The breakdown of *Tetrahymena* ribosomes *in vivo*

The effects of inhibitors

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1. When *Tetrahymena* were deprived of nutrients 50% of the polysomes disaggregated within 20 min and 20% of the total RNA broke down in 2 h. Ribosomal RNA accounted for 75% of the RNA breakdown. 2. RNA labelled by a long incubation with [¹⁴C]uridine was stable in growing cells and in the presence of actinomycin D, but broke down at the same rate as bulk RNA in starved cells. 3. The following substances inhibited the loss of RNA during starvation: cycloheximide (which inhibited both polysome disaggregation and protein synthesis), inhibitors of energy metabolism and puromycin (all of which caused polysome disaggregation and inhibited protein synthesis), and chloroquine and 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK') (neither of which affected polysomes or protein synthesis). 4. Starvation appears to activate a ribosome degradation mechanism that may involve lysosomal and non-lysosomal enzymes.

In many types of cell the total amount of ribosomal material decreases during starvation (e.g. Munro, 1964). In rat liver this decrease is partly due to an enhanced rate of ribosome degradation (Hirsch & Hiatt, 1966). The mechanism of ribosome breakdown remains obscure. The protein and RNA components turn over with similar half-lives in liver (Hirsch & Hiatt, 1966) and other tissues (summarized by Kristiansen & Krüger, 1979), suggesting that the ribosome is degraded as a unit. During starvation the proportion of ribosomes in the form of polysomes often decreases because the attachment of ribosomes to messenger RNA to initiate protein synthesis is inhibited (summarized in Clemens, 1980). As a result there is an increase in the proportion of free 80S ribosomes and it has been proposed that these could be the target for destruction (Perry, 1973). In certain circumstances the amount of 80S ribosomes does not change when the rate of breakdown is altered and it has been suggested that degradation mechanisms act on the ribosomal subunits formed by dissociation of the 80S ribosomes (Kumar *et al.*, 1976; Melvin *et al.*, 1976; Melvin & Keir, 1978).

In *Tetrahymena pyriformis* phosphorylation of a specific protein in the 40S ribosomal subunit is correlated with ribosome destruction (Kristiansen

et al., 1978; Kristiansen & Krüger, 1979). This may reflect a specialized mechanism that is not found for example in mammalian cells, but several other features of ribosome breakdown are similar in *Tetrahymena* and mammalian cells. Thus starvation causes a rapid disaggregation of polysomes to 80S ribosomes (Klemperer & Rose, 1974) and leads to a decrease in RNA (Conner & Koroly, 1974; Kristiansen & Krüger, 1979; Eckert & Kaffenberger, 1980) and total ribosomes (Hallberg & Sutton, 1977). Cycloheximide prevents the run-down of polysomes in starvation (Klemperer & Rose, 1974) and might therefore be expected to inhibit ribosome breakdown. The effects of cycloheximide and other inhibitors have suggested possible mechanisms of ribosome destruction.

Materials and methods

Tetrahymena pyriformis (strain T) was cultured at 28°C on an orbital shaker in a growth medium consisting of 2% proteose peptone, 0.2% yeast extract, 0.5 mM-CaCl₂, 50 µM-MnCl₂ and 50 µM-FeSO₄. The cells were harvested while still in exponential phase (density 10⁵ cells/ml) by rapidly chilling the culture with crushed ice and then centrifuging for 3 min at 4°C and 200g. The cells were washed by resuspension in ice-cold starvation medium consisting of 1 mM-MgCl₂, 2 mM-KCl, 2 mM-potassium phosphate (pH 7.2) and 50 mM-

Abbreviation used: Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one, 'TLCK'.

