Onset of Ribosome Degradation during Cessation of Growth in BHK-21/C13 Cells

By WILLIAM T. MELVIN and HAMISH M. KEIR

*Department of Biochemistry, University of Aberdeen, Marischal College,
Aberdeen AB9 1AS, Scotland, U.K.*

(Received 16 May 1978)

When BHK-21/C13 cells growing exponentially in 10% serum are transferred to a medium containing only 0.25% serum, cell growth is decreased. After initial changes in RNA synthesis and degradation, protein content of the cultures reaches a plateau and eventually DNA synthesis is arrested. rRNA is relatively stable in exponentially growing cells. Immediately after 'step-down' rRNA degradation commences, but poly(A)-containing RNA does not appear to be degraded any faster than in control cells. Reutilization of RNA precursors has been independently measured and amounts to less than 1%/h for rRNA, insufficient to influence the conclusion that rRNA degradation begins almost immediately after 'step-down'. The degree of reutilization of uridine is much greater for poly(A)-containing RNA than for poly(A)-free RNA.

The cellular ribosome complement of non-dividing mammalian cells in culture is less than that of actively growing cells (Becker *et al.*, 1971) and the mechanisms by which such alterations are brought about may be of importance in the regulation of cell growth, in analogy to the stringent response of *Escherichia coli*. Moreover, alterations in metabolism of ribosomes, particularly as regards their cytoplasmic degradation, may be implicated in the maintenance of the transformed state of tumour cells (Liebhaber *et al.*, 1978).

rRNA is constantly degraded and resynthesized in resting cells, but degradation ceases or is slowed down when cell growth commences. This has been observed both for fibroblasts in culture (Abelson *et al.*, 1974) and in the intact animal during renoprival growth after contralateral nephrectomy (Melvin *et al.*, 1976). It has also been shown that ribosome turnover commences as cells in culture approach confluence (Weber, 1972; Kolodny, 1975). Such studies have all used the technique of pulsing cells with a radioactive RNA precursor and then following the fate of radioactive 28S RNA and 18S RNA. Correction for possible reutilization of radioactive precursor (arising from breakdown of RNA) during the chase period has not been attempted, because until recently there has been no method of determining the extent of reutilization in these studies. However, reutilization is known to occur, since on labelling with different precursors different half-lives are obtained for rRNA (Melvin *et al.*, 1976). Moreover, rRNA labelled with radioactive orotic acid shows different turnover rates for UMP and CMP residues (Seifert & Vácha, 1974).

Assuming that the observed alterations in rate of loss of label from rRNA really do reflect alterations in rRNA degradation (i.e. altered reutilization does not

explain any differences), there is no known mechanism to account for altered rates of ribosome degradation. In resting cells a larger proportion of ribosomes is found 'out-of-cycle' as single ribosomes or as subunits (Rudland *et al.*, 1975; Stanners & Becker, 1971), and it has been suggested that these out-of-cycle ribosomes provide a pool for ribosome degradation and hence ribosome degradation might be expected to increase in resting cells (Perry, 1973).

Most studies in this area have either been concerned with the transition of resting cells to growing cells, the so-called 'step-up' system, or have compared the properties of already resting cells with growing cells. To gain further information on the regulation of cell growth, we have studied the opposite phenomenon, cessation of growth or the 'step-down' system. In addition, the influence of reutilization on the measurement of rRNA degradation has been assessed.

Materials and Methods

Materials

6-Thioguanosine was from Sigma (London) Chemical Co., Poole, Dorset, U.K. [2-³H]Adenosine (21 Ci/mmol), [5-³H]uridine (28.1 Ci/mmol) and L-[4,5-³H]leucine (40 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K., and oligo(dT)-cellulose (T-3) was from Collaborative Research Inc., Waltham, MA, U.S.A. Allyl glycidyl ether and mercuric acetate were from Aldrich Chemical Co., Gillingham, Dorset, U.K., and ashless cellulose powder was from Whatman Biochemicals, Maidstone, Kent, U.K. Materials for cell culture were

obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K., and calf serum screened for mycoplasma from Gibco Bio-Cult, Paisley, Scotland, U.K. Sarkosyl NL-35 was from Ciba-Geigy (U.K.), London, U.K.

