Polyribosomes in Isolated Liver Cells

PREPARATIVE PROCEDURES, EFFECTS OF INCUBATION AND CORRELATION WITH PROTEIN SYNTHESIS

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Methods have been derived which permit the isolation of undegraded polyribosomes from isolated rat liver cells. Under the conditions used the polyribosome profile of hepatocytes immediately after isolation was essentially identical with that from intact liver. However, during incubation of cells in complex physiological media there was a progressive dissociation of polyribosomes. The addition of a variety of factors that produce reaggregation of polyribosomes in rat liver *in vivo* did not prevent dissociation during cell incubations. Although large polyribosomes were lost most rapidly, the albumin-synthesizing capacity of isolated cells was not selectively lost when compared with total protein synthesis. The significance of these results for the use of isolated hepatocytes in the study of liver protein synthesis is discussed.

Although there are many successful reports of the isolation of polyribosomes from rat liver, there is no standardized method (Blobel & Potter, 1967; Clemens & Pain, 1974; Ramsey & Steele, 1977; Wall *et al.*, 1977). Each paper professes advantages for the isolation of undegraded polyribosomes. Allowing for this variation in preparation methods, all results have indicated that the aggregation state of polyribosomes is under nutritional and hormonal control (Wunner *et al.*, 1966; Swan *et al.*, 1971; Clemens & Pain, 1974). Isolated hepatocytes have been used to investigate the action of such factors on a variety of metabolic parameters (Seglen, 1976). There have been two reports on the aggregation state of polyribosomes in isolated rat hepatocytes (Grant & Black, 1974; Tanaka *et al.*, 1978), both indicating that ribosomes in liver parenchymal cells immediately after isolation exist mainly as monomers, dimers and other small aggregates. Although the measured polyribosome profile contrasts sharply with that of intact liver, the ability of isolated hepatocytes to synthesize proteins does not appear to be impaired when compared with that of the liver *in vivo* (Jeejeebhoy *et al.*, 1975; Crane & Miller, 1977; Feldhoff *et al.*, 1977).

Because of the variation in methodology for isolation of polyribosomes from intact liver, we considered it essential to carry out a systematic study of factors required for analysis of polyribosome profiles in isolated parenchymal cells. We report here methodology for the isolation of highly aggregated

polyribosome profiles in such cells and the effects of long-term incubation on aggregation state in the presence and absence of a variety of factors known to influence aggregation states *in vivo*.

Materials and Methods

Male Sprague–Dawley rats (CD strain, body wt. 180–250 g) were used throughout this study. They were housed under normal animal-house conditions and allowed either free access to food and water or deprived of food only for 48 h before cell isolation.

Dextran sulphate (type 500-S), Triton X-100, RNA (type VI), triamcinolone, glucagon and all amino acids were obtained from Sigma, Poole, Dorset, U.K. L-[4,5-³H]Leucine (113 Ci/nmol), L-[*side chain*-2,3-³H]tyrosine (10 Ci/mmol), NCS and PCS were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ribonuclease-free sucrose was purchased from Schwarz–Mann through Uniscience, Cambridge, U.K. Monocomponent insulin was a gift from Wellcome Research Laboratories, Dartford, Kent, U.K., and specific anti-(rat serum albumin) serum was the kind gift of Professor J. D. Judah, University College, London, U.K. The sources of chemicals used for preparation of isolated hepatocytes have been specified in a previous paper (Dickson & Pogson, 1977). Sodium deoxycholate and all other chemicals of the highest possible grade were obtained from standard chemical suppliers.

Hepatocyte isolation

Essentially, hepatocytes from fed and 48 h-starved

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rats were isolated by the method described earlier (Dickson & Pogson, 1977). Livers were either perfused with Krebs–Henseleit buffer alone, as described in the reference, or in Krebs–Henseleit buffer containing ten times the normal plasma amino acid concentrations (East *et al.*, 1973) plus 20 mM-glucose and 10 nM-insulin (amino acid perfusion). As suggested by Seglen (1973), Ca^{2+} was added back to the recirculating perfusion to give a final concentration of 0.5 mM. Hepatocytes were washed and resuspended in Eagle's minimum essential medium, containing 2.54 mM- CaCl_2 plus 10% (v/v) foetal calf serum (charcoal-treated, then extensively dialysed against Krebs–Henseleit buffer minus Ca^{2+}) (Dickson & Pogson, 1977). Incubations for analysis of polyribosome profiles consisted of 5 ml of cell suspension (80–120 mg dry wt. of cells) plus 25 ml of medium in 100 ml silicone-treated flasks, but for all other parameters 0.2 ml of cell suspension and 1.0 ml of medium were incubated in 20 ml silicone-treated scintillation vials. The incubation medium was either the same as for resuspension or consisted of Eagle's medium modified to contain 5 times the normal amino acid concentration of Eagle's medium or 10 times the normal rat plasma amino acid concentrations (East *et al.*, 1973) plus 20 mM-glucose, 10 nM-insulin and 2.5 mM-tryptophan.

