Preparation and partial characterization of cell-free protein-synthesizing extracts from *Tetrahymena pyriformis*

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1. We have examined methods necessary for preparing post-mitochondrial supernatants from *Tetrahymena pyriformis* strain HSM that are capable of efficient cell-free protein synthesis. 2. The requirements for optimum synthesis in these extracts are described. 3. Data relating to the kinetics of protein synthesis and the initiation capacity of these supernatants are presented.

Cell-free protein-synthesizing systems from higher eukaryotes, e.g. mammalian cells, tissues, and their tumours and plant tissues, have been available for a relatively long time. Hence, they have been widely studied and analysed (Moldave & Grossman, 1974). On the other hand, active counterparts from single-celled eukaryotic organisms such as algae, protozoa and fungi have proved extremely difficult to prepare. Thus, despite intensive effort during the decade, it is only in the last few years that publications describing the preparation and properties of cell-free protein-synthesizing systems from *Saccharomyces cerevisiae* capable of efficient translation of added mRNA have been reported (Gasior *et al.*, 1979*a,b*). Despite an equal amount of effort, equivalent preparations from protozoan sources are still not available. For example, the literature generally describes rather poor, inefficient run-off systems with *Tetrahymena* extracts (Allen & Suyama, 1972; Conklin & Chou, 1971). In the present paper we describe the preparation and properties of *Tetrahymena pyriformis* postmitochondrial supernatants which are capable of translating endogenous mRNA. Nearly 40% of the total hot-acid-insoluble material synthesized by these extracts is due to re-initiation.

Materials and methods

Materials

Proteose-peptone and yeast extract were purchased from Difco (Detroit, MI, U.S.A.). Poly(I) and baker's-yeast tRNA were purchased from Sigma Chemical Co (Poole, Dorset, U.K.) and Celite was obtained from Koch-Light Laboratories (Colnbrook, Bucks., U.K.). All radioactive chemicals

were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). T-2 toxin was a gift from Dr. M. Cannon, King's College, University of London.

Maintenance and growth of *Tetrahymena pyriformis*

Stocks of *Tetrahymena pyriformis* strain HSM were maintained as 100 ml standing cultures at 18°C in a medium composed of 1% proteose-peptone and 0.25% yeast extract. The medium was contained in lightly stoppered 250 ml conical flasks. After incubation for 1 week, the cells were sub-cultured by transferring 3 ml of uniform suspension into 100 ml of fresh medium.

Experience has taught us that the best results are obtained if cells are conditioned before an experiment. This was achieved by removing 3 ml of stock culture and inoculating into 100 ml of fresh medium. The cells were grown for 16 h at 25°C as standing cultures. Then 3 ml of the overnight culture was removed and again transferred to 100 ml of fresh medium. The cells were grown at 28°C for a further 16 h in a reciprocal shaking water bath operated at 130 rev./min. These cells are in mid-exponential phase of growth at the end of the 16 h incubation and are usually at a density of about 6×10^4 cells/ml.

Counting of cells

For this 1 ml of uniform cell suspension was mixed with 0.1 ml of aq. 2% (v/v) formaldehyde and incubated for 2 min at room temperature. The formaldehyde treatment immobilizes the cells, thus allowing accurate counting with a haemocytometer.

Harvesting of cells

Cells from overnight cultures were harvested by centrifuging at room temperature for 10 min in an

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MSE Chilspin 6×100ml angle rotor spun at 3600 rev./min. The cell pellets from four separate 100ml cultures were resuspended and transferred to 100ml of fresh medium pre-warmed to 28°C. After 1h additional incubation at 28°C the cells were harvested. The contents of each culture flask were quickly added to 50g of finely crushed frozen growth medium and thoroughly mixed, so that the temperature of the cells was lowered to 0–2°C almost instantaneously. Cells were harvested at 2°C as described above.

Preparation of Tetrahymena postmitochondrial extracts

Cell pellets were resuspended and washed once in 50ml of ice-cold buffer A (25 mM-KCl, 7 mM-magnesium acetate, 1 mM-dithiothreitol, 0.1 mM-EDTA, made up in 20 mM-triethanolamine/HCl buffer, pH 7.4). The volume of the washed cell pellets was measured, and an equal amount of ice-cold buffer A was added. The cells were then gently resuspended with a glass rod. Powdered washed Celite was added to the cell suspension in the ratio of 0.2g of Celite for each 1ml of suspension. The mixture was transferred to a glass Dounce homogenizer and immersed in an ice-bath. Cells were broken by using approximately ten vertical and even strokes of the hand-powered plunger.

