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The Limiting Factors of a Cell-Free Protein-Synthesizing System from Rat Brain

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The limiting factors of a cell-free system from rat brain for incorporating amino acids into protein were studied. The initial more rapid incorporation by microsomes, as opposed to that by ribosomes, is suggested to be due to damage of the ribosomes by detergent. The defect is rectifiable by incubation of the ribosomes in cell sap, so that ribosomes eventually incorporate more amino acid than do microsomes. This may be because ribonuclease, which is associated with the microsomes but removed by detergent treatment, inactivates the microsomal system. The factor that causes incorporation by microsomes to cease abruptly within 1h is not the lack of any precursor or of adenosine triphosphate, of the inactivation of cell-sap factors or the accumulation of inhibitory substances, but is a deficiency of usable messenger ribonucleic acid. Chain initiation in the system is negligible. Ribosomes also become jammed at the end of messenger ribonucleic acid molecules, unable to terminate protein chains. This eventually leads to jammed polyribosomes, which can be partially relieved by very low concentrations of puromycin. A study of the release of polypeptides synthesized in response to the addition of synthetic messengers did not provide any conclusive information on chain-termination sequences, but did indicate some phenomena that were artifacts. It is concluded that ribonuclease action is sufficient to account for all the deficiencies of the cell-free system, but a lack of chain initiation may be a contributory factor.

To understand the significance of the extent of incorporation of amino acids into protein in a cell-free system, it is important to know the character of the incorporation process and in particular the factors limiting the process. This is especially so since in general cell-free systems work at a very small fraction of the rate *in vivo*.

The differences observed by some authors between the incorporating abilities of microsomes and of ribosomes derived from microsomes by detergent treatment offered a convenient tool to study this. Thus Acs, Neidle & Schneiderman (1962) suggested that brain microsomal incorporation was terminated prematurely by the release from the microsomal membrane of fatty acids, which they showed would inhibit amino acid incorporation by ribosomes. However, Korner (1961), working with a rat liver cell-free system, suggested that it was the microsomal ATPase† that limited microsomal incorporation, since ribosomes, unlike microsomes, did not display a requirement for an ATP-regenerating system.

The results obtained with the rat brain cell-free system studied here show that neither of the above mechanisms was operative. Ribonuclease activity, in particular on mRNA, was sufficient to explain all the limiting factors of the system.

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† Abbreviation: ATPase, adenosine triphosphatase.

MATERIALS AND METHODS

L-[G-^{3}H]Arginine (420mCi/mmol), L-[U-^{14}C]isoleucine (16mCi/mmol), DL-[1-^{14}C]leucine (32mCi/mmol), L-[U-^{14}C]lysine (222mCi/mmol), DL-[1-^{14}C]phenylalanine (42mCi/mmol), L-[U-^{14}C]phenylalanine (244mCi/mmol), L-[U-^{14}C]valine (155mCi/mmol), ^{14}C-labelled reconstituted protein hydrolysate and [6-^{14}C]orotic acid were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.) and [5-^{3}H]UDP was from Schwarz BioResearch Inc. (Orangeburg, N.Y., U.S.A.). Puromycin was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.), the sodium salts of ADP, ATP, GDP, GTP and UDP from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and creatine phosphate and creatine phosphokinase from C. F. Boehringer and Soehne G.m.b.H. (Mannheim, Germany). Poly U, poly A and poly (U,C) were obtained from Miles Chemical Co. (Stoke Poges, Bucks., U.K.). Other polyribonucleotides, including [^{3}H]poly U, were synthesized from the nucleoside diphosphates by the method of Steiner & Beers (1961), by using polynucleotide

phosphorylase prepared from spray-dried cells of *Micrococcus lysodeikticus* (obtained from Calbiochem, Los Angeles, Calif., U.S.A.), as described by the same authors. Polymers were purified of free nucleotides by chromatography on Sephadex G-100 and stored dry after being freeze-dried.

Microsomes, ribosomes, polyribosomes and cell sap. These were prepared by a method similar to that of Munro, Jackson & Korner (1964) for rat liver. Female rats (4–6 weeks old) of an albino strain bred in the laboratory were killed by decapitation, the brains rapidly excised and the cortexes separated and dropped into ice-cold 0.25M-sucrose in medium A [20mM-tris–HCl (pH 7.6)–100mM-KCl–40mM-NaCl–5mM-magnesium acetate] containing 6mM-2-mercaptoethanol. They were then homogenized in 1.5vol. of the same medium in a precision-bore glass homogenizer with a loose-fitting Perspex ball plunger. The homogenate was freed of cell debris, nuclei and mitochondria by centrifugation for 15min at 10000g in an MSE 18 centrifuge at 0°C.

Microsomes were prepared by layering 6.2ml of the 10000g supernatant over 6ml of 1M-sucrose in medium A in Polyallomer centrifuge tubes of the Spinco no. 40 rotor. The tubes were then centrifuged for 4h at 40000rev./min in the model L or L2 ultracentrifuge at 4°C. For the preparation of ribosomes, the 10000g supernatant was made 1% with respect to Triton X-100 from a stock solution of 10% (v/v) in water before this centrifugation (Hunter & Korner, 1966). Polyribosomes were prepared similarly but with layers of 3ml of 1M-sucrose and 3ml of 2M-sucrose in medium A instead of 6ml of 1M-sucrose. After centrifugation the tubes were rinsed with medium A and the walls dried with Kleenex tissue. Pellets were resuspended in medium A at 0°C by gentle agitation immediately before use. The RNA/protein ratios of the various preparations were: microsomes, 0.12–0.33; ribosomes, 0.53–0.75; polyribosomes, 0.73–0.85.

Cell sap was made from the 10000g supernatant by centrifugation in the Spinco no. 40 rotor at 40000rev./min for not less than 2h. The upper four-fifths of the supernatant was removed by aspiration and passed through a column of Sephadex G-25 to remove free amino acids, nucleotides and sucrose, and to standardize the concentrations of salts (Mansbridge & Korner, 1963). The column, of total volume not less than ten times the volume of cell-sap to be treated, was equilibrated at 4°C in medium M (Munro *et al.* 1964; medium M is medium A with the addition of 5mM-magnesium acetate and 6mM-2-mercaptoethanol).