Cell culture

BHK-21/C13 cells were routinely passaged in mono-layer culture at 37°C in CO₂/air (1:19) in 2.24-litre roller bottles in Eagle's medium, Glasgow modification, containing 10% (v/v) calf serum and 10% (v/v) tryptose/phosphate broth (medium ETC) and were checked regularly for mycoplasma contamination (McPherson & Stoker, 1962; Craig & Keir 1975). Trypsin-treated cells were replated at 2×10^4 cells/cm² in 10cm-diameter plastic dishes and left to grow in medium ETC overnight. 'Step-down' cells had the medium replaced with one containing 0.25% serum and no tryptose/phosphate broth (medium EC_{0.25}) (Bürk, 1970). Cells were harvested by washing the cell sheet with ice-cold phosphate-buffered saline (137mM-NaCl, 3mM-KCl, 8mM-Na₂HPO₄, pH 7.2, sterilized by autoclaving) and stored frozen until required. Cell number was determined by using an improved Neubauer haemocytometer.

Determination of DNA, RNA and protein

Cells in 10cm-diameter plastic dishes were washed *in situ* with 3 × 5 ml of ice-cold phosphate-buffered saline, and were scraped off the dishes in 5 ml of ice-cold 0.5M-HClO₄. Acid-insoluble material was collected by centrifugation at 200g_{av.} for 5 min at 4°C and washed twice more with 5 ml of 0.5M-HClO₄. Protein, RNA and DNA were extracted from the precipitate as described by Munro & Fleck (1966) and measured respectively by the method of Lowry *et al.* (1951) against a standard of bovine serum albumin, by the A₂₆₀ (1 A₂₆₀ unit = 32 µg of RNA/ml), and by Burton's (1956) diphenylamine method.

Free polyribosome profiles

Cells in 2.24-litre roller bottles were washed with ice-cold phosphate-buffered saline *in situ* and scraped off the glass in 20 ml of ice-cold phosphate-buffered saline. After pelleting at 200g_{av.} for 5 min to remove residual serum, and washing with phosphate-buffered saline, cells were swollen for 10 min at 0°C in 2 ml of buffer consisting of 10mM-NaCl, 1.5mM-MgCl₂ and 0.01M-Tris/HCl, pH 7.4. Cells were lysed by homogenization in a Dounce homogenizer and after centrifugation at 10000g_{av.} for 10 min at 4°C the supernatant was applied to 7–47% (w/v) sucrose gradients in 0.5M-NaCl/50mM-MgCl₂/10mM-Tris/HCl, pH 7.4. After centrifugation in a Beckman SW-27 rotor at 23000 rev./min for 4 h, gradients were

scanned in a Gilford spectrophotometer at 260 nm and separated into polyribosomal and post-polyribosomal (monomeric ribosomes and ribosomal subunits) fractions.

Extraction of RNA

Cell sheets were dissolved in 4 ml of buffer consisting of 0.1M-LiCl, 10mM-EDTA, 10mM-Tris/HCl, pH 7.4, and 0.5% (w/v) sodium dodecyl sulphate, and 4 ml of liquified phenol was immediately added, followed by 4 ml of chloroform containing 4% (v/v) 3-methylbutan-1-ol. After mixing for 30 s followed by centrifugation at 1000g_{av.} for 2 min, the organic phase was removed (Penman, 1969). The aqueous phase was extracted with 2 vol. of phenol/chloroform/3-methylbutan-1-ol (25:24:1, w/v/v) until no inter-phase was present. After three further extractions (each of equal volume) with chloroform/4% (v/v) 3-methylbutan-1-ol, the RNA was precipitated from the aqueous phase by addition of 2 vol. of ethanol and stored at -20°C. RNA was collected by centrifugation at 12000g_{av.} for 12 min and dried under vacuum before being dissolved in 3 ml of buffer consisting of 10mM-NaCl, 10mM-EDTA, 10mM-Tris/HCl, pH 7.4, and 0.2% (w/v) Sarkosyl NL-35. Then 2 ml of 5M-LiCl was added and the mixture stored overnight at 4°C before centrifugation at 12000g_{av.} for 12 min. The pellet, containing high-molecular-weight RNA (Penman *et al.*, 1968), was redissolved in 4 ml of buffer consisting of 0.1M-LiCl, 10mM-EDTA, 10mM-Tris/HCl, pH 7.4, and 0.2% (w/v) sodium dodecyl sulphate, and the RNA was reprecipitated by addition of 2 vol. of ethanol and storage at -20°C as above.

