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Turnover of Nucleic Acids in a Multiplying Animal Cell

1. INCORPORATION STUDIES

By J. W. WATTS

Department of Cell Biology, John Innes Institute, Bayfordbury, Hertford, Herts.

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It is widely accepted that RNA is involved in the transfer of information from the DNA to the rest of the cell, but opinions differ about the precise way in which it is involved (Sirlin, 1963). A specific model based on experimental evidence has been proposed for those cells which have well-defined nuclei. The cytoplasmic RNA is thought to be made in the nucleus on the DNA and then transferred to the cytoplasm (Goldstein & Plaut, 1955). This 'translocation' model is based mainly on the evidence obtained from radioautographic studies. These showed, in general, that, when animal or plant cells were grown in a medium containing radioactive precursors of RNA, the RNA of the nucleus became labelled much more rapidly than that of the cytoplasm. In some cases there was virtually no incorporation of radioactivity into cytoplasmic RNA during the first 30 min. of labelling, although incorporation into nuclear RNA was extensive. This observation has been widely assumed to show that most of the RNA in the cytoplasm originates in the nucleus (Zalokar, 1959; Perry, Hell & Errera, 1960).

Perry, Errera, Hell & Durwald (1961) found with radioautographic techniques that the incorporation of label into the nuclear RNA reached a maximum steady value after 2–3 hr., whereas incorporation into the cytoplasmic RNA, after an initial lag, continued to rise steadily throughout the experiment. This result was also considered to support the view that the cytoplasmic RNA was synthesized in the nucleus.

It is known, however, that animal and plant cells normally synthesize the constituents of RNA from relatively simple precursors (Brown & Roll, 1955), and the introduction of preformed bases, nucleosides and nucleotides does not necessarily suppress endogenous synthesis immediately or entirely (Henderson, 1962). It is also known that cells often contain relatively large 'pools' of metabolites which may have to be diluted by added exogenous precursors before the specific radioactivities of the immediate precursors of RNA become the same as those of the added compounds (Harris & Watts, 1962). It has been pointed out (Harris, 1959; Harris & Watts, 1962) that these factors make it

almost impossible to decide on the basis of radioautographic and simple analytical studies whether or not translocation of RNA does take place. The magnitude of these 'pool effects' can be very large; and the observed kinetics of incorporation of radioactive precursors into RNA are further complicated by the presence of turnover of RNA.

Our knowledge of the precise contributions that 'pool effects', turnover and similar factors may make to the observed kinetics of incorporation of radioactive precursors into nucleic acids is very fragmentary, although clearly a detailed interpretation of the kinetic data is impossible without this knowledge. The present work provides information about the origin of some of the complex features of the kinetics of incorporation of precursors into the RNA of the HeLa cell, and indicates some of the difficulties likely to be encountered in this type of study.

EXPERIMENTAL

Conditions of cell culture. Stocks of HeLa cells were maintained in suspension culture (McLimans, Davis, Glover & Rake, 1957) in 'complex medium' which consisted of 0.25% (w/v) Bacto yeast extract (Difco Laboratories Inc.), 0.25% (w/v) Nutrient Broth no. 2 (Oxoid Division of Oxo Ltd.), 5% (v/v) pig serum and 95% (v/v) Earle's (1943) saline from which CaCl_2 was omitted. 'Eagle's medium' was used in all experiments with suspension cultures and was that described by Eagle, Oyama, Levy, Horton & Fleischman (1956) with the modifications that the amino acids were present at three times the concentrations recommended, 5% (v/v) pig serum was added, and CaCl_2 was omitted. The medium was kept at about pH 7.2 by passing a continuous stream of CO_2 + air (5:95) over the surface of the suspension culture. The cells grew as single and double cells with virtually no clumping.

When the cells were grown as monolayers adherent to glass, the culture vessels were Carrel flasks (diam. 8.5 cm.). The conditions of growth were those described by Watts & Harris (1959). The monolayers were prepared from cells grown in suspension culture. The cells were spun down at 220g for 3 min. and resuspended in Eagle's medium to which had been added 10% (v/v) rabbit serum and CaCl_2 to a final concentration of 2 mM. Samples containing about 3×10^6 cells in 10 ml. of medium were put in each flask and left for 1 hr. to allow the cells to settle and adhere to the glass. The medium was replaced with 12 ml. of fresh medium, and the cultures were allowed to grow overnight before being used for experimental purposes.