Cell-free incorporation of amino acids. Cell-free incorporation of amino acids was done in medium M in a total volume of 0.5ml at 37°C. The incubation mixture contained ribosomes or microsomes (10–50μg of RNA), cell sap (about 2mg of protein), 5mM-ATP, 0.5mM-GTP, 5μM of the radioactive amino acid and 10μM of the remaining amino acids of the following: alanine, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. When incubation periods longer than 1h were used, benzylpenicillin (100 units/ml) and streptomycin sulphate (50μg/ml) were also included. All additions were at neutral pH and made so as not to disturb the concentrations of salts. Polynucleotides were added, where applicable, at 100μg/tube, which was a saturating amount for poly U.

Incorporation was stopped by the addition of 2.5ml of cold 0.5M-$HClO_4$ containing 0.1% of non-radioactive amino acid corresponding to the radioactive amino acid in the incubation mixture. After not less than 2h at 4°C, the precipitates were collected by centrifugation and dissolved in 2.5ml of 0.3M-NaOH, also containing 0.1% of the non-radioactive amino acid. The RNA was hydrolysed by incubation for 1h at 37°C and the proteins were reprecipitated by the addition of 1ml of 2M-$HClO_4$ and kept overnight at 4°C. The proteins were collected by filtration on Oxoid membrane filters, washed with four 5ml portions of cold 0.5M-$HClO_4$ containing the non-radioactive amino acid and fixed with rubber solution to cardboard discs for radioactivity counting in a Nuclear-Chicago end-window gas-flow counter (efficiency about 20%, background less than 2c.p.m.). Washing with lipid solvents did not decrease the net count rate recorded.

All results are the mean of the counts recorded in three separate incubation tubes and have been corrected for the c.p.m. recorded in control incubation mixtures for each time-point in which no ribosomes or microsomes were included. This latter was a small fraction of the total c.p.m. observed in the presence of ribosomes or microsomes.

Sucrose gradients. Linear 15–30% (w/v) sucrose gradients in medium A were used for the separation of ribosomes and polyribosomes. They were centrifuged in the SW 50 rotor of the Spinco model L2 ultracentrifuge at 50000rev./min at 2°C. Gradients were fractionated by upward displacement with a dense sucrose solution from an injection pump, the gradient was passed through a flow cell of total vol. 0.1ml fitted to a Beckman DK2 recording spectrophotometer to monitor the E_{260}, and fractions were collected with a time-based fraction collector from the continuous flow.

Where quantitative recovery of radioactivity was required, a 'cushion' of 2M-sucrose was used in the centrifuge tube below the gradient to prevent polyribosomes from pelleting. Fractions were precipitated with 2.5ml of 5% (w/v) trichloroacetic acid after the addition of 200μg of bovine serum albumin. They were then filtered on glass-fibre filters (Whatman GF/C), dried in an oven at 60°C and counted in a scintillation counter with a scintillation fluid of 4g of 2,5-diphenyloxazole (PPO)/l of toluene.

In the experiments on release of radioactive peptides by synthetic polynucleotide messengers, trichloroacetic acid saturated with tungstic acid was used as the precipitating reagent (Gardner *et al.* 1962). Control experiments had shown that, in order to precipitate polylysine, it was essential to maintain a saturating concentration of tungstic acid for all precipitations. The procedure used was as follows. First, 2ml of 0.3M-NaOH was added to each gradient fraction, which was then incubated for 1h at 37°C. Then 200μg of bovine serum albumin and a sufficient volume of 5% sodium tungstate solution to cause just a very fine precipitate of tungstic acid after the addition of the trichloroacetic acid were added to each tube followed by 1ml of 20% (w/v) trichloroacetic acid. The samples were then filtered on glass-fibre filters, washed with 5% trichloroacetic acid saturated in tungstic acid and counted as above.

Determinations. RNA was determined by the method described by Munro *et al.* (1964) but with an extinction coefficient of 32.2 for 1 mg of hydrolysed RNA (calculated from the base composition of brain ribosomal RNA). No interference by microsomal components was observed, insofar as reaction of the hydrolysed bases with orcinol gave comparable results. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard, special care being taken to allow for the reactions produced by tris and mercaptoethanol.

ATPase was assayed by the release from ATP of P_i, assayed by the method of Mokrasch (1961). This method gave results identical with those obtained with the method of Schwarz, Bachelard & McIlwain (1962). Phosphatase was assayed by the method of Ohmori (1937) and phosphodiesterase by that of Sinsheimer & Koerner (1952) with a molar extinction coefficient of 17000 for *p*-nitrophenol (Burch, 1957). Ribonuclease was assayed by the loss of acid-precipitable radioactivity on incubation of [^{3}H]poly U.

RESULTS AND DISCUSSION

Fig. 1 shows the time-course of the incorporation of amino acids by brain microsomes and ribosomes. All the conditions of incubation had previously been optimized and were found to be identical for microsomes, ribosomes and total 20000*g* supernatant (Dunn & Korner, 1966; Dunn, 1968). Three differences are apparent in the time-course; the microsomes are initially more active than ribosomes, the incorporation by microsomes ceases fairly abruptly in less than 1h whereas the ribosomes incorporate at a steadily decreasing rate for at least 3h, and the ribosomes are more active in terms of their RNA content than the microsomes.

A higher initial rate of incorporation by microsomes was observed in rat liver by Korner (1961) and in rat brain by Zomzely, Roberts & Rapaport (1964) and Takahashi, Mase & Abe (1966), but not by Acs *et al.* (1962). Zomzely *et al.* (1964) suggested that the effect could be explained if the amino acid-activating enzymes were located within microsomal vesicles. In support of this, Clouet, Ratner & Williams (1966) noted that microsomes were less dependent on added cell sap or pH 5 fraction than were ribosomes, a result confirmed in the present study. However, brain ribosomes are not predominantly membrane-bound, as shown by sucrose-gradient analysis (Fig. 2) or in electron micrographs (Ekholm & Hydén, 1965), and in particular the microsomes used here were comparatively free of membrane after centrifugation through 1M-sucrose. Also, since the ribosomes are located principally on the outside of microsomal vesicles (Palade & Siekevitz, 1956), a more likely explanation is that the detergent removes or damages some component necessary for full activity. If this component was normally present in cell sap or the defect was rectified by incubation in it, the observed effect of cell sap concentration would be explained.

