Free Pyrimidine Nucleotide Pool of Ehrlich Ascites-Tumour Cells

CHARACTERISTICS RELATED TO QUANTITATIVE STUDIES OF RNA METABOLISM

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Ehrlich ascites-tumour cells incubated in a mineral medium supplemented with glucose and glutamine intensely incorporated labelled uridine into free nucleotides and RNA. Detailed kinetic studies of the labelling of total acid-soluble pools of uridine and cytidine nucleotides and of RNA under standard conditions and after chase with non-radioactive uridine were carried out. The relative distribution of the radioactivity between the individual uridine phosphates as well as their total cellular contents were also determined under standard and chase conditions. The labelling kinetics of the mononucleotides and RNA by radioactive uridine and orotate were compared, and some turnover parameters were estimated by mathematical analysis of a steady-state model. The following conclusions were drawn. (1) The observed chase effect of addition of non-radioactive uridine is due to a rapid expansion of the acid-soluble uridine nucleotide pool and consequently a severalfold lowering of its specific radioactivity. (2) The free uridine nucleotides are partly separated into a large and a small compartment, labelled preferentially by exogenous orotate and uridine respectively. (3) The half-life of the mononucleotides, at least those located in the smaller compartment, is a few minutes only, owing to a rapid exchange with the uridylic acid residues of unstable RNA species. (4) This process of reutilization of RNA-degradation products accounts for 85% (a minimum estimate) of the overall turnover of the nucleotide pool in resting cells.

In animal cells two main techniques are used for determination of the turnover parameters of RNA: (a) measurements of the incorporation of radioactive precursors into acid-soluble nucleotides and RNA (e.g. Brandhorst & Humphreys, 1971; Brandhorst & McConkey, 1974; Hunt, 1976) and (b) pulse-labelling of RNA followed by a chase with an excess of the non-radioactive precursor (for references see below). Such studies require a detailed knowledge of the characteristics of the free nucleotide precursor pools. Despite numerous investigations the available information is still controversial. The following closely interrelated aspects must be considered as being of primary importance for quantitative studies on RNA metabolism.

(1) It is not clear why with only a few mammalian cells an effective chase with the non-radioactive precursor is attained (Scherrer & Marcaud, 1968; Plagemann, 1971; Von Tigerstrom, 1973), whereas in most cases RNA labelling is not easily arrested (Warner *et al.*, 1966; Cooper, 1968; Abelson *et al.*, 1974; Cowan & Milstein, 1974).

(2) Although labelled pyrimidines are the most often used RNA precursors, the turnover of the free uridine nucleotides has not been studied in detail, and the available values for their half-lives vary between a few hours and several days (Blobel & Potter, 1968; Cowan & Milstein, 1974; Vácha & Seifert, 1975; Wiegiers *et al.*, 1976).

(3) Reutilization of RNA-degradation products may be of critical importance for determinations of turnover rates of free nucleotides and unstable RNA species. However, the contribution of this process has never been evaluated quantitatively, and conclusions about its importance are controversial (see Rovera *et al.*, 1970; Bölcsföldi *et al.*, 1971; Abelson *et al.*, 1974; Wiegiers *et al.*, 1975).

(4) In some cases evidence was obtained that cellular mononucleotide pools are not compartmentalized (Brandhorst & McConkey, 1974; Puckett & Darnell, 1977). However, the opposite conclusion was reached in numerous studies (Plagemann, 1971, 1972; Bölcsföldi *et al.*, 1971; Goody & Ellem, 1975; Losman & Harley, 1978). In any case

a role of compartmentation in estimating the rates of RNA synthesis has not yet been demonstrated.

In the present paper the results of experiments aimed at the elucidation of the above points are reported. Preliminary communications of some findings have been given (Genchev & Hadjiolov, 1978; Genchev, 1979).