Radioactive compounds. [2- ^{14}C]Thymidine, [8- ^{14}C]adenine, [2- ^{14}C]uracil and [^{32}P]orthophosphate were obtained from The Radiochemical Centre, Amersham, Bucks. [2- ^{14}C]Uracil was converted into [2- ^{14}C]uridylic acid by a uracil-requiring mutant of *Escherichia coli*.

Preparation of nucleic acid fractions. The procedures used to isolate adenine and guanine from the RNA and DNA of cells growing as monolayers on glass were as described by Watts & Harris (1959). The following methods refer to the treatment of suspension cultures.

Preparation of the total ribonucleic acid fraction. A volume of culture containing about 1.5×10^7 cells was spun at

220g for 3 min., the pellet of cells was washed with 10 ml. of 0.85% (w/v) NaCl soln. and spun down again. The cells were resuspended in 5 ml. of 10% (w/v) trichloroacetic acid at 0°, spun at 2000g for 15 min. and washed with water. The pellet was extracted for 30 min. at 70° with aq. 70% (v/v) ethanol, followed by 30 min. at 40° with ethanol-ether (1:1, v/v). It was then hydrolysed with 1 ml. of 0.3N-KOH for 18 hr. at 37°. The hydrolysate was cooled to 0° and acidified to pH 2 with 10N-HClO₄. The precipitate was spun down at 2000g for 15 min. at 0° and the supernatant, which contained the RNA in the form of mononucleotides, was collected and neutralized with 0.1N-KOH.

Preparation of the cytoplasmic ribonucleic acid, nuclear ribonucleic acid and deoxyribonucleic acid fractions. A volume of culture containing about 3.5×10^7 cells was spun at 220g for 3 min., and the cells were washed with 0.85% (w/v) NaCl soln. as above. The cells were resuspended in 5 ml. of water which had been saturated with phenol and which contained about 0.2 ml. of phenol saturated with water. The suspension was stirred for 1 hr. at 4°, after which it was spun at 2500g for 30 min. at 4°. The clear supernatant was collected; it contained the cytoplasmic RNA. The pellet was resuspended in 10 ml. of water and spun down at 2500g for 15 min.; it contained the nuclear RNA, DNA and most of the cell protein. About 25% of the total RNA was found in this pellet (Harris & Watts, 1962).

The supernatant containing the cytoplasmic RNA was cooled to 0°, 1 ml. of ethanol and 0.1 ml. of N-HCl were added, and the mixture was spun at 2000g for 20 min. at 0°. The pellet of cytoplasmic RNA was washed by decantation with 10 ml. of ice-cold 0.1N-HCl and then hydrolysed with 1.0 ml. of 0.3N-KOH for 18 hr. at 37°. The hydrolysate was acidified to pH 2 with 10N-HClO₄ at 0°, spun at 2000g for 20 min., and the supernatant was collected and neutralized with 0.1N-KOH.

The pellet containing the nuclear RNA and DNA was extracted for 1 hr. at 70° with 10 ml. of aq. 70% (v/v) ethanol and then for 30 min. at 40° with ethanol-ether (1:1, v/v). The residue was then hydrolysed with 3 ml. of 0.3N-KOH for 18 hr. at 37°. The hydrolysate was acidified to pH 2 with 10N-HClO₄ at 0° and spun at 2000g for 15 min. at 0°. The supernatant, which contained the nuclear RNA in the form of mononucleotides, was collected and neutralized with 0.1N-KOH. The precipitate was washed twice with 10 ml. of water and extracted with 2 ml. of 0.3N-trichloroacetic acid for 1 hr. at 90°. The extract contained the DNA bases, mainly as free purines and pyrimidine deoxyriboside phosphates.