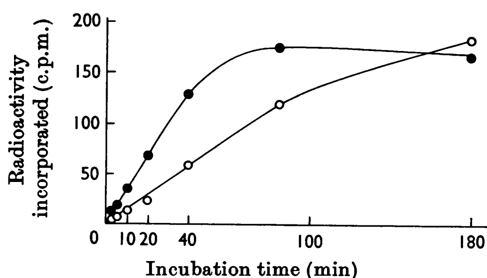

Fig. 1. Time-course of amino acid incorporation by microsomes or ribosomes. Microsomes or ribosomes prepared from equal amounts of the same 10000*g* supernatant were incubated under the conditions for the incorporation of amino acids ([^{14}C]leucine) described in the Materials and Methods section. ●, Microsomes (48 μg of RNA); ○, ribosomes (34 μg of RNA).

Most workers have observed that microsomes cease amino acid incorporation within 1h but that ribosomes may continue for considerably longer (Korner, 1961; Acs, Neidle & Waelsch, 1961; Bondy & Perry, 1963; Zomzely *et al.* 1964; Murthy & Rappoport, 1965), but not Stenzel, Aronson & Rubin (1966), working with rabbit brain. It is pertinent to note that if ribosomes and microsomes are prepared from the same amount of 10000*g* supernatant and the stoicheiometry is preserved (as in the experiments of Fig. 1), the absolute final incorporations are of the same order although the ribosomes are consistently slightly more active, so that the initial low activity of ribosomes is compensated by a longer time-course. This is also consistent with the hypothesis outlined above.

It is striking that in Fig. 1 the yields of RNA in equivalent preparations of microsomes and ribosomes are so different. This difference enhances the greater incorporation activity of ribosomes in terms of RNA content, which is a better measure of ribosome content than the protein content used by most other authors. This discrepancy is not due to interference by microsomal components in the RNA assay, but is because not all the ribosomes are pelleted in the 4h centrifugation period, pelleting being slower after detergent treatment. Since the unpelleted components are most likely monomeric ribosomes and ribosomal subunits, which are probably comparatively inactive in cell-free amino acid incorporation (Munro *et al.* 1964), the preparation is relatively activated. Little evidence for the presence of membrane RNA was obtained since similar amounts of RNA were pelleted in both the presence and the absence of detergent after 15h centrifugation, and also very little material absorbing at 260nm was released from microsomes by detergent treatment, shown by sucrose-gradient analysis.

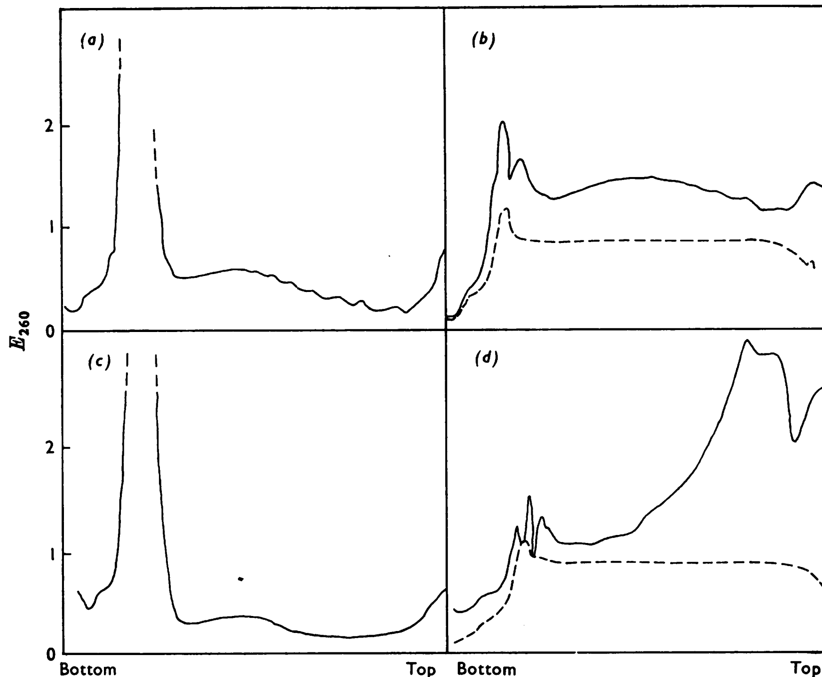

Fig. 2. Sedimentation profile of heavy microsomes of brain and liver. Heavy microsomes (membrane-bound) were prepared from rat liver and brain by centrifugation of the 10000g supernatants for 1h at 20000rev./min in the Spinco no. 40 rotor (Campbell, Serck-Hanssen and Lowe, 1965). Analysis on 4ml 15–30% sucrose gradients with a cushion of 1ml of 2M-sucrose centrifuged for 15min at 50000rev./min in the Spinco SW50 rotor. Extinction profiles at 260nm. of brain (a and b) and liver (c and d) heavy microsomes are shown. Gradients (b) and (d) contained 0.1% Triton X-100; the broken line shows the profile of a control gradient with no sample. Light microsomes of either tissue showed little, if any, change in profile after Triton treatment.

Exhaustion of energy or amino acids. Korner (1961) suggested that the longer incorporation time observed with liver ribosomes than with microsomes might be attributable to the removal of the membrane-associated ATPase, especially since ribosomes did not display a requirement for an ATP-regenerating system. In the brain system studied here, however, no requirement for an ATP-regenerating system could be shown with microsomes or total 20000g supernatant (Dunn & Korner, 1966). This may be a consequence of the lower proportion of endoplasmic reticulum in brain cells (Palade, 1955).