Debris was removed by centrifuging the homogenized cells in a 6×5.5ml aluminium swing-out rotor for 10min at 25 000 rev./min (40 000 $g_{av.}$) and 2°C in an MSE 65 Mk. II ultracentrifuge. The supernatant was removed from the debris and stored in 1 ml batches under liquid nitrogen.

Protein synthesis in Tetrahymena postmitochondrial supernatants

Incubations were carried out at 28°C unless otherwise indicated. The first experiments were performed as follows. Each 150µl incubation mixture consisted of 75µl of *Tetrahymena* postmitochondrial supernatant (containing 40–60 A_{260} units/ml in buffer A), 20 mM-triethanolamine/HCl buffer, pH 7.6, 25 mM-KCl, 7 mM-magnesium acetate, 1 mM-dithiothreitol, 0.1 mM-EDTA, 5 mM-phosphocreatine, 2 mM-ATP, 1 mM-GTP, creatine kinase (EC 2.7.3.2) (20 µg/ml), 30 µg of baker's-yeast tRNA and 0.01 mM of each of 16 unlabelled protein amino acids (i.e. minus leucine, lysine, phenylalanine and valine). The incubations were supplemented with a mixture of L-[³H]leucine (sp. radioactivity 57 Ci/mmol), L-[³H]lysine (sp. radioactivity 21 Ci/mmol), L-[³H]phenylalanine (sp. radioactivity 19 Ci/mmol) and L-[³H]valine (sp. radioactivity 33 Ci/mmol), each at a concentration of 1 µCi/150µl incubation mixture. Assays were performed in duplicate. The assay was modified as the optimum incubation

conditions were determined. These changes are described at the appropriate places in the text.

Incubations were terminated after 12min incubation by pipetting 100µl samples into 2ml of ice-cold 5% (w/v) trichloroacetic acid containing 0.1% (w/v) unlabelled leucine, lysine, phenylalanine and valine.

All samples were heated for 30min at 95°C to hydrolyse aminoacyl-tRNA. After cooling to 0°C, the protein precipitates were collected on to 2.5cm Whatman GF/C glass-fibre filter discs, which were then washed with 2×2.5ml of ice-cold 5% trichloroacetic acid (containing the four unlabelled amino acids) followed by two washes with 2.5ml of aq. 70% (v/v) ethanol. The filter discs were dried and placed in 5ml plastic insert tubes contained in scintillation vials. Then 4ml of a scintillation 'cocktail' was added to the insert tubes. The 'cocktail' was made up by dissolving 5g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of toluene. The samples were counted for radioactivity in a Beckman liquid-scintillation counter, model-LS-150.

Final procedures for preparing and analysing protein synthesis in Tetrahymena postmitochondrial extracts

Mid-exponentially growing cells were harvested, washed and resuspended in ice-cold 25 mM-KCl/7 mM-magnesium acetate / 1 mM-dithiothreitol/0.1 mM-EDTA made up in 20 mM-triethanolamine/HCl buffer, pH 7.4, and 10% (v/v) glycerol as described in the preceding sections. Postmitochondrial-supernatant extracts were prepared as described previously from cells homogenized with washed and treated Celite (prepared as described in the next section). Between 1 and 2ml of postmitochondrial-supernatant extract was layered on top of 10ml columns of Sephadex G-25 (medium grade) made up and equilibrated with buffer A supplemented with 10% glycerol. The extract was eluted with the same buffer at a flow rate of 1.4 ml/min; 0.5 ml fractions were collected. The A_{260} of each fraction was measured and the most concentrated fractions were combined; 0.2 ml portions were frozen and stored in liquid nitrogen.

Protein-synthesis assays were performed by using 100µl incubation volumes comprising 50µl of the Sephadex-G-25-'de-salted' extract (20–30 A_{260} units/ml), supplemented with 50µl of assay solution made up to give final concentrations of 20 mM-triethanolamine/HCl, pH 7.4, 80 mM-KCl, 4.5 mM-magnesium acetate, 1 mM-dithiothreitol, 0.1 mM-EDTA, 2.5 mM-phosphocreatine, 1 mM-ATP, 0.5 mM-GTP, 10 µg of creatine kinase/ml, 40 µg of baker's-yeast tRNA and 10 µM of each of 18 unlabelled protein amino acids minus either [³H]-valine + [³H]phenylalanine or [³H]leucine + [³H]-

phenylalanine. Each labelled amino acid was diluted to a specific radioactivity of 3 Ci/mmol, and 1.5 μ Ci was added to the incubations, giving a final concentration of 5 μ M. Incubations were performed and analysed as described above.