Oligo(dT)-cellulose chromatography

Samples of RNA were dissolved in 10mM-Tris/HCl, pH 7.4, and adjusted to 0.5M-NaCl before application to columns (0.4cm × 1cm) of 50 mg of oligo(dT)-cellulose equilibrated in 0.5M-NaCl/10mM-Tris/HCl, pH 7.4, after treatment with 0.1% (w/v) diethyl pyrocarbonate. After washing with the same buffer to elute poly(A)-free RNA, poly(A)-containing RNA was eluted with 10mM-Tris/HCl, pH 7.4, into tubes containing 1 ml of buffer consisting of 0.1M-LiCl/10mM-EDTA/10mM-Tris/HCl, pH 7.4, and 0.2% (w/v) sodium dodecyl sulphate. Fractions containing radioactivity were pooled and RNA was precipitated by addition of 2 vol. of ethanol.

Mercurated cellulose chromatography

Mercurated cellulose was prepared by the method of Shainoff (1968). Esterified cellulose was prepared by addition of allyl glycidyl ether to alkaline ash-free cellulose and was washed thoroughly and dried.

Mercuric acetate was added to the etherified cellulose in acid solution and the mercurated cellulose washed free of mercuric acetate and dried. All steps were conducted in a fume hood because of the known toxicities of the reagents.

Mercurated cellulose was packed in columns (5.5 cm × 0.7 cm) and stored in a buffer consisting of 10 mM-NaCl/10 mM-EDTA/10 mM-Tris/HCl, pH 8.0. All steps were performed at room temperature (20°C). Before use, columns were equilibrated with buffer A [60% (v/v) formamide, 40 mM-LiCl, 4 mM-EDTA, 4 mM-Tris/HCl, pH 7.4, 0.08% (w/v) sodium dodecyl sulphate]. RNA samples were applied in buffer A; the column was washed with the same buffer in order to elute non-thiol RNA and 1 ml fractions were collected. Thiol-containing RNA was then eluted with 50 mM-dithiothreitol in buffer A. After completion of the operation, the column was recharged by running 100 mM-mercuric acetate through and washing with 10 mM-NaCl/10 mM-EDTA/10 mM-Tris/HCl, pH 8.0 (Melvin & Keir, 1977).

Sucrose gradient sedimentation analysis of RNA

RNA samples were dissolved in 1 ml of a buffer consisting of 0.1 M-LiCl, 10 mM-EDTA, 10 mM-Tris/HCl, pH 7.4, and 0.2% (w/v) sodium dodecyl sulphate and applied to 36 ml linear 15–30% (w/v) sucrose gradients in the same buffer. After centrifugation at 23 000 rev./min in a Beckman SW-27 rotor for 17 h at 20°C, gradients were scanned with a Gilford spectrophotometer, fractionated and radioactivity was measured.

Measurement of [³H]leucine incorporation

Cells on 10 cm-diameter plastic dishes were 'stepped-down' for up to 24 h before labelling with [³H]leucine (2 μ Ci/ml) for 30 min. Cells were washed with ice-cold phosphate-buffered saline and scraped into conical centrifuge tubes and washed with 3 × 5 ml of ice-cold 5% (w/v) trichloroacetic acid, with the precipitate collected by centrifugation at 1000 g_{av} for 5 min each time. Acid-precipitable material was dissolved in 1 ml of 0.3 M-NaOH and 0.5 ml measured for radioactivity in 10 ml of Triton X-100/toluene scintillation fluid with 0.5 ml of 5% (w/v) trichloroacetic acid.

Measurement of radioactivity

Fractions were counted for radioactivity in 10 ml of a Triton X-100/toluene (1:2, v/v) scintillation fluid containing 4 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/litre in an Inter technique SL40 liquid-scintillation spectrometer with approx. 40% efficiency for ³H and 95% efficiency for ¹⁴C.

Results

Basic characteristics of the 'step-down' system

When sub-confluent BHK-21/C13 cells growing in 10% serum have the medium replaced with one containing only 0.25% serum, growth is stopped. These 'resting' cells may be maintained for several weeks and more than 95% of the cells will participate in the wave of DNA synthesis observed on restimulation of the culture with 10% serum (Howard *et al.*, 1974). After 'step-down', DNA synthesis ceases after 5 days, as does the increase in cell number.

It would appear that the term 'cessation of growth' is unsatisfactory in describing the observed changes, since abrupt cessation of accumulation of RNA is followed much later by a halt in the accumulation of DNA in the culture and cell division (Fig. 1). When cells which have been in low-serum medium for 3 days are given 10% serum, RNA, protein, DNA and cell number per culture all increase, the response for RNA being, again, the most dramatic. This demonstrates the readily reversible nature of the 'step-down' system.

