The Loss of Rapidly Labelled Ribonucleic Acid from Isolated HeLa Cell Nuclei

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1. The loss of nucleic acids and protein from isolated HeLa-cell nuclei was studied. During 4 hr. incubation at 37° DNA was conserved, but appreciable amounts of RNA and protein were lost. 2. Two classes of nuclear RNA were distinguished: at least 75% of the RNA was lost from the nuclei relatively slowly through degradation to acid-soluble fragments; the rest of the RNA was lost much more rapidly, not only through degradation to acid-soluble fragments but also through diffusion of RNA out of the nuclei into the incubation medium. 3. The RNA that was preferentially lost was the fraction of nuclear RNA that was rapidly labelled when intact HeLa cells were grown in a medium containing radioactive precursors of RNA. 4. The RNA appearing in the incubation medium was apparently partially degraded and had a sedimentation coefficient of about that of transfer RNA. 5. Both the degradation of RNA and the loss of RNA from the nuclei were sensitive to bivalent cations. Low concentrations of Mg^{2+} and Mn^{2+} greatly increased the rate of degradation of the rapidly labelled RNA to acid-soluble fragments, and produced a corresponding decrease in the amount of RNA diffusing into the medium. At higher concentrations they suppressed both degradation and diffusion of RNA. The cations Ca^{2+} , Cu^{2+} , Zn^{2+} and Ni^{2+} all progressively inhibited both forms of loss of RNA. 6. Salts of univalent cations produced appreciable effects only at ionic strengths of about 0.2, when degradation to acid-soluble fragments was preferentially inhibited. 7. Both ADP and ATP inhibited loss of RNA at about 30 mM. 8. It was concluded that the diffusion of rapidly labelled RNA out of the isolated nuclei was not related to the movement of RNA from nucleus to cytoplasm *in vivo*, but reflected the ease with which the rapidly labelled RNA detached from the chromatin and the permeability of the membranes of isolated nuclei.

Several different experimental approaches have provided evidence that the RNA in the cell is made in the nucleus (Prescott, 1964; Georgiev, 1967) and then translocated to different parts of the cell. Thus the nucleolus is thought to be the primary site of synthesis of ribosomal RNA (Perry, 1967). The evidence for the translocation of RNA was originally largely derived from radioautographic and chemical studies of the pattern of incorporation and retention of labelled precursors of RNA within the cell. The kinetics of these processes were thought to show a precursor-product relationship between nuclear and cytoplasmic RNA, but evidence has accumulated that the results are complicated by turnover of a fraction of RNA that is probably restricted to the nucleus (Harris, 1963*a*; Watts, 1964*b*; Roberts, 1965; Adams, 1966; Attardi, Parnas, Hwang & Attardi, 1966; Shearer & McCarthy, 1967).

Isolated nuclei have been used to study the mechanism of intranuclear turnover of RNA, and it

is clear that some features of RNA catabolism in the isolated nucleus resemble those observed in the intact cell (Harris, Fisher, Rodgers, Spencer & Watts, 1963; Harris, 1963*b*). The use of isolated nuclei is open to criticism because of the methods of isolation and incubation, and ignorance of the effects of cytoplasm on nuclear behaviour. Isolated nuclei retain, however, a considerable degree of structural integrity and function. The properties of isolated HeLa-cell nuclei were therefore examined further to see whether they can provide more information about the metabolism of nuclear RNA.

METHODS

HeLa cells were grown in suspension culture by the methods described by Watts (1964*a*).

Isolated nuclei were prepared by the method of Fisher & Harris (1962) modified for use with a Dounce homogenizer. Unless otherwise stated, approximately 10^8 HeLa cells were suspended in 60 ml. of freshly prepared 0.1% (v/v) Tween 80 and enucleated by about eight strokes in a stainless-steel

Dounce homogenizer at 4°. The nuclei were centrifuged at 300g for 3 min. and washed twice with 50 ml. volumes of 0.25 M-sucrose containing 0.09 M-tris-HCl buffer, pH 7.5 at 21° (sucrose-tris medium). They were finally suspended in about 15 ml. of sucrose-tris medium for use in experiments. The nuclear preparations were kept at 4° until used. All incubations were done in a water bath at 37°.

The number of nuclei in each sample was determined by measuring the DNA content by the method of Burton (1956) and assuming a mean content of 22 pg. of DNA/nucleus (Salzman, 1959).

Materials. [8-¹⁴C]Adenine (specific radioactivity 30 mc/m-mole), [1-¹⁴C]valine (specific radioactivity 5 mc/m-mole), [G-¹⁴C]uridine (specific radioactivity 30 mc/m-mole), [8-¹⁴C]ATP (specific radioactivity 18 mc/m-mole), [2-¹⁴C]-thymidine (specific radioactivity 30 mc/m-mole) and [2,8-³H₂]adenine (specific radioactivity 5 c/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

The sodium salts of AMP, ADP and ATP were Sigma grade from Sigma Chemical Co., St Louis, Mo., U.S.A.