NaCl followed by centrifugation, and were finally suspended in starvation medium at 28°C to give a density of approx. 10^5 cells/ml. For chemical determination aliquots of the culture were rapidly chilled in ice and centrifuged for 3 min at 4°C and 1000g. Protein was determined (Lowry *et al.*, 1951) after the cells had been washed in ice-cold starvation medium. For RNA determination the cells were washed twice by resuspension in 10% (w/v) trichloroacetic acid at 4°C, followed by centrifugation for 10 min at 1000g. Extracts prepared at 90°C in 5% (w/v) trichloroacetic acid were taken for the orcinol reaction (Schneider, 1957).

To measure the proportion of polysomes and ribosomes the cells were harvested as above and lysed in medium A (5 mM-2-mercaptoethanol, 50 mM-Tris/HCl, pH 7.4, 100 mM-KCl and 250 mM-sucrose) containing 5 mM-MgCl₂, 0.2% Nonidet P40, 1% sodium deoxycholate and heparin (100 µg/ml). Samples of the lysate (0.2 ml, equivalent to 8 ml of cell culture) were layered over 12 ml non-isokinetic gradients (Noll, 1969) of 10–50% (w/v) sucrose in medium A containing 5 mM-MgCl₂ (made by running 12 ml of medium A containing 5 mM-MgCl₂ into a constant volume mixer containing 8 ml of 50% sucrose in the same medium). The gradients were centrifuged for 2.5 h at 4°C and 200 000g in an MSE 6 × 14 ml titanium swing-out rotor (40 000 rev./min, r_{av} 111 mm) and the distribution of material that absorbed at 254 nm was analysed by pumping the gradient from the bottom of the tube through a u.v. spectrophotometer (LKB Uvicord) connected to a linear-output chart recorder. The peaks of monomeric ribosomes, large and small subunits are designated 80S, 60S and 40S respectively.

To measure the distribution of the bulk species of RNA on sucrose gradients the cells from 7 ml of starvation medium were harvested by centrifugation and lysed in 0.4 ml of 2% (w/v) sodium dodecyl sulphate/20 mM-Tris/HCl (pH 7.4)/300 mM-NaCl. The lysate was layered over a 12 ml non-isokinetic gradient of 10–30% sucrose in a medium consisting of 0.1% sodium dodecyl sulphate, 20 mM-Tris/HCl (pH 7.4) and 300 mM-NaCl (made by running 12 ml of medium into a constant volume mixer containing 17 ml of 30% sucrose in the same medium). The gradients were centrifuged at 23°C (4 h, 40 000 rev./min) and analysed as above.

To measure turnover of RNA the cells were allowed to grow for six generation times after taking up labelled uridine. The cells (10^5 /ml) were first shaken for 2 h at 28°C in 3 ml of a medium consisting of 1 part growth medium and 9 parts starvation medium containing 2 µCi of [2-¹⁴C]-uridine (57 µCi/µmol). The cells were then collected by centrifugation, washed in starvation medium, cultured overnight at 28°C in 200 ml of growth

medium, harvested (10^5 cells/ml) as before, and incubated in various media. Acid-insoluble material, precipitated by mixing 1 ml of culture with 5 ml of 10% (w/v) trichloroacetic acid at 0°C, was collected by filtration on a glass-fibre disk (Whatman GF/C). After drying the disk, radioactivity was measured in a liquid-scintillation system (efficiency 66%, background 30 d.p.m.). Essentially all of the radioactivity was in the form of RNA as judged by acid-solubility after hydrolysis with NaOH.

Protein synthesis *in vivo* was measured by shaking 3 ml of culture (approx. 10^5 cells/ml) with 1 µCi of L-[U-¹⁴C]leucine (20 µCi/µmol) at 28°C. At 20 min intervals 0.5 ml samples were withdrawn into 5 ml of 5% (w/v) trichloroacetic acid containing 0.1% leucine and heated for 20 min at 90°C. Radioactivity in the acid-insoluble material was collected by filtration on glass-fibre disks and measured as above.