That the energy source or amino acid supply may not be limiting was also suggested by the fact that the total incorporation observed was linearly dependent on the quantity of microsomes or ribosomes incubated. Direct measurement of the ATPase activity in an incubation system (Table 1) showed that only a fraction of the added ATP would be destroyed during the incubation time. Moreover added ATP, or an ATP-regenerating system, did not revive the incorporation after it had ceased (Table 2). As a definitive test, extra microsomes added to the incubation tube after the original incorporation had ceased stimulated the incorporation to something approaching initial values (Fig. 3). There was, however, some decrease in the stimulatory capacity of the extra microsomes with time, which may indicate a slow exhaustion of soluble factors or the accumulation of inhibitory

Table 1. *ATPase activity of microsomes and ribosomes*

Samples (0.1ml) of microsomes, ribosomes or cell sap were incubated in medium M with 5mM-ATP at 37°C. The P_i released in a 5min incubation was assayed as described in the Materials and Methods section.

	RNA (μg/ml)	Protein (mg/ml)	ATPase (nmol of P_i/min)	Cell-free incorporation of [^{14}C]leucine in 4h (c.p.m./mg of RNA)
Cell sap	21	6.4	2.2	—
Microsomes	383	2.4	10.4	6300
Ribosomes	393	0.74	1.2	8100

Table 2. *Effect of the addition of ATP or an ATP-regenerating system on amino acid incorporation by the cell-free system*

Additions	[^{14}C]leucine incorporated (c.p.m.)	
	20 min	60 min
None	89	97
ATP (2.5 μmol)		100
ATP + Mg^{2+} (2.5 μmol each)		97
Creatine phosphate (1 mg) + creatine phosphokinase (0.1 mg)		107
Medium M		101

The incorporation of leucine into protein by a standard microsomal cell-free system (16 μg of RNA) was measured. Additions were made at 20 min in a volume of 0.1 ml.

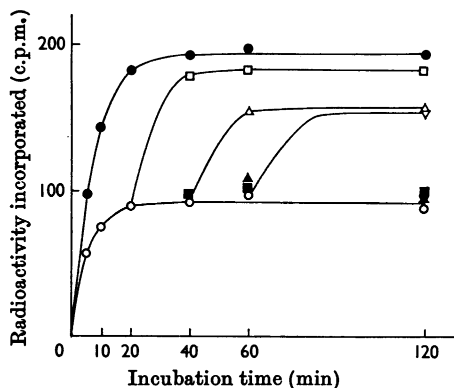

Fig. 3. Effect of the addition of extra microsomes to the incubation. Microsomes (16 μg of RNA) were incubated under standard conditions with [^{14}C]leucine as described in Table 2 (○). An extra, equal portion of microsomes was added to the incubations at: 0 min (●); 20 min (□); 40 min (△); or 60 min (▽). An equal volume (0.1 ml) of medium M was added at: 20 min (■); 40 min (▲); or 60 min (▼).

substances such as amino acids diluting the added labelled precursor; but clearly this could not account for the cessation of incorporation by the original microsomes. This slow decrease could also be due to decay of the resuspended microsomes at 0°C.

Accumulation of inhibitory substances or inactivation of cell-sap factors. Acs *et al.* (1962). showed that fatty acids would inhibit amino acid incorporation by brain ribosomes and that microsomes released fatty acids on incubation. The time-course observed in the present study is very similar to that observed by Acs *et al.* (1961) but cannot be explained by the release of inhibitory substances from the microsomes, since in the experiment shown in Fig. 3 the extra micro-

Table 3. *Effect of poly U or puromycin or both after incubation on amino acid incorporation by the cell-free system*

An S-20 (Dunn & Korner, 1966) cell-free system was incubated with [^{14}C]phenylalanine under standard conditions. Microsomes were isolated from a parallel incubation mixture that did not include [^{14}C]phenylalanine after 30 min incubation, and from the original unincubated 20000g supernatant. A part of both the incubated and the unincubated 20000g supernatants were made 5 μg/ml with respect to puromycin before isolation of the microsomes. The four sets of microsomes were then incubated under standard cell-free incorporation conditions for 30 min. All incubation mixtures contained approximately equal quantities of ribosomal RNA. The poly U, puromycin or both were added to the S-20 system at 30 min.

	Incorporation of [^{14}C]-phenylalanine (c.p.m.)	
	30 min	60 min
S-20	1050	1130
+poly U (100 μg)		2740
+puromycin (1 μg)		1020
+poly U + puromycin		3120
	No puromycin treatment	Puromycin treated
Unincubated microsomes	395	383
+poly U (100 μg)	2970	3180
Incubated microsomes	58	94
+poly U (100 μg)	4850	4870

somes showed a similar time-course. In addition, the incorporating activity was not revived if the incubation system was passed through a Sephadex G-25 column and incubated with fresh ATP, GTP and amino acids. The difference may be ascribed to the far greater concentrations of microsomes used by Acs *et al.* (1961) in their incubation mixtures, but their own numerical results do not support the hypothesis that inhibition by fatty acids accounts for the shorter incorporation time with microsomes.

Microsomes or ribosomes isolated from a microsomal incubation system at 30 min, when incorporation had ceased, did not stimulate further incorporation when incubated with fresh cell sap, amino acids and an energy source. It may be concluded that cell-sap factors do not account for the cessation of incorporation, neither does the microsomal membrane limit the incorporation since its removal by detergent treatment did not restore any incorporation. The first conclusion may also be deduced from the results of the experiments shown in Fig. 3.

Inactivation of the ribosomes. It is clear from the above experiments that the defect that causes the incorporation to cease resides in the ribosomes or microsomes themselves. One possibility is an exhaustion of mRNA. To test this a synthetic mRNA,

poly U, was added to the system after it had ceased incorporation (Table 3). Not only was the system still active but the additional incorporation was greater than that originally observed. This is presumably because ribosomes had become released from the mRNA during the course of incubation.