Washing and treatment of the Celite

Celite (5 g) was suspended for 1–2 h in freshly made 0.1 M-HCl. The HCl was discarded and the Celite was rinsed in three changes of 0.1 M-NaOH before resuspension in 0.1 M-NaOH for 1–2 h. The NaOH was discarded and the Celite washed thoroughly in several changes of distilled water. Each time the pH of the suspension was adjusted to pH 7. After the final wash, the Celite was dried at 75°C.

The washed Celite was then treated as follows. A 5 g portion was suspended in 100 ml of a solution containing 1% (w/v) each of spermine, casein and proteose-peptone made up in 1.5 mM-EGTA. The Celite was stirred for approx. 4 h before it was collected and dried.

Results

Protein-synthesizing ability of Tetrahymena post-mitochondrial supernatants

Our first attempt at assaying *Tetrahymena* protein synthesis *in vitro* was in a postmitochondrial supernatant (S-40 extract, prepared by centrifuging homogenized cells for 10 min at 40 000 g_{av} at 2°C). The cells were disrupted by Dounce homogenization with Celite as described in the Materials and methods section. This cell-disruption technique had been developed previously for the isolation of undegraded high-molecular-weight aggregates of polyribosomes (David & Smith, 1981). However, we found that these S-40 extracts were capable of incorporating only extremely low amounts of 3 H-labelled amino acids into protein after a 12 min incubation at 28°C (Table 1, line 1). We considered the possibility that the poor synthesis activity was caused by protein-factor inactivation during extract

preparation and decided to test this theory by adding glycerol to all our preparation and protein-synthesis assay buffers. When glycerol was used throughout at a final concentration of 10% (v/v), protein synthesis in the resultant S-40 extracts was about six times that in the extracts prepared without glycerol (Table 1, cf. lines 1 and 2).

Further work has shown that the optimum final concentration of glycerol required is 10% and that at this concentration S-40 extracts survive freezing and thawing without loss of activity (results not shown).

The incorporation by the glycerol-containing S-40 extracts is still very low, especially when compared, for example, with that obtainable with equivalent extracts from rabbit reticulocytes (Pelham & Jackson, 1976). We suspected that the free amino acid concentration of the *Tetrahymena* S-40 extracts was extremely high, as the cells were cultured in media containing proteose-peptone and yeast extract. Thus we examined the protein-synthesizing activity of our extracts after removing the free amino acids and other small molecules either by 'de-salting' through Sephadex G-25 columns or by dialysis against buffer A supplemented with 10% glycerol. The Sephadex 'de-salting' procedure greatly improved the incorporating ability of the S-40 extracts (Table 1, line 3), whereas dialysis inactivated much of it (Table 1, line 4).

Characterization of the conditions for protein synthesis in S-40 extracts

The KCl requirement for protein synthesis was investigated and the concentration curve obtained is shown in Fig. 1(a). The optimum was between 80 and 100 mM, at which concentrations synthesis was twice that at the lowest (15 mM) and highest (150 mM) KCl concentrations tested.

Changes in magnesium acetate concentration affected the protein-synthetic capacity more dramatically than did changes in KCl concentration. Activity increased sharply as the magnesium acetate concentration was raised up to the optimum

Table 1. *Effect of glycerol treatment and of removing the endogenous free amino acids on the protein-synthetic ability of Tetrahymena S-40 extracts*

The S-40 extracts were prepared and assayed as described in the Materials and methods section. The incubations were for 12 min at 28°C and contained 3.5 A_{260} units of S-40 extract in a final incubation volume of 0.15 ml.

Procedure	Incorporation of 3 H-labelled amino acids into protein (c.p.m./ A_{260} unit of S-40 extract)
(1) S-40 extract prepared in buffer A	294
(2) S-40 extract prepared in buffer A with 10% glycerol	1824
(3) S-40 extract prepared in buffer A with 10% glycerol and passed through a Sephadex G-25 column made up in buffer A with 10% glycerol	60 735
(4) S-40 extract prepared in buffer A with 10% glycerol and dialysed for 6 h against buffer A with 10% glycerol	1032

Fig. 1. Characterization of *Tetrahymena* cell-free protein-synthesizing S-40 extracts

Tetrahymena S-40 extracts from glycerol-treated cells were prepared as described in the Material and methods section. The 150 μl protein-synthesis incubation mixtures contained 75 μl of S-40 extract and the reagents described in the Materials and methods section, except: in (a) KCl concentration was varied; in (b) magnesium acetate concentration was varied; in (c) the pH of the extract preparation and assay buffers was varied; and in (d) the concentration of baker's-yeast tRNA was varied. Incubations were for 12 min at 28°C.

at 4.5 mM, and then declined equally sharply thereafter (Fig. 1b).