Measurement of the loss of RNA from isolated nuclei. When the loss of total RNA was determined, isolated nuclei were incubated in sucrose-tris medium. Samples (1 ml.) were taken at intervals and centrifuged at 300g for 3 min., and the pellet of nuclei was then extracted with 2.5 ml. of 0.6 M-HClO₄ for 15 min. at 0°. After centrifugation at 1000g for 5 min. the extract was discarded and the pellet was hydrolysed with 1 ml. of 0.3 M-KOH overnight at 37°. The hydrolysate was cooled to 0°, acidified with M-HClO₄ and centrifuged at 1000g for 10 min. The supernatant was collected and its *E*₂₆₀ was measured in a Unicam SP.800 spectrophotometer.

The disappearance of labelled RNA from nuclei was determined with nuclei prepared from cells that had been grown in medium containing 0.02 μCi of [8-¹⁴C]adenine or [1-¹⁴C]uridine/ml. When the cells had been labelled for less than 2 hr., there was negligible radioactivity in the DNA. The isolated nuclei were incubated in sucrose-tris medium, samples (0.5 ml.) were taken at intervals, 2.5 ml. of 0.6 M-trichloroacetic acid was added at 0°, the precipitate was centrifuged at 1000g for 5 min. and washed with 3 ml. of water, and the radioactivity counted. This procedure was modified when the appearance of RNA in the incubation medium was investigated. After incubation, samples of nuclei (1 ml.) were centrifuged at 300g for 3 min. and the supernatant medium was collected. Yeast RNA (1 mg.; British Drug Houses Ltd., Poole, Dorset) was added as carrier to the supernatant, and 2.5 ml. of 0.6 M-trichloroacetic acid was added at 0° to both the pellet of nuclei and the supernatant. The samples were centrifuged at 1000g for 5 min., and the radioactivity in the precipitates was counted.

When the nuclei had been prepared from cells that had been labelled for several hours, there was appreciable radioactivity in DNA. This was eliminated by hydrolysing the precipitates obtained after treatment with 0.6 M-trichloroacetic acid with 0.5 ml. of 0.3 M-KOH for 4 hr. at 37°. The hydrolysate was then acidified with M-HClO₄ at 0° and centrifuged at 1000g for 10 min., and the supernatant was collected and counted.

Measurement of the loss of radioactive DNA and protein from isolated nuclei. HeLa cells were grown in medium containing [2-¹⁴C]thymidine (0.02 μCi/ml.) to label DNA or

[1-¹⁴C]valine (0.04 μCi/ml.) to label protein. Nuclei were prepared and incubated in sucrose-tris medium. Samples (1 ml.) were taken at intervals and centrifuged at 300g for 3 min., and the supernatant was removed from the pellet; 0.6 M-trichloroacetic acid (2.5 ml.) was added to both pellet and supernatant at 0°, the samples were centrifuged at 1000g for 5 min., and the radioactivity in the precipitates was counted.

Measurement of radioactivity. The samples were dissolved in 0.25 ml. of M-cetyltrimethylammonium hydroxide in methanol, added to 4 ml. of methanol and mixed with 10 ml. of a solution of 4 g. of 2,5-diphenyloxazole and 0.05 g. of 1,4-bis-(5-phenyloxalol-2-yl)benzene in 1 l. of toluene, and counted in a liquid-scintillation counter (Nuclear-Chicago Corp. model 720). All counts were corrected for internal quenching.

Preparation of radioautographs. About 2.5 × 10⁷ cells were grown for 20 min. in 50 ml. of medium containing [2,8-³H₂]adenine (10 μCi/ml.). The nuclei were isolated and incubated in 5 ml. of sucrose-tris medium. Samples (2 ml.) were taken before and after 20 min. incubation and centrifuged at 300g for 3 min., and the pellet of nuclei was fixed with methanol. The nuclei were embedded in rubber wax and sections were cut on water at 2 μm. Radioautographs were prepared in the usual way from Kodak V1042 stripping film, exposed for 2 weeks at 4° and developed. Radioautographs of whole cells labelled and then incubated in sucrose-tris medium in the same way were also prepared.

Sucrose-density-gradient examination of the RNA leaving isolated nuclei. Cells (10⁸) were grown for 15 min. in medium containing [8-¹⁴C]adenine (0.02 μCi/ml.), and nuclei were then prepared, incubated for 15 min. in 15 ml. of sucrose-tris medium and centrifuged at 300g for 3 min.; the supernatant was collected and the RNA precipitated with 2 vol. of acetone. The nuclei and the acetone precipitate were deproteinized by the method of Philipson (1961), which gave almost quantitative recovery of nuclear RNA (Harris *et al.* 1963; Bramwell & Harris, 1967). The pellets were treated with 10 ml. of aq. 2% (w/v) sodium dodecyl sulphate, followed by 10 ml. of aq. 80% (w/v) phenol, and shaken at 4° for 15 min. The mixture was centrifuged at 2000g for 15 min., the lower phenol phase was discarded, and the residue was extracted a second time with aq. 80% phenol. The upper layer was collected, and 2 vol. of ethanol was added. The precipitate was washed twice with ethanol and dissolved in 0.5 ml. of 0.1 M-MgCl₂-10 mM-tris-HCl buffer, pH 7.3 at 20°. The solution (0.25 ml.) was layered on to a linear 5–20% sucrose gradient in the same buffer in a 5 ml. centrifuge tube, and centrifuged for 3 hr. at 36 000 rev./min. in the SW 39 rotor of a Spinco model L ultracentrifuge. The contents of the centrifuge tube were fractionated by puncturing the bottom of the tube with a hypodermic needle and collecting fractions (15 drops). Each fraction was diluted with 0.5 ml. of water, *E*₂₆₀ was measured with a Unicam SP.800 spectrophotometer, and the radioactivity in a sample was measured in the usual way.

