Calculation of Absolute Rates of RNA Synthesis, Accumulation, and Degradation in Tobacco Callus in Vivo†

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ABSTRACT: Uptake and incorporation data were used to calculate an absolute rate of ribonucleic acid (RNA) synthesis by assuming that incorporation of radioactive nucleosides into RNA was a first-order function of the nucleoside triphosphate cellular pool. Homogenized tissue was separated into an RNA and cellular pool fraction. Methods were developed to directly measure the specific radioactivity of specific nucleosides in each fraction. Exogenous [^{3}H]uridine was taken up by the cells, converted to [^{3}H]uridine triphosphate ([^{3}H]UTP) and [^{3}H]cytidine triphosphate ([^{3}H]CTP), and incorporated into RNA. All of the radioactivity in RNA was contained in either uridine or cytidine. The average rate of RNA synthesis (20 mg of RNA (g of RNA)$^{-1}$ h^{-1}) was calculated from changes in the specific radioactivity of uridine or cytidine in RNA and UTP or CTP in the pool. Since ribosomal RNA does not turn over in exponentially growing tissue, its rate of synthesis was measured (14 mg g^{-1} h^{-1}) and compared with the rate of accumulation of RNA (12 mg g^{-1} h^{-1}). The similarity between ribosomal RNA synthesis and total RNA accumulation confirmed the assumption that these methods measured an absolute rate of RNA synthesis.

The absolute rates of synthesis of protein, deoxyribonucleic acid (DNA),[1] or ribonucleic acid (RNA) can be determined by analysis of the uptake of an appropriate radioactive compound into a precursor pool and the incorporation of the precursor into the product. In many cases, because of the difficulties encountered in measuring precursor pools, many assume that measurements of incorporation of radioactivity into the product alone will reflect the true rate of synthesis. The pitfalls in such an assumption are numerous (Kemp and Sutton, 1971; Kemp and Sutton, 1972; Kemp and Sutton, 1975).

Similarly, other investigators have addressed themselves to the problems encountered when equating rates of RNA synthesis with the rate of [^{3}H]uridine incorporation into RNA (Stambrook and Sisken, 1972; Epstein and Daentl, 1971; Roller et al., 1974; Weber, 1972; Daentl and Epstein, 1971). Therefore, before a rate of RNA synthesis can be calculated, it is imperative that the specific radioactivity of the nucleoside triphosphate precursors of RNA be measured.

This investigation was undertaken to develop procedures for accurately determining the specific radioactivity of both the nucleoside triphosphate pool and the nucleosides in RNA. Once these specific activities are measured as a function of time, an absolute rate of RNA synthesis can be calculated.

Materials and Methods

Callus tissue derived from the pith of *Nicotiana tabacum* L. var. Wisconsin No. 38 was maintained on Linsmaier and Skoog's (1965) medium supplied with 1 μM kinetin and 11.5 μM IAA. Growth rate determinations were made as described by Helgeson et al. (1969).

Exponentially growing tissues (3 days after transfer) were labeled for various lengths of time by moving them to an identical agar medium supplemented with 0.19 μM [5-^{3}H]-uridine (26 Ci/mmol, New England Nuclear).[2] Tissues were homogenized in 0.3 volume (w/v) of 0.1 M Tris-HCl, pH 7.5, and 1 volume (w/v) of H_2O-saturated redistilled phenol, pH 7.5, containing 0.01% 8-hydroxyquinoline. After addition of an equal volume of 3 mM EDTA, the nucleic acids were separated from the soluble nucleotides by precipitation from cold 70% ethanol. The precipitated nucleic acids were washed three times with cold 70% ethanol, and the washes were added to the soluble nucleotide pool fraction.