An equally dramatic effect on protein synthesis in our S-40 extracts was obtained by varying the pH of both the preparation and assay buffers (Fig. 1c). The curve obtained was similar to the magnesium acetate curve, being sharp and bell-shaped. Optimum synthesis was at pH 7.4.

Uncharged baker's-yeast tRNA was found to stimulate the protein-synthetic capacity of our extracts (Fig. 1d), although the increase was only about 2-fold and at the relatively high concentration of 60 $\mu\text{g}/0.15 \text{ ml}$ assay mixture. We are confident, however, that the tRNA genuinely stimulates protein synthesis and does not merely protect against nuclease attack, because we have found that polyribosomes remain stable, regardless of the presence of tRNA, in incubated extracts prevented from synthesizing protein (results not shown).

The unlabelled amino acid concentration requirements for protein synthesis in S-40 extracts were tested, and the results are presented in Fig. 2(a). There was an approx. 50% stimulation in synthesis over a range from 1 μM to the optimum at 50 μM

(solid line). Slight inhibition was observed above the optimum concentration. Similarly, the ^3H -labelled amino acid concentration affected the incorporation obtained (dashed line). In these experiments a value of 0.75 $\mu\text{Ci}/0.15 \text{ ml}$ assay represented an absolute concentration of about 2 μM for each labelled amino acid added. Thus all concentrations tested in this part of the experiment were slightly on the sub-optimum side of the solid-line curve, and were between 2 and 8 μM .

Other experiments, the results of which are not presented here, have shown approximately equal incorporation when [^3H]leucine, [^3H]lysine, [^3H]phenylalanine or [^3H]valine was included in the incubations as the sole ^3H -labelled amino acid.

The extent of protein synthesis was influenced by the amount of S-40 extract added to the assay (Fig. 2b). The best results were obtained by mixing 1 vol. of extract with an equal volume of assay buffer containing the amino acid and energy 'cocktails'. Further increases in the ratio of S-40 extract added were inhibitory. This decline in protein-synthesis activity may reflect the presence of an inhibitor in the S-40 extract. Indeed, inhibition of protein

Fig. 2. Effect of concentrations of amino acid and S-40 extract on protein synthesis

The S-40 extracts were prepared from glycerol-treated cells and 'de-salted' on Sephadex G-25 columns as described in the Materials and methods section. The protein-synthesis assays were performed as described in the Results section, except that in (a) the concentration of 18 unlabelled amino acids was varied, and the ^3H -labelled amino acid mixture was kept constant and was composed of $1.50 \mu\text{Ci}$ each of [^3H]leucine and [^3H]phenylalanine (solid line). The dashed line represents the effect of increasing the amount of [^3H]leucine and [^3H]phenylalanine in the assays when the concentration of the other 18 unlabelled amino acids was held constant at $10 \mu\text{M}$. The values of μCi of ^3H label added represent the amount of each ^3H -labelled amino acid. Each $100 \mu\text{l}$ incubation contained $2.85 A_{260}$ units of S-40 extract and was performed at 28°C for 15 min. In (b) the volume of S-40 extract added to the incubations was varied. The amino acid 'cocktail' was added to give 16 unlabelled amino acids at a final concentration of $10 \mu\text{M}$ each and $0.66 \mu\text{Ci}$ each of [^3H]leucine, [^3H]lysine, [^3H]phenylalanine and [^3H]valine. The concentration of the S-40 extract was $57 A_{260}$ units/ml. The volumes of extract added to $50 \mu\text{l}$ incubations are shown in the Figure. Incubations were performed at 28°C for 15 min.

Table 2. Effect of altering the concentration of the energy-generating system on protein synthesis in *Tetrahymena* S-40 extracts

The S-40 extracts were prepared from glycerol-treated cells and assayed as described in the Materials and methods section. The incubations were for 12 min at 28°C and contained $3.6 A_{260}$ units of S-40 extract in a final incubation volume of 0.15 ml .

Final concentration of energy-generating system in the cell-free system	Incorporation of ^3H -labelled amino acids into protein (c.p.m./ A_{260} unit of S-40 extract)
(1) 2 mM-ATP, 1 mM-GTP, 5 mM-phosphocreatine and $20 \mu\text{g}$ of creatine kinase	7494
(2) 4 mM-ATP, 2 mM-GTP, 10 mM-phosphocreatine and $40 \mu\text{g}$ of creatine kinase	508
(3) 1 mM-ATP, 0.5 mM-GTP, 2.5 mM-phosphocreatine and $10 \mu\text{g}$ of creatine kinase	7385

synthesis was obtained when ribosome-free cytosol was added to assays containing optimum concentrations of post-mitochondrial supernatant (results not shown). It is noteworthy that aminoacyl-tRNA synthetase inhibitors have been reported in *Tetrahymena* post-ribosomal supernatants (Chua *et al.*, 1977).