DNA interfered with attempts to locate RNA in the sucrose density gradient by *E*₂₆₀, but could be removed, in the case of unincubated nuclei, by the method of Bramwell & Harris (1967). The gel obtained when nuclei were treated with 2% sodium dodecyl sulphate was digested at room temperature with 100 μg. of deoxyribonuclease I (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) for about 3 min., and then extracted with phenol as

described above. The precipitate of RNA with DNA fragments obtained with ethanol was extracted with 10ml. of 2M-LiCl at 0°, washed twice with ethanol, dissolved in 0.5ml. of 5% sucrose-0.1 mM-MgCl₂-10 mM-tris-HCl buffer, pH 7.3 at 20°, and centrifuged in a sucrose density gradient as described above.

RESULTS

Loss of nucleic acids from isolated nuclei. There was no loss of radioactivity when nuclei with labelled DNA were incubated for 4 hr. at 37° (Fig. 1). This confirms the observation by Harris (1963b). In contrast with the retention of DNA, there was a loss of almost 50% of the total nuclear RNA during the same time. Fig. 1 shows that there was a rapid loss of about 20% of the RNA during the first 30 min. incubation, followed by a much slower loss during the next 3.5 hr. of about 8%/hr. The loss of nuclear RNA, but not DNA, has also been observed in the intact cell grown in conditions that inhibit RNA synthesis, e.g. in the presence of actinomycin D or proflavin (Weisner, Acs, Reich & Shafiq, 1965; Watts & Davis, 1966).

Although the loss of RNA clearly involved the destruction of nuclear components, examination by

both light microscopy and electron microscopy showed no obvious changes in the nuclear and nucleolar structure of isolated nuclei during the first 60 min. of incubation at 37° in sucrose-tris medium.

The loss of RNA was also examined by following the disappearance of radioactivity from nuclei prepared from cells that had been grown for 30 min. in a medium containing [8-¹⁴C]adenine. Under these conditions over 90% of the radioactivity was in RNA (Watts, 1964a). The loss of labelled RNA was much faster than the loss of total RNA (Fig. 1). About 50% of the radioactivity initially present was lost during the first 15 min. of incubation. Nuclei from cells grown in a medium containing [¹⁴C]-uridine gave similar results. The difference between the rates of loss of total and labelled RNA shows that there was a preferential loss of the rapidly labelled fraction of RNA (Harris, 1963b). The rate at which other, less rapidly labelled, fractions were lost was considerably lower.

Radioautography was used to determine the site of the rapidly labelled RNA lost from the nuclei during incubation. The radioactivity in nuclei prepared from cells that had been grown for 20 min. in a medium containing [³H]adenine was found mainly in the extranucleolar regions. There was no significant redistribution of label after the nuclei had been incubated for 20 min. in sucrose-tris medium, although some 50% of the radioactivity had been lost during this time. It therefore appears that under these conditions the rapidly labelled RNA lost from the nuclei was principally non-nucleolar, presumably associated with the chromatin. Thus the bulk of the radioactivity was not the 45s RNA, which is localized in the nucleolus, but rather in the polydisperse extranucleolar fraction that is preferentially labelled by purine precursors (Harris, 1959) and turns over rapidly (Soeiro, Birnboim & Darnell, 1966). The result gives no real indication of the relative absolute amounts of rapidly labelled RNA lost from the nucleolus and from the rest of the nucleus, because the distribution of radioactivity between the two regions depends on the labelled precursor used (Harris, 1959; Newsome, 1966).

A parallel experiment with intact cells that had been labelled with [³H]adenine and then incubated in sucrose-tris medium for 20 min. showed no detectable increase in the radioactivity in the cytoplasm. The loss of rapidly labelled RNA from isolated nuclei was thus not apparently related to any equivalent movement of rapidly labelled RNA in a stable form into the cytoplasm of the intact cell.

Loss of RNA from isolated nuclei. The rate of loss of rapidly labelled RNA shown in Fig. 1 was considerably greater than that observed by Harris

Fig. 1. Loss of DNA and RNA from isolated nuclei. The loss of DNA was determined with nuclei prepared from cells that had been grown for 30 min. in medium containing [2-¹⁴C]thymidine. The loss of total RNA was determined from *E*₂₆₀. The loss of rapidly labelled RNA was determined with nuclei prepared from cells that had been grown for 30 min. in medium containing [8-¹⁴C]adenine. O, [2-¹⁴C]-Thymidine in DNA; ▲, total nuclear RNA (arbitrary units); ●, [8-¹⁴C]adenine in nuclear RNA.