Table 4. *Breakdown of pulse-labelled RNA of ribosomes on incubation*

Polyribosomes were isolated from the brain of a rat 60min after the intracranial injection of 5μCi of [14C]-orotic acid and incubated with cell sap under the standard conditions for the incorporation of amino acids into protein. Samples were precipitated at various times with 5% trichloroacetic acid and filtered on to glass-fibre filters for counting in a scintillation counter. In the absence of cell sap, loss of acid-precipitable radioactivity was negligible.

Time (min)	Trichloroacetic acid-precipitable radioactivity (c.p.m.) Complete system	Complete system less ATP, GTP and amino acids
0	227	
10	173	193
20	162	182
30	154	167

Munro & Korner (1964) have presented evidence that the radioactivity associated with rat liver polyribosomes at short times after the administration of radioactive precursors of RNA is predominantly in mRNA. These observations have also been extended to rat brain (Jacob, Samec, Stévenin, Garel & Mandel, 1967; Dunn, 1968). Table 4 shows that if such polyribosomes are incubated under the standard conditions for incorporation of amino acids into protein, the radioactive RNA is destroyed, a process that is accelerated when translation is allowed to occur. An important defect in the cell-free system therefore lies in the mRNA.

Table 5 shows the phosphatase, phosphodiesterase and ribonuclease activities of ribosomes and microsomes. The much greater activity of these enzymes in the microsome preparation could well account for the differences in final incorporation between microsomes and ribosomes. Since these enzymes are also activated by Triton X-100, it is clear that the ribosomes become particularly vulnerable during their preparation, which may minimize these differences.

Table 5. *Phosphatase, phosphodiesterase and ribonuclease activities of microsomes and ribosomes*

Microsomes (58μg of RNA) or ribosomes (32μg of RNA) were incubated in 1ml of medium A at 37°C and the enzymes assayed as described in the Materials and Methods section.

	Phosphatase (nmol of *p*-nitrophenol)		Phosphodiesterase (nmol of *p*-nitrophenol)		Ribonuclease (c.p.m. of [3H]poly U)		
	30min	60min	30min	60min	0min	10min	20min
Microsomes	12.5	26.1	1.4	5.5	1130	802	727
+0.2% Triton	21.2	32.3	3.7	6.5	1080	774	472
Ribosomes	0.4	1.9	0	0	1050	1140	1060
+0.2% Triton	—	—	—	—	1130	1070	1050

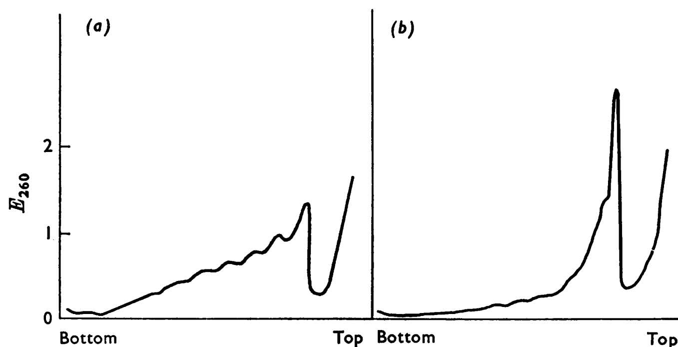

Fig. 4. Sedimentation profiles of incubated and unincubated microsomes. Microsomes before (*a*) and after (*b*) 30min incubation under standard incorporation conditions were centrifuged on 5ml 15–30% sucrose gradients for 30min at 50000rev./min in the Spinco SW 50 rotor.

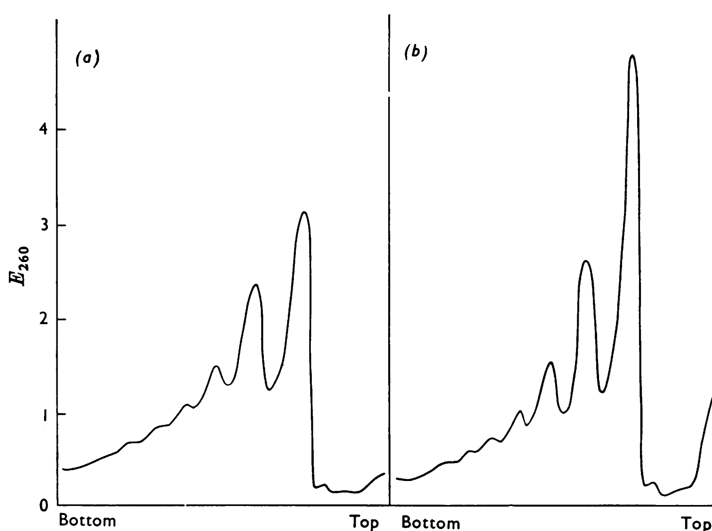

Fig. 5. Effect of puromycin on the sedimentation profile of ribosomes. Ribosomes were incubated for 15min at 37°C in medium A in the presence (*b*) and absence (*a*) of 10 μg of puromycin/ml before centrifugation on 15–30% sucrose gradients for 40min at 50000rev./min in the Spinco SW50 rotor.

Table 6. *Effect of puromycin on microsomal amino acid incorporation*

	Incorporation of [^{3}H]arginine (c.p.m.)		
	10min	30min	60min
Microsomes alone	2760	4860	6290
+1 μg of puromycin at zero time	2770	4710	6140
+1 μg of puromycin at 10min		5220	7500
+1 μg of puromycin at 30min			8150

However, Fig. 4 shows that polyribosomes still exist even after incorporation has ceased, so that presumably not all the mRNA is destroyed. Ribonuclease could totally destroy the mRNA, but even a more limited action could result in the excision of initiation or termination regions, or both, which may be of specific sequence. The excision of initiation regions, by preventing initiation, renders the messenger useless for translation so that it will be further degraded by ribonuclease action, since it is unprotected by ribosomes. The excision of termination regions may result in ribosomes becoming jammed at the end of the messenger, unable to terminate the chain or translate the messenger further (see Bretscher, 1968). This jammed ribosome would physically block the translation of the message by other ribosomes so that a jammed polyribosome would result.