Methods and Materials

Theory

For a steady-state system of the pools u and r (Fig. 1) the differential equation describing the movement of radioactivity in pool u , which is instantaneously labelled at zero time, is

$$dq_u/dt = k_{ru} q_r - k_{uu} q_u \quad (1)$$

where q_u and q_r are quantities of radioactivity in the respective pools and k values are the rate constants of transfer (for the meaning of subscripts see Fig. 1); k_{uu} (and analogous) denotes the sum of all rate constants of output from the corresponding pool, i.e. $k_{uu} = k_{uo} + k_{ur}$. Eqn. (1) gives on integration

$$q_u/q_{u0} = H_1 e^{-g_1 t} + H_2 e^{-g_2 t} \quad (2)$$

where q_{u0} is the radioactivity introduced at zero time, and H and g may be determined as normalized intercepts and exponential slopes, respectively, by graphical analysis of the curve for radioactivity decay of pool u . Then the rate constants may be calculated from H and g by solving a group of simultaneous equations (see Shipley & Clark, 1972).

A second type of equation was used when $S_u = ct$ (S is specific radioactivity, c is a constant and v is flow rate; for the meaning of subscripts see above). The equation was derived as follows:

$$dq_r/dt = v_{ur} S_u - v_{rr} S_r = v_{rr} (ct - S_r)$$

or

$$dS_r/dt = k_{rr} (ct - S_r)$$

Fig. 1. Model to be analysed

The model is used for the mathematical analysis of uridine nucleotides and RNA-labelling kinetics. Single cellular pools of free uridine nucleotides (u) and of uridylic acid residues in unstable RNA (r) are assumed.

After integration and suitable substitutions this gives

$$\frac{S_r}{S_u} = \frac{1}{k_{rr} t} (k_{rr} t - 1 + e^{-k_{rr} t}) \quad (3)$$

from which k_{rr} may be numerically calculated.

The knowledge of the rate constants permits the turnover parameters of a pool to be estimated according to well-known relations (Shipley & Clark, 1972).

Labelling procedure

Ehrlich ascites-tumour cells (a hyperdiploid line) were collected on day 7 or 8 after implantation into mice and washed twice with 0.14 M-NaCl by low-speed centrifugation (700g for 3 min) at room temperature. The cells were suspended at about 15×10^6 cells/ml in a standard medium, consisting of phosphate-buffered saline (Dulbecco & Vogt, 1954) supplemented with 18 mM-glucose and 2 mM-L-glutamine, and preincubated with continuous shaking at 37°C for 10 min. Then [14 C]uridine or [14 C]orotate was added to start the labelling.

Determination of total radioactivity incorporated into uridine and cytidine mononucleotides

At specified time intervals, samples of the cell suspension were rapidly mixed with 2 vol. of cold 0.6 M-HClO₄. After 30–40 min the precipitate was removed by centrifugation for 15 min at 2500g in the cold and re-extracted with 0.4 M-HClO₄. To the combined acid-soluble extracts carrier UMP and CMP were added, the solution was adjusted to 1 M-HClO₄ and heated for 30 min at 100°C to hydrolyse the pyrimidine nucleotides to monophosphates. The latter were collected by charcoal treatment (Hadjiolov *et al.*, 1967), and ethanol and ammonia removed from the eluate by evaporation. The concentrate was suitably diluted, acidified and fractionated on a Dowex 50 column as previously described (Genchev & Mandel, 1974). UMP, which is not retained by the column, was collected from the effluent by charcoal as described above and further purified by chromatography on a column (7 cm $\times$ 0.9 cm) of Dowex 1 (formate form; X8). The column was washed with water and then developed with 0.4 M-ammonium formate. In some cases the same purification was applied for CMP (except that the charcoal step was omitted). In some experiments additional purification of UMP and CMP was accomplished by t.l.c. on silica-gel plates in the following solvents: 95% (v/v) ethanol/1 M-ammonium acetate, pH 7.5 (5:2, v/v), butan-1-ol/water (43:7, v/v) and isobutyric acid/conc. NH₃ (sp.gr. 0.880)/water (66:1:33, by vol.). The total amount of radioactivity incorporated into uridine or cytidine nucleotides was then calculated from the known

quantity of the added carrier and the specific radioactivity of UMP or CMP.

