mRNA TURNOVER STUDIES APPLYING LABELED URIDINE REQUIRE AN EVALUATION OF SPECIFIC RADIOACTIVITIES OF UTP AND RNA-U

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SUMMARY

In (^{3}H)uridine labeling experiments as applied for the determination of RNA turnover, several parameters have been widely neglected which influence considerably the interpretation of the data:

- The specific radioactivity of the UTP pool in HeLa cells attained steady state level only after two - three hours.
- Even 10^{-5} exogenous (^{3}H)uridine did not guarantee a constant supply of the label in long-term ($\gtrsim$ 6 hours) experiments as indicated by a decrease in exogenous uridine and a decrease in UTP specific radioactivity. This, however, could be overcome by continuous infusion of (^{3}H) uridine.
- Pyrimidine nucleotide synthesis de novo was only partially suppressed by exogenous uridine (- 10^{-5}M caused 55% inhibition -). Thus, steady state values of UTP specific radioactivity were considerably lower than those of exogenous uridine.
- (^{3}H)uridine labeling led to a rather rapid equilibration of the UTP and CTP pools with an equal labeling of U and C in (total) RNA after 6 hours of incubation. Separation and analysis of individual RNA bases, therefore, are required, when labeling kinetics are to be used to evaluate RNA turnover.

The analysis of mRNA turnover in mammalian cells has gained new interest when it became clear that the recommended use of actinomycin (cf. 1) led to erroneous results because of interference with RNA metabolism (cf. 2) and of non-selectivity towards inhibition of ribosomal RNA formation (3, 4). All new approaches to the problem (cf. 5,6,7), however, rely on several assumptions which, to our knowledge, have not been verified. This paper demonstrates that

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kinetic analyses of uridine incorporation as applied to RNA turnover studies require - in addition to a selective determination of the RNA species - the analysis of the UTP pool and the kinetics of the (^{3}H)U $\longrightarrow$ (^{3}H)C conversion.

MATERIALS AND METHODS

(^{3}H)uridine (uridine-5-T; spec.act. 30 Ci/mmole) and (^{14}C)aspartate (uniformly labeled; spec.act. 230 mCi/mmole) were purchased from The Radiochemical Centre, Amersham, England; modified Joklik medium (F-13) from Grand Island Biological Comp., USA; cofactors and enzymes from E. Merck, Darmstadt, and from Boehringer, Mannheim, Germany; NP 40 was obtained from Shell Comp.; Proteinase K (chromatographically pure) was a generous gift from Dr. H. Lang (E. Merck, Darmstadt).

HeLa S3 cells were grown in suspension culture or as monolayers as described previously (8). Mean generation time was 24 hrs.

UTP specific radioactivity was determined by the method of 'enzymic displacement' (electrophoresis $\pm$ enzymic conversion to UDPG) as described previously (8). An aliquot of the cell suspension (3 - 5 x 10^6 cells) was centrifuged, washed with saline, and the pellet was extracted with 1.0 ml 4% $HClO_4$ (10 min, 0°). After centrifugation the supernatant was neutralized with solid $KHCO_3$ at 0°. $KClO_4$ was removed by centrifugation. The supernatant was used for UTP analysis (8).

Analysis of base labeling in RNA: The acid insoluble residue (see above) was washed with cold 4% $HClO_4$, and incubated with 0.5 N KOH at 20° overnight. After precipitation of DNA and protein by $HClO_4$, the supernatant obtained by centrifugation was analyzed for base composition and base specific radioactivity using electrophoretic separation of the nucleotides at pH 3.5.