Purification and Specific Radioactivity of RNA. The nucleic acid fraction was dissolved in 0.025 M sodium phosphate, pH 6.8, and further purified on a hydroxylapatite column as described by Merlo and Kemp (1976). The nucleic acids were adsorbed to the hydroxylapatite (Bio-Rad Laboratories) at 60 °C and eluted with a linear gradient of sodium phosphate (0.025 to 0.3 M). RNA eluted between single- and double-stranded DNA. The quantity of RNA in each fraction was estimated by measuring its absorbance at 257 nm. Fractions containing RNA were combined, dialyzed against H_2O, and evaporated to dryness. RNA was dissolved in 1 ml of 3 mM Tris-HCl, pH 7.5, and incubated for 30 min at room temperature with 0.05 mg of heat-treated pancreatic ribonuclease (Worthington Biochemicals); then, 0.05 unit of phosphodiesterase from *Crotalus adamantus* venom (Sigma Chemical Company) was added, and incubation was continued for 30 min. Finally, 0.5 unit of alkaline phosphatase from *Escherichia coli* (Sigma Chemical Company) was added, and incubation was continued for 2 h. After incubation, the proteins were removed by precipitation with chloroform–1-octanol (10:1) and the pH of the supernatant was adjusted to 5 with HCl. These

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[1] Abbreviations used: cAMP, cyclic adenosine monophosphate; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; rRNA, ribosomal RNA; UMP, uridine monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; IAA, indole-3-acetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetracetic acid; uv, ultraviolet; ATP, adenosine triphosphate; GTP, guanosine triphosphate.

[2] Mention of companies or commercial products does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

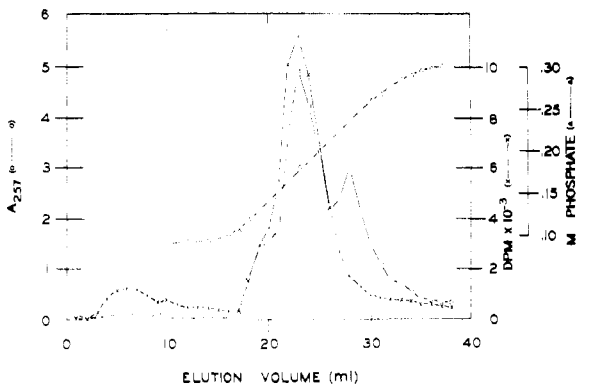


FIGURE 1: Chromatography of nucleic acids from callus tissue on hydroxylapatite. A nucleic acid preparation isolated from 3-day-old tissue labeled 5 h on media containing [^{3}H]uridine was chromatographed on a column of hydroxylapatite. Elution was accomplished at 60 °C with a linear 0.025 to 0.3 M sodium phosphate gradient (Δ-Δ). Nucleic acids were detected by their absorption at 257 nm (O-O). RNA was localized by measuring radioactivity (X-X) in an aliquot of each fraction.

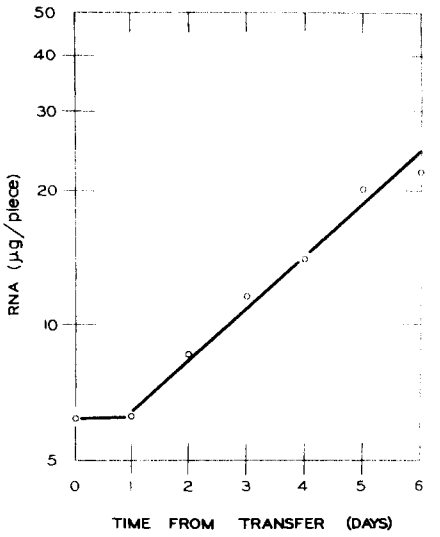


FIGURE 2: Accumulation of RNA in pieces of tobacco tissue at various times after transfer. Tissues were cut into pieces of approximately 20 mg fresh weight and transferred to fresh medium (Linsmaier and Skoog, 1965) containing 11.5 µM indoleacetic acid and 1.0 µM kinetin. At the times indicated, pieces were taken and total RNA was estimated as described in the Results. RNA accumulation was calculated directly from the doubling time (2.5 day) estimated between 1 and 5 days.

procedures did not hydrolyze the small amount of DNA that was occasionally present in RNA preparations.