The energy requirements were tested by varying simultaneously the concentrations of all the components in the 'cocktail' (Table 2). It was found that the

standard 'cocktail' containing 2 mM-ATP, 1 mM-GTP, 5 mM-phosphocreatine and $20 \mu\text{g}$ of creatine kinase/ml gave the best incorporation. Half-strength 'cocktail' was as good as the standard energy 'cocktail', whereas double-strength 'cocktail' was strongly inhibitory.

Effect of cell-breakage method on the capacity of postmitochondrial supernatants to synthesize protein

The method chosen to disrupt the *Tetrahymena*

cells has a profound effect on the ability of the subsequent S-40 supernatants to synthesize protein. Thus, of the methods investigated (Table 3), four of them produced very poor protein-synthesizing extracts and two produced very active extracts. Generally, the cell-breakage methods that produced poor protein-synthesizing extracts yielded concentrated S-40 supernatants (lines 1–3), whereas the breakage methods that produced good protein-synthesizing extracts yielded more dilute S-40 supernatants (lines 4–5).

First analysis would suggest that a compromise is reached between efficiency of cell breakage and protein-synthetic activity with the Celite-aided Dounce-homogenization technique (Table 3, line 5). However, we deemed it necessary to continue this particular line of investigation, because we were concerned by the activity loss of these extracts (cf. lines 4 and 5). Moreover, further experiments highlighted additional problems that are not immediately apparent from the data presented. For example, we observed (i) a high degree of variability in the incorporation ability of the various S-40 extracts (cf. the results in Table 1, line 3, in Fig. 2 and in Table 3), (ii) a variability in the degree of protein-synthesis inhibition with initiation inhibitors and (iii) an occasional lack of correlation between the protein-synthetic capacity and the nature of the polyribosome aggregation in the S-40 supernatants. We considered that these problems had two possible causes. Either inhibitory material was being washed from the Celite, or protein-synthesis factors were being adsorbed from the cytosol on to the Celite during the cell homogenization.

Celite contains bound Ca^{2+} ions in a magnesium/

aluminium silicate matrix. The washing procedure that we use will remove loosely bound Ca^{2+} ions. However, we thought it possible that residual free Ca^{2+} ions were leaking from the Celite into the homogenized cell suspension, and despite the presence of 0.1 mM-EDTA in the preparation buffers was inhibiting protein synthesis. Consequently, we tested the effect of adding EGTA to S-40 supernatants. A large stimulation of incorporation was obtained (Table 4).

Table 4. *Effect of EGTA on protein synthesis in Tetrahymena S-40 extracts prepared by Dounce homogenization with Celite*

The S-40 extracts were prepared from glycerol-treated cells by Dounce homogenization with 0.2 g of acid-washed Celite/ml of washed cell suspension as described in the Materials and methods section. The extracts were 'de-salted' through Sephadex G-25 columns and the incubation mixtures were made up as described in the legend to Table 3, except that the ^3H -labelled amino acids were added to the incubations at concentrations of 1.5 μCi each of valine and phenylalanine at 5 Ci/mmol. Each incubation contained 1.25 A_{260} units of S-40 extract and was performed at 28°C for 15 min.

	Concn. of EGTA added to S-40 extract (mM)	Incorporation of ^3H -labelled amino acids into protein (c.p.m./ A_{260} unit of S-40 extract)
(1)	(i) 0	62 297
	(ii) 0.75	88 318
	(iii) 1.5	88 509
(2)	(i) 0	47 354
	(ii) 1.5	97 446

Table 3. *Protein-synthetic ability of S-40 extracts prepared from Tetrahymena cells disrupted by different methods*

The S-40 extracts were prepared from glycerol-treated cells as described below. Each extract was 'de-salted' by passage through Sephadex G-25 columns (10 cm $\times$ 1.2 cm) made up in buffer A. The final incubation volume of 0.1 ml contained 0.05 ml of S-40 extract at the concentration described in the Table and 0.05 ml of incubation mixture made up to give the concentration of components described in the Results section. The ^3H -labelled amino acids were added to the incubations at concentrations of 1 μCi each of leucine, lysine, phenylalanine and valine at the specific radioactivity listed in the Materials and methods section. The incubations were for 15 min at 28°C.