Isolation of ribonucleic acid nucleotides. When the RNA had been labelled with [^{32}P]phosphate, the radioactivity of free orthophosphate in the solution of mononucleotides was decreased by adding 0.2 ml. of 5% (w/v) MgNH_4PO_4 in 0.01N-HCl and 1 vol. of ethanol. The solution was mixed and 0.2 ml. of NH_3 soln. (sp.gr. 0.880) was added. The precipitate of MgNH_4PO_4 was spun down at 0° and the supernatant was collected. The nucleotides were then precipitated by adding 0.5 vol. of ethanol and 0.2 ml. of 20% (w/v) BaCl_2 soln. The barium salts of the ribonucleotides were spun down at 0° and freed from Ba^{2+} ions by dissolving them in 1 ml. of water in the presence of 0.2 g. of Dowex 50 (NH_4^+ form) resin. The resin was allowed to settle and the supernatant was collected and evaporated to dryness *in vacuo*. The nucleotides were separated by electrophoresis for 5 hr. at 9v/cm. on Whatman no. 1 filter

paper in 0.05M-ammonium formate buffer, pH 3.5, by using an apparatus similar to that described by Flynn & de Mayo (1951). After electrophoresis the paper was dried and placed in contact with X-ray film (Kodak; Blue Brand) for 7 days, and the film was then developed. The film showed darkening which coincided precisely with the ultraviolet-absorbing spots of the mononucleotides. There was no sign of contamination of the nucleotides by ^{32}P in non-nucleotide compounds of the type described by Davidson & Smellie (1952).

Isolation of ribonucleic acid and deoxyribonucleic acid purines. The solution of ribonucleotides or the trichloroacetic acid extract of DNA was diluted to 5 ml. with water, and 0.5 ml. of $\text{N-H}_2\text{SO}_4$ and 0.1 ml. of N-HCl were added. The solution was heated for 1 hr. at 100° to hydrolyse the purine derivatives to the free bases, and 0.4 ml. of 20% (w/v) AgNO_3 soln. was then added. The precipitate of silver salts was spun at 500g for 5 min. at 0° , the supernatant was discarded, and the purines were extracted by heating the pellet with 1 ml. of N-HCl for 15 min. at 70° . The solution was spun at 500g for 5 min. and the supernatant was collected and evaporated to dryness *in vacuo*. The purines were then separated from one another by chromatography for 4 hr. on Whatman no. 1 filter paper with a descending solvent system consisting of methanol-ethanol-water-conc. HCl (50:25:19:6, by vol.) (Kirby, 1955).

Isolation of thymine. The trichloroacetic acid extract of DNA was heated for 1 hr. at 100° with 1 ml. of 0.2N- HCl . The solution was adjusted to pH 8 by the addition of N-KOH , and 2 vol. of ethanol and 0.2 ml. of 20% (w/v) BaCl_2 were added. The precipitate was spun down at 0° and redissolved in 1 ml. of water containing 0.2 g. of Dowex 50 (H^+ form). The resin was spun down and the supernatant was collected and evaporated to dryness. The dry residue was hydrolysed with 1 ml. of 98% (w/v) formic acid for 2 hr. at 175° and the hydrolysate was evaporated to dryness, redissolved in 70% (v/v) ethanol and subjected to chromatography overnight on sheets of Whatman no. 1 filter paper with a descending solvent system consisting of butan-1-ol saturated with water and containing 1% (v/v) of aq. NH_3 soln. (sp.gr. 0.880) (MacNutt, 1952).

Determination of specific radioactivity. The ultraviolet-absorbing regions on the chromatograms were located by the method of Holiday & Johnson (1949) with a Chromatolite lamp (Engelhard Hanovia Lamps Ltd.). These regions were cut out and eluted overnight into 0.8 ml. of 0.01N- HCl . The ultraviolet-absorption spectra of the eluates were measured in 1 cm. micro-cells in a Hilger Uvispek (model H. 700). Known volumes of the eluates were dried on glass coverslips, and the radioactivity was measured with a thin-end-window counter (Nuclear-Chicago Corp. model D-47).

Measurement of cell growth. Direct measurement of the growth of suspension cultures of HeLa cells by means of cell counts gave a mean generation time of about 20 hr. However, the direct method for measuring growth was found to be unreliable under the present conditions because of the difficulty of obtaining a representative sample from the culture vessel. The method finally adopted assumed that there was no turnover of DNA in the multiplying cell. HeLa cells were grown for 6 hr. in medium which contained about $0.25\text{ }\mu\text{C}$ of $[2\text{-}^{14}\text{C}]\text{thymidine/ml}$. Under these conditions only the thymine in DNA became labelled. The cells were transferred to non-radioactive medium and grown for a further period of 18 hr., after which they were used for

experimental purposes. Growth was estimated from the rate at which the specific radioactivity of the thymine in DNA fell during the experiment. The method gave results in good agreement with those obtained by the direct method. It was not used in experiments in which $[2\text{-}^{14}\text{C}]\text{-uridylic acid}$ was used to label the cells.