Determination of the labelling of the individual pyrimidine nucleotides

The acid-soluble extract, prepared as described above but not hydrolysed, was neutralized with 2.5 M-KOH in the cold, and after removal of KClO_4 by centrifugation for 15 min at 2500 g was fractionated directly on a Dowex 1 column by the method of Hurlbert *et al.* (1954) modified as described in the legend to Fig. 4.

Determination of the cellular content of pyrimidine nucleotides

An isotope-dilution method was used. To the acid extract prepared as above from a large sample of the incubation medium, containing 10^9 or more unlabelled cells, known quantities of $[^{14}\text{C}]\text{UTP}$ and $[^3\text{H}]\text{CTP}$ were added. UMP and CMP were then isolated by the hydrolysis and purification steps described above and the specific radioactivities of these nucleotides were determined. The reliability of this method was verified by appropriate control measurements of added marker compounds.

Extraction and determination of total RNA

The acid-insoluble residue was washed several times with cold 0.2 M- HClO_4 , RNA was extracted with 0.3 M-KOH at 37°C for 90 min, and its quantity estimated spectrophotometrically, by assuming that 1 A_{260} unit is equivalent to 32 μg of RNA (see details in Munro & Fleck, 1966).

Other determinations

The cells were counted in a Neubauer haemocytometer and their viability was assessed by Trypan Blue exclusion. DNA was determined as described by Burton (1956). Radioactivity of aqueous samples was measured in toluene/Triton X-100 scintillation mixture (Patterson & Greene, 1965) in a LKB Ultrabeta liquid-scintillation counter. All radioactivity measurements were corrected for quenching by an automatic computer program by using the external-standard channels-ratio calibration method.

Materials

Analytical-grade reagents were used throughout. Nucleotides and t.l.c. silica-gel 60F₂₅₄ plates were from E. Merck A.G. (Darmstadt, W. Germany), and 2,5-diphenyloxazole, 1,4-bis-(5-phenyloxazol-2-yl)-benzene and Triton X-100 from Koch-Light Laboratories (Colnbrook, Bucks., U.K.). $[2\text{-}^{14}\text{C}]\text{-Uridine}$ (60 mCi/mmol), $[4\text{-}^{14}\text{C}]\text{-UTP}$ (46 mCi/mmol) and $[5\text{-}^3\text{H}]\text{-CTP}$ (25 Ci/mmol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.), and $[6\text{-}^{14}\text{C}]\text{-orotic acid}$ (61 mCi/mmol) was from the Institute for Research

Production and Uses of Radioisotopes (Prague, Czechoslovakia).

Results

General characteristics of the cell system and of the incorporation of radioactive precursors

The average content of DNA and RNA was 12.8 and 20.9 pg/cell respectively. The quantities of nucleic acids and the cell number and viability were checked periodically, and no statistically significant changes were observed during the incubation periods used. As expected, in the medium used the cells are non-growing and may be considered as a steady-state system.

In preliminary experiments radioactive uridine, uracil and orotate were compared as RNA precursors. It was found that, even when present in trace amounts, uridine is efficiently incorporated, uracil is a very poor precursor, and orotate used in somewhat higher concentrations gives satisfactory labelling.

Labelling kinetics of RNA and pyrimidine nucleotides by $[^{14}\text{C}]\text{uridine}$ and chase experiments

The uridine incorporation into RNA is characterized by a high initial rate, which is considerably slowed down after 10–20 min incubation (Fig. 2). Similar kinetics have been observed

Fig. 2. Incorporation of $[^{14}\text{C}]\text{uridine}$ into RNA of Ehrlich ascites-tumour cells and the effect of addition of unlabelled uridine

Cells were labelled with 0.073 μCi of $[^{14}\text{C}]\text{uridine/ml}$, and total RNA radioactivity per ml of cell suspension was determined as described in the Methods and Materials section. At the times indicated with an arrow a sample of the suspension was transferred into a flask containing unlabelled uridine (final concn. 0.4 mM). O, Standard incubation; ●, incubation in the presence of unlabelled uridine (0.4 mM).

previously with Ehrlich tumour cells incubated in phosphate-buffered saline (Harwood & Itzhaki, 1973) or richer media (Rovera *et al.*, 1970). It has been also demonstrated that in these cells further incorporation of radioactive uridine into RNA can be arrested by the addition of 2 mM non-radioactive uridine (Von Tigerstrom, 1973). A typical experiment in which 0.4 mM unlabelled uridine was used as a chasing agent is shown on Fig. 2. This addition causes not only cessation of RNA labelling, but after some relatively short lag a disappearance of 40–60% of the radioactivity already incorporated, thus indicating the metabolic instability of newly synthesized RNA. No significant effects on RNA labelling were noted on addition of unlabelled uracil or orotate.