Puromycin causes the release of nascent protein chains from ribosomes by displacing the tRNA (Allen & Zamecnik, 1962), and ribosomes that do not carry nascent protein chains are less stable on the mRNA than those that do (Munro *et al.* 1964; Dunn, 1968). It might therefore be expected that ribosomes that have reacted with puromycin will detach from the mRNA. Fig. 5 shows that low concentrations of puromycin do cause ribosomes to detach from polyribosomes in the absence of protein synthesis. Table 3 showed that the stimulation by poly U of phenylalanine incorporation was enhanced by puromycin, which suggests removal of active ribosomes from mRNA. If ribosomes jammed at the end of messengers without a terminating signal can be released by puromycin in this way, further translation of the messenger should be possible by the queueing ribosomes. Table 6 shows that the addition of extremely low concentrations of puromycin to a microsomal incubation system after it had ceased incorporation does in fact stimulate the incorporation. This effect was greatest with arginine but was also observed with leucine. A similar effect has been shown by Hunter & Korner (1969) with the rat liver cell-free system.

Chain initiation. Assuming a requirement for a specific initiation sequence in the mRNA such as exists in *Escherichia coli* (Lengyel, 1967), then, as mentioned above, excision of this sequence by ribonuclease will curtail initiation. It is possible, however, that other conditons necessary for chain initiation are not fulfilled in the cell-free system. Initiation with

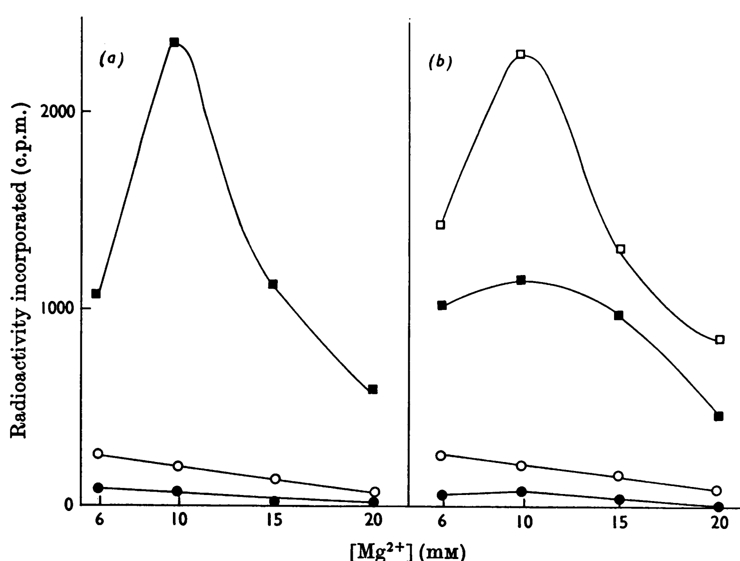

Fig. 6. Effect of NaF on amino acid incorporation by the cell-free system. Ribosomes (36 μg of RNA) incubated under standard conditions with [14C]phenylalanine but with the stated concentrations of magnesium acetate and 2mM-NaF. Incubation for 5min (●) or 60min (○, □ and ■). □, 100 μg of poly U added before incubation and the NaF added at 5min; ■, NaF added before incubation and 100 μg of poly U added after 5min. (a), Control; (b), +2mM-NaF.

poly U and other synthetic polynucleotide messengers may not be of the same kind as in the endogenous system, a situation that has been demonstrated in *E. coli* (Lengyel, 1967). It is relevant that very high concentrations of the synthetic messengers are required in mammalian systems.

Fig. 6 shows the result of an experiment in which sodium fluoride was added to the cell-free system. Clearly this compound inhibits the initiation but not the subsequent translation of poly U, as has been shown in reticulocytes (Ravel, Mosteller & Hardesty, 1966). Dreyfus & Shapira (1966) and Hunt, Hunter & Munro (1968) have presented evidence that fluoride blocks the initiation of the haemoglobin messenger in the same tissue, so the effect is apparently not specific for poly U. Sodium fluoride was not observed to inhibit the endogenous incorporation by brain ribosomes or polyribosomes by more than 5% after correction for the Mg^{2+} concentration has been made. Chain initiation does not therefore play an important part in the cell-free incorporation system studied here.

As a direct measure, the amount of radioactive amino acid incorporated from a ^{14}C-labelled amino acid mixture into *N*-terminal positions in polypeptides was measured by using 1-fluoro-2,4-dinitrobenzene (Sanger, 1945). No appreciable *N*-terminal incorporation was detected.

Chain termination. Experiments presented above showed that ribosomes apparently do become jammed at the end of messengers, unable to terminate, though it is not known whether this is because of a lack of a proper terminating sequence or a defective terminating mechanism.

Fig. 7 shows the results of an experiment in which the release of incorporated radioactive amino acid was studied. The results are very similar to those of Bondy & Perry (1963) and Zomzely *et al.* (1964). However, some observations suggested caution in the interpretation of these results. First, the results were only precisely reproducible if the experiments were performed in exactly the same way, and, secondly, the absolute amount of release often decreased slightly with the time of incubation and occasionally with Triton treatment. The results of the above-mentioned authors also show a decrease of release with incubation time.

Bretscher (1964) showed that polylysine synthesized by an *E. coli* cell-free system in the presence of poly A remained in the supernatant attached to tRNA after sucrose-gradient analysis. Nascent protein chains, either free or attached to tRNA, may be released if the ribosomes become damaged, by treatment with sodium dodecyl sulphate (Gilbert, 1963) or EDTA (Tashiro & Siekevitz, 1965), or by activity of nuclease (Gilbert, 1963) or protease (Malkin & Rich, 1967). Conversely, insoluble proteins may be synthesized that sediment with the ribosomes. In particular, Lim & Adams (1967) found that an appreciable proportion of the protein synthesized by a brain cell-free system from 4-day-old rats was easily