RESULTS

Choice and concentration of radioactive precursor. $[8\text{-}^{14}\text{C}]\text{Adenine}$ was the precursor used in most of the incorporation studies. It has the advantages of cheapness and availability at high specific radioactivity, it is stable in solution in cell cultures, and it is used by the cells only to label adenine and guanine in the nucleic acids. Henderson (1962) has also shown that adenine at concentrations around 0.1 mM efficiently inhibits endogenous purine biosynthesis. The free pyrimidine bases are only poorly utilized by HeLa cells and it is necessary to use labelled nucleosides or nucleotides to obtain rapid incorporation of radioactivity into RNA (Salzman & Sebring, 1959). $[32\text{P}]\text{Orthophosphate}$ gives general rather than specific labelling of cell components. For purposes of comparison, however, the cells in some of the experiments were labelled with $[2\text{-}^{14}\text{C}]\text{uridylic acid}$ or $[32\text{P}]\text{orthophosphate}$.

The suspension cultures of HeLa cells used in these experiments contained about 10^6 cells/ml. and had a net requirement of about $0.5\text{ }\mu\text{g}$. of purine/ml./hr. Since Henderson's (1962) work also indicated that an exogenous concentration of about 0.1 mM-adenine was required to produce 90% inhibition of endogenous purine biosynthesis, the minimum initial concentration of adenine was set at 0.1 mM in experiments lasting 12 hr., and at 0.15 mM in experiments lasting 24 hr. These concentrations ensured that a high extracellular concentration of adenine was maintained throughout the experiment.

Effect of concentration of labelled precursor on the incorporation of radioactivity into nucleic acids. When the cells were grown in medium containing excess of $[8\text{-}^{14}\text{C}]\text{adenine}$, radioactivity entered the adenine in the RNA at a rate which did not vary greatly during the experiment (Fig. 1). The incorporation of radioactivity into the adenine in the DNA became linear after about 2 hr., but showed a characteristic lag during the first 2 hr. A more detailed analysis of incorporation of $[8\text{-}^{14}\text{C}]\text{adenine}$ into adenine in the different fractions of RNA is shown in Fig. 2. Incorporation of label into the total RNA was again substantially linear during the 12 hr. of the experiment, but the rate of incorporation into nuclear RNA fell sharply after a few hours to a steady value that was only 30% of that of the first hour. The incorporation of label into the adenine of the cytoplasmic RNA resembled the

incorporation curve for DNA shown in Fig. 1; there was a lag initially before incorporation reached a maximum steady rate after about 1 hr. The incorporation of radioactivity into the guanine of the RNA fractions in the same experiment is shown in Fig. 3. These three curves each showed a marked lag initially, the magnitude of the effect being greatest for the cytoplasmic guanine and least for the nuclear guanine.

When the cells were grown in medium which contained much smaller amounts of $[8-^{14}\text{C}]$ adenine, the observed pattern of incorporation was considerably modified. The initial concentration of $[8-^{14}\text{C}]$ adenine used in the experiment illustrated by Fig. 4 was $3\text{ }\mu\text{g./ml. (0.02 mM)}$. Two features of the curves require comment at this stage:

(i) The rate of incorporation of radioactivity into the nuclear RNA fell rapidly after the first hour, but the rate of incorporation into the cytoplasmic RNA was maintained throughout the experiment.

(ii) The rate of incorporation of label into the total RNA also fell away rapidly after the first hour, showing that it was the radioactivity in the medium that was being exhausted.

This result is of interest since it resembles some of the results Perry *et al.* (1961) obtained by radioautographic procedures. Perry *et al.* (1961) explained their results in terms of rapid equilibration of the nuclear RNA with the exogenous labelled precursor. This clearly is not the case in the present

Fig. 1. Incorporation of $[8-^{14}\text{C}]$ adenine into the adenine of the RNA and DNA of HeLa cells. The cells were grown as monolayers adherent to glass in medium containing $0.1\text{ mM-}[8-^{14}\text{C}]$ adenine (initial specific radioactivity $10^6\text{ counts/min./}\mu\text{mole}$). Samples of the cells were harvested at intervals and the specific radioactivity of the adenine in the RNA and DNA was determined. O, RNA adenine; ●, DNA adenine.