Stability of RNA after 'step-down'

BHK-21/C13 cells were labelled with [³H]uridine for 1 day in a 2.24-litre bottle and the label was removed for the next day of growth to chase the radioactivity into relatively stable RNA molecules such as rRNA. After 'step-down', the total cellular RNA was isolated and poly(A)-free RNA and poly(A)-containing RNA were separated by chromatography on oligo(dT)-cellulose columns. Poly(A)-free RNA had the sedimentation pattern characteristic of rRNA and poly(A)-containing RNA had the heterogeneous sedimentation pattern characteristic of mRNA (Fig. 2). Within 1 h of 'step-down', the label in rRNA [poly(A)-free RNA] decayed with an apparent half-life of 23 h, although the rRNA was stable in control exponentially growing cells (Fig. 3). However, no such dramatic response was observed with poly(A)-containing RNA, which appeared just as stable in 'step-down' cells as in the growing cells. The relative proportions of radioactivity in the 28S and 18S rRNA peaks remained constant after 'step-down', which suggests that 28S RNA and 18S RNA are degraded at the same rate.

It may be regarded as surprising that 'step-down' appeared not to affect the stability of mRNA, but it must be remembered that the 24 h chase selected for mRNA molecules that were relatively stable. To investigate the fate of less stable mRNA molecules after 'step-down', cells were labelled with [³H]-adenosine for 1 h and were then washed with unlabelled medium to remove [³H]adenosine shortly before 'step-down'. After 10 h in the serum-depleted

Fig. 1. Characteristics of the 'step-down' system

BHK-21/C13 cells were seeded on 10cm-diameter dishes at 2×10^4 cells/cm² and left to grow in medium containing 10% serum overnight. Medium was again changed to fresh medium containing 10% serum (○) or medium containing 0.25% serum (●), and cell number (a), DNA content per culture (b), protein content per culture (c) and RNA content per culture (d) were analysed over 6 days. After 3 days in low-serum medium some cultures were 'stepped-up' by addition of serum to 10% (■).

medium rRNA radioactivity had decayed by about 30%, but radioactivity in poly(A)-containing RNA was identical with that in control (exponentially growing) cells (Table 1). Consequently, it appears that mRNA molecules of both long and shorter half-lives are degraded at the same rate in cells newly deprived of serum as in growing cells.

Utilization of ribosomes in protein synthesis

The onset of ribosome degradation appears to be a very early consequence of 'step-down' and is therefore probably under some fine control mechanism. To investigate the possible involvement of 'out-of-cycle' ribosomes in controlling ribosome degradation, polyribosome profiles of growing, 'step-down' and confluent cultures were obtained (Fig. 4). The proportion of ribosomes that existed as single

ribosomes or subunits was small in growing cells and did not increase significantly in cells deprived of serum for 6 h, when ribosome degradation was already well established (Fig. 2). On the other hand, in confluent cells, which were growing only slowly in 10% serum, and were probably partially deprived of amino acids owing to the high cell density, many of the ribosomes were found as monomers and as subunits (Fig. 4).

The rates of synthesis of rRNA and mRNA and their participation in protein synthesis were measured in growing cells and in 'step-down' cells by following the distribution of radioactivity in the polyribosomal region of sucrose gradients after a 2 h pulse with [³H]adenosine. (Adenosine was used for this experiment since transport of uridine was found to be increased after 'step-down'; W. T. Melvin & H. M. Keir, unpublished work.) Incorporation of [³H]-

Fig. 2. Sedimentation of poly(A)-containing and poly(A)-free RNA

BHK-21/C13 cells were labelled for 24 h in the presence of $2.5 \mu\text{Ci}$ of $[^3\text{H}]$ uridine/ml in 10% serum. After trypsin treatment, cells were replated at 2×10^4 cells/cm² in 10-cm-diameter plastic dishes and allowed to grow in 10% serum for a further 24 h. Cell sheets were washed and RNA was extracted as described in the text. Poly(A)-containing and poly(A)-free RNA were separated by oligo(dT)-cellulose chromatography and sedimented on 15–30% sucrose gradients as described in the text. Fractions (1.2 ml) were collected and measured for radioactivity. $\square$, Poly(A)-free RNA; $\blacksquare$, poly(A)-containing RNA.

adenosine into cytoplasmic poly(A)-containing RNA was the same in growing cells as in 'step-down' cells, but there was 20% lower incorporation into poly(A)-free RNA (rRNA) in 'step-down' cells compared with growing cells (Table 2). Of the rRNA newly synthesized in 'step-down' cells, 31% was found in the post-polyribosomal region of the sucrose gradient as against 28% in growing cells, again showing that there is no significant increase in the proportion of post-polyribosomal ribosomes after 'step-down'.