To determine ATP, 30 ml of cell suspension were placed in a special centrifuge tube (Hems *et al.*, 1975) the upper part of which was separated by a capillary containing starvation medium from a lower bulb filled with 0.6 ml of 5% (w/v) trichloroacetic acid at 4°C. The tube was immediately centrifuged for 30 s at 4°C and 800g (r_{av} 170 mm) which displaced the cells quantitatively into the lower bulb. The liquid in the upper part of the tube was discarded and the trichloroacetic acid together with the cells were transferred by Pasteur pipette to an all-glass homogenizer in which the cells were broken up. The volume of the homogenate was adjusted to 1.75 ml with 5% (w/v) trichloroacetic acid, the acid-insoluble material was removed by centrifugation, and the supernatant was extracted five times with 2.5 ml of diethyl ether. Aliquots diluted 20-fold with 0.1 M-Tris/HCl (pH 7.4) were taken for ATP determination by the luciferin/luciferase method as described by Lundin & Thore (1975). Values for duplicate samples in one experiment agreed within 10%. Values for replicate experiments carried out under similar conditions on different days showed appreciable variation (see legend to Table 2), though not as much as was found by Scherbaum *et al.* (1962).

Cycloheximide was obtained from the Upjohn Co. Actinomycin D (used as a 50 mg/ml solution in dimethylformamide), puromycin, and Tos-Lys-CH₂Cl were Sigma products.

Results

RNA breakdown in starvation

During starvation there was a decrease in total cellular RNA and in RNA relative to total protein (rather than to DNA, the cellular content of which varies in different growth states: McDonald, 1958; Bolund & Ringertz, 1966). The initial RNA values

varied considerably in apparently similar cultures taken on separate occasions over a period of several months, but the data from different experiments agreed when expressed as % change. Table 1 shows that cycloheximide (1 µg/ml) had little effect on the RNA of cells in growth medium but decreased the rate of RNA loss in starved cells.

Sucrose-density-gradient analysis of postmitochondrial supernatants from cells that had been starved for 2 h showed a large increase in 80S monomeric ribosomes and in ribosome subunits (60S and 40S) at the expense of polysomes (Fig. 1). These effects were the same after 20 min of starvation as after 2 h (results not shown) and they were prevented by cycloheximide which is known to slow down the transit of ribosomes over the messenger (see, e.g. Klemperer & Rose, 1974).

Fractionation of the total cell RNA on sucrose gradients showed that all of the main bulk species of RNA broke down during starvation (Fig. 2) and that this was partially inhibited by cycloheximide. Ribosomal RNA represented at least 75% of the RNA on these gradients and it accounted for the major part (75%) of the decrease during starvation.

Breakdown of radioactive RNA

Experiments with cells labelled with [¹⁴C]uridine suggested that there was little turnover of RNA (Fig. 3). Growing cells lost little or no radioactivity in the initial 2–4 h period (i.e. before the growth rate became limited by cell density). In starvation medium the cells lost acid-precipitable radioactivity at approximately the same rate as RNA (in Table 1). All of the acid-precipitable radioactivity in these cell suspensions was intracellular because, in other experiments, the values for cells collected by centrifugation were the same as for the unfractionated cell suspension. Cycloheximide (1 µg/ml) had no effect on the radioactivity of cells in growth medium but inhibited the loss in starvation medium. When actinomycin D (50 µg/ml, which is known to inhibit RNA synthesis by at least 95%) was added to prevent re-utilization of possible RNA precursors produced by RNA breakdown there was little effect on the radioactive RNA in cells in growth medium. However, after 2 h under these conditions protein synthesis was inhibited by 60% (legend to Fig. 3) and the proportion of ribosomes in the form of polysomes was approximately halved (Klemperer & Rose, 1974). Therefore a decrease in polysomes was not associated with a loss of RNA in the presence of actinomycin. Moreover, Fig. 3 shows that actinomycin inhibited the loss of radioactive RNA from cells in starvation medium.