To elucidate the mechanism of uridine chase, several types of experiments were carried out. First it was demonstrated that, at least during the time intervals studied, the excess of unlabelled uridine does not display any toxic effects, judging from the microscopic observation of the cells and from the incorporation of labelled amino acids into their proteins (results not shown). There are numerous reports that this nucleoside, used even in higher concentrations than here, is not inhibitory for nucleic acid synthesis (Plagemann, 1971, 1972; Von Tigerstrom, 1973). Hence it would be expected that the radioactivity is rapidly washed out from the acid-soluble pool shortly after the addition of unlabelled uridine. The data presented in Fig. 3 show that this is not the case. It is evident that this pool is labelled very rapidly by [^{14}C]uridine, most of the radioactivity being in uridine nucleotides, whereas only a few per cent are associated with cytidine nucleotides. Soon after the introduction of unlabelled uridine there is even a marked increase of total label in both UMP and CMP. This increase indicates reutilization of RNA-degradation products under conditions of much diminished amounts or specific radioactivities of its immediate nucleoside triphosphate precursors.

Labelling of individual uridine nucleotides under standard and chase conditions

Since the above data are for total incorporation into all uridine phosphates (isolated as UMP; see the Methods and Materials section), a possible explanation of the chase effect of unlabelled uridine could be a dephosphorylation of UTP or its conversion into other compounds. To test this possibility, a series of experiments was run in which the individual pyrimidine nucleotides were isolated, as illustrated in Fig. 4. The results of the experiments (Table 1) disclose that essentially no redistribution of the label among the uridine phosphates occurs under chase conditions. These data further suggest that UMP, UDP, UTP and UDP-glucose are rapidly

Fig. 3. Incorporation of [^{14}C]uridine into RNA and free pyrimidine nucleotides and the effect of chase with unlabelled uridine

Cells were labelled with 0.012 μCi of [^{14}C]uridine/ml, and radioactivity per ml of cell suspension in RNA (Δ , $\blacktriangle$), UMP ($\circ$, $\bullet$) and CMP ($\square$, $\blacksquare$) was determined. UMP and CMP were obtained through hydrolysis of the acid-soluble extracts and purified as described in the Methods and Materials section. At the time indicated with an arrow a sample of the cell suspension was chased with unlabelled uridine as described in the legend to Fig. 2. Open symbols, standard incubation medium; closed symbols, incubation in the presence of unlabelled uridine (0.4 mM).

Table 1. Distribution of radioactivity among the free uridine nucleotides after labelling with [^{14}C]uridine
Unhydrolysed acid-soluble extracts obtained from cells labelled with [^{14}C]uridine for the specified times were fractionated on a Dowex 1 column (for details see the legend to Fig. 4). When indicated, the chase was accomplished at 10 min of labelling by the addition of unlabelled uridine (final concn. 0.4 mM) as described in the legend to Fig. 2.

Time of labelling and conditions of incubation	Distribution of radioactivity (% of total radioactivity in uridine nucleotides) among			
	UMP	UDPG*	UDP	UTP
10 min; standard	11	14	14	61
20 min; standard	13	15	11	61
40 min; standard	11	20	15	54
40 min; chase	10	14	13	63
60 min; standard	17	22	10	51
60 min; chase	7	25	12	56

* UDPG (UDP-glucose) refers to the compound(s) co-eluted with an UDP-glucose marker. No further identification was attempted.