Fig. 2. Incorporation of $[8-^{14}\text{C}]$ adenine into the adenine of the RNA of HeLa cells. The cells were grown in suspension culture in medium containing $0.1\text{ mM-}[8-^{14}\text{C}]$ adenine (initial specific radioactivity $4 \times 10^5\text{ counts/min./}\mu\text{mole}$). Samples of the cells were harvested at intervals and the specific radioactivity of the adenine in the different RNA fractions was determined. O, Nuclear RNA adenine; ●, cytoplasmic RNA adenine; Δ, total RNA adenine.

Fig. 3. Incorporation of $[8-^{14}\text{C}]$ adenine into the guanine of the RNA of HeLa cells. The experiment was that described under Fig. 2. The specific radioactivity of the guanine in the different RNA fractions was determined. O, Nuclear RNA guanine; ●, cytoplasmic RNA guanine; Δ, total RNA guanine.

work, and it is possible that the results obtained by Perry *et al.* (1961) reflected exhaustion of the supply of labelled precursor in the medium, rather than equilibration of the nuclear RNA with the labelled precursor. This experiment illustrates one of the difficulties that may be encountered in experiments in which small amounts of radioactive precursors of very high specific radioactivity are used.

Incorporation of [2-¹⁴C]uridylic acid. When cells were grown in medium which contained [2-¹⁴C]-uridylic acid, radioactivity entered both uracil and cytosine in the RNA. The incorporation of radioactivity into RNA uracil is shown in Fig. 5. The curves were essentially similar to those shown in Fig. 2. The main difference between the two was the fall in the rate of incorporation of label into the nuclear RNA after the first hour: for RNA uracil the rate of incorporation fell to a value that was only 10 % of that observed initially. Since both adenine and uridylic acid gave this effect, it is reasonable to assume that the nuclear RNA contained at least two fractions, one of which was labelled much more rapidly than the other. The difference between the two precursors presumably reflected the ability of the cells to use uridylic acid more easily than adenine. The effect with uridylic acid was so marked that the rate of incorporation into total RNA uracil also showed a similar fall, although the rate of incorporation into cytoplasmic RNA uracil was increasing during this period. The

sharp fall in the rate of incorporation of label into nuclear RNA uracil cannot, therefore, be due to a fraction of the nuclear RNA being transferred in a stable form to the cytoplasm. It is, however, consistent with the rapid turnover of a minor fraction of nuclear RNA.

Incorporation of [³²P]orthophosphate into ribonucleic acid. The pattern of incorporation of ³²P into the nucleotides of RNA is shown in Figs. 6, 7 and 8. To get reasonable amounts of radioactivity into RNA it is usual to add to the culture medium only trace amounts of orthophosphate of very high specific radioactivity. In the present experiments, however, the [³²P]orthophosphate was added directly to cells growing in medium which already contained 'physiological' concentrations of phosphate, i.e. about 1 mM-orthophosphate. Since the exogenous concentration of precursor may have a considerable effect on the pattern of labelling of RNA, it may not be valid to compare these experiments with those in which different concentrations of phosphate have been used.

The illustrated curves show several interesting features.

(i) All curves show an initial lag in the incorporation of ³²P. This lag is presumably due to the fact that the cells cannot incorporate phosphate directly into macromolecular RNA, so that the phosphorus must pass through various intermediate pools of metabolites. The specific radioactivity of the phosphorus entering the RNA would thus rise during the

Fig. 4. Incorporation of [8-¹⁴C]adenine at low concentration into the adenine of the RNA of HeLa cells. The cells were grown in suspension culture in medium containing 0.02 mM-[8-¹⁴C]adenine (initial specific radioactivity 3.5×10^6 counts/min./μmole). Samples of the cells were harvested at intervals and the specific radioactivity of the adenine in the different RNA fractions was determined. O, Nuclear RNA adenine; ●, cytoplasmic RNA adenine; Δ, total RNA adenine.