Method of breaking cells	Concn. of S-40 extracts (A_{260} units/ml)	Incorporation of ^3H -labelled amino acids into protein (c.p.m./ A_{260} unit of S-40 extract)
(1) Washed cell suspension frozen in liquid N_2 and quickly thawed	61.0	1072
(2) Washed cell suspension incubated in 0.5% Triton X-100 for 5 min at 0°C	51.0	359
(3) Washed cell suspension ultrasonicated for 2 s at 25 W output	77.0	1415
(4) Washed cell suspension homogenized with 50 strokes of a hand-operated glass Dounce homogenizer	18.0	38 754
(5) Washed cell suspension (1 ml) mixed with 0.2 g of acid-washed Celite and homogenized with ten strokes of a hand-operated glass Dounce homogenizer	31.0	17 674
(6) Washed cell suspension incubated in 0.5 M-sucrose for 5 min at 0°C, centrifuged and cells disrupted by a further 10 min incubation at 0°C in buffer	24.0	4018

In the light of this finding we investigated the protein-synthesis ability of S-40 extracts made by using various abrasives, and compared the data obtained with results produced by using coated Celite, as described in the legend to Table 5. Coating the Celite with protein material, as well as washing it with EGTA and including EGTA in the homogenization step, resulted in a 3-fold stimulation of protein synthesis over the untreated-Celite controls (Table 5, lines 1 and 3). The need to add additional EGTA to the final S-40 extracts was also obviated (lines 2 and 4). In fact, adding EGTA to the final S-40 extracts was not as effective as including it throughout the preparative steps. The efficiency of cell breakage was unaltered by the Celite treatment.

Although all abrasives tested produced approximately equally concentrated extracts, the protein-synthetic capacities were vastly different. Indeed, both aluminium oxide powder and carborundum powder gave poorer-incorporating extracts than that produced by untreated Celite (Table 5, lines 1, 5, 7). Moreover, in these preparations EGTA was inhibitory (Table 5, lines 2, 6, 8).

Kinetics of protein synthesis

Time courses of protein synthesis were determined for five temperatures in the range 20–32°C (Fig. 3). The data show that the initial rate of protein synthesis increased as the temperature increased. In contrast, the duration of synthesis shortened as the

incubation temperature was raised. For example, S-40 extracts still incorporated amino acids after 50 min at 20°C, albeit at a somewhat lower rate than that initially recorded, whereas it ceased somewhere between the 10 and 15 min time points at 32°C. Intermediate temperatures gave intermediate results both for initial-rate values and for the duration of synthesis.

Are *Tetrahymena* S-40 supernatants capable of initiation of protein synthesis?

Naturally we asked the question whether our S-40 extracts were capable of initiation. To answer this we compared protein synthesis in extracts incubated with two known initiation inhibitors, T-2 toxin (Smith *et al.*, 1975; Carter & Cannon, 1977) and poly(I) (Pain, 1973), with an untreated control and one containing the elongation inhibitor cycloheximide.

Almost identical extents of protein synthesis were obtained with the two initiation inhibitors, and these were nearly 60% of the uninhibited control (Table 6). As these inhibitors were added at their respective saturation concentrations, we conclude that approx. 40% of the incorporation observed resulted from initiation events. Further experiments concerned with the effect of initiation inhibitors on the kinetics of protein synthesis and on poly-ribosome distributions confirm this conclusion (K. E. Smith & E. T. David, unpublished work).

Table 5. *Effect of using different abrasives and abrasive-washing methods on the protein-synthesis activity of Tetrahymena S-40 extracts*

The S-40 extracts were prepared from glycerol-treated cells by ten strokes on a Dounce homogenizer with the abrasives described in the Table. Each abrasive was used in the ratio of 0.2 g/ml of washed cell suspension. The S-40 extracts were 'de-salted' through Sephadex G-25 columns and the incubations performed as described in the legend to Table 4. Each incubation contained 0.05 ml of S-40 extract/0.1 ml of final assay volume. The incubations were performed at 28°C for 15 min.

Cell breakage and assay procedure	Concn. of S-40 extract (A_{260} units/ml)	Incorporation of ^3H -labelled amino acids into protein (c.p.m./ A_{260} unit of S-40 extract)
(1) (i) Dounce homogenization using acid-washed Celite	14	45 869
(ii) Dounce homogenization using acid-washed Celite and 1.5 mM-EGTA added to the S-40 extract	14	56 203
(2) (i) Celite washed with acid and then equilibrated in a solution containing 1.5 mM-EGTA and 1% (w/v) each of casein, spermine and proteose-peptone before use in the Dounce- homogenization procedure	14	125 871
(ii) Celite washed and equilibrated as in 2(i) before use in the Dounce-homogenization procedure; 1.5 mM-EGTA added to the S-40 extract	14	122 682
(3) (i) Dounce homogenization using acid-washed alumina	9	27 809
(ii) Dounce homogenization using acid-washed alumina and 1.5 mM-EGTA added to the S-40 extract	9	11 916
(4) (i) Dounce homogenization using acid-washed carborundum powder	12	34 133
(ii) Dounce homogenization using acid-washed carborundum powder and 1.5 mM-EGTA added to the S-40 extract	12	27 417