Fig. 4. Ion-exchange chromatography of an acid-soluble extract from Ehrlich tumour cells labelled with [^{14}C]-uridine

The unhydrolysed acid-soluble extract obtained from cells labelled for 60 min with [^{14}C]uridine ($0.075 \mu\text{Ci/ml}$) was fractionated on a column ($11.6 \text{ cm} \times 0.75 \text{ cm}$) of Dowex 1 (X8, formate form), fractions of about 15 ml being collected. Linear gradient elution was employed; mixer and reservoir each contained about 150 ml of solution: fractions 1–4 (water), fractions 5–21 (water to 3 M-formic acid), fractions 22–40 (3 M-formic acid to 4 M-formic acid/0.8 M-ammonium formate). The identity of the radioactive compounds was ascertained by co-chromatography of marker nucleotides and by t.l.c. The peak denoted CMP is heterogeneous and only a fraction of its radioactivity is associated with CMP. The peak denoted UDPG refers to the compound(s) co-eluted with an authentic UDP-glucose marker.

interconverted, since only slight changes of their relative radioactivities are found (see also Kramer *et al.*, 1972).

Determination of pyrimidine nucleotide pool sizes

By the isotope-dilution techniques (see the Methods and Materials section), the normal content of uridine nucleotides in Ehrlich ascites-tumour cells is $1040 \text{ pmol}/10^6$ cells, about twofold higher than the amount of cytidine nucleotides. It was found that incubation in the presence of 0.4 mM-uridine brings about an extremely rapid increase of the concentration of cellular uridine nucleotides (Table 2). That the amount of UTP, serving as immediate RNA precursor, does increase abruptly under chase conditions is indicated also by experiments in which cells were first preincubated for 10 min in the presence of unlabelled uridine (0.4 mM) and then labelled: a considerably delayed incorporation of radioactivity into RNA was recorded (results not shown). Therefore the obvious explanation of the chase effect of uridine is the rapid decrease of the

Table 2. Uridine nucleotide content of Ehrlich ascites-tumour cells as a function of the presence of unlabelled uridine or orotate in the medium

After the specified times of incubation of the cells in the standard medium without a labelled precursor and with the indicated additions, the total quantity of their acid-soluble uridine nucleotides was determined by isotope dilution as described in the Methods and Materials section. Unlabelled uridine or orotate, when added, was at a final concentration of 0.4 mM. All values given are means obtained from two to five independent measurements.

Incubation time (min)	Addition to the standard medium	Uridine nucleotide content	
		(pmol/ 10^6 cells)	(% of the control)
0–60*	None (control)	1040	100
5	Uridine	1810	174
10	Uridine	2530	243
10	Orotate	1190	114
30	Uridine	2690	259
30	Orotate	1120	108

* No statistically significant deviations from the mean value ($1040 \text{ pmol}/10^6$ cells) were observed in several determinations done at different time points throughout this time interval.

specific radioactivity of the precursor pool, owing to its expansion. This explanation is in line with the inability, mentioned above, of orotate to reproduce the effect of uridine (cf. Table 2), whereas [^{14}C]orotate-labelled cells may be successfully chased with unlabelled uridine (Genchev *et al.*, 1980).

Comparison of the labelling kinetics with [^{14}C]uridine and [^{14}C]orotate as precursors

As the previous observations suggested that [^{14}C]uridine enters the nucleotide pool very rapidly, a more detailed study of the labelling kinetics at early times was performed by sampling the incubation mixture at 1 min intervals. The results (Fig. 5) indicate that the incorporation into RNA begins without appreciable lag and that the labelling kinetics of the uridine nucleotide pool display a pulse-labelling pattern (see also Rovera *et al.*, 1970; Harwood & Itzhaki, 1973). The latter should not be surprising, since (i) the labelled precursor is used in very low concentrations, and (ii) the ascites-tumour cells possess an extremely active uridine kinase [see Table 2, and Sköld (1960)] as well as a high ability to split exogenous uridine to uracil (Jacquez, 1962), itself a poor labelling agent. It can be estimated that up to 60–70% of the uridine initially present in the medium is incorporated, and about one-half of that may be found in RNA after 15 min incubation.