Effects of other inhibitors

The loss of radioactive RNA was measured in the presence of several other inhibitors. These were all

Table 1. *Effect of starvation, cycloheximide and puromycin on RNA content of Tetrahymena*

Tetrahymena (10⁵ cells/ml) were incubated at 28°C in starvation medium or in growth medium. Cycloheximide (1 µg/ml) was added as shown. RNA and protein were determined at 0, 2 and 4 h. The values are means of three experiments in which the results (expressed as % of value at 0 h) agreed within 10%. Initial values for RNA varied between 49 and 85 µg/ml and for protein between 0.30 and 0.34 mg/ml.

Incubation medium Time (h)	...	RNA (% of initial)			
		(µg/ml)		(µg/mg of protein)	
		2	4	2	4
Starvation medium		82	68	90	80
Starvation medium + cycloheximide		96	85	95	95
Growth medium		165	255	100	95
Growth medium + cycloheximide		100	95	100	95

Fig. 1. *Effect of starvation and cycloheximide on polysomes*

Ribosomal material in postmitochondrial supernatants from cells in growth medium (—) or incubated for 2 h in starvation medium (----) or starvation medium plus 1 µg of cycloheximide/ml (····) was fractionated on 12 ml sucrose gradients.

Fig. 2. Effect of starvation on different species of RNA from *Tetrahymena*

Cells in starvation medium were harvested at 0h (—) and after 4h (---), lysed in sodium dodecyl sulphate, and the RNA was fractionated on 12ml sucrose gradients. Curves are averages of duplicates that agreed within 3%. Total material under the curve at 4h was 70% of that at 0h. In the presence of 1 μ g of cycloheximide/ml the 4h value (not shown) was 85% of the 0h value. Peaks corresponding to the major RNA species of the large and small subunits are designated 26S and 17S respectively (Chi & Suyama, 1970).

studied at concentrations where they had a significant effect on labelled RNA without causing visible changes in morphology or motility during the first 1–2h of incubation (Fig. 4). To provide a contrast with cycloheximide, inhibitors of energy metabolism and of protein synthesis were used to disaggregate the polysomes. Sodium azide (1.5 mM), 2,4-dinitrophenol (2 mM) and puromycin (1 mM) all caused a rapid fall in polysomes of cells in growth medium (proportion after 20 min approximately same as after 60 min; results not shown), but contrary to expectation the radioactive RNA was unchanged for at least 60 min. Moreover, these substances inhibited the loss of labelled RNA from cells in starvation medium, although the effect of dinitrophenol (tested at 1 mM since higher concentrations caused visible damage to cells in starvation medium) may not be significant in these circumstances ($P < 0.2$). The inhibitor of lysosome function chloroquine (0.3 mM) had similar effects on [14 C]RNA (Figs. 4b and 4d).

Fig. 3. Loss of labelled RNA from cells during incubation

Cells that had taken up [14 C]uridine were allowed to grow for 16h in growth medium to a density of 10^5 cells/ml. The cells were then washed and incubated at the same density in growth medium (○) or starvation medium (●), or in the presence of 50 μ g of actinomycin D/ml in growth medium (□) or starvation medium (■), or of 1 μ g of cycloheximide/ml in starvation medium (▲). Samples (1 ml) were taken at intervals for determination of trichloroacetic acid-precipitable radioactivity. The values (expressed as % of radioactivity present at zero time) are averages of the data from two experiments which agreed within 10%. Initial radioactivity (taken as 100%) was approx. 4.5×10^3 d.p.m./ml of culture. Cycloheximide had essentially no effect on radioactivity in cells during the first 4h in growth medium (results not shown for clarity). The [14 C]-leucine incorporation during a 20 min incubation period in growth medium was inhibited 60% and 80% respectively after 2h and 4h in the presence of actinomycin.

Table 2 compares the effects of cycloheximide and other inhibitors on polysomes, ATP concentration and protein synthesis. Also included are data for the proteinase inhibitor Tos-Lys-CH₂Cl (30 μ M). Cellular ATP was decreased in all instances by the inhibitors of energy metabolism (sodium azide and dinitrophenol) but was actually increased in cells in starvation medium by cycloheximide and puromycin, and the common feature of these inhibitors was that they all inhibited protein synthesis. By contrast, chloroquine and Tos-Lys-CH₂Cl, both of which protected the RNA of cells in starvation medium, had little or no effect on polysomes or protein synthesis.

