extracts for the purification and characterization of the heat-labile protein component was 0.05 M Hepes[1] buffer, pH 7.5. Better recoveries of the heat-labile component were obtained during the purification procedure with the Hepes buffer system.

Column Chromatographic Materials. Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, CA) was swollen and equilibrated in deionized, glass-distilled water. AG1-X2 anion-exchange resin (Bio-Rad Laboratories, Richmond, CA) was obtained in the acetate form and was washed extensively in deionized, glass-distilled water prior to use. AG 50W-X2 cation-exchange resin (Bio-Rad Laboratories, Richmond, CA) was obtained in the hydrogen form and was prepared as described by Bergmann & Dikstein (1958). DEAE-Sephadex A-50 and Sephadex G-200 (Pharmacia Fine Chemicals, Pis-

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[1] Abbreviations used: Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DEAE, diethylaminoethyl; Tf, transferrin; S-105, supernatant of intestinal mucosal homogenates following centrifugation at 105000*g* for 1 h.

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