As mentioned above, with [^{14}C]orotate at higher

Fig. 5. Time-course of incorporation of [^{14}C]uridine into RNA and uridine mononucleotides

The labelling with [^{14}C]uridine ($0.012\ \mu\text{Ci/ml}$) was performed under standard incubation conditions. The total free uridine nucleotides were converted into UMP by acid hydrolysis, and RNA (O) and UMP (●) were isolated as described in the Methods and Materials section.

Fig. 6. Time-course of incorporation of [^{14}C]orotic acid into RNA and uridine mononucleotides

The labelling with [^{14}C]orotate ($6.84\ \mu\text{Ci/ml}$) was performed under standard conditions and UMP (O) and RNA (●) were isolated as described in the legend to Fig. 5.

concentrations, an extensive labelling of both RNA and acid-soluble uridine nucleotides can be obtained (Fig. 6). It is worth noting that the orotate

Fig. 7. Graphical analysis of the disappearance of radioactivity from the acid-soluble uridine nucleotide pool

The values for the labelling of uridine nucleotides by [^{14}C]uridine under standard conditions are taken from the experiments shown in Fig. 3 (O), Fig. 5 (●) and from an independent similar experiment (Δ). They were normalized by setting each 10 min value as 100. The tail of the resulting experimental curve (1) was extrapolated back to obtain the slow component (2). To find the early rapid component, the corresponding values along the extrapolated line were subtracted arithmetically from the experimental points, and the new points thus obtained (denoted by the same symbols as the experimental ones) define the fast component (3). The slopes of the straight lines (2) and (3) were determined as 0.0043 and 0.104, and their ordinate intercepts as 76 and 80, respectively.

incorporation into RNA seems considerably delayed in comparison with the incorporation of uridine.

Quantitative analysis of labelling kinetics

In the mathematical analysis based on a relatively simple model (Fig. 1), two general assumptions are made: (1) the cells are under steady-state conditions; (2) all acid-soluble uridine phosphates are in equilibrium (or, alternatively, the bulk of them is UTP). It must be added also that the calculations given below should give the average rate of synthesis of different unstable RNA species, as their uridylic acid residues are considered as a single pool r .

As seen from Figs. 3 and 5, the sum of radioactivity in RNA and free pyrimidine nucleotides does not increase after 6–7 min of incubation. If we accept that all label has entered the

pool u at 6 min, the 'curve peeling' technique (Shipley & Clark, 1972) can be applied (see legend to Fig. 7). Accordingly, the experimental curve for disappearance of radioactivity from pool u may be described by the equation (in normalized form):

$$q_u/q_{u0} = 0.51 e^{-0.104t} + 0.49 e^{-0.004t} \quad (4)$$

which corresponds to eqn. (2) (see under 'Theory'). It should be stressed at this point that the graphical analysis does not prove that the curve for q_u is really built up only of two exponential terms. It is so because the curve-analysis technique displays insensitivity to disclose more than two components, especially when the observational errors are to be tolerated (see Koch, 1962; Shipley & Clark, 1972). By using eqn. (4) the model of Fig. 1 may be solved unequivocally with respect to the half-life of the pool u and in rather narrow ranges with respect to the rates of transfer and the size of pool r . It appears (Table 3) that under steady-state conditions an intensive RNA synthesis takes place and the half-life of the pool u is only about 12 min. The solution of the model further discloses that the sizes of the pools u and r are approximately equal and that an extensive exchange between these pools (i.e. re-utilization) occurs. Taking into account the quantity of total RNA ($20.9 \mu\text{g}/10^6$ cells) and its base composition (Genchev *et al.*, 1980), it may be

calculated that 7–8% of the cellular RNA is unstable and is synthesized with a rate of about 90 fg/cell per min. Both values are very close to those reported by Brandhorst & McConkey (1974) for L cells, but considerably lower rates of RNA synthesis have been recorded in sea-urchin embryo (Brandhorst & Humphreys, 1971) and in erythroid cells (Hunt, 1976). The value obtained in the present determination could be only underestimated for at least two reasons: (a) an incorporation of some label into pool u probably continues after the 6 min of incubation (although such residual labelling is obviously quite slow), and (b) under experimental conditions the label is not instantaneously introduced into pool u , as theoretically required.