Fig. 5. Incorporation of [2-¹⁴C]uridylic acid into the uridylic acid of the RNA of HeLa cells. The cells were grown in suspension culture in medium containing 0.2 mM-[2-¹⁴C]-uridylic acid (initial specific radioactivity 3×10^6 counts/min./μmole). Samples of the cells were harvested at intervals and the specific radioactivity of the uridylic acid in the different RNA fractions was determined. O, Nuclear RNA uridylic acid; ●, cytoplasmic RNA uridylic acid; ▲, total RNA uridylic acid.

Fig. 6. Incorporation of [^{32}P]orthophosphate into the cytidylic acid of the RNA of HeLa cells. The cells were grown in suspension culture in medium containing 1 mM- ^{32}P orthophosphate. Samples were harvested at intervals and the specific radioactivity of the cytidylic acid in the different RNA fractions was determined. ○, Nuclear RNA; ●, cytoplasmic RNA; △, total RNA.

Fig. 7. Incorporation of [^{32}P]orthophosphate into the nucleotides of the nuclear RNA of HeLa cells. The experiment was that described under Fig. 6. The specific radioactivities of the nucleotides of the nuclear RNA were determined. △, Adenylic acid; ▲, cytidylic acid; ○, uridylic acid. The curve for guanylic acid closely follows that for cytidylic acid.

early stages of incorporation as these pools become more and more radioactive, finally attaining a steady maximum level. This process would give incorporation curves of the shape shown.

(ii) The specific radioactivities of the RNA nucleotides did not tend to become equal. The striking thing about the pattern of labelling of the nuclear RNA is not so much the size of the discrepancy between uridylic acid and the other nucleotides, as the fact that only uridylic acid has a different specific radioactivity. In contrast, after the first 3 hr., the specific radioactivities of all four nucleotides of cytoplasmic RNA show consistent and clearly significant differences.

The most widely held view of the nature of the precursors of RNA is that they are phosphorylated at the 5'-position of the ribose molecule. Alkaline hydrolysis is thought to split RNA into 2'- and 3'-nucleotides, with the result that phosphorus which was originally attached to one riboside before incorporation into the RNA molecule is transferred to the neighbouring riboside in the RNA molecule during alkaline hydrolysis of RNA. Alkaline hydrolysis thus has the effect of 'randomizing' the distribution of ^{32}P among the nucleotides. It is therefore difficult to understand how the uridylic acid isolated from the nuclear RNA could have a specific radioactivity so much higher than the other nucleotides unless the preparations were contaminated with uridylic acid that did not come from RNA, or uridylic acid occupied rather special

Fig. 8. Incorporation of [^{32}P]orthophosphate into the nucleotides of the cytoplasmic RNA of HeLa cells. The experiment was that described under Fig. 6. The specific radioactivities of the nucleotides of the cytoplasmic RNA were determined. △, Adenylic acid; ●, guanylic acid; ▲, cytidylic acid; ○, uridylic acid.

positions in the nuclear RNA. The fact that the specific radioactivities of the nucleotides did not tend to become equal during the experiment indicates that labelling with ^{32}P cannot safely be used to measure base ratios (Volkin, 1962; Harris, Fisher, Rodgers, Spencer & Watts, 1963).

Initial lag in the incorporation of radioactivity into cytoplasmic ribonucleic acid. The experiments described above show that the kinetics of incorporation usually seen in the labelling of cytoplasmic RNA are also characteristic of incorporation of radioactivity into DNA (Fig. 1) and can, under certain circumstances, also be observed in the labelling of nuclear RNA (Figs. 3 and 7). The appearance of an initial lag in the incorporation of radioactivity into nuclear RNA was associated with the use of a precursor that required either considerable modification before it was incorporated into RNA, or that had to dilute large intracellular pools of endogenous precursors. It is possible that considerations of this sort may also be responsible for the lag in incorporation of radioactivity into cytoplasmic RNA. Fig. 4 shows that it is possible to devise an experiment in which the kinetics of incorporation of label into the cytoplasmic RNA remain linear during the first 5 hr. of labelling. As a contrast Fig. 9 shows an experiment in which incorporation of label into the cytoplasmic RNA was still curvilinear after 5 hr. of labelling. In the experiment shown in Fig. 4 the cells were grown in 'Eagle's medium' which contained no preformed purines, whereas in the experiment shown in Fig. 9 the cells were grown continuously in 'complex medium' which contained considerable amounts of preformed purines (approx. 0.1 mM with respect to adenine and related purines). There is little doubt that in the experiment shown in Fig. 9 the $[8\text{-}^{14}\text{C}]$ -adenine had to compete with large intracellular accumulations of purine derivatives, and also with exogenous unlabelled purine derivatives. In the experiment shown in Fig. 10 the cells were grown in 'Eagle's medium' for 24 hr. before the radioactive precursor was introduced. Under these conditions, where competition from other sources of adenine was at a minimum:

(i) there was no detectable lag in the appearance of label in the adenine of the cytoplasmic RNA;

(ii) the rate of incorporation of label into the adenine in the cytoplasmic RNA showed little tendency to increase with time.

In view of the results obtained by many workers with radioautographic procedures, the experiment shown in Fig. 10 is remarkable in demonstrating that after only 15 min. in radioactive medium the cytoplasmic RNA contained 25–30 % of the radioactivity in the nuclear RNA.

Losses of ribonucleic acid during phenol extraction. The phenol method has been shown to afford a

remarkably clean separation of nuclear and cytoplasmic RNA in several animal cells (Georgiev, Mantieva & Zbarsky, 1960; Sibatani, Yamana, Kimura & Okagaki, 1958; Harris & Watts, 1962). A difficulty experienced in the present work arose

Fig. 9. Incorporation of $[8\text{-}^{14}\text{C}]$ adenine into the adenine of the RNA of HeLa cells grown in medium containing preformed purines. The cells were grown in suspension culture in non-radioactive complex medium containing adenine and hypoxanthine derivatives at about 0.1 mM. After 18 hr. 0.2 mM- $[8\text{-}^{14}\text{C}]$ adenine was added and samples were then harvested at intervals and the specific radioactivity of the adenine of the different RNA fractions was determined. ○, Nuclear RNA adenine; ●, cytoplasmic RNA adenine.

Fig. 10. Incorporation of $[8\text{-}^{14}\text{C}]$ adenine into the adenine of the RNA of HeLa cells during the first 75 min. in radioactive medium. The cells were grown in suspension culture in Eagle's medium. After 18 hr. 0.2 mM- $[8\text{-}^{14}\text{C}]$ adenine was added and samples were then harvested at intervals and the specific radioactivity of the adenine in the different RNA fractions was determined. ○, Nuclear RNA adenine; ●, cytoplasmic RNA adenine.

Table 1. *Recovery of radioactivity from labelled cells after treatment with phenol or acid*

The cells were grown for 1 hr. in medium containing 0.02 mM-[8-¹⁴C]adenine. One sample of cells was treated with 0.3N-trichloroacetic acid at 0°; the solid residue was then dissolved in 2 ml. of N-KOH and the total radioactivity determined. Another sample of cells was treated with phenol; the residual pellet was dissolved in 2 ml. of N-KOH and the total radioactivity determined; the supernatant from the phenol extraction was acidified at 0°, the precipitate was collected, dissolved in 2 ml. of N-KOH, and the total radioactivity determined.

	Total radioactivity (counts/min.)
Pellet from the phenol extraction	1671
Acid-insoluble material in the supernatant from the phenol extraction	305
Acid-insoluble material from whole cells	2476

during preliminary studies of the incorporation of radioactivity into nuclear RNA, when the values for the specific radioactivities of the RNA bases showed much more scatter than could be accounted for by analytical errors. Closer examination of the phenomenon, which did not affect total RNA or cytoplasmic RNA measurements, showed that the scatter could be eliminated by the procedure described in the Experimental section, i.e. by treating the cells with phenol for at least 1 hr., followed by a careful water wash. The scatter appears to be due to a variable loss of RNA during short periods of extraction with phenol. When the recovery of radioactivity by the phenol method was compared with the recovery after ordinary acid precipitation of whole cells, this discrepancy was revealed. Table 1 gives the radioactivity found in the different fractions after 1 hr. labelling, and shows that the total acid-precipitable RNA contained about 25 % more radioactivity than did the combined fractions after phenol extraction. The loss may be due to the action of a ribonuclease that is not completely destroyed by the action of phenol (Huppert & Pelmont, 1962).