Fig. 4. Effects of inhibitors on the loss of ^{14}C -labelled RNA from cells

Cells were incubated as in Fig. 3 in growth medium (panels *a* and *b*) or starvation medium (panels *c* and *d*) without additions (○) or in the presence of 1.5 mM-sodium azide (□), dinitrophenol at a concentration of 2 mM in growth medium and 1 mM in starvation medium (■), 1 mM-puromycin (△) or 0.3 mM-chloroquine (▲). Values are expressed as in Fig. 3 and are means of four separate experiments. S.E.M. in *a* and *b* are ± 1.0 at 1 and 2 h, ± 2.0 at 4 h; in *c* and *d* ± 2.5 at 1 and 2 h, ± 4.5 at 4 h.

Discussion

In agreement with previous reports ribosomal RNA decreased in nutrient-deprived *Tetrahymena* (see the Introduction). There was little or no loss of radioactive RNA from growing cells even in the presence of actinomycin D, suggesting that ribo-

somal RNA did not turn over. The parallel loss of radioactivity and of RNA from starved cells therefore seemed to be due to a breakdown mechanism that was activated during starvation. Ribosomal RNA represented approx. 75% of the total RNA lost during starvation. Cycloheximide inhibited the breakdown of total RNA and of ribo-

Table 2. *Effects of inhibitors on the amount of [¹⁴C]RNA, % of polysomes, concentration of ATP, and [¹⁴C]leucine incorporation after 1 h of incubation*

Tetrahymena were incubated for 1 h in growth medium or starvation medium as described in Figs. 3 and 4. The values are expressed as a percentage of the value for cells in growth medium. The [¹⁴C]RNA values (means $\pm$ s.e.m. for four separate experiments) are taken from Fig. 4 and from additional experiments with cycloheximide (1 μ g/ml) and Tos-Lys-CH₂Cl (30 μ M). The probability that the observed difference in [¹⁴C]RNA was due to chance was calculated by Student's *t* test. Values for polysomes, ATP and [¹⁴C]leucine incorporation are each averages of two experiments in which the % change in the presence of inhibitor agreed within 10%. Polysomes measured as in Fig. 1 were 80% of the total ribosomal material in growing cells (taken as 100% value in the Table). The 100% value for ATP varied between 9.9 and 12.6 nmol/mg of protein in control cells in growth medium. The 100% value for [¹⁴C]leucine incorporation into acid-precipitable material in 60 min was approx. 10⁴ d.p.m./ml of control culture in growth medium.

Additions	Values after 60 min incubation (% of control in growth medium)						
	Growth medium				Starvation medium		
	[¹⁴ C]RNA	Polysomes	ATP	[¹⁴ C]Leucine	[¹⁴ C]RNA	Polysomes	ATP
None	100	100	100	100	84 $\pm$ 2.5	50	100
Cycloheximide	99 $\pm$ 1.2	110	96	39	94 $\pm$ 2.2 (<i>P</i> < 0.05)	85	185
Puromycin	100 $\pm$ 0.5	50	59	27	96 $\pm$ 1.9 (<i>P</i> < 0.01)	25	180
Sodium azide	99 $\pm$ 0.5	25	44	25	96 $\pm$ 1.6 (<i>P</i> < 0.01)	<5	35
Dinitrophenol	99 $\pm$ 0.9	65	74	24	89 $\pm$ 1.5 (<i>P</i> < 0.2)	30	42
Chloroquine	99 $\pm$ 0.5	100	88	100	94 $\pm$ 2.1 (<i>P</i> < 0.02)	40	81
Tos-Lys-CH ₂ Cl	100 $\pm$ 0.5	100	—	85	99 $\pm$ 0.8 (<i>P</i> < 0.01)	35	—

somal RNA while other inhibitors were shown only to inhibit the loss of radioactive RNA from starved cells. However, a decrease of at least 50% in the rate of breakdown in all instances (except dinitrophenol) implied that ribosomal RNA breakdown was included in this inhibition.