The rate of RNA synthesis may be calculated alternatively from eqn. (3) (see under 'Theory'). It can be applied for both [^{14}C]uridine- and [^{14}C]orotate-labelling kinetics, because with both precursors the specific radioactivity of pool u is increasing almost linearly with time during the initial periods (Figs. 5 and 6), so that the condition $S_u = ct$ is fulfilled. The eqn. (3) requires the ratio S_r/S_u to be known, but, as the sizes of the pools u and r seem to be approximately equal (see above), the ratio should be about the same as q_r/q_u , which is determined experimentally. To calculate k_{rr} and hence v_{rr} , which is in fact RNA-turnover (or synthesis) rate, the time

Table 3. Rates of RNA synthesis and half-lives of the pool of free uridine nucleotides

The parameters of the model (see Fig. 1) were estimated by using the directly determined size of pool u (1040 pmol of uridine nucleotides/ 10^6 cells) and the rate constants calculated from eqn. (2) (the values given in the first row of the Table) or eqn. (3) (all other values). Except when noted, it was assumed that the RNA radioactivity is only in its uridylic acid residues.

Radioactive precursor	Source of the data	Time interval analysed	Rate of RNA synthesis (v_{ur}) (pmol of UMP/min per 10^6 cells)	Half-life of the pool u (min)	Size of the pool r (pmol of UMP/ 10^6 cells)
[^{14}C]Uridine	Fig. 7	6–40 min	49–58*	12.5	923–1090*
[^{14}C]Uridine	Fig. 5	0–5 min	260	≤ 2.8	1040†
			222	≤ 3.2	2080‡
			114§	$\leq 3.2§$	1040†
			42	$\leq 3.4 $	1040†
[^{14}C]Orotate	Fig. 6	0–80 min	12	≤ 60	1040†
			10¶	$\leq 72¶$	1040†

* The ranges within which the 'true values' lie are given. The other rates calculated (in pmol of UMP/min per 10^6 cells) are: $v_{ou} = 9$, $v_{ru} = 49$, $v_{ro} + v_{uo} = 9$.

† The size of the pool r is accepted as equal to that of the pool u on the basis of the results obtained (see the value in the first row) and literature data (Brandhorst & McConkey, 1974).

‡ Provisionally accepted as twice the above value (cf. preceding footnote) to illustrate that the real size of the pool r is not of critical importance for the determination of the RNA-synthesis rate.

§, || These values are obtained by assuming that [^{14}C]uridine is labelling only an independent compartment of the free uridine nucleotide pool, which contains 50% (§) or 20% (||) of the total cellular free uridine nucleotides and supplies all RNA synthesis.

¶ A correction for the labelling of cytidylic acid residues in RNA is done in this case because of the relatively long incubation time employed. The data used for this correction are taken from Genchev *et al.* (1980).

points of [^{14}C]uridine incorporation up to 5 min of incubation are used (see Fig. 5 and Table 3). In this time interval the label in total RNA may be safely assumed as representing the radioactivity of its uridylic acid residues only, i.e. q_u . A very high turnover rate of RNA (260 pmol of UMP/ 10^6 cells per min) is found. This result, as exemplified in Table 3 (see footnotes to the Table), is crucially dependent on the real size of the pool u , but not of the pool r . It is noteworthy that in any case the determined half-life of the pool u appears to be well below 10 min.

Quite a different result is obtained when the same calculations are done for [^{14}C]orotate-labelling kinetics (Fig. 6). Assuming, as above, an equality of pool sizes, the apparent rate of RNA synthesis is measured as 10–12 pmol of UMP/ 10^6 cells per min (Table 3), a value more than 20-fold lower than that derived from the [^{14}C]uridine-labelling study. A difference of the same order was repeatedly observed in this type of experiment, and any possible artifacts, caused for instance by an incomplete purification of the nucleotides or RNA, may be excluded (see the Methods and Materials section). Therefore the only explanation of these results is that the free uridine nucleotides are subdivided into a larger and a smaller compartment, which are labelled predominantly by orotate and uridine respectively.