Nucleosides from hydrolyzed RNA were chromatographed by a procedure modified from that of Uziel et al. (1968). Approximately 2 µmol of nucleosides was adsorbed to Aminex Q-15S cation exchange resin (Bio-Rad Laboratories) equilibrated at 50 °C with 0.4 M ammonium formate, pH 4.65, and eluted in 1-ml fractions with the same equilibration buffer. Each nucleoside was quantitated using its uv spectrum as described previously (Kemp and Sutton, 1976). This procedure separates all of the common ribonucleosides and deoxyribonucleosides. Radioactivity was measured in an aliquot from each fraction and specific radioactivity (defined as the number of curies of radioactivity co-chromatographing with uridine or cytidine per mole of nucleoside) was calculated.

Isolation of 28S Ribosomal RNA. An RNA preparation was centrifuged 15 h at 65 000 *g* through a linear gradient of sucrose, 7.5 to 30%, in 0.025 M sodium phosphate, pH 6.8. The gradient was fractionated and those fractions containing 28S RNA were combined, dialyzed, and hydrolyzed as described before.

Purification and Specific Radioactivity of Nucleoside Triphosphate Pools. The ethanol-soluble nucleotide pool fraction was evaporated in vacuo and the residue was dissolved in H_2O. Remaining traces of phenol were removed by extraction with diethyl ether.

The nucleotides were separated by a modification of a procedure described by Lindsay et al. (1968). A 1 × 9-cm column containing AGMP-1 anion exchange resin (Bio-Rad Laboratories) was washed with 1 M ammonium hydroxide and then equilibrated with 0.05 M ammonium chloride, pH 7.6. The sample was adjusted to pH 8 with HCl and adsorbed to the resin. The nucleotides were eluted at room temperature with a linear gradient of ammonium chloride, pH 7.6 (0.05 to 0.3 M). The eluate was collected in 2-ml fractions and an aliquot removed from each for measuring radioactivity. The nucleoside triphosphates eluted together between 0.22 and 0.26 M ammonium chloride. The fractions containing triphosphates were pooled and dialyzed against distilled water for 6 h. This procedure removed more than 90% of the salt but less than 20% of the nucleoside triphosphates. The sample was evaporated to dryness, dissolved in 3 mM Tris, pH 7.5, and finally hydrolyzed to nucleosides by the addition of 0.5 unit of *E. coli* alkaline phosphatase. Protein was removed with chloroform–1-octanol (10:1), the pH adjusted to 5 with HCl, and the sample adsorbed to the same Aminex Q-15S resin used in determining the base composition of the RNA. Each nucleoside was quantitated from its ultraviolet spectrum and specific radioactivity calculated as before.

Results

RNA Accumulation. RNA eluted from hydroxylapatite at 0.18 M sodium phosphate and was separated from either single- or double-stranded DNA as illustrated by a preparation from 3-day-old tissue labeled 5 h (Figure 1). The small peak of nucleic acid eluting at 0.23 M sodium phosphate was double-stranded DNA as documented in previous work (Merlo and Kemp, 1976). Ninety percent of the radioactivity co-chromatographed with RNA with most of the remaining 10% eluting at the void volume for the column. The amount of RNA in tissues was calculated from the sum of the absorbances at 257 nm of fractions eluting from the hydroxylapatite column between 0.1 and 0.2 M sodium phosphate. For these calculations RNA at a concentration of 50 µg/ml was assumed to have an absorbance at 257 nm of 1.0.

Callus tissue did not begin accumulating RNA until after 24 h from the time of transfer (Figure 2). The tissue then began accumulating RNA exponentially with a doubling time equal to 2.5 days. This doubling time is equal to a rate of 12 mg of RNA accumulated (g of RNA)$^{-1}$ h^{-1}.