Table 6. *Effect of protein-synthesis inhibitors on amino acid incorporation into protein in Tetrahymena S-40 extracts*
 The S-40 extracts were prepared from glycerol-treated cells by using the EGTA-protein-treated-Celite homogenization technique described in Table 5. The S-40 extracts were 'de-salted' through Sephadex G-25 columns and the incubations performed as described in the legend to Table 4. Each incubation contained 1.2 A_{260} units of S-40 extract and was performed at 28°C for 15 min.

Addition to incubation	Incorporation of ^3H -labelled amino acids into protein (c.p.m./ A_{260} unit of S-40 extract)	Inhibition (%)
(1) No addition	292 250	
(2) T-2 toxin (10 $\mu\text{g}/\text{ml}$)	182 801	37.5
(3) Poly(I) (600 $\mu\text{g}/\text{ml}$)	187 405	36
(4) Cycloheximide (100 $\mu\text{g}/\text{ml}$)	4456	98.5
(5) Cycloheximide (10 $\mu\text{g}/\text{ml}$)	9192	97

Fig. 3. *Kinetics of protein synthesis in S-40 extracts*
 The S-40 extracts were prepared from glycerol-treated cells and 'de-salted' through Sephadex G-25 columns by using the protein-coated/EGTA-washed-Celite Dounce-homogenization technique as described in Table 5, line 2(i). Assays were performed as described in the Results section, except that 50 μl samples were removed from 600 μl master incubations at the times indicated. The incubation temperatures were: 20°C (●); 25°C (○); 28°C (▲); 30°C (△); 32°C (■). Each 50 μl assay sample contained 0.98 A_{260} unit of S-40 extract.

In summary, we report that very active *Tetrahymena* cell-free protein-synthesizing S-40 extracts, which are capable of a reasonable amount of initiation, may be prepared as described in this paper by using the treated-Celite homogenization technique.

Discussion

In this paper we have described the preparation and properties of a postmitochondrial-supernatant extract (S-40) from *Tetrahymena pyriformis* that is highly active in cell-free protein synthesis and is capable of at least limited re-initiation.

This work started because we found that the widely reported methods used for preparing most mammalian cell-free protein-synthesizing extracts failed to produce active counterparts from *Tetrahymena* cells. We conclude from the results presented in this paper that there were a number of reasons for this initial failure. For example, it is imperative that the S-40 extracts are prepared from cells that have been harvested, washed and disrupted in glycerol-containing buffers (Table 1). The effect of the glycerol is to preserve protein-factor activity, since the state of polyribosome aggregation is unaffected by the presence of the glycerol. Indeed, we have repeatedly found that high-molecular-weight polyribosome aggregates may be prepared routinely by homogenizing cells in buffer A with or without the addition of the glycerol, i.e. under conditions that yield completely inactive protein-synthesizing extracts. In this respect the procedures described in this paper have little effect on the state of polyribosome aggregation, as can be seen by comparing the polyribosome profiles of S-40 extracts (Fig. 4) prepared by the methods that produced the inactive S-40 extracts (Table 1, line 1) with those used to prepare the most active S-40 extracts [Table 5, line 2(i)].

Much of the protein-synthesizing potential of the crude S-40 extracts is masked because of the dilution of the input ^3H -labelled precursor amino acids by the large endogenous pool of unlabelled amino acids. When these unlabelled amino acids are removed by molecular-sieve chromatography through Sephadex G-25 columns, and the resulting extracts supplemented with an energy-generating system and a fresh amino acid 'cocktail', the incorporation by the S-40 extract is greatly enhanced, and an apparent stimulation of several tensfold is seen (Table 1).