Cell viability was assessed by cell ATP content (Dickson & Pogson, 1977). For all experiments reported here initial ATP contents were 8–10 nmol/mg dry wt., and did not fall by more than 10% over 6 h.

Polyribosome isolation

Buffers with the following compositions were used throughout: buffer A, 20 mM-Tris/HCl, pH 7.6, containing 0.25 M-sucrose, 25 mM-KCl, 30 mM- MgCl_2 and 3 mM- CaCl_2 ; buffer B, 20 mM-Tris/HCl, pH 7.6, containing 20 mM- MgCl_2 and 100 μg of dextran sulphate/ml; buffer C, 20 mM-Tris/HCl, pH 7.6, containing 100 mM-KCl, 40 mM-NaCl and 10 mM- MgCl_2 . All solutions used in these procedures were autoclaved before use.

(a) *Preparation of high-speed supernatant.* Livers from rats starved for 18 h were minced finely, then homogenized by ten passes in a Teflon/glass homogenizer (clearance 0.10–0.15 mm) in 2 vol. of ice-cold buffer A. After centrifugation at 16000g (20 min, 2°C), the postmitochondrial supernatant was filtered through glass wool and re-centrifuged at 100000g (4 h, 2°C). The resultant supernatant contains a cell-sap glycoprotein inhibitor of liver ribonuclease (Gribnau *et al.*, 1969) and was stable stored at –20°C.

(b) *Polyribosome isolation from isolated hepatocytes.* The 5 ml portion of cell suspension was pelleted by centrifugation at 50g for 2 min; the pellet was homogenized in 3 ml of ice-cold buffer A (30 passes in a 10 ml Teflon/glass homogenizer, clearance 0.10–0.15 mm) containing specified proportions of

high-speed supernatant to which was added (where noted) MgCl_2 , Triton X-100 and sodium deoxycholate [final concentrations 50 mM, 1.33% (w/v) and 0.44% (w/v) respectively]. The resulting homogenate was left on ice to lyse for 5–15 min. After further centrifugation (Eppendorf 3200; 12000g; 5 min; 4°C) a 2.5 ml portion of the postmitochondrial supernatant was immediately layered over a discontinuous sucrose gradient consisting of 0.7 ml of 1.0 M-sucrose overlaid by 1.3 ml of 0.4 M-sucrose in 5 ml polycarbonate centrifuge tubes (type 34411-111 0877; MSE, Crawley, Sussex, U.K.). The sucrose solutions were dissolved in buffer B. The gradient was centrifuged at 160000g for 90 min. at 2°C in a 6 × 5.5 ml swing-out rotor (MSE Superspeed 65 centrifuge). After aspiration, the pellet was either analysed immediately or frozen and stored in liquid nitrogen until analysis the following day.

(c) *Polyribosome isolation from whole liver.* Portions of rat liver (300 mg) were homogenized in 3 ml of buffer A as described for isolated cells, and all further treatment was also as described above. Gloves were, however, worn during the handling of liver pieces to decrease the possibility of contamination of the liver with ribonuclease.

(d) *Preparation of continuous sucrose gradients.* Linear 10–40% (w/v) sucrose gradients in buffer C were made by a slight modification of the method of Stone (1974). In 5 ml polycarbonate centrifuge tubes 2.3 ml of 10% sucrose was overlaid on to 2.3 ml of 40% sucrose, the tube turned to an angle of 30° and placed in the cold-room for 18 h. Diffusion over this time resulted in a linear gradient of sucrose throughout the entire tube length.

(e) *Analysis of polyribosome aggregation state.* The ribosome pellet from isolated cells or whole liver was resuspended in 150 μl of buffer C by leaving at 37°C for 1 min and by gentle shaking and gentle pipetting. The tubes were rinsed with a further 100 μl of buffer C and the total suspension of 250 μl was clarified by centrifuging at 200g for 3 min (MSE Super Minor bench centrifuge). Portions (100 μl) of the final suspension were layered over continuous sucrose gradients and were then centrifuged at 160000g at 2°C for 30 min in a 6 × 5.5 ml swing-out rotor (MSE Superspeed 65 centrifuge) and allowed to slow without brake.

Gradients were scanned in a Gilford density-gradient scanner 2480 attached to a Gilford 240 spectrophotometer (Gilford Instruments, Oberlin, OH, U.S.A.) by upward elution and the A_{260} profiles recorded. The A_{260}/A_{280} ratio was also recorded for each sample.

