RAPID LABELING OF DNA-C DURING INCUBATION OF HELA CELLS WITH (5-T) URIDINE

Ulrich WIEGERS* and Karin KLAPPROTH
Institut für Physiologische Chemie der Universität Hamburg, 2 Hamburg 20, Martinistraße 52, West Germany

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1. Introduction

(5-T) uridine is a widely used radioactive precursor for the analysis of RNA metabolism. It has been proposed as a specific marker for RNA formation, cf. [1] because its label is lost during conversion from U to dT. As this may be valid for very short incubation times, it certainly introduces serious errors in studies exceeding 1/10 of the cell doubling time. As shown in this paper, the radioactivity very soon enters DNA via [^{3}H]UTP→[^{3}H]CTP→[^{3}H]dCTP conversion, thus labeling DNA exclusively in C bases. The U to C conversion was analysed in HeLa suspension cultures labeled with (5-T) uridine at different concentrations. C bases in RNA became rapidly labeled, though with some delay to U bases. The time needed to reach equilibrium depended on the concentration of the exogenous uridine indicating profound differences of UTP and CTP pools under these conditions. Base analysis of DNA showed a time-dependent increase in radioactivity which was exclusively located in the cytosine residues.

2. Material and methods

(5-T) uridine (spec.act. 25 Ci/mmole) was purchased from Buchler-Amersham, Braunschweig; modified Joklik medium (F-13) from Grand Island Biological Comp., USA; Nucleotides, nucleosides, and bases from Boehringer, Mannheim. HeLa S3 cells were grown in suspension culture as described previously [2]. Labeling was performed in suspension culture ($\sim$0.3 × 10^6 cells/ml) at the given concentrations of (5-T) uridine.

2.1. *Acid-insoluble fraction*

At the times indicated aliquots of the cell suspension (1–3 × 10^7 cells) were taken and centrifuged at 1200 *g*. The pellet was extracted and washed twice with ice-cold 0.4 N $HClO_4$. The acid-insoluble residue was incubated with 0.5 N KOH for 18 hr at 25°C. DNA and proteins were precipitated by acidification with $HClO_4$. RNA nucleotides in the supernatant were analysed by paper electrophoresis at pH 3.5 [3]. The pellet was washed twice with 0.4 N $HClO_4$ and analysed for DNA content according to Burton [4]. For radioactivity determination the alkali-resistant residue was hydrolysed with 3 N $HClO_4$ for 30 min at 95°C.

2.2. *DNA base analysis*

For analysis of DNA bases labeling an aliquot of the cell suspension (0.5–1.5 × 10^7 cells) was centrifuged off and the pellet was extracted with 5% TCA. The pellet was hydrolysed with 0.5 N KOH for 18 hr at 25°C to remove RNA. DNA and proteins were precipitated with 10% TCA. The pellet was washed twice with 5% TCA and dissolved in 0.5 ml of 98% formic acid. DNA was hydrolysed for 45 min at 175°C. After hydrolysis the formic acid was evaporated and the residue dissolved in water. Insoluble material was removed by centrifugation, and radioactivity determined in the supernatant. Aliquots (10–100 µl) were taken and the bases were separated by descending paper chromatography (Schleicher and Schüll 2043 b) according to Chandra [5], using isopropanol– conc. HCl–H_2O (65/16.5/18.5; v/v/v) as solvent. Non-label-

* Present address: I. Medizinische Universitätsklinik Hamburg, 2 Hamburg 20, Martinistraße 52, West Germany.

ed bases were added as u.v. markers. The chromatography was run up to 30 cm from the origin. For analysis of the radioactivity the paper was cut into 2 cm stripes, eluted with 1.0 ml 1N HCl and 0.2 ml were analysed for cpm. All radioactivity determination were corrected for quenching by the internal standard method.

3. Results

HeLa S3 suspension cultures were labeled with (5-T) uridine at three different concentrations (1.6×10^{-8} M, 1×10^{-5} M and 2×10^{-4} M uridine). The radioactivity in the acid-insoluble fraction was analysed. At all times of incubation radioactive material was found which proved resistant to extensive alkaline hydrolysis (0.5 N KOH for 18 hr at 25°C) which therefore was not identical with RNA [6]. The resistant material could be split into acid-soluble products by incubation with DNase or by incubation with 3 N $HClO_4$ at 95°C for 30 min indicating labeled DNA. Table 1 shows the distribution of radioactivity between the alkali labile (RNA) and the alkali resistant (DNA) fraction collected after one hour of incubation with (5-T) uridine. About 90% of the acid-insoluble fraction was found in the RNA fraction while about 10% resisted alkali treatment at this time of incubation. The ratio was only slightly influenced by changes of the exogenous uridine concentration.