Fig. 2. Loss of rapidly labelled RNA from isolated nuclei. Nuclei were prepared from cells that had been grown for 1 hr. in medium containing $[8\text{-}^{14}\text{C}]\text{adenine}$, and incubated in sucrose-tris medium. Samples (2×10^6 nuclei) were harvested at intervals, and the acid-precipitable radioactivity in the nuclei and the medium was determined. $\circ$, Radioactivity in nuclei; $\bullet$, radioactivity in medium; $\blacktriangle$, total radioactivity in nuclei and medium.

(1963b), who found the half-time for degradation of RNA to acid-soluble fragments to be about 2.5 hr. The difference was due to the methods by which the nuclei were treated after incubation, and not to difference in conditions of incubation. The methods used in the experiment shown in Fig. 1 measured the loss of rapidly labelled RNA through both degradation to acid-soluble fragments and diffusion of macromolecular RNA out of the nuclei; the methods used by Harris (1963b) measured only degradation to acid-soluble material. When the RNA contents in the nuclei and medium were separately determined, the results shown in Fig. 2 were obtained. In addition to degradation of RNA to acid-soluble fragments, there was a very large movement of rapidly labelled RNA in an acid-precipitable form out of the nuclei into the incubation medium. The content of radioactive RNA in the medium reached a maximum after about 30 min. incubation and then remained substantially constant for the rest of the experiment. Well over 50% of the labelled RNA lost from the nuclei during the first 30 min. was recovered from the medium in an acid-precipitable form. After 45 min. incubation there was actually more labelled RNA in the medium than in the nuclei. The process of degradation to acid-soluble fragments was much slower and was similar to that described by Harris (1963b), with a half-time in excess of 2 hr.

Experiments were also done with nuclei prepared from cells that had been labelled for shorter and

Table 1. Loss of RNA from isolated nuclei after different periods of labelling *in vivo*

Cells were grown for the stated time of labelling in medium containing $[8\text{-}^{14}\text{C}]\text{adenine}$; the cells labelled for 4 hr. were subsequently grown a further 18 hr. in non-radioactive medium. The nuclei were prepared and incubated in sucrose-tris medium, samples were taken at intervals and the loss of RNA by degradation and diffusion was determined.

Time of labelling (hr.)	Time of incubation (min.)	Loss of labelled RNA (%)	
		Diffusion	Degradation
$\frac{1}{4}$	20	39	21
	90	35	48
1	20	30	17
	90	33	45
4	20	25	11
	90	27	13

longer times. Table 1 shows that the rate of degradation of labelled RNA was not significantly increased by using a shorter period of labelling, but the relative amount of labelled RNA appearing in the medium was considerably increased. When nuclei were prepared from cells that had been labelled for 18 hr., there was still a fraction of labelled RNA that was rapidly and preferentially lost from the isolated nuclei. Table 1 also shows the loss of radioactive RNA from nuclei prepared from cells that had been grown for 4 hr. in medium containing $[8\text{-}^{14}\text{C}]\text{adenine}$ and then for 18 hr. in non-radioactive medium. These conditions should have decreased the contribution of the rapidly labelled fraction to the radioactivity in the RNA. There was still, however, a rapid loss of radioactivity from the nuclei during the first 30 min., most of which was recovered as RNA in the incubation medium. After this the rate of loss of radioactivity from the nuclei was much lower, and was accounted for entirely by degradation of RNA to acid-soluble fragments; further, degradation occurred at a much lower rate than in the nuclei prepared from pulse-labelled cells.

The results thus demonstrate the following points. (i) There was a large loss of RNA from the nucleus, by both diffusion and degradation. (ii) The RNA that diffused into the medium included the rapidly labelled fraction of RNA. (iii) All fractions of nuclear RNA were degraded, but the rapidly labelled fraction was preferentially degraded. (iv) The fraction of RNA that diffused into the medium comprised not more than 25% of the total nuclear RNA.

Character of the RNA appearing in the medium. Most of the radioactivity in nuclear RNA after a short period of labelling is in molecules with

Table 2. *Recovery from the medium of radioactive RNA lost from isolated nuclei*

Nuclei, prepared from cells that had been grown for 20 min. in medium containing [8-¹⁴C]adenine, were incubated for 10 min. in sucrose-tris medium. Samples were taken before and after incubation, and centrifuged at 300g for 3 min. The pellets and supernatants were treated with 2 vol. of ethanol or of 0.6M-trichloroacetic acid, with or without the addition of 1 mg. of carrier RNA. The samples were allowed to stand 1 hr. at 0° and centrifuged at 2000g for 5 min., and the radioactivity of the precipitates was counted.