The initial target for breakdown has not been defined. The rapid disaggregation of polysomes during starvation suggests that the 80S monomers or the ribosomal subunits rather than polymers are the substrate for degradative enzymes. However there is no proof that polysome disaggregation is an essential initial step because the inhibitory effect of cycloheximide on ribosome breakdown could be attributable to an inhibition of protein synthesis (see below) rather than to the preservation of polysomes.

The inhibitor studies suggest mechanisms that may be involved in ribosome destruction. Several different types of metabolic inhibitor prevented RNA breakdown in starved cells. Puromycin and inhibitors of energy metabolism increased polysome disaggregation but, like cycloheximide, all these substances inhibited protein synthesis. Chloroquine, an inhibitor of lysosomal function (de Duve *et al.*, 1974) and Tos-Lys-CH₂Cl, an inhibitor of proteolysis, also decreased RNA breakdown although they had little or no effect on protein synthesis or on polysomes. RNA breakdown may therefore depend not only on ribonuclease-like enzymes but also on other enzymes that act on ribosomes either directly or as part of a lysosomal digestion process. Any of

these enzymes could turn over rapidly in starved cells and so be a target for inhibitors of protein synthesis. Alternatively, depressed protein synthesis may raise the amino acid concentration in starved cells, so decreasing some intracellular signal for ribosome breakdown.

Possible mechanisms of ribosome destruction include ribosome-bound enzymes such as the ribonuclease that occurs in *Tetrahymena* and other cells (e.g. Lazarus & Scherbaum, 1967; Bransgrove & Cosquer, 1978; Krechetova *et al.*, 1972) and a trypsin-like proteinase as has been found associated with mammalian ribosomes (Korant, 1977; Lewis & Sabatini, 1977). Degradation may also depend on lysosome-related autophagic vacuoles which ingest cellular components in *Tetrahymena* during starvation (Levy & Elliott, 1968; Nilsson, 1970). The inhibitor studies do not distinguish between these mechanisms. Thus Tos-Lys-CH₂Cl and the lysosomotropic agent chloroquine inhibit lysosomal cathepsin B1 (Barrett, 1973; Wibo & Poole, 1974), but Tos-Lys-CH₂Cl also inhibits the ribosome-associated proteinase. Cycloheximide inhibits the synthesis of a ribosome-associated ribonuclease that has been implicated in ribosome breakdown in yeast (Swida *et al.*, 1981), but inhibitors of protein synthesis also suppress autophagic mechanisms (see below).

Ribosome breakdown in *Tetrahymena* shows an interesting resemblance to mammalian intracellular protein breakdown insofar as the latter is stimulated

by nutrient deprivation and suppressed by inhibitors of proteinases and of protein synthesis (Goldberg & St. John, 1976). Protein digestion in autophagic vacuoles which form during starvation (Neely *et al.*, 1977; Mortimore & Schworer, 1977) is decreased by inhibitors of lysosomal cathepsins (Huisman *et al.*, 1974; Dean, 1975; Knowles & Ballard, 1976; Hopgood *et al.*, 1977; Seglen *et al.*, 1979) and by inhibitors of protein synthesis (Knowles & Ballard, 1976; Hopgood *et al.*, 1977; Amenta *et al.*, 1977; Kovacs & Seglen, 1981). Inhibitor experiments have also implicated non-lysosomal enzymes in protein turnover. In some instances these enzymes may inflict an initial critical cleavage that makes a protein susceptible to digestion by less specific mechanisms (e.g. Schimke, 1975; Segal & Doyle, 1978). Progress towards defining the role of analogous systems for ribosome breakdown in *Tetrahymena* will require experiments with subcellular preparations.

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