When the RNA was degraded by treatment with alkali and the resulting nucleotides were subjected to paper electrophoresis at pH 3.5, a kinetic analysis of the labeling of RNA-U and RNA-C* could be perform-

* No significant label (< 2% of RNA-U) was detected in A and G bases.

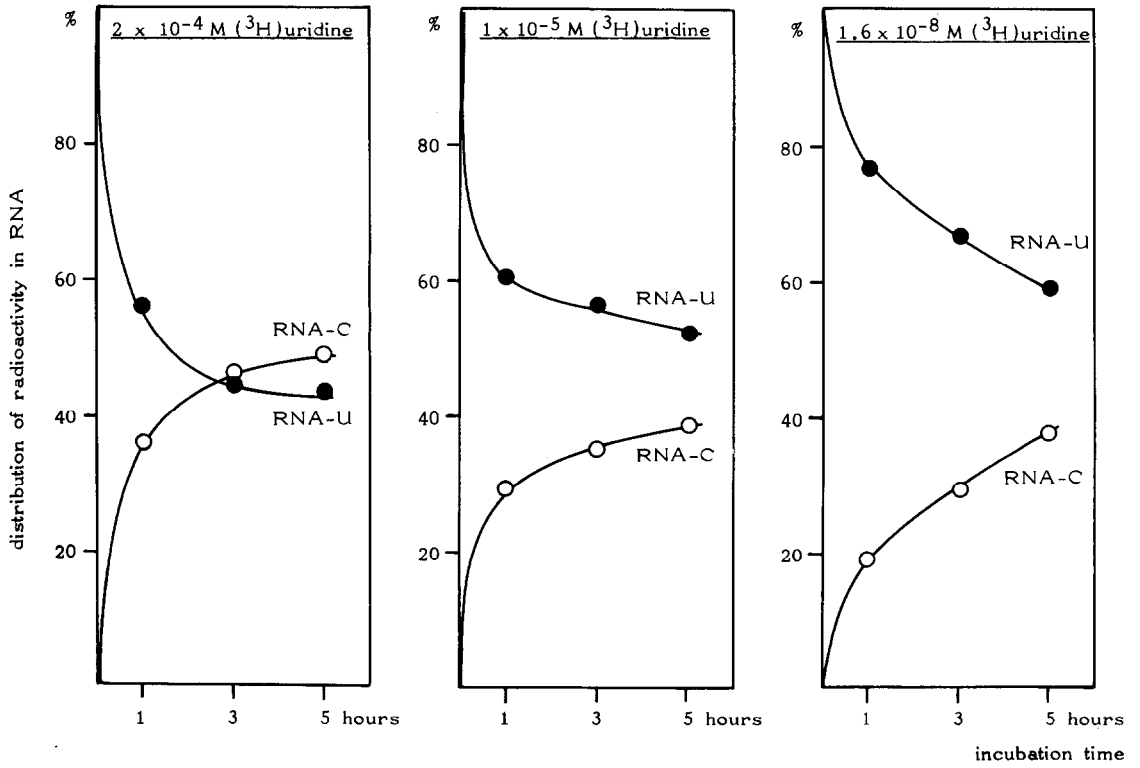

Fig.1. Labeling kinetics of RNA-U and RNA-C at three different concentrations of (5-T) uridine. 1000 ml HeLa suspension cultures ($\sim 0.3 \times 10^{-6}$ cells/ml) were labeled at three different concentrations (1.6×10^{-8} M, 1×10^{-5} M and 2×10^{-4} M) of (5-T) uridine. At the times indicated 100 ml of the cells suspensions were taken, the acid insoluble fractions prepared, and processed for RNA base labeling as described under Materials and methods.

Table 1
Distribution of radioactivity/10^6cells after one hour of labeling with (5-T) uridine between alkali labile and alkali resistant fraction

Uridine conc. (moles)		RNA (=alkali labile)	DNA (=alkali stabile)
1.6×10^{-8} M	cpm	4.74×10^6	5.32×10^5
	%	89.9%	10.1%
1×10^{-5} M	cpm	6.10×10^5	7.17×10^4
	%	89.5%	10.5%
2×10^{-4} M	cpm	5.2×10^4	6.5×10^3
	%	88.9%	11.1%

HeLa S3 suspension cultures (∿0.3 X 10^6 cells/ml) were labeled at three different concentrations (1.6 X 10^{-8} M, 1 X 10^{-5} M and 2 X 10^{-4} M) for one hr with (5-T) uridine. 100 ml of the cell suspensions were centrifuged off and the pellets taken for analysis of the acid insoluble radioactivity as described in Materials and methods.