Time of incubation (min.)	Fraction	Reagent for precipitation	Carrier RNA	Radio-activity (c.p.m.)
0	Nuclei	Acid	Absent	14425
20	Nuclei	Acid	Absent	9170
20	Medium	Acid	Absent	2170
20	Medium	Acid	Present	4950
20	Medium	Ethanol	Absent	3020
20	Medium	Ethanol	Present	4890

sedimentation coefficients above 16s (Georgiev, 1967). Molecules of this size are readily precipitated by ethanol or trichloroacetic acid. Table 2 shows the efficiency with which the RNA that appeared in the medium was precipitated by these reagents. Carrier RNA was necessary to obtain relatively complete precipitation, and recovery was the same with both ethanol and trichloroacetic acid when carrier was present. In this particular experiment, after 10 min. incubation the nuclei had lost about 36% of their radioactivity, and about 94% of this loss was recovered in the medium as acid- or ethanol-precipitable material.

Some idea of the size of the RNA appearing in the medium was obtained by centrifuging it on a sucrose density gradient. Irrespective of whether the material was applied directly to the gradient, or first purified by treatment with phenol, both the radioactivity and the ultraviolet-absorption profiles were the same: the RNA was in a zone that moved only slowly down the gradient (Fig. 3). Most of the RNA initially present in the nuclei moved much more rapidly; the ultraviolet absorption showed the characteristic major peak at about 28s, whereas the radioactivity was in a polydisperse fraction that occupied most of the gradient (Harris *et al.* 1963; Bramwell & Harris, 1967). Fig. 3 shows, however, that after 15 min. incubation the radioactive RNA remaining in the nuclei had undergone considerable degradation; the radioactivity and ultraviolet-absorption profiles then coincided. It would therefore seem probable that the RNA that appeared in the medium had been degraded within the nuclei, presumably by an endonuclease.

Loss of protein from isolated nuclei. It is possible

Fig. 3. Sucrose-density-gradient profile of the rapidly labelled RNA appearing in the medium. Nuclei, prepared from cells that had been grown for 15 min. in medium containing [8-¹⁴C]adenine, were incubated for 10 min. in sucrose-tris medium. The RNA in the medium, and in the nuclei before and after incubation, were prepared and centrifuged for 3 hr. at 36 000 rev./min. on a sucrose density gradient in the SW 39 rotor of the Spinco model L ultracentrifuge. The recovery of radioactivity from the gradients was better than 90%. ○, Radioactivity in nuclei before incubation; ●, radioactivity in nuclei after incubation; ▲, radioactivity in medium.

that the loss of RNA was associated with a loss of protein; for completeness therefore the loss of radioactive protein from isolated nuclei was examined (Fig. 4). There was a progressive loss of protein from the nuclei and only a small proportion of this was recovered from the medium in an acid-precipitable form; the bulk of the loss into the medium occurred in the first 30 min. incubation, as in the case of appearance of labelled RNA in the medium (Fig. 2). The significance of this result is uncertain and there was no evidence of a strong association between the RNA and protein in the medium. Although DNA and RNA are conserved, an appreciable fraction of the nuclear dry weight is lost during the isolation of nuclei (Fisher & Harris, 1962); the loss illustrated in Fig. 4 may therefore have been the continuation of this process. The fact that much of the protein lost was acid-soluble suggests that histones were present in it.

Fig. 4. Loss of protein from isolated nuclei. Nuclei, prepared from cells that had been grown for 40 min. in medium containing $[1-^{14}\text{C}]$ valine, were incubated in sucrose-tris medium. Samples (2.5×10^6 nuclei) were taken at intervals, and the acid-precipitable radioactivity in the nuclei and the medium was determined. $\circ$, Radioactivity in nuclei; $\bullet$, radioactivity in medium; $\blacktriangle$, total radioactivity in nuclei and medium.

Effect of cations on the loss of RNA. Both the degradation of rapidly labelled nuclear RNA and the appearance of labelled RNA in the medium were inhibited by the bivalent cations examined (Table 3). The effect of Mg^{2+} is shown in Fig. 5. The lower concentrations of Mg^{2+} stimulated the degradation of nuclear RNA to acid-soluble fragments, but the appearance of RNA in the incubation medium was quite strongly inhibited even by the lowest concentrations of the cation. Rather lower concentrations of Mn^{2+} (0.5 mM) produced a similar effect. In view of the increased degradation of RNA in the presence of low concentrations of Mg^{2+} , the effect of 6 mM-magnesium chloride on the loss of RNA from isolated nuclei was examined. Fig. 6 shows that there was little effect on the total loss of RNA from the nuclei, but the degradation to acid-soluble fragments was markedly increased, and there was a similar large decrease in the amount of labelled RNA appearing in the medium. There therefore appears to be a close link between the processes of release of labelled RNA and its degradation, and this coupling is in some way Mg^{2+} -dependent.

The cations Ca^{2+} , Cu^{2+} , Ni^{2+} and Zn^{2+} all had similar effects, progressively inhibiting both the degradation of rapidly labelled RNA and its appearance in the incubation medium. Cu^{2+} , Ni^{2+} and Zn^{2+} were, however, active at much lower concentrations than Ca^{2+} (Table 3). This was perhaps not surprising in view of the ability of these cations to precipitate nucleic acids and their components.