(^{3}H)uridine in the medium was analyzed in 1.0 ml aliquots by precipitating proteins with 150 µl 16% $HClO_4$, centrifuging after 10 min in ice, neutralizing 1.0 ml of the supernatant with 35 µl 10 N KOH, and centrifuging again. An aliquot of the supernatant was taken for determination of radioactivity. 30 µl were cochromatographed with 120 nmoles of uracil and 120 nmoles of uridine (Schleicher and Schüll paper no. 2043; butanol / H_2O (86/14 v/v) for 16 hours). The uracil and uridine spots as well as blanks were eluted with 1.0 ml 0.1 N HCl for one hour at room temperature. Radioactivity and A_{260} were determined in an aliquot of the eluates.
Determination of specific radioactivity of polysome-associated mRNA was performed as described previously (9). Isolated polysomes (6 A_{260}-units/ml) were incubated at 0° with pancreatic ribonucle-

ase (2×10^{-1} µg/ml) for 0, 20 and 40 min in duplicates, and precipitated with $HClO_4$. A_{260} and radioactivity of the acid-soluble mRNA split products were determined in the supernatant. The high specificity of this test is documented in a preceeding paper (9).

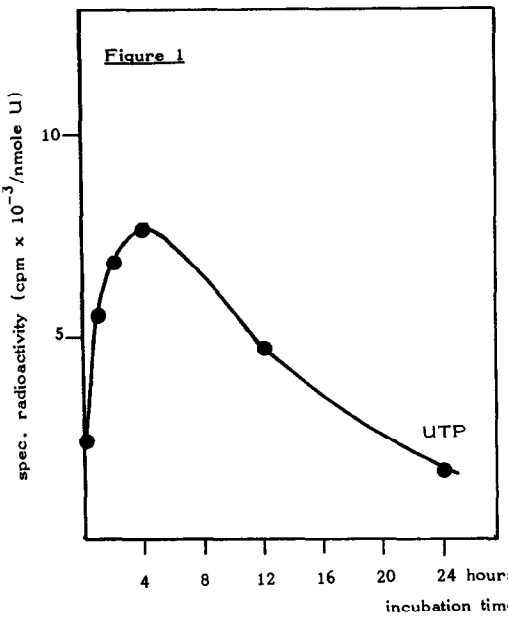
Determination of specific radioactivity of ribosomal RNA: An aliquot of the ribonuclease-treated polysomes (20 min; see above) was incubated with proteinase K (10,4) in order to inactivate the enzyme and to isolate the RNA, which was then precipitated with alcohol and kept overnight at -20°. The different RNA species were separated by sucrose gradient centrifugation (5 - 20% w/v in TNE buffer (10); Spinco SW 40 rotor for 18 hrs at 25 000 rpm and 4°). Fractions of 0.5 ml were collected and incubated with 0.5 ml 0.5 N KOH at room temperature overnight. After addition of 0.5 ml ice-cold 8% $HClO_4$ and centrifugation, an aliquot of the supernatant was taken for determination of A_{260} and radioactivity. Counts were corrected for quenching by the internal standard method. Percentage of cpm in U was determined after electrophoresis of hydrolyzed (total) RNA. Specific radioactivity of U (cpm/nmole U) was calculated assuming 28 A_{260} units per mg hydrolyzed RNA, and 16.5% U in 28 S RNA (11) and 23.8% U in mRNA (12).

RESULTS

1. Specific radioactivity of UTP never reaches specific radioactivity of exogenous (^{3}H)uridine in standard labeling experiments.

When HeLa suspension cultures were incubated with (^{3}H)uridine below 10^{-6}M concentrations, a rapid exhaustion of the labeled precursor took place due to uptake by the cells and to degradation (8). This resulted - after an initial rise - in a rapid decrease in UTP specific radioactivity. Even at a level of 2×10^{-5}M, no constant supply was guaranteed for incubation times exceeding 6 hours as measured by chromatographic analysis of the medium and a decrease in specific radioactivity of UTP (fig. 1). To keep the exogenous concentration of uridine at a constant level a continuous infusion of medium containing (^{3}H)uridine of the same specific radioactivity had to be applied. Thus, a constant specific radioactivity of UTP over extended periods was finally reached (fig. 2). When experiments were performed under these conditions (fig.2), two unexpected results were obtained:

- It took two - three hours ($\sim$1/10 of the generation time) until UTP reached steady state labeling.