Protein synthesis per culture, as measured by incorporation of $[^3\text{H}]$ lysine or $[^3\text{H}]$ leucine, did not increase after 'step-down', and after 24 h had fallen relative to protein synthesis at zero time (Fig. 5). The extent of protein synthesis over the first 24 h of 'step-down' paralleled the content of rRNA per culture, thus showing again that the efficiency of protein synthesis per ribosome is the same as in exponentially growing cells even 24 h after 'step-down'.

Reutilization of $[^3\text{H}]$ uridine radioactivity

The rate of reutilization of an RNA precursor resulting from degradation of RNA can be measured by physical isolation of RNA synthesized during the period of the 'chase'. A technique for physical isolation of newly synthesized RNA containing incorporated 6-thioguanosine on mercurated cellulose has been described (Melvin & Keir, 1977). Cells were labelled with $[^3\text{H}]$ uridine and chased for 24 h as for the experiments described in Fig. 3 and were incubated

Fig. 3. Stability of RNA after 'step-down' of BHK-21/C13 cells

Cells were labelled and chased as described in the legend to Fig. 2 and the medium was then replaced with either fresh medium containing 10% serum ($\square$) or medium containing 0.25% serum ($\blacksquare$). At various times thereafter cultures were removed and processed as described in the text for analysis of radioactivity remaining in poly(A)-free RNA (a) and poly(A)-containing RNA (b).

for 4h with 100 μ M-6-thioguanosine 4h after either 'step-down' or replacement of the medium with fresh medium containing 10% serum. RNA was separated into poly(A)-containing and poly(A)-free RNA and the fraction containing thiol groups (newly synthesized RNA) was isolated on mercurated cellulose columns. After correction for the proportion of newly synthesized RNA that was retained on mercurated cellulose (71% as measured with RNA extracted from a separate culture labelled with [3 H]uridine for 4h in the presence of 6-thioguanosine) and binding of non-thiol RNA (0.6%), the reutiliz-

ation of label during the 4h period was calculated. Table 3 shows the extent of reutilization per hour for poly(A)-containing and poly(A)-free RNA in 'step-

Table 1. *Stability of RNA labelled for 1 h with [3 H]adenosine 10 h after transfer to 10% serum or 0.25% serum*
Cells on 10cm-diameter plastic dishes were labelled with 50 μ Ci of [3 H]adenosine/ml for 1h, and the radioactive medium was removed and the cells washed before replacement with medium containing either 10% serum or 0.25% serum for 10h. Cells were harvested and RNA isolated as described in the text. Poly(A)-free RNA and poly(A)-containing RNA were isolated by oligo(dT)-cellulose chromatography and the radioactivity was measured. RNA had a similar sedimentation distribution to that described in Fig. 1.

	$10^{-4} \times$ Radioactivity (c.p.m.)	
	10% Serum	0.25% Serum
Poly(A)-free RNA	41.5	29.1
Poly(A)-containing RNA	2.5	2.5

Table 2. *Synthesis and cytoplasmic distribution of poly(A)-containing and poly(A)-free RNA in growing cells and after 'step-down'*

BHK-21/C13 cells were seeded in 2.24-litre roller bottles (15×10^6 cells/bottle) and grown for 24h in medium containing 10% serum. Medium was replaced with fresh medium containing 10% serum or 0.25% serum and [3 H]adenosine (10 μ Ci/ml) was added 2.5h later. After a further 2h labelling period cells were harvested and polyribosomes obtained as described in the text by sedimentation on 7-47% sucrose gradients. Ribonucleoprotein from polyribosomal and post-polyribosomal regions was precipitated by addition of 2vol. of ethanol and storage at -20°C . RNA was extracted and separated into poly(A)-containing RNA and poly(A)-free RNA as described in the text.