Distribution of Radioactivity and Nucleosides from RNA. As pointed out earlier, it is necessary to measure the specific radioactivity of a particular nucleoside from RNA if an absolute rate of RNA synthesis is to be calculated. The specific radioactivity of uridine and cytidine in RNA was calculated after the RNA was purified on a hydroxylapatite column and hydrolyzed and the resulting nucleosides were separated on an Aminex Q-15S column. As illustrated with tissue cultured 3 days and labeled 5 h, the four nucleosides were well separated (Figure 3) and easily identified by their characteristic uv absorption spectra. Nucleoside concentrations were calculated from the molar extinction coefficient at the λ_{max} as determined for authentic compounds in 0.4 M ammonium formate, pH

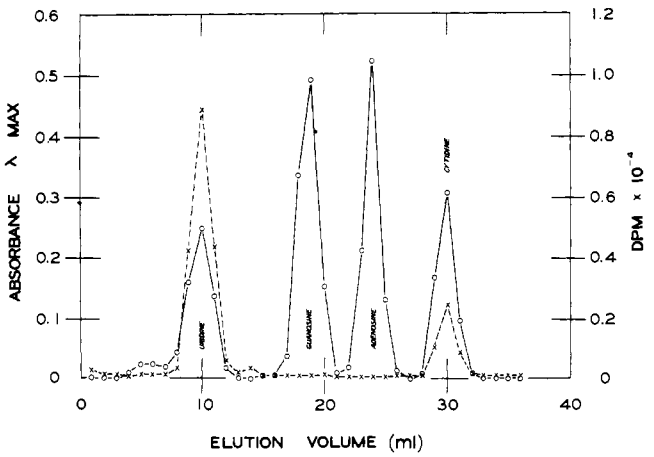


FIGURE 3: Separation of ribonucleosides from hydrolyzed radioactive RNA. RNA from 3-day-old tissue labeled 5 h on medium containing [^{3}H]uridine was enzymically hydrolyzed and chromatographed on Aminex Q-15S resin as described in Materials and Methods. Absorption (O-O) of each nucleoside measured at its λ_{max} in formate buffer (262 nm, uridine; 253 nm, guanosine; 258 nm, adenosine; and 272 nm, cytidine). The RNA from tobacco callus contained 22% uridine, 31% guanosine, 24% adenosine, and 23% cytidine. Radioactivity (X- - -X) was measured in an aliquot of each fraction.

4.65. Radioactivity in the hydrolyzed RNA co-chromatographed with both uridine and cytidine. Uridine contained approximately 80% of the radioactivity and cytidine 15%, with the remaining 5% in uracil or other derivatives of uridine (Figure 3). If tissues were labeled for shorter periods of time (1 h), uridine contained 94% of the radioactivity and cytidine only 4%. This result is understandable if UTP is a precursor for CTP as proposed by others (Ross and Cole, 1968; Long et al., 1969). Specific radioactivities of uridine (SA_u RNA) and cytidine (SA_c RNA) were 0.51 and 0.13, respectively.

Distribution of Radioactivity in Nucleoside Triphosphate Pool. Another measurement necessary for calculating a rate of RNA synthesis is the specific radioactivity of the precursor pool. Because tissues were labeled with [^{3}H]uridine, this investigation centered on the UTP and CTP pools. Authentic UMP, UDP, and UTP were chromatographed on AGMP-1 resin (Figure 4) to determine the order and point of elution of these nucleotides. All three nucleotides were well separated. Other nucleoside triphosphates (CTP, ATP, GTP) co-chromatographed with UTP while uridine and UDP-glucose co-chromatographed with UMP.

Seven percent of the radioactivity contained in the ethanol-soluble pool fraction of labeled tissue eluted from the AGMP-1 resin with the nucleoside triphosphates (Figure 4). The large percentage of radioactivity (75%) that eluted with UMP was probably associated with uridine with a small part of the total in UMP or UDP-glucose.

The fractions eluting from the AGMP-1 resin between 54 and 62 ml (Figure 4) were pooled and hydrolyzed by alkaline phosphatase. Chromatography of the hydrolysate on Aminex Q-15S resin (Figure 5) revealed that radioactivity co-chromatographed with both uridine and cytidine as it did with hydrolyzed RNA. Again, specific radioactivities were calculated for both uridine (SA_u pool = 9.5) and cytidine (SA_c pool = 3.2) with the ratio of these specific radioactivities about the same as those found in RNA (SA_u pool/SA_c pool = 3). The amount of UTP and CTP in the tissue was approximately 0.03 and 0.01 μmol/g fresh weight, respectively, and remained constant throughout tissue growth. The large uv-absorbing peak at the void volume was due to organic solvents.