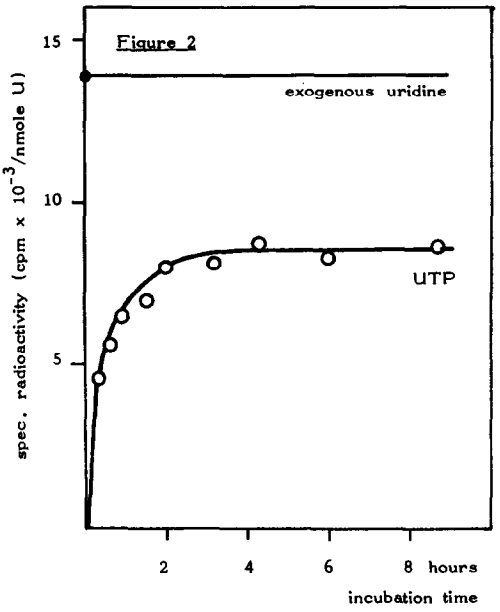


Figure 1: Kinetics of UTP specific radioactivity in HeLa cells labeled with (^{3}H)uridine. - 2.4 ml (^{3}H)uridine were added to 3500 ml HeLa suspension culture (0.27 x 10^6 cells/ml) to give a final concentration of 2 x 10^{-5} M uridine and a specific radioactivity of 5.1 x 10^4 cpm/nmole U. At the times indicated 50 ml suspension were taken to determine UTP specific radioactivity as described in methods.

Figure 2: Kinetics of UTP specific radioactivity in HeLa cells under continuous labeling with (^{3}H)uridine. - 1.9 ml (^{3}H)uridine were added to a 4000 ml HeLa suspension culture (0.3 x 10^6 cells/ml) to give a final concentration of 1 x 10^{-5} M uridine and a specific radioactivity of 1.4 x 10^4 cpm per nmole. 3.8 ml (^{3}H)uridine were added to 40 ml medium (final concentration 2 x 10^{-3}M uridine; 1.4 x 10^4 cpm per nmole U) and pumped into the culture at a velocity of 1.0 ml/hour. At the times indicated two aliquots of 10 ml each were taken to determine UTP specific radioactivity as described under methods. The level of (^{3}H)uridine in the medium was analyzed at different times as described in methods.

- The final specific radioactivity of UTP remained at a value which was only about 2/3 of the specific radioactivity of the constantly supplied exogenous (^{3}H)uridine.

Usually, the 'labeling-through' of precursor molecules appears to be rapid. The rather slow labeling of UTP apparently was due to

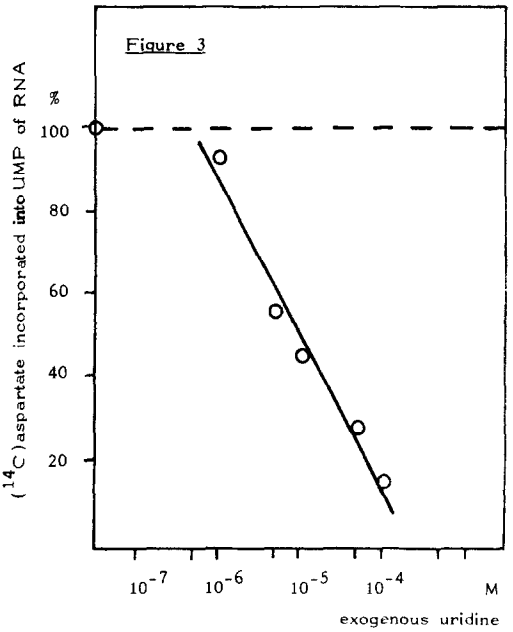


Figure 3: Uncomplete suppression of UTP synthesis de novo by exogenous uridine. -
HeLa monolayer cultures (2.7×10^7 cells/culture containing 80 ml medium) were incubated for 30 min with cold uridine at the concentrations indicated. 200 ul (^{14}C)aspartate/culture were then added and incubation continued for another 2 hours. Controls were run without uridine. Incubation was stopped by pouring off the medium, washing the cells once with isotonic saline and adding 2.5 ml cold 4% $HClO_4$. The acid-insoluble fraction was hydrolyzed, and radioactivity in UMP was determined electrophoretically as described in methods. In each case the percentage of radioactivity found in UMP was calculated. This value in the controls without exogenous uridine was than taken as 100%.