	$10^{-4} \times$ Radioactivity (c.p.m.)	
	10% Serum	0.25% Serum
Poly(A)-free RNA		
Polyribosomal	44.0	34.2
Post-polyribosomal	17.7	15.9
Total	61.7	50.1
Poly(A)-containing RNA		
Polyribosomal	4.18	3.64
Post-polyribosomal	1.43	1.88
Total	5.62	5.52

Fig. 4. *Free polyribosome profiles from BHK-21/C13 cells*

Cells in 2.24-litre bottles in 10% serum were 'stepped-down' in medium containing only 0.25% serum for 4h (a), left in 10% serum (b) or left to reach confluence over 4 days (c). Cells were harvested and polyribosomes were sedimented on 7-47% sucrose gradients as described in the text.

Fig. 5. Efficiency of ribosomes in protein synthesis

Cells on 10cm-diameter plastic dishes were left in fresh 10% serum (□) or were 'stepped-down' to medium containing 0.25% serum (■) at zero time. Protein synthesis was measured by a 30 min pulse with 2 μ Ci of [3 H]leucine/ml (a) and rRNA contents were estimated from the absorption of poly(A)-free RNA isolated as described in the text (b).

Table 3. Reutilization of [3 H]uridine radioactivity during incubation of growing and 'step-down' cells in unlabelled medium

BHK-21/C13 cells were labelled with [3 H]uridine and then incubated in unlabelled medium as described in the legend to Fig. 2. Medium was replaced with fresh medium containing 10% serum or 0.25% for 4h, then again with medium containing 100 μ M-6-thioguanosine for a further 4h. Cells were harvested and poly(A)-containing and poly(A)-free RNA obtained as described in the text. RNA was chromatographed on mercurated cellulose and the radioactivity found in the second (thiol-containing) peak was taken as a direct measure of reutilization in the 4h period after correction for the proportion of newly synthesized RNA retained in the second peak (71%) and the retention in the second peak of non-thiol-containing RNA (0.6%).

	Reutilization (%/h)	
	10% Serum	0.25% Serum
Poly(A)-free RNA	0.5	0.7
Poly(A)-containing RNA	6.0	3.0

down' and growing cells. The reutilization for poly(A)-free RNA of about 0.6%/h is insufficient to affect the conclusion about increased degradation of rRNA after 'step-down', but when allowance is made

for reutilization the half-life in 'step-down' cells becomes 18h rather than 23h. Reutilization for poly(A)-containing RNA appears to be much greater than for poly(A)-free RNA. This degree of reutilization for poly(A)-containing RNA, 3-6%, suggests that the half-life for poly(A)-containing RNA is considerably shorter than observed, perhaps about 18h.

Discussion

Degradation of rRNA appears to be an early consequence of serum deprivation (Fig. 2). After correction for reutilization, the half-life of rRNA degradation is 18h, much shorter than has been reported previously in cells in culture or in the intact animal, although reutilization was not taken into account in these earlier studies (Loeb *et al.*, 1965; Hirsch & Hiatt, 1966; Blobel & Potter, 1968; Quincey & Wilson, 1969; Barka, 1972; Weber, 1972; Abelson *et al.*, 1974; Kolodny, 1975; Rudland *et al.*, 1975; Melvin *et al.*, 1976; Bowman & Emerson, 1977; Liebhaber *et al.*, 1978). The degree of reutilization measured here, 0.7%/h, when the bulk of cell RNA is decaying with a half-life of 18h, is very low and shows that the rate of exploitation of salvaged precursors is not affected significantly by 'step-down'.

On the other hand polyadenylated RNA appears to be much more subject to reutilization than rRNA. This may indicate simply that poly(A)-containing RNA turns over more rapidly than poly(A)-free RNA or that nucleolar and nucleoplasmic polymerases draw on separate UTP pools, as has been shown in HeLa cells by using exogenous label (Wieggers *et al.*, 1976), the pool for nucleoplasmic synthesis drawing to a greater extent on the salvage pathway.

When 6-thioguanosine is used to measure reutilization it must be remembered that 6-thioguanosine inhibits synthesis of nucleic acid precursors *de novo* and may therefore cause an overestimation of the extent of reutilization. The estimation of reutilization for rRNA is further complicated by the fact that only about 70% of mRNA molecules are polyadenylated (Milcarek *et al.*, 1974; Buckingham & Gros, 1975; Greenberg, 1976). Therefore the poly(A)-free RNA may consist of predominantly rRNA, but with a small amount of poly(A)-free RNA having the same characteristics as poly(A)-containing RNA with respect to reutilization. Calculations show that there might be a 10% overestimate for reutilization for rRNA. However, the main conclusion, that reutilization has little effect on the measurements of rRNA degradation, is not influenced by these considerations.