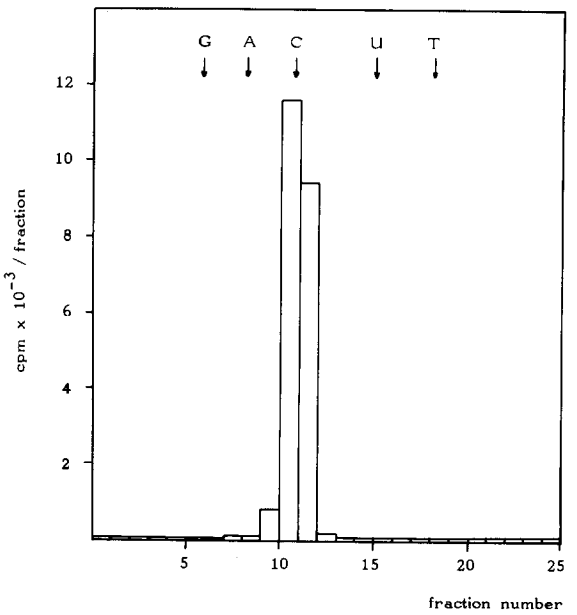

Fig.2. Analysis of the alkali resistant radioactive material in the acid insoluble fraction after incubation with (5-T) uridine. The alkali resistant material of the acid insoluble fraction obtained after three hours labeling of HeLa S3 suspension cultures at 1 X 10^{-5} M (5-T) uridine was hydrolysed with 98% formic acid. The DNA bases were separated by descending paper chromatography as described under Materials and methods. Arrows indicate the positions of the u.v. spots of the added markers.

ed (fig.1). Surprisingly, the extent as well as the rate of labeling were strongly influenced by the concentration of the exogenous uridine. At 2 X 10^{-4} M uridine – a concentration required for complete supression of pyrimidine synthesis de novo [3] – there was a rapid equilibration between RNA-U and RNA-C. The ratio of U to C label in equilibrium being 0.8 corresponding to the molar base ratio in total RNA [7]. Even at 1.6 X 10^{-8} M uridine, with no detectable feedback inhibition of pyrimidine synthesis de novo [3], an extensive labeling was seen in RNA-C, although the time needed to reach equilibrium was clearly prolonged at this low uridine concentration.

The analysis of base labeling of the alkali resistant (DNA) fraction was performed by reprecipitation of the resistant material with 10% TCA and extensive washing with 5% TCA in order to remove low molecular weight material derived from RNA hydrolysis. The precipitate was then hydrolysed in 98% formic acid resulting in a complete hydrolysis of DNA with liberation of the free bases. The bases were then separated by descending paper chromatography [5]. Fig.2 shows a typical analysis of the label in the alkali resistant fraction after 3 hr incubation with 1 X 10^{-5} M (5-T) uridine. 95% of the radioactivity was detected in the cytidine position of the chromatogram indicating selective DNA-C labeling. In some of the analyses trace amounts of label (< 5%) were found in the uracil position, presumably due to a slight contamination with RNA hydrolysis products (not shown). Fig.3 shows the incorporation of tritium from (5-T) uridine into the pyrimidine bases of RNA and DNA. At 2 X 10^{-4} M and 1 X 10^{-5} M exogenous uridine – concentrations sufficient for a constant supply of the labeled precursor during the incubation period [3] – a steady increase of label in RNA-U, RNA-C and DNA-C was observed. At 2 X 10^{-4} M uridine the radioactivity found in DNA-C is relatively constant about 1/3 of that found in RNA-C, whereas at 1 X 10^{-5} M uridine between 1/3 and 1/2 of the radioactivity is found in DNA-C compared to RNA-C. The radioactivity of DNA-C in percent of the total acid-insoluble material increased at 2 X 10^{-4} M uridine from 11.1% at one hr of incubation only slightly to values of about 12%