Fig. 5. Effect of MgCl_2 on the loss of rapidly labelled RNA from isolated nuclei. Nuclei (2×10^6 /sample), prepared from cells that had been grown for 20 min. in medium containing $[8-^{14}\text{C}]$ adenine, were incubated for 20 min. in sucrose-tris medium containing MgCl_2 , and the acid-precipitable radioactivity in the nuclei and the medium was determined. $\circ$, Radioactivity in nuclei; $\bullet$, radioactivity in medium; $\blacktriangle$, total radioactivity in nuclei and medium; ----, radioactivity in nuclei before incubation.

Fig. 6. Effect of 6 mM- MgCl_2 on the loss of rapidly labelled RNA from isolated nuclei. The experiment was similar to that in Fig. 2. Nuclei (4×10^6 /sample), prepared from cells that had been grown for 15 min. in medium containing $[8-^{14}\text{C}]$ adenine, were incubated in sucrose-tris medium containing 6 mM- MgCl_2 , and the acid-precipitable radioactivity in the nuclei and the medium was determined. $\circ$, Radioactivity in nuclei; $\bullet$, radioactivity in medium; $\blacktriangle$, total radioactivity in nuclei and medium.

The univalent cations Na^+ , K^+ and NH_4^+ had little effect on the loss of rapidly labelled RNA until concentrations above 0.1 M were used. The effects

Table 3. *Effect of bivalent cations on the loss of radioactive RNA from isolated nuclei*

Nuclei, prepared from cells that had been grown for 20 min. in medium containing $[8-^{14}\text{C}]$ adenine, were incubated for 20 min. in sucrose-tris medium containing different concentrations of the various cations. The total loss of labelled RNA from the nuclei was then determined, i.e. the sum of diffusion and degradation of RNA. The results given in the table show the concentrations of cations that decreased the loss of RNA by 50%.

Salt	Concn. of salt that decreased the loss of RNA by 50% (mM)
MgCl ₂	22
CaCl ₂	9
MnCl ₂	2
NiCl ₂	0.2
CuSO ₄	0.1
ZnSO ₄	0.05

Fig. 7. Effect of $(\text{NH}_4)_2\text{SO}_4$ on the loss of rapidly labelled RNA from isolated nuclei. The experiment was similar to that in Fig. 5. Each sample contained 3.5×10^6 nuclei. The acid-precipitable radioactivity in the nuclei and the medium was determined. $\circ$, Radioactivity in nuclei; $\bullet$, radioactivity in medium; $\blacktriangle$, total radioactivity in nuclei and medium; ----, radioactivity in nuclei before incubation.

produced by these higher concentrations were presumably related to high ionic strengths rather than to any specific effects of individual cations or anions. Fig. 7 shows the effect of ammonium

sulphate; sodium chloride and potassium chloride produced similar effects: as the salt concentration increased, degradation to acid-soluble material at first increased and then was progressively inhibited. In contrast, the appearance of labelled RNA in the medium was stimulated slightly. The experiment shown in Fig. 7 is of some interest because the highest concentration of ammonium sulphate used (0.125 M) almost completely suppressed degradation without producing any significant decrease in the appearance of RNA in the medium. This provides a clear demonstration that the processes by which RNA was released from the nuclei and degraded could be separated from one another.

Harris (1963b) used an incubation medium containing Mg^{2+} and Ca^{2+} in addition to potassium dihydrogen phosphate and sodium chloride to examine the degradation of rapidly labelled RNA in isolated HeLa-cell nuclei. In view of the marked effects of these ions on the degradation and movement of nuclear RNA, the experiment shown in Table 1 was repeated with the 'High K' medium described by Harris *et al.* (1963) in place of sucrose-tris medium. The results were virtually the same as those shown in Table 1.

Effect of Tween 80 on the loss of RNA. When nuclei are isolated in presence of detergents, the outer of the two nuclear membranes is removed (Fisher & Harris, 1962; Hubert, Favard, Carasso, Rozenecwajg & Zalta, 1962), and the detergent may have other less obvious effects. Nuclei were therefore prepared in water without Tween 80, and the loss of rapidly labelled RNA was examined. These nuclei retained the outer nuclear membrane, but electron microscopy showed that it was badly fragmented. The degradation of rapidly labelled RNA and the appearance of labelled RNA in the medium were indistinguishable from those observed with nuclei treated with detergent (Table 1).

As a further check, nuclei were isolated in 0.25 M-sucrose; this method, however, introduces another complication since bivalent cations (Ca^{2+} , Mg^{2+} or Mn^{2+}) must be present at about 2 mM during enucleation to prevent the nuclei bursting. The presence of bivalent cations during isolation, even when the nuclei were washed several times to remove them, had a marked effect on the subsequent behaviour of the nuclei when incubated in sucrose-tris medium. Table 4 shows the loss of RNA from nuclei isolated in 0.25 M-sucrose and incubated in sucrose-tris medium containing Mg^{2+} or EDTA. The behaviour of the nuclei was essentially the same as that observed with nuclei isolated in Tween 80 solutions: there was large-scale diffusion of RNA into the medium when bivalent cations were absent, and very rapid degradation of RNA when Mg^{2+} was present. This result is in agreement with the work of Scholtissek & Potter

Table 4. *Loss of RNA from nuclei isolated in 0.25M-sucrose*

Cells, grown for 15 min. in medium containing $[8-^{14}\text{C}]$ -adenine, were enucleated in 0.25M-sucrose containing CaCl_2 or MgCl_2 . The nuclei were washed twice with sucrose-tris medium, and incubated for 20 min. in sucrose-tris medium with and without added MgCl_2 or EDTA. The loss of labelled RNA by diffusion into the medium or degradation to acid-soluble fragments was then determined.