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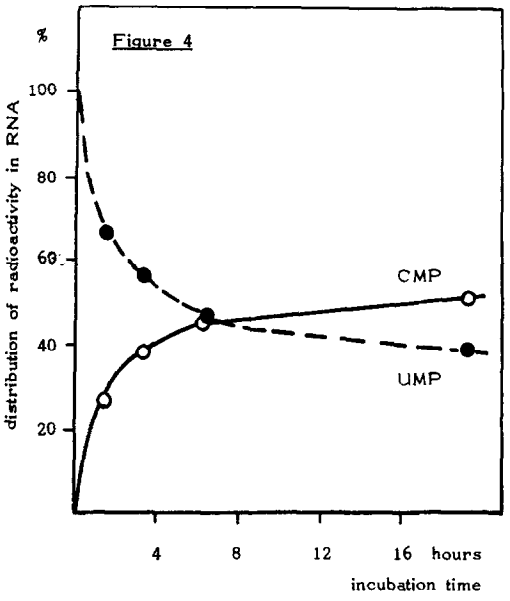
its relatively slow consumption in RNA formation which in turn is the consequence of the relatively high UTP concentration in these cells (8): Normal levels per cell would allow the formation of 1/10 of the total RNA-U of a daughter cell which means that only after 1/10 of the generation time the unlabeled UTP could be consumed for the formation of new RNA and be replaced by labeled UTP (not considering RNA turnover). In addition there is a (rapid) expansion of the UTP pool by a factor of two at these concentrations of exogenous uridine (cf.8).

More important, however, is the observation that the steady state



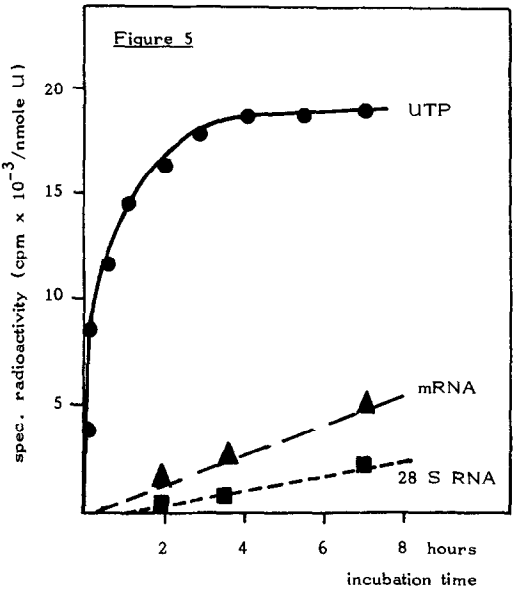


Figure 4: Labeling of CMP in RNA on incubation of HeLa cells with (^{3}H)uridine.
0.55 ml (^{3}H)uridine was added to 1000 ml suspension culture (0.17 x 10^6 cells/ml) to a final concentration of 5 x 10^{-6}M and 3.6 x 10^4 cpm/nmole U). This (^{3}H)uridine concentration was kept constant by continuous infusion of 5.8 ml/hour of medium containing 2.65 x 10^6 cpm and 73 nmoles (^{3}H)uridine /ml medium. At the indicated times 40 ml of the culture were centrifuged. The acid-insoluble fraction was prepared, and the RNA bases analyzed as described in methods.

Figure 5: Labeling kinetics of UTP, polysome-associated mRNA, and ribosomal 28 S RNA. -
A 4000 ml culture (0.23 x 10^6 cells/ml) was labeled with (^{3}H)uridine (2.8 x 10^4 cpm; final concentration = 1.2 x 10^{-5}M). 2 x 10 ml were withdrawn for the determination of UTP specific radioactivity at the times indicated. Specific radioactivities of mRNA and 28 S RNA were determined from 400 ml aliquots of the culture as described under methods.