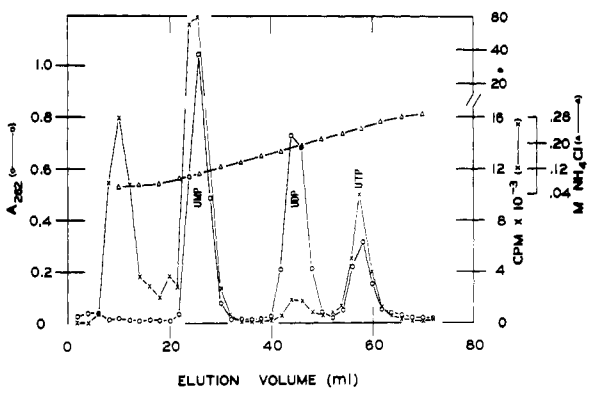


FIGURE 4: Separation of nucleotides. Authentic UMP, UDP, and UTP were separated on AGMP-1 resin with a linear gradient of 0.05 to 0.3 M ammonium chloride (Δ-Δ). Nucleotides were detected by their absorption at 262 nm (O-O). After regeneration of the AGMP-1 resin, the nucleotides in the ethanol soluble pool fraction from tobacco callus tissue described in Figure 3 were separated. Radioactivity (X-X) was measured in an aliquot of each fraction. The nucleoside triphosphates eluting between 54 and 62 ml were pooled for further analysis.

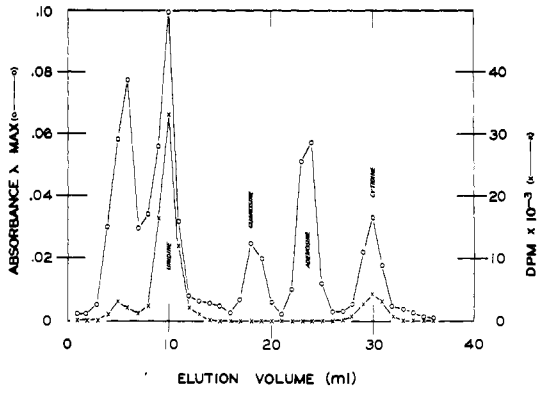


FIGURE 5: Nucleoside triphosphates purified from the column in Figure 4 were hydrolyzed with alkaline phosphatase and chromatographed on the same column as described in Figure 3. The open circles represent the absorbance at the λ_{max} for each of the nucleosides. The X's represent the radioactivity in an aliquot of each fraction.

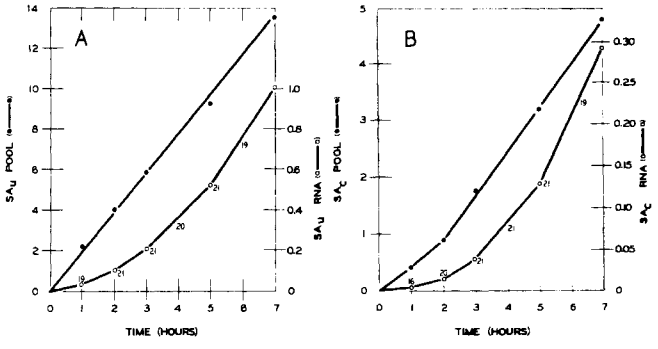


FIGURE 6: Changes with labeling time in the specific radioactivities of uridine (A) and cytidine (B) present in RNA (O-O) and in the precursor pool (●-●) of exponentially growing 3-day-old tobacco callus tissue. The numbers shown adjacent to the SA RNA curves are the rates of synthesis in mg of RNA (g of RNA^{-1}) h^{-1} calculated as described in Results.

Synthetic Rates. The specific radioactivity of UTP in the pool from tissue placed on medium containing [^{3}H]uridine increased linearly from 1 through 7 h of labeling (Figure 6A). Over the same labeling period, the increase in the specific radioactivity of uridine in RNA followed a curve the shape of which was predictable from the following set of equations

(Figure 6A). Similarly, the shape of the curve generated by the increase in the specific radioactivity of cytidine in RNA was predictable (Figure 6B). The equations used in our calculations assume that the only source of radioactivity for uridine in RNA or for cytidine in RNA was the UTP and CTP pools, respectively.