Salt present during enucleation	Additions to incubation medium	Loss of RNA (%)	
		Degradation	Diffusion
2mM- CaCl_2	—	2.5	20
2mM- MgCl_2	—	16	22
3mM- MgCl_2 *	—	5	18
	5mM- MgCl_2	38	5
	10mM-EDTA	2	54

* Nuclei isolated in sucrose containing 3mM- MgCl_2 were washed before use with sucrose-tris medium containing 20mM-EDTA, and then with sucrose-tris medium.

Fig. 8. Effect of ADP on the loss of rapidly labelled RNA from isolated nuclei. The experiment was similar to that in Fig. 5. Each sample contained 1.5×10^6 nuclei. The acid-precipitable radioactivity in the nuclei and the medium was determined. ○, Radioactivity in nuclei; ●, radioactivity in medium; ▲, total radioactivity in nuclei and medium; ----, radioactivity in nuclei before incubation.

(1960) with liver-cell nuclei isolated in sucrose solutions, and indicates that the effects are general, and not an artifact caused by the use of detergents.

Effect of AMP, ADP and ATP on the loss of RNA. Harris (1963b) found that ADP inhibited the degradation of RNA in isolated nuclei; Fig. 8 confirms and extends this observation. AMP and ATP produced similar effects.

Table 5. *Effect of ammonium sulphate on RNA synthesis in isolated nuclei*

Nuclei (5×10^6) were incubated for 15 min. at 37° in sucrose-tris medium containing 5mM- MgCl_2 , CTP, GTP and UTP each at 0.6mM, $[8-^{14}\text{C}]$ ATP (0.1 $\mu\text{C}/\text{ml.}$) and various amounts of $(\text{NH}_4)_2\text{SO}_4$. The sample volume was 1 ml. The nuclei were centrifuged and RNA was precipitated in the usual way with 10% trichloroacetic acid. The precipitates were washed three times with trichloroacetic acid and the radioactivity was counted. Concentrations greater than about 250mM- $(\text{NH}_4)_2\text{SO}_4$ caused the nuclei to gel after a few minutes' incubation; above this concentration, therefore, the values given represent total incorporation.

Concn. of $(\text{NH}_4)_2\text{SO}_4$ in medium (mM)	$[^{14}\text{C}]$ ATP incorporated (pmoles/mg. of DNA)	
	Nuclei	Medium
0*	14	5
0	68	27
50	189	75
100	249	65
150	264	69
200	366	83
300	510	
400	442	
500	455	

* Incubation without GTP, UTP and CTP.

Isolated nuclei can be stimulated to synthesize RNA by the addition of the four ribonucleoside triphosphates and 5mM-magnesium chloride; the optimum pH of the reaction is about 8.5 (Weiss & Gladstone, 1959; Goldberg, 1961). The conditions of incubation used in the present work were not optimum, but significant RNA synthesis was observed when nuclei were incubated in sucrose-tris medium containing 5mM-magnesium chloride and the ribonucleoside triphosphates (Table 5). Under these conditions, however, the loss of rapidly labelled RNA was essentially the same as shown in Fig. 6: there was extensive degradation of labelled RNA, and very little diffusion of RNA into the medium.

Goldberg (1961) and Widnell & Tata (1966) showed that the synthesis of RNA by isolated nuclei is stimulated by the presence of the salts of univalent cations, especially ammonium sulphate, at about 0.4M. Table 5 shows that this was also the case with RNA synthesis by nuclei isolated in Tween 80 solution. It is probable, therefore, that at least part of the stimulation of RNA synthesis was due to the suppression of degradation of RNA.

DISCUSSION

One feature that emerges from the results is the way in which the subsequent behaviour of isolated nuclei depends on the conditions used during

isolation. If the presence of bivalent cations during enucleation can produce effects on the subsequent catabolism of RNA on the scale described here, it is probable that other aspects of the behaviour of isolated nuclei will also be affected.

There appear to be two main classes of RNA in isolated nuclei: the first was rapidly lost from the nuclei during incubation at 37°, partly through degradation to acid-soluble fragments, and partly through diffusion out of the nuclei into the incubation medium, and it contained most, if not all, of the rapidly labelled RNA, and comprised not more than 25% of the total nuclear RNA; the second comprised about 75% of the total nuclear RNA, and there was no evidence that any of it appeared in the incubation medium as RNA; *in vivo* it incorporated radioactive precursors relatively slowly, and *in vitro* was degraded to acid-soluble fragments relatively slowly. The reasons for these differences in behaviour are not known, but there is a great deal of evidence that in the intact cell the nucleus contains several classes of RNA, as judged by such criteria as turnover (Watts, 1964b) and competitive hybridization with DNA (Georgiev, 1967; Perry, 1967; Shearer & McCarthy, 1967). There are thus grounds for thinking that the differences between the two groups are related to processes that occur in the intact cell.