Discussion

The present investigation reveals new data about the labelling kinetics and other characteristics of the pyrimidine nucleotide pool in Ehrlich tumour cells, which are often used for studies on RNA metabolism.

This study discloses that the mechanism underlying the changes of the RNA labelling on addition of unlabelled uridine (Fig. 2) is a rapid expansion of the free uridine nucleotide pool. According to my knowledge, such a fast increase in a ribonucleotide pool by an exogenous nucleoside has not yet been recorded (cf. Table 2; Kramer *et al.*, 1972; Plagemann, 1972). This observation explains why the 'cold chase' appears to be especially effective with Ehrlich tumour cells as compared with other types of cells (see the introduction for references). It must be pointed out that neither immediate nor complete removal of the label from the free nucleotide pool occurs on addition of the unlabelled precursor. This consideration shows that a straightforward analysis of the decay curve of RNA radioactivity is not reliable. Further, in the interpretation of our 'chase' kinetics it should be remembered that (1) the labelled RNA is heterogeneous; (2) some radioactivity is incorporated (especially after longer incubation

times) into its cytidylic acid residues; (3) a pronounced stimulation of the biosynthesis of CTP from UTP occurs when the intracellular concentration of the latter is elevated (D. D. Genchev, unpublished work). Yet it may be concluded that even in resting cells some RNA species are so rapidly turning over that their radioactivity equilibrates with that of the free nucleotide precursors: the rate with which the label is chased from RNA is comparable with the rate of pool expansion (cf. Figs. 2 and 3 with Table 2).

Although the model shown on Fig. 1 is certainly oversimplified, it is pertinent to discuss on its basis the turnover parameters of the free nucleotide pool. An important fact emerging from the present results is the high intensity of the process of reutilization of RNA-degradation products, which in turn determines very short half-lives of the mononucleotide pool (see Table 3). In previous studies the existence of such a reutilization often remained undiscovered, and the recorded half-lives of the free uridine nucleotides were several hours or even longer (see the introduction). The main reason for the discrepancies between the present and some of the earlier findings lies in the fact that the extremely important theoretical requirement for an instantaneous chase, or the instantaneous introduction of the label into a nucleotide pool, cannot be fulfilled in practice (see also Cowan & Milstein, 1974; Puckett & Darnell, 1977). Hence the most informative early components of the radioactivity-decay curves of unstable RNA or of the free nucleotide pool are rarely observed. This results in the erroneous assumption of simple exponential loss of radioactivity and leads to conclusions that the reutilization is insignificant, or alternatively to a severe overestimation of the half-lives of free nucleotides.

The present analysis indicates that in resting cells the reutilization is the main factor determining the turnover of the free uridine nucleotide pool. The irreversible outflow (synthesis of cytidine nucleotides, degradation etc.) represents no more than 15% of the overall turnover of this pool (a maximum estimate; see Table 3). The results of Fig. 3 are in accordance with this conclusion, as they show almost directly that a considerable reutilization does exist, and that the rate of synthesis of cytidine nucleotides, in particular, is very slow compared with the rate of RNA synthesis.

It is appropriate to remark that there are no reasons to believe that in growing cells the half-lives of the mononucleotides are longer than recorded here. In these cells, owing to the accumulation of stable nucleic acids, the irreversible outflow from the acid-soluble pool should proceed with a higher rate (see Kaminskis, 1979). In addition, a significant reutilization has to occur too, as it is well known that at least half of the molecules of the precursor to

rRNA and a considerably greater proportion of those to mRNA are rapidly degraded.

The present study supplies evidence for a compartmentation of the mononucleotide pool. The results (Table 3) demonstrate the difficulties in measurement of the true rate of RNA synthesis and also that such estimates may be strongly dependent on the radioactive precursor used, evidently because of its potential ability to label to some extent specifically one or the other compartment of the pool. The compartmentation of the RNA-precursors pool is discussed in detail in the accompanying paper (Genchev *et al.*, 1980).

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