The rate of RNA synthesis (R) was related to changes in SA pool and SA RNA data by the following equation:

$$\frac{d(\text{SA RNA})}{dt} = R(\text{SA pool}) \quad (1)$$

Since SA_u pool increased linearly with time, SA_u pool = kt, kt can be substituted for the SA_u pool in eq 1. Integrating eq 1 after substitution

$$SA_u\ RNA = kRt^2/2 \quad (2)$$

where t is time in hours. We empirically determined that k equaled 2 (Figure 6A) and substituted this value into eq 2:

$$SA_u\ RNA = Rt^2 \quad (3)$$

Rates of RNA synthesis calculated for each time point in Figure 6A were scattered ranging from 18 to 22 mg g^{-1} h^{-1} with an average of 20 mg g^{-1} h^{-1}.

Rates of RNA synthesis were also calculated from the changes in SA_c pool and SA_c RNA (Figure 6B). Since SA_c pool was not a simple function of t, an alternate method was chosen for estimating synthesis. This method measures an instantaneous rate of change of SA_c RNA by drawing a tangent to the SA_c RNA curve (Figure 6B) at any particular time, and estimating synthesis by dividing the instantaneous rate of change by the value for SA_c pool at that time. This method follows directly from eq 1 and has been used successfully in the past (Kemp and Sutton, 1972). The numbers next to the SA RNA curves in both Figures 6A and 6B were derived in this manner. In both cases the average rate of RNA synthesis was identical with that calculated from eq 3, 20 mg g^{-1} h^{-1}. Therefore, the same rate of synthesis could be calculated from either UTP or CTP incorporation data.

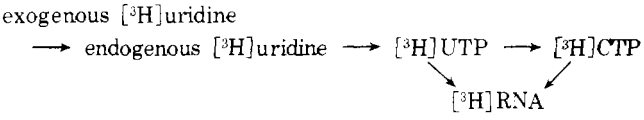
The difference between the rate of RNA synthesis (20 mg g^{-1} h^{-1}) and accumulation (12 mg g^{-1} h^{-1}) was taken as the average rate of RNA degradation in this tissue. The low rate of degradation (8 mg g^{-1} h^{-1}) either represented a small amount of turnover of all classes of RNA or, more likely, a high rate of turnover of some small specific class of RNA molecules and little turnover in the bulk of the RNA. We measured the rate of synthesis of 28S rRNA because ribosomal RNA composes 75% of the total RNA and was reported in other organisms not to turn over (Emerson, 1971; Weber, 1972; Wiegers et al., 1975).

Three-day-old tissue was labeled for 2 and 5 h on medium containing [^{3}H]uridine and the total RNA purified as before. A fraction was removed and SA_u and SA_c RNA were determined as before. The average rate of synthesis was again about 20 mg g^{-1} h^{-1}. The various RNA species in the remaining fraction were separated by sedimentation through sucrose. The fractions containing 28S rRNA were collected and dialyzed, and SA_u and SA_c were determined. The results were a reduction of 29% in both SA_u and SA_c of rRNA, indicating that the rate of 28S rRNA synthesis was 14 mg g^{-1} h^{-1}. The similarity between the rate of synthesis of rRNA and the rate of accumulation of total RNA suggested that we were measuring an absolute rate. Therefore, the measured turnover may be due to a high rate of turnover in some small specific class of RNA molecules.