RNA assay

RNA was assayed by the spectrophotometric method of Blobel & Potter (1968); 1 A_{260} unit $\equiv$ 32.1 μg of RNA/ml. Total RNA content of isolated

hepatocytes at required times of incubation was obtained by centrifuging cell suspensions for 2 min at 50g and analysing pellet RNA. Assays were also performed on samples from various stages of the polyribosome isolation procedure, namely total homogenates, postmitochondrial supernatant and resuspended ribosome pellet. The RNA content of the high-speed supernatant was also calculated for use as a blank where required.

Ribonuclease assay (EC 3.1.4.22/23)

Ribonuclease activity was measured essentially as outlined by Reddi (1975). The activity in portions of isolated hepatocytes was assayed after three cycles of freezing and thawing; a similar scheme was used for whole liver homogenates.

Synthesis of total proteins and albumin

The rate of synthesis was measured by the incorporation of L-[4,5-³H]leucine; 2 μ Ci of [³H]leucine was added to incubations in normal Eagle's medium (final specific radioactivity 4.2 μ Ci/ μ mol; the specific radioactivity was constant throughout, even in media containing increased amino acid concentrations). In some experiments the total leucine concentration was increased to 5 mM (while maintaining constant specific radioactivity) both to expand the intracellular pool and to ensure similar specific radioactivities in the intracellular and extracellular water (Khairallah & Mortimore, 1976; Feldhoff *et al.*, 1977). At specified times, synthesis was terminated by freezing in liquid nitrogen, and, after three cycles of freezing and thawing, sodium deoxycholate was added to each sample to give a final concentration of 1% (w/v).

Total protein synthesis was measured by precipitating 50 μ l portions of suspensions with 50 μ l of ice-cold 10% (w/v) trichloroacetic acid containing 10 mM-L-leucine. The reactions were carried out in 400 μ l Beckman centrifuge tubes, and the pellets were washed with 3 $\times$ 250 μ l of ice-cold 5% (w/v) trichloroacetic acid containing 5 mM-leucine and centrifuged (14000g, 2 min) in a Beckman 152 microfuge (Beckman-RIC Ltd).

Portions of the deoxycholate-treated supernatant (100 μ l) were mixed with 4 μ g of partially purified rat serum albumin (Debro *et al.*, 1957) and sufficient specific anti-(rat serum albumin) to precipitate 20 μ g of albumin. After incubation for 1 h at 37°C and 16 h at 4°C, the immunoprecipitates were collected by centrifuging (14000g, 4 min) and three cycles of washing with 250 μ l of 20 mM-sodium phosphate, pH 7.4, containing 150 mM-NaCl. Controls were carried out by addition of further portions of rat serum albumin and antialbumin to the supernatant recovered from the initial immunoprecipitation reaction. These controls were incubated as detailed for the samples; the pellets obtained and radioactivity

incorporated were found to be less than 2% of those in the initial immunoprecipitation.

The bottom 6–7 mm of the Beckman centrifuge tubes were cut off and 30 μ l of NCS was added to all the pellets. After stirring, all material was digested within 1 h and the entire tips were added to minivials (Hopkin and Williams, Chadwell Heath, Essex, U.K.) with 1 ml of PCS and counted for radioactivity in a Packard scintillation counter (models 3375 or 3003; Packard Instrument Co., Downers Grove, IL, U.S.A.).

Tyrosine aminotransferase assay (EC 2.6.1.5)

Cell suspensions were centrifuged at 50g for 2 min and the pellets were frozen and stored in liquid nitrogen. Just before assay, 200 μ l of 0.1 M-sodium phosphate, pH 6.9, was added to each sample and the suspensions were treated by three cycles of freezing and thawing. Tyrosine aminotransferase in portions of the supernatant obtained after centrifugation (Eppendorf 3200; 12000g, 2 min, 4°C) was assayed by the radiometric method of Marston & Pogson (1977).

Expression of results

All results are initially expressed in terms of mg dry wt., although in some cases values were converted into wet wt. by using the conversion factor of 3.7 (Elliott *et al.*, 1976).

Values are means $\pm$ S.E.M. for the number of experiments detailed.

Results and Discussion

Effects of isolation conditions on state of polyribosomes in isolated hepatocytes

In the analysis of polyribosomal aggregation state it is essential to ensure quantitative removal of RNA from the tissue to permit a representative sample to be obtained for analysis. Shearing forces incurred during homogenization are not in themselves sufficient to release all the intracellular constituents of isolated hepatocytes, and most workers use freezing-thawing or detergent lysis (Grant & Black, 1974; Thomas *et al.*, 1977). We found that complete release of hepatocyte RNA occurred only if detergents were included with homogenization (Table 1). The necessity for the addition of deoxycholate for cell lysis makes it impossible to distinguish between free and bound ribosomes, and only total polyribosomes can be assessed.