Most of the rapidly labelled RNA is thought to be degraded within the nucleus *in vivo* (Harris, 1963a; Roberts, 1965; Attardi *et al.* 1966; Soeiro *et al.* 1966). It is therefore unlikely that the diffusion of RNA out of the isolated nuclei is related to the translocation of RNA from nucleus to cytoplasm *in vivo*; it probably reflected the ease with which the rapidly labelled RNA could be dissociated from the chromatin, and the permeability of the membranes of isolated nuclei. This view is supported by the sensitivity of both degradation and diffusion to the presence of bivalent cations. Diffusion was substantially independent of degradation of RNA, since it could occur in the almost complete absence of degradation to acid-soluble fragments. The suppression of diffusion of RNA out of the isolated nuclei may largely be due to the ability of bivalent cations to coagulate the nuclear contents, since the chromatin in nuclei incubated in medium containing bivalent cations such as Mg^{2+} and Ca^{2+} at about 5 mM visibly becomes more granular. Presumably it was the release of RNA from chromatin, rather than the subsequent diffusion out of the nucleus, that was affected by bivalent cations.

Lazarus & Sporn (1967) described an exonuclease in animal-cell nuclei that is dependent on Mg^{2+} or Mn^{2+} , but not on Ca^{2+} , which preferentially degrades the rapidly labelled fraction of nuclear RNA to 5'-mononucleotides. The stimulation of degradation of rapidly labelled RNA by Mg^{2+} and Mn^{2+} , but not by Ca^{2+} , observed in the present work, together with the previous report that degradation of rapidly labelled RNA in isolated nuclei produces only 5'-mononucleotides (Harris *et al.* 1963), indicate that an enzyme of this type was probably responsible.

REFERENCES

- Adams, D. H. (1966). *Biochem. J.* **98**, 636.
 Attardi, G., Parnas, H., Hwang, M. & Attardi, B. (1966). *J. molec. Biol.* **20**, 145.
 Bramwell, M. E. & Harris, H. (1967). *Biochem. J.* **103**, 816.
 Burton, K. (1956). *Biochem. J.* **62**, 315.
 Fisher, H. W. & Harris, H. (1962). *Proc. Roy. Soc. B*, **156**, 521.
 Georgiev, G. P. (1967). *Progr. Nucleic Acid Res. molec. Biol.* **5**, 259.
 Goldberg, I. H. (1961). *Biochim. biophys. Acta*, **51**, 201.
 Harris, H. (1959). *Biochem. J.* **73**, 362.
 Harris, H. (1963a). *Progr. Nucleic Acid Res.* **2**, 19.
 Harris, H. (1963b). *Proc. Roy. Soc. B*, **158**, 79.
 Harris, H., Fisher, H. W., Rodgers, A., Spencer, T. & Watts, J. W. (1963). *Proc. Roy. Soc. B*, **157**, 177.
 Hubert, M.-T., Favard, P., Carasso, N., Rozenowajg, R. & Zalta, J.-P. (1962). *J. Microsc.* **1**, 435.
 Lazarus, H. M. & Sporn, M. B. (1967). *Proc. nat. Acad. Sci., Wash.*, **57**, 1386.
 Newsome, J. (1966). *Biochim. biophys. Acta*, **114**, 36.
 Perry, R. P. (1967). *Progr. Nucleic Acid Res. molec. Biol.* **5**, 219.
 Philipson, L. (1961). *J. gen. Physiol.* **44**, 899.
 Prescott, D. M. (1964). *Progr. Nucleic Acid Res. molec. Biol.* **3**, 33.
 Roberts, W. K. (1965). *Biochim. biophys. Acta*, **108**, 474.
 Salzman, N. P. (1959). *Biochim. biophys. Acta*, **31**, 158.
 Scholtissek, C. & Potter, V. R. (1960). *Z. Naturf.* **15b**, 453.
 Shearer, R. W. & McCarthy, B. J. (1967). *Biochemistry*, **6**, 283.
 Soeiro, R., Birnboim, H. C. & Darnell, J. E. (1966). *J. molec. Biol.* **19**, 362.
 Watts, J. W. (1964a). *Biochem. J.* **93**, 297.
 Watts, J. W. (1964b). *Biochem. J.* **93**, 306.
 Watts, J. W. & Davis, M. A. F. (1966). *Biochem. J.* **100**, 467.
 Weisner, R., Acs, G., Reich, E. & Shafiq, A. (1965). *J. Cell Biol.* **27**, 47.
 Weiss, S. B. & Gladstone, J. (1959). *J. Amer. chem. Soc.* **81**, 4118.
 Widnell, C. C. & Tata, J. R. (1966). *Biochim. biophys. Acta*, **123**, 478.