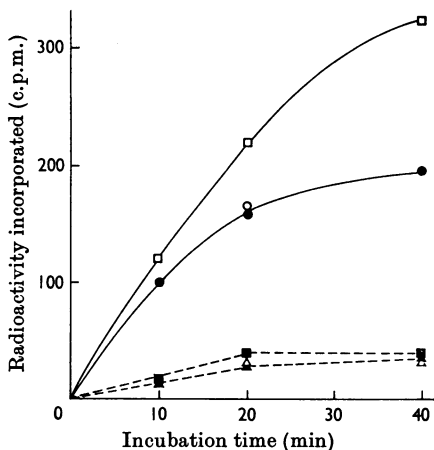

Fig. 7. Release of radioactive protein from the ribosomes during incubation. Microsomes and ribosomes were incubated in standard incubation systems, but of larger volume, with [^{14}C]phenylalanine. At various times 0.3ml samples were removed and added to 0.1ml of 1% phenylalanine in medium A. Then 0.3ml of each of these samples was centrifuged on 4ml 15–30% sucrose gradients with a cushion of 1ml of 2M-sucrose for 80min at 50000rev./min in the Spinco SW50 rotor. After centrifugation the tube was sliced at its mid-point (previously shown to be above the sedimentation position of the ribosomal subunits) and the top and bottom halves of the gradient were separately precipitated with 4mg of serum albumin as a carrier and 0.5M-$HClO_4$. They were then treated as the products of a normal cell-free incorporation experiment (see the Materials and Methods section). Total incorporation (——): ○, microsomes; ●, microsomes treated with 1% Triton after incubation; □, ribosomes. Released radioactivity (----): △, microsomes; ▲, microsomes treated with Triton after incubation; ■, ribosomes.

sedimentable, but this proportion decreased with older rats. It would therefore be unwise to assume that release measured by centrifugal separation represents genuine termination, as some workers with rat liver cell-free systems have done (Hultin, 1961, 1962; Hultin & Abraham, 1964; Khairallah & Pitot, 1967). However, if the release observed in the present paper does represent proper chain termination or an approximation to it, it seems unlikely that the chain-terminating mechanism is itself limiting the system, in view of the limited translation that occurs (very approximately 30 amino acids per ribosome).

It was decided to study the release of polypeptides synthesized in response to the addition of synthetic polynucleotides, since this may provide information on chain-termination sequences if such exist in mammalian systems. The technical obstacles were, however, considerable. It is difficult to decrease the endogenous incorporation of mammalian cell-free systems to very low values while retaining ribosome activity. When mixed polynucleotides are used, the incorporation of individual amino acids is low, an effect that cannot be improved by using several amino acids together since this increases the background incorporation value and in particular polymers not containing uridylic acid produce poor responses. In order to get good quantitative results it was also necessary to use sucrose-gradient separation, thus limiting the number of samples that could be handled in one experiment. Centrifuge tubes in angle rotors leak slightly and pellets are impossible to resuspend quantitatively for washing procedures, and a Millipore assay was not found reproducible and may not be valid with products, like polyphenylalanine, that are very insoluble. Finally, polynucleotides containing a high proportion of terminating sequences are likely to promote the synthesis of very short peptides that may not be precipitated by trichloroacetic acid, an effect that will be enhanced by the lysine content if terminating codons similar to those operative in *E. coli* exist (Stretton & Brenner, 1965).

However, Table 7 presents some of the results from experiments in which significant stimulation by polynucleotides occurred. Several observations can be made from these results. The extent of release depends on the labelled amino acid used. The product of phenylalanine incorporation stimulated by poly U was never significantly released from the ribosomes (radioactivity was associated principally with the monomer and dimer peaks and was not pelleted). Some apparent release was observed with all the other polymers tested, excepting, possibly, poly (U,C). The results obtained with an amino acid mixture are particularly significant because here a substantial proportion of the product of poly U-stimulated incorporation was released. Since poly U has been observed to stimulate the incorporation by polyribosomes of amino acids other than phenylalanine, an effect unrelated to mistranslation of poly U (Hunter & Korner, 1969; Dunn, 1968), it is possible that this stimulated release is also artifactual. Poly (U,C) did not give this effect, or at least not to the same extent.

This result severely questions the feasibility of experiments with mammalian systems similar to those performed with *E. coli* by Takanami & Yan (1965) and Bretscher, Goodman, Menninger & Smith (1965). Certainly the results presented in Table 7 do not provide any good evidence for particular terminating codons.

It may therefore be concluded that the cell-free incorporation of amino acids into protein stops when there is no more mRNA available for translation. Ribosomes released after translation do not become reattached to messengers to any appreciable extent either because the free initiating regions of mRNA molecules are destroyed or because the initiation

Table 7. *Release of radioactive protein from ribosomes labelled during cell-free incorporation*

Polyribosomes were incubated under standard conditions with the stated radioactive amino acid and polynucleotide additions. After 20min incubation, non-radioactive amino acid was added and the mixture was immediately centrifuged on a sucrose gradient as described in the legend to Fig. 7. After centrifugation, the gradients were fractionated and the radioactivity was counted as described in the Materials and Methods section. Released radioactivity is the radioactivity sedimenting in the gradient above the subunit peaks, bound radioactivity is that in the remainder of the gradient. Release (%) is the former expressed as a percentage of the total radioactivity recovered from the gradient and is corrected for an unincubated sample with the endogenous incorporation (except in Expts. 1, 2 and 3) and for the endogenous incorporation with polynucleotides. In Expts. 3, 4, 5 and 6, the polyribosomes were preincubated in a complete incorporation system without radioactivity for 1h and then passed through a small column of Sephadex G-25 equilibrated in medium M before incubation with fresh cell sap, ATP, GTP and amino acids. In Expt. 6 the gradient tubes were sliced after centrifugation and treated as described in the legend to Fig. 7. The ratios of nucleotides in the polynucleotides refer to the input ratio of nucleoside diphosphates in the polynucleotide-synthesis process.