Discussion

Radioactive incorporation of exogenous, labeled nucleosides or amino acids into acid-precipitable material is repeatedly used to calculate at least a relative rate of synthesis. Many times such data are compared from one experiment with the next and differences interpreted as changes in rates of synthesis when in fact they may be due to differences in the synthesis of unrelated, acid- or ethanol-precipitable material or to a change in the distribution of radioactivity within precursor pools. For example, we previously demonstrated that a tenfold increase in the rate of incorporation of [^{3}H]leucine into protein of cultured tobacco pith was due to a tenfold change in the specific radioactivity of the precursor pool, not to an increase in the rate of synthesis (Kemp and Sutton, 1972). Similarly, Roller et al. (1974) demonstrated that an increase in the rate of incorporation of [^{3}H]uridine into RNA of CV-1 cells when treated with cAMP was due to a change in the precursor pool rather than an increase in RNA synthesis. We, therefore, want to emphasize the importance of examining an individual precursor in both the pool and product whether one measures protein or nucleic acid synthesis.

Our data can be explained as follows if we assume that the flow of radioactivity from the medium to RNA is:



If the uptake of exogenous [^{3}H]uridine is diffusion limited (as was shown for leucine uptake, Kemp and Sutton, 1971), then the SA of endogenous [^{3}H]uridine should be constant over the labeling period. With a constant uridine pool, the rate of incorporation of radioactivity into UTP should be linear with time, as was found (Figure 6A). Finally, the rate of incorporation of radioactivity from UTP into CTP and RNA should be nonlinear (Figures 6A and 6B). This model can be expressed mathematically by assuming first-order rate kinetics (eq 1) and will quantitatively predict the shape of SA RNA curves from SA pool curves. As an example, SA_u pool changed linearly with time (Figure 6A) predicting that SA_u RNA should change linearly with the square of time (eq 3). Such a replotting of the data from Figure 6A confirmed this prediction. Others (Rutherford et al., 1974; Wiegers et al., 1975) have also shown similar kinetics and have argued in favor of using radioactive uptake and incorporation data for measuring rates of synthesis of RNA.

Some investigators, even those who consider precursor pools, still differ in their conclusions concerning the validity of calculating rates of synthesis from radioactive data (Steward and Bidwell, 1966; Oaks and Bidwell, 1970; Plagemann, 1972; Goody and Ellem, 1975). The differences usually center around the question of whether kinetic changes in incorporation can be explained by concurrent changes in uptake. As an example, Goody and Ellem (1975) concluded that the pool of UTP forms a compartment within the cell and has kinetics of labeling distinct from the general UTP pool. Their conclusion was based primarily on data indicating that the change with labeling time in SA_u pool formed a biphasic curve, whereas the change with time in the incorporation of label into RNA was linear. However, their data are difficult to interpret because only three data points are shown with the earliest point at 10 min. As documented by Rutherford et al. (1974), appropriate labeling times for liquid cell cultures can be quite short (several minutes). This is in contrast to labeling on agar surface where times can be much longer (several hours). Although we are not

implying that separate UTP pools cannot exist, we suggest that, if separate pools do exist, they are in rapid equilibrium and have the same specific radioactivities.

The predictability of the shape of SA RNA curves from SA pool curves strongly suggests that if separate UTP and CTP precursor pools do exist, they must be in the same ratio to the total UTP and CTP pools at all labeling times. Further, the estimation of the same rate of RNA synthesis (20 mg g^{-1} h^{-1}) from both cytidine and uridine incorporation data suggests that these ratios must be the same for both UTP and CTP. If this ratio were different from one, it would have the effect of raising or lowering the measured rate of synthesis by a constant factor (this ratio). Since the rate of 28S rRNA synthesis was the same as the rate of RNA accumulation, we concluded that the pool ratios discussed above must be one (i.e., if a second precursor pool exists, it must be in equilibrium with, and have the same specific radioactivity as, the total pool). Therefore, the rates of syntheses (20 mg g^{-1} h^{-1} for total RNA and 14 mg g^{-1} h^{-1} for rRNA) measured by radioactive uptake and incorporation were absolute rates.

An advantage of our procedure for measuring a rate of RNA synthesis is that it allows us to calculate specific activities from direct measurements of the number of curies of radioactivity per mole of nucleoside. The concentration of nucleoside triphosphate may be so low in some systems that it is not practical to measure it directly. Therefore, there will always be some doubt as to validity of reported rates of synthesis in these systems. Another advantage of our system is that appropriate labeling times are practical, whereas some systems may require such short times that accurate experimentation is not possible.

Acknowledgment

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