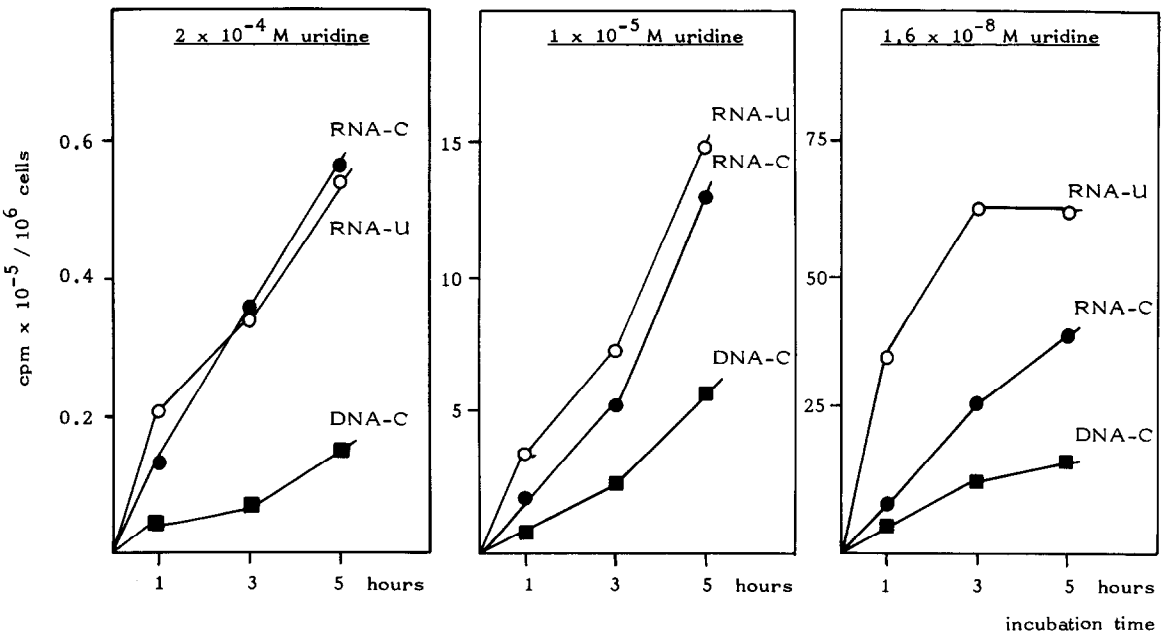

Fig.3. Kinetics of incorporation of radioactivity into the different RNA and DNA bases. 1000 ml HeLa S3 suspension cultures were labeled at three different (see legend fig.1) concentrations with (5-T) uridine. At the times indicated 100 ml were taken for RNA analysis and 50 ml for DNA bases analysis. For experimental details see Materials and methods.

at 5 hr. At 1×10^{-5} M uridine an increase from 10.5% to 17.5% for the time period was observed. At 1.6×10^{-8} M uridine the rapid exhaustion of the precursor in the medium (not shown) caused an early slow down of labeling in all three fractions. But this did not affect the fractions to the same extent. While RNA-U even showed a slight decrease between three and five hr, RNA-C and DNA-C still continued to rise leading to an increase of the label in DNA-C in percent of the total acid-insoluble radioactivity from 10.1% at one hr to 15% at five hr of incubation.

4. Discussion

(5-T) uridine is commonly used for specific analysis of RNA formation because of its inability to give rise to labeled thymidine nucleotides. But as shown in these experiments the conversion from UTP to CTP must be taken into account as it leads to radioactive DNA too. In this case, the label is found exclusively in cytidine residues. Incubation at three different concentrations of (5-T) uridine exhibiting different degrees of feedback inhibition of pyrimidine nucleotide synthesis de novo cf. [3], resulted in substantial labeling (10%–18%) of DNA. Analysis of RNA-C and RNA-U show that the CTP pool is in delayed equilibrium with the UTP pool. The extent of delay depends on the concentrations of the extracellular uridine (cf. fig.2) which may indicate its dependence on the degree of the feedback inhibition of the pyrimidine nucleotide synthesis de novo [3]. For these reasons (5-T) uridine cannot simply be taken as a specific precursor for RNA labeling. The specificity is only valid in short time experiments when the labeling of the CTP pool is negligible compared to the UTP pool. Exact analyses of RNA formation with (5-T) uridine therefore require corrections for DNA label, which could be done by alkaline hydrolysis of RNA and subsequent analysis of nucleotides rendered acid-soluble. Furthermore, due to the delayed equilibration of UTP and CTP pools, separation of the RNA bases is neccessary when half-life determination of RNA species are intended cf. [7]. Such analyses also require rather high

concentrations of the labeled precursor in order to avoid rapid depletion of the label in the medium with subsequent changes in UTP and also CTP specific radioactivities.

Serious errors in the determination of RNA synthesis with (5-T) uridine will arise especially in situations where the ratio of DNA and RNA formation will be altered as by the use of certain drugs and in synchronized cell cultures.

Acknowledgement

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