In contrast with rRNA, mRNA is degraded at the same rate in 'step-down' cells as in growing cells (Fig. 3, Table 1). This finding agrees with other work showing that mRNA has the same stability in resting mouse 3T6 and 3T3 cells as in restimulated cells (Abelson *et al.*, 1974; Rudland *et al.*, 1975). In these studies, however, the fate of mRNA synthesized in growing cells was followed in growing cells, and fate of mRNA synthesized in resting cells was followed in resting cells. In our experiments mRNA was labelled in growing cells and the rate of degradation of this mRNA was found to be the same in both growing and 'step-down' cells. This suggests that the gross composition of the mRNA population is not an important control in cell growth, although a few specific mRNA molecules might easily be involved and would be undetectable by these techniques. This view is supported by the observation that the bulk of the mRNA populations in resting and growing cells are composed of identical mRNA molecules (Williams & Penman, 1975; Getz *et al.*, 1976).

After labelling with [³H]uridine followed by prolonged incubation of cells in unlabelled medium a class of polyadenylated RNA with a very long apparent half-life was observed. Half-lives for poly(A)-containing cytoplasmic RNA in excess of 24h have been reported in a variety of cell types (Murphy & Attardi, 1973; Berger & Cooper, 1975; Aviv *et al.*, 1976), although shorter half-lives have

also been reported, of 6–24h (Greenberg, 1972; Singer & Penman, 1973; Schultz, 1973, 1974; Perry & Kelley, 1973; Abelson *et al.*, 1974; Ouellette & Malt, 1976) and 1–2h (Puckett *et al.*, 1975; Ouellette *et al.*, 1976). In BHK-21/C13 cells the half-life of the apparently stable poly(A)-containing RNA is decreased to around 18h when correction for reutilization is made. It is also possible that some of the radioactivity in poly(A)-containing RNA (Fig. 3) may be due to incorporation of reutilized radioactivity into a class of nuclear RNA molecules which does not act as precursor for mRNA, as had been demonstrated in human lymphocytes (Berger & Cooper, 1978).

The lack of alteration in polyribosome profiles combined with the information on efficiency of ribosomes in protein synthesis shows that the proportion of the ribosomes which are 'out-of-cycle' is probably not involved in the control of ribosome degradation, although such ribosomes may still be the target for degradation. Baenziger *et al.* (1974) have also shown that BHK-21/C13 cells did not show a reduced efficiency of protein synthesis following growth restriction. Moreover, in the growing kidney ribosome degradation is decreased with no concurrent alteration in the polyribosome pattern (Melvin *et al.*, 1976). In the temperature-sensitive mutant *ts422E* of BHK-21/C13 cells, at the non-permissive temperature the small ribosomal subunit is degraded rapidly, whereas the large subunit is stable (Kumar *et al.*, 1976), which suggests that perhaps the individual subunits may be the target for degradation. However, in normal cells small and large subunits decay at the same rate (Melvin *et al.*, 1976). Since ribosomes are degraded as a unit of both RNA and protein (Tsurugi *et al.*, 1974; Lastick & McConkey, 1976) it may be useful to look for alterations in proteinase activity, ribonuclease activity and lysosomal activity within the first hour of 'step-down'. We have found that total protein degradation commences soon after 'step-down' with a half-life similar to that for rRNA (W. T. Melvin & H. M. Keir, unpublished work).

We are grateful to the Faculty of Medicine of the University of Aberdeen for support of this work. W. T. M. is the Georgina McRobert Fellow in Cancer Research of the University. The skilled technical assistance of Mrs. Alison Blair is warmly appreciated.