Expt. no.	Radioactive amino acid	Polynucleotide	Radioactivity (c.p.m.)		Release (%)
			Bound	Released	
1	Phenylalanine	None	35000	20300	37
		Poly (U_5,A,G)	69000	24300	11
		Poly U	106000	22500	3
2	Lysine	None	2030	7390	78
		Poly (U,A)	2210	7890	74
		Poly (U,A_2)	2250	7720	60
	Phenylalanine	None	5520	2840	34
		Poly U	13700	2790	0
3	Valine	None	370	5620	94
4	Reconstituted protein hydrolysate	None	15800	20900	57
		Poly U	27100	28600	41
		Poly (U,C)	41700	21900	4
5	Lysine	None	1530	675	31
		Poly A	6190	1010	7
		Poly (A_2,G)	9590	1220	6
6	Phenylalanine	None	33	50	60
		Poly U	1640	86	2
		Poly (U_3,A)	480	108	12
	Isoleucine	None	0	0	
		Poly (U_3,A)	49	16	24

process is unable to function properly in the system. Other ribosomes become jammed at the end of messengers unable to terminate and jammed polyribosomes may form. Ribonuclease action would be sufficient to account for all these defects but may not necessarily be solely responsible.

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REFERENCES

Acs, G., Neidle, A. & Schneiderman, N. (1962). *Biochim. biophys. Acta*, **56**, 373.

Acs, G., Neidle, A. & Waelsch, H. (1961). *Biochim. biophys. Acta*, **50**, 403.

Allen, D. W. & Zamecnik, P. C. (1962). *Biochim. biophys. Acta*, **55**, 865.

Bondy, S. C. & Perry, S. V. (1963). *J. Neurochem.* **10**, 603.

Bretscher, M. S. (1964). Ph.D. Thesis: University of Cambridge: quoted by Bretscher *et al.* (1965).

Bretscher, M. S. (1968). *J. molec. Biol.* **34**, 131.

Bretscher, M. S., Goodman, H. M., Menninger, J. R. & Smith, J. D. (1965). *J. molec. Biol.* **14**, 634.

Burch, H. B. (1957). In *Methods in Enzymology*, vol. 3, p. 948. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Campbell, P. N., Serck-Hanssen, G. & Lowe, E. (1965). *Biochem. J.* **97**, 422.

Clouet, D. H., Ratner, M. & Williams, N. (1966). *Biochim. biophys. Acta*, **123**, 142.

Dreyfus, J. C. & Shapira, G. (1966). *Biochim. biophys. Acta*, **129**, 601.

Dunn, A. J. (1968). Ph.D. Thesis: University of Cambridge.

Dunn, A. J. & Korner, A. (1966). *Biochem. J.* **100**, 76P.

Ekholm, R. & Hydén, H. (1965). *J. Ultrastruct. Res.* **13**, 269.

Gardner, R. S., Wahba, A. J., Basilio, C., Miller, R. S., Lengyel, P. & Speyer, J. F. (1962). *Proc. natn. Acad. Sci. U.S.A.* **48**, 2087.

Gilbert, W. (1963). *J. molec. Biol.* **6**, 389.

Hultin, T. (1961). *Biochim. biophys. Acta*, **51**, 219.

Hultin, T. (1962). *Biochim. biophys. Acta*, **61**, 916.

Hultin, T. & Abraham, K. A. (1964). *Biochim. biophys. Acta*, **87**, 232.

Hunt, R. T., Hunter, A. R. & Munro, A. J. (1968). *J. molec. Biol.* **36**, 31.

Hunter, A. R. & Korner, A. (1966). *Biochem. J.* **100**, 73P.

Hunter, A. R. & Korner, A. (1969). *Biochim. biophys. Acta*, **179**, 115.

Jacob, M., Samec, J., Stévenin, J., Garel, J. P. & Mandel, P. (1967). *J. Neurochem.* **14**, 169.

Khairallah, E. A. & Pitot, H. C. (1967). *Biochem. biophys. Res. Commun.* **29**, 269.

Korner, A. (1961). *Biochem. J.* **81**, 168.

Lengyel, P. (1967). In *Molecular Genetics*, part 2, p. 194. Ed. by Taylor, J. H. London: Academic Press (Inc.) Ltd.

Lim, L. & Adams, D. H. (1967). *Biochem. J.* **104**, 229.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **217**, 111.

Malkin, L. I. & Rich, A. (1967). *J. molec. Biol.* **26**, 329.

Mansbridge, J. & Korner, A. (1963). *Biochem. J.* **89**, 15P.

Mokrasch, L. C. (1961). *Analyt. Chem.* **33**, 432.

Munro, A. J., Jackson, R. J. & Korner, A. (1964). *Biochem. J.* **92**, 289.

Munro, A. J. & Korner, A. (1964). *Nature, Lond.*, **201**, 1194.

Murthy, M. R. V. & Rappoport, D. A. (1965). *Biochim. biophys. Acta*, **95**, 132.

Ohmori, Y. (1937). *Enzymologia*, **4**, 217.

Palade, G. E. (1955). *J. biophys. biochem. Cytol.* **1**, 59.

Palade, G. E. & Siekevitz, P. (1956). *J. biophys. biochem. Cytol.* **2**, 69.

Ravel, J. M., Mosteller, R. D. & Hardesty, B. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56**, 701.

Sanger, F. (1945). *Biochem. J.* **35**, 507.

Schwartz, A., Bachelard, H. S. & McIlwain, H. (1962). *Biochem. J.* **84**, 626.

Sinsheimer, R. L. & Koerner, J. F. (1952). *J. biol. Chem.* **198**, 293.

Steiner, R. F. & Beers, R. F. (1961). *Polynucleotides*, p. 375. Amsterdam: Elsevier Publishing Co.

Stenzel, K. H., Aronson, R. F. & Rubin, A. L. (1966). *Biochemistry, Easton*, **5**, 930.

Stretton, A. O. W. & Brenner, S. (1965). *J. molec. Biol.* **12**, 456.

Takahashi, Y., Mase, K. & Abe, S. (1966). *J. Biochem., Tokyo*, **60**, 363.

Takanami, M. & Yan, Y. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 1450.

Tashiro, Y. & Siekevitz, P. (1965). *J. molec. Biol.* **11**, 166.

Zomzely, C. E., Roberts, S. & Rapaport, D. (1964). *J. Neurochem.* **11**, 567.