Further evidence (Watts, 1964) supports the idea that the scatter in the specific radioactivities of the bases in the nuclear RNA is due to the presence in the total RNA of the cells of a fraction that is normally lost during the phenol procedure and that is metabolically extremely active, turning over with a half-life of less than 1 hr.

DISCUSSION

The present results show that the observed kinetics of incorporation of radioactivity into both nuclear and cytoplasmic RNA of HeLa cells are complex functions and vary with the conditions of

previous cell growth, the concentration of the exogenous labelled precursor, the efficiency with which the precursor can inhibit endogenous synthesis of precursor and the rate at which it can enter and dilute any intracellular pools of precursors that may exist. These factors are to a greater or smaller extent related in their effects, and cannot easily be resolved from one another. The present results indicate that the nucleus of the HeLa cell contains at least three different classes of RNA that can be distinguished by the different rates at which they become labelled and by their differing susceptibilities to enzymic attack within the cell. However, the different fractions of RNA have not been isolated free from contaminating protein and other cell material. The results are therefore open to the criticism that all the radioactivity may not be in the nucleic acids. Unfortunately the methods of isolation of the different fractions of RNA in HeLa cells (Harris *et al.* 1963) are unsuitable for use in the type of study described above. Until the behaviour of the three fractions has been examined separately, a detailed analysis of the kinetics of labelling of nuclear RNA remains difficult.

The initial lag in the incorporation of radioactivity into cytoplasmic RNA, in contrast with the immediate rapid incorporation of many labelled precursors into nuclear RNA, has been accepted as evidence that cytoplasmic RNA has a nuclear origin (Goldstein & Plaut, 1955; Woods & Taylor, 1959; Fitzgerald & Vinijchaikul, 1959; Zolokar, 1959). It may be correct that some cytoplasmic RNA is derived from the nucleus, but the ease with which the magnitude of the observed lag in the incorporation of radioactivity into cytoplasmic RNA can be varied within wide limits by the control of factors like precursor concentration and growth conditions shows that the presence of the lag does not in itself mean that cytoplasmic RNA has a nuclear origin. It is likely that the initial absence of incorporation of radioactivity into the cytoplasmic RNA that many workers have observed in radioautographic studies has been due to factors of the type examined in the present work; it is no demonstration that cytoplasmic RNA is synthesized in the nucleus. Harris & La Cour (1963) have, indeed, published radioautographic findings that show that radioactivity can enter cytoplasmic RNA without a detectable lag. It is clear that we do not at present have enough information to permit a detailed analysis of the observed kinetics of incorporation of radioactivity into the RNA of HeLa cells. However, the present results show that the simple model of translocation of RNA from the nucleus to cytoplasm is not in itself adequate to account for the observed kinetics.

SUMMARY

1. The incorporation of [8-¹⁴C]adenine, [2-¹⁴C]-uridylic acid and [³²P]orthophosphate into the nucleic acids of HeLa cells was studied.

2. Radioactivity entered the nuclear RNA much more rapidly than the cytoplasmic RNA, and incorporation into cytoplasmic RNA usually attained its maximum rate only after 1 hr.

3. The observed kinetics of incorporation were dependent on the concentration of radioactive precursor in the culture medium. Trace amounts of radioactive precursor were soon exhausted and incorporation into the nuclear RNA then ceased, although radioactivity continued to enter the cytoplasmic RNA for some hours.

4. The initial lag in the incorporation of radioactivity into cytoplasmic RNA could be eliminated by a suitable choice of experimental conditions. When the intracellular pools of unlabelled precursors were diminished as far as possible, 20 % of the total radioactivity in RNA was found in cytoplasmic RNA within 15 min.

5. The kinetics of incorporation of radioactive adenine and uridylic acid indicated that the nuclear RNA contained three fractions that could be distinguished by the rates at which they became radioactive. A rapidly labelled fraction was selectively degraded during the phenol extraction of RNA.

6. When the cells were labelled with [³²P]orthophosphate, the specific radioactivities of the cytoplasmic RNA nucleotides obtained after alkaline hydrolysis of the RNA were all different and showed no tendency to become equal even after 6 hr. labelling. The specific radioactivities of three of the nuclear RNA nucleotides were equal after only 1 hr. labelling, but the uridylic acid consistently had a much higher specific radioactivity than had the other nucleotides.

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