Fig. 4. Comparison of polyribosome-distribution patterns in *Tetrahymena* S-40 extracts prepared by two different methods

In (a) the S-40 extracts were prepared by the method used to obtain the protein-synthesis result of Table 1, line 1, i.e. virtually inactive extracts, whereas (b) the S-40 extracts were prepared by the method described in Table 5, line 2(i). S-40 extract (4 A_{260} units) was diluted to 0.2 ml and layered on to 5 ml linear 10–40% sucrose gradients made up in buffer A. The gradients were centrifuged for 55 min

The protein-synthetic activity of the S-40 extracts is greatly affected by the final pH and Mg^{2+} concentration of the assay, and only marginally by the K^+ and tRNA concentrations (Fig. 1). The Mg^{2+} curve is probably the consequence of two interacting phenomena. The sharp rise in protein-synthetic activity up to the 4.5 mM- Mg^{2+} optimum may be explained by the effect of Mg^{2+} on polyribosome aggregation, since we have found that at 4 mM- Mg^{2+} nearly 80% of the ribosomes are in polyribosome structures which extend into the octomer region of the gradient and beyond (Fig. 4), whereas at 2 mM- Mg^{2+} only 55% of the ribosomes are present as polyribosomes and these are predominantly dimers and trimers (results not shown). Increasing the Mg^{2+} from 4 mM to 15 mM does not alter the state of polyribosome aggregation. Thus the sharp decline in protein-synthesis activity seen above 4.5 mM- Mg^{2+} (Fig. 2b) must be attributed to an effect on protein-factor activity. In contrast, quite wide variations in pH and KCl concentration have little effect on polyribosome aggregation.

Other factors that affect the extent of protein synthesis obtained include the concentration of S-40 extract added and the final concentration of amino acids included in the incubation (Fig. 2).

The method used to disrupt the cells substantially influences the protein-synthesizing activity of the resultant S-40 extract. Precisely why this happens is not clear. We find little effect of cell-breakage method on polyribosome aggregation, although ribosome yields are affected (Table 3). Thus each method described in Table 3 is capable of producing polyribosomes containing around 80% of the total ribosomes in high-molecular-weight aggregation states. It is noteworthy that the least active S-40 protein-synthesizing extracts were obtained from the most efficiently disrupted cells (Table 3, lines 1–3). Whether complete cellular disruption releases more compounds injurious to protein synthesis than does partial disruption is not known. However, the situation is extremely subtle, because abrasives that produce equivalent degrees of cell breakage do not yield equally active protein-synthesizing extracts (Table 5).

at 45 000 rev./min and 2°C in a 6 × 5.2 ml aluminium swing-out rotor in an M.S.E. 65 Mk. II ultracentrifuge. The gradients were analysed by upward displacement with a 60% sucrose cushion through a 0.1 cm flow cell mounted in a Pye Unicam SP.8-100 automatic recording spectrophotometer. Note the similarity of the profiles and the presence of high-molecular-weight polyribosome aggregates. However, in (b) the distribution is toward slightly larger polyribosomes.

Although the Celite increases cell-disruption yields, it also decreases the activity of the protein-synthesizing extracts (Table 3, lines 4–5). Because the unaided Dounce homogenization proved to be very variable in cell-breakage efficiency, we decided to try to improve the activity of the extracts prepared by Celite homogenization.

In this connection we considered the possibilities that (i) the Celite was absorbing protein-synthesis factors and (ii) that inhibitory material was leaking from it during the homogenization. The results of experiments designed to test these points are consistent with both explanations (Tables 4 and 5). In the light of these findings we now wash the Celite and then further treat it with an EGTA solution containing 1% (w/v) each of casein, proteose-peptone and spermidine before it is used to homogenize *Tetrahymena* cells.

The modifications in the procedure used for preparing S-40 extracts have resulted in an enormous increase in the efficiency of protein synthesis. Thus a value of nearly 61 000 c.p.m./ A_{260} unit of S-40 extract was obtained in our first experiment (Table 1, line 3) when the assay contained 1 μ Ci each of L-[3 H]-leucine, -lysine, -phenylalanine and -valine each of specific radioactivity in the region of 30 Ci/mmol, whereas in later experiments (Table 6, line 1) incorporation values of 200 000 c.p.m./ A_{260} unit of S-40 extract were obtained by using 1.5 μ Ci each of L-[3 H]-phenylalanine and -valine of specific radioactivity 5 Ci/mmol.

We have demonstrated that the *Tetrahymena* S-40 extracts prepared by the treated-Celite-homogenization method are capable of at least limited initiation. Both poly(I) and T-2 toxin produced

nearly 40% inhibition of synthesis when added to the S-40 extract at above optimum concentration. In contrast, the control assays containing excess of the elongation inhibitor cycloheximide produced virtually 100% inhibition (Table 6). Moreover, these inhibitors produced incorporation kinetics and poly-ribosome profiles in the *Tetrahymena* extracts which are consistent with a significant initiation capacity (K. E. Smith & E. T. David, unpublished work).

As far as we are aware, the results presented in this paper describe the most efficient and the best-initiating *Tetrahymena* cell-free protein-synthesis system in the literature to date.

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