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level of specific radioactivity finally reached by UTP was only about 60% of that of exogenous (^{3}H)uridine. These findings should have consequences for the evaluation of certain RNA turnover experiments which are based on the assumption of identical specific radioactivities of exogenous uridine and UTP (cf.5).

2. Pyrimidine nucleotide synthesis de novo is suppressed only to 60% by 10^{-5}M exogenous uridine.

The fact that UTP specific radioactivity remained at a distinctly lower value than exogenous uridine, cannot be explained by the presence of two UTP pools (cf.13). If present, they might turnover at different rates. However, they should be in equilibrium in less than one generation time. Furthermore, Wu and Soeiro have provided evidence for a single ribonucleotide pool for (nuclear) RNA in HeLa cells (14). Rather, an incomplete inhibition of pyrimidine nucleotide synthesis de novo appeared to be responsible for the lower specific radioactivity of UTP by providing 1/3 of the UTP needed in unlabeled form. The validity of this interpretation has been verified by measuring uridine nucleotide formation de novo in the presence of exogenous uridine (fig.3): Although incorporation of (^{14}C) aspartate into RNA-U (and into total RNA) showed an increasing suppression by increasing uridine concentrations, inhibition was only partial. Even 10^{-4}M exogenous uridine could not inhibit synthesis de novo completely.

3. (^{3}H)uridine labeling leads also to a rapid labeling of the CTP pool and of CMP residues in RNA.

CTP levels in mammalian cells are considerably lower (2 - 8 times) than UTP concentrations (cf.13,15-17). As C is present in RNA in similar amounts as U, RNA formation, then, requires much higher rates of synthesis of CTP than of UTP. The higher turnover of the CTP pool would favor a rather rapid equilibration with the UTP-pool. This was indeed indicated by the labeling pattern of CTP in (^{3}H)uridine labeled HeLa cells (not shown). The rapid appearance of ^{3}H in CTP led to a fast labeling of C in total RNA (fig.4): Within 6-7 hours, radioactivity in RNA-C reached the same percentage as RNA-U indicating again fairly rapid equilibration of UTP and CTP pools in HeLa cells. Label in AMP and GMP residues was less than 5%. These data confirm similar observations made by Plageman with respect to C-labeling in RNA (17). They show that long term (^{3}H)uridine incorporation into RNA also reflects C labeling.

4. Turnover studies of mRNA and of ribosomal RNA with (^{3}H)uridine require methods to analyze UTP specific radioactivity and C-labeling of RNA.

Obviously, any analysis of RNA turnover based solely on uridine labeling (cf. 5) will lead to erroneous results. As shown in fig. 2 and 5, it took more than two hours until UTP had attained a constant specific radioactivity. Furthermore, the steady state level of UTP specific radioactivity remained considerably below that of the exogenous (^{3}H)uridine (cf. fig. 2). In addition, the rather rapid equilibrium of the CTP pool which is indicated by the labeling kinetics of C in RNA (fig. 4), precludes simple deductions based on an assumed specificity of the radioactive precursor (cf. 5,7). In long-term experiments, even DNA will become labeled via CTP⟶dCTP inspite of using 5-T-uridine. An exact analysis of the turnover of RNA species therefore requires a selective evaluation of the specific radioactivity of the RNA fraction in question as well as a determination of the percentage of label in the different RNA bases. Methods for a direct and selective determination of specific radioactivities of polysome-associated mRNA and 28 S RNA without interference by inhibitors have been worked out in our laboratory (9,4). The distribution of label in RNA bases is described in this communication. Fig. 5 shows a labeling experiment taking into account these parameters. Labeled with these rates, specific radioactivity of mRNA would reach 50% of UTP specific radioactivity after 14 hours. This indicates rather slow turnover of polysome-associated mRNA (but definitely faster than the values reported by Murphy and Attardi (7)). Specific radioactivity of 28 S RNA would reach 50% of UTP specific radioactivity after 26 hours, which is about one generation time of HeLa cells. This may indicate that 28 S RNA is a stable RNA species with no detectable turnover.

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