References

- Abelson, H. T., Johnson, L. F., Penman, S. & Green, H. (1974) *Cell* **1**, 161–165
- Aviv, H., Voloch, Z., Bastos, R. & Levy, S. (1976) *Cell* **8**, 495–503
- Baenziger, N. L., Jacobi, C. H. & Thach, R. E. (1974) *J. Biol. Chem.* **249**, 3483–3488

- Barka, T. (1972) *Exp. Cell Res.* **74**, 439–449
- Becker, H., Stanners, C. P. & Kudlow, J. E. (1971) *J. Cell. Physiol.* **77**, 43–50
- Berger, S. L. & Cooper, H. L. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3873–3877
- Berger, S. L. & Cooper, H. L. (1978) *Biochim. Biophys. Acta* **517**, 84–98
- Blobel, G. & Potter, V. R. (1968) *Biochim. Biophys. Acta* **166**, 48–57
- Bowman, L. H. & Emerson, C. P. (1977) *Cell* **10**, 587–596
- Buckingham, M. E. & Gros, F. (1975) *FEBS Lett.* **53**, 355–359
- Bürk, R. R. (1970) *Exp. Cell Res.* **63**, 309–316
- Burton, K. (1956) *Biochem. J.* **62**, 315–323
- Craig, R. K. & Keir, H. M. (1975) *Biochem. J.* **145**, 215–224
- Getz, M. J., Elder, P. K., Benz, E. W., Stephens, R. E. & Moses, H. L. (1976) *Cell* **7**, 255–265
- Greenberg, J. R. (1972) *Nature (London)* **240**, 102–104
- Greenberg, J. R. (1976) *Biochemistry* **15**, 3516–3522
- Hirsch, C. A. & Hiatt, H. H. (1966) *J. Biol. Chem.* **241**, 5936–5940
- Howard, D. K., Hay, J., Melvin, W. T. & Durham, J. P. (1974) *Exp. Cell Res.* **86**, 31–42
- Kolodny, G. M. (1975) *Exp. Cell Res.* **91**, 101–106
- Kumar, A., Bandman, E. & Melvin, W. T. (1976) *Nature (London)* **259**, 692–694
- Lastick, S. M. & McConkey, E. H. (1976) *J. Biol. Chem.* **251**, 2867–2875
- Liebhaber, S. A., Wolf, S. & Schlessinger, D. (1978) *Cell* **13**, 121–127
- Loeb, J. N., Howell, R. R. & Tomkins, G. M. (1965) *Science* **149**, 1093–1095
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McPherson, J. A. & Stoker, M. G. P. (1962) *Virology* **16**, 147–151
- Melvin, W. T. & Keir, H. M. (1977) *Biochem. J.* **168**, 595–597
- Melvin, W. T., Kumar, A. & Malt, R. A. (1976) *J. Cell Biol.* **69**, 548–556
- Milcarek, C., Price, R. & Penman, S. (1974) *Cell* **3**, 1–10
- Munro, H. N. & Fleck, A. (1966) *Methods Biochem. Anal.* **14**, 159–160
- Murphy, W. & Attardi, G. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 115–119
- Ouellette, A. J. & Malt, R. A. (1976) *Biochemistry* **15**, 3358–3361
- Ouellette, A. J., Reed, S. L. & Malt, R. A. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2609–2613
- Penman, S. (1969) in *Fundamental Techniques of Virology* (Salzman, N., ed.), pp. 49–58, Academic Press, New York
- Penman, S., Vesco, C. & Penman, M. (1968) *J. Mol. Biol.* **34**, 49–69
- Perry, R. P. (1973) *Biochem. Soc. Symp.* **37**, 105–116
- Perry, R. P. & Kelley, D. E. (1973) *J. Mol. Biol.* **79**, 697–708
- Puckett, L., Chambers, S. & Darnell, J. E. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 389–393
- Quincey, R. V. & Wilson, S. H. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 981–988
- Rudland, P. S., Weil, S. & Hunter, A. R. (1975) *J. Mol. Biol.* **96**, 745–766
- Schultz, G. A. (1973) *Can. J. Biochem.* **51**, 1515–1520
- Schultz, G. A. (1974) *Exp. Cell Res.* **86**, 190–193
- Seifert, J. & Vácha, J. (1974) *Arch. Biochem. Biophys.* **160**, 285–288
- Shainoff, J. R. (1968) *J. Immunol.* **100**, 187–193
- Singer, R. H. & Penman, S. (1973) *J. Mol. Biol.* **78**, 321–334
- Stanners, C. P. & Becker, H. (1971) *J. Cell. Physiol.* **77**, 31–42
- Tsurugi, K., Morita, T. & Ogata, K. (1974) *Eur. J. Biochem.* **45**, 119–126
- Weber, M. J. (1972) *Nature (London) New Biol.* **235**, 58–61
- Wiegers, U., Kramer, G., Klapproth, K. & Hilz, H. (1976) *Eur. J. Biochem.* **64**, 535–540
- Williams, J. G. & Penman, S. (1975) *Cell* **6**, 197–206