Initial isolations of liver cells from fed rats, by using buffer A unfortified with rat liver supernatant, resulted in polyribosome profiles with a high degree of disaggregation (Fig. 1). This occurred even though the liver was perfused with the amino acid medium, which had been shown to be essential to maintain

Table 1. *Extraction of total RNA from isolated rat hepatocytes*

Fed-rat hepatocytes (36 mg dry wt.) were lysed by using the buffer system specified in the text with modifications as noted. For calculation of extraction efficiency, RNA was assayed in total cell fractions and in portions of each postmitochondrial supernatant. Results are the means of duplicate determinations in each case.

Number of homogenization passes	Final concn. (% w/v)		Time of lysis (min)	RNA extraction efficiency (%)
	Triton X-100	Deoxycholate		
—	0.33	0.44	5	82.1
—	0.33	0.44	5	78.6
3	1.33	0.44	5	94.0
3	1.33	0.44	15	95.5
30	—	—	5	17.3
30	1.33	—	5	96.9
30	—	0.44	5	95.1
30	1.33	0.44	5	102.1

Fig. 1. Polyribosome scans from isolated fed-rat liver cells (—) immediately after isolation and from intact fed-rat liver (----)

Both livers were perfused for the same length of time with Krebs–Henseleit buffer containing $10\times$ normal concn. of rat plasma amino acids and 10 nM-insulin; isolated cells were dispersed in Krebs–Henseleit buffer containing $10\times$ normal concn. of plasma amino acids and 10 nM-insulin plus 2% bovine serum albumin. Ribosomes were isolated as described in the Materials and Methods section, but rat high-speed supernatant was not included in buffer A.

polyribosome aggregation state in the isolated perfused liver (Jefferson & Korner, 1969). Similar perfusion of a control liver for the same period (30–35 min), but without added collagenase, did result in a reasonably aggregated profile (Fig. 1). A piece of liver removed from the collagenase perfusion, just before release of isolated hepatocytes, gave a profile identical with that of the isolated cells.

These results suggested that disaggregation was occurring because of either contamination by ribonuclease present in collagenase or actions of collagenase on the liver which might result in activa-

tion of ribonuclease. It is possible, however, that the mere release of cells from the matrix might impair their polyribosome profile. Homogenates of intact liver left in contact with collagenase (0.10 mg/ml) for 5 min at 37°C did not show disaggregated polyribosomes, and this correlated with the absence of ribonuclease activity measured in collagenase preparations. Unlike Tanaka *et al.* (1978), we found no increase in tissue activity of ribonucleases after isolation of hepatocytes by collagenase perfusion.

The requirement for both homogenization and detergents lysis for RNA release might alter the subsequent ability to isolate polyribosomal RNA. With fixed detergent conditions, increased homogenization resulted in greater amounts of polyribosomal RNA passing through the discontinuous gradient on centrifugation (Fig. 2). No improvement occurred with greater than 30 homogenization passes, nor with increased lysis time over 5 min. Although Triton or deoxycholate alone were equally effective in releasing RNA from cells (Table 1), neither alone was as effective for the final yield of polyribosomal RNA. Thus for all subsequent isolations a mixture of 1.33% Triton X-100 and 0.44% deoxycholate (final concentrations) was used.

The presence of detergents means not only that only total polyribosomes can be measured but also that ribonucleases from nucleus or lysosomes may be released into the postmitochondrial supernatant and result in polyribosomal degradation. This fact in itself discouraged us from using the Mg^{2+} precipitation method now widely used for intact rat liver (Palmiter, 1974; Clemens & Pain 1974). Inclusion of dextran sulphate in buffer A as a ribonuclease inhibitor (Philipson & Kaufman, 1964) had little effect on aggregation state. However, the addition of rat liver high-speed supernatant to buffer A resulted in good states of polyribosome aggregation (Fig. 3). The polyribosome profile was progressively improved by increased amounts of high-speed supernatant in

Fig. 2. Effects of different extraction procedures on polyribosome profiles from isolated fed-rat hepatocytes

The methodology was as given in the legend for Fig. 1. The extraction medium contained 1.33% (w/v) Triton and 0.44% (w/v) deoxycholate. The extent of homogenization varied as follows: $\cdots$, 3 passes; $----$ 15 passes; $—$, 30 passes.

Fig. 3. Isolation of liver cell polyribosomes: effects of inclusion of high-speed supernatant in the homogenization step

Fed-rat liver cells were isolated by the procedures given in the legend to Fig. 1. Homogenization buffers were: 2 vol. of buffer A, 1 vol. of high-speed supernatant ($\cdots$), 1 vol. of buffer A, 2 vol. of supernatant ($----$) or entirely high-speed supernatant ($—$) (see the Materials and Methods section for details).

the homogenization buffer. Best profiles were obtained by preparing high-speed supernatant in buffer A (without Triton or deoxycholate) and then using that preparation directly with added detergents in the lysis of hepatocytes. This medium itself gave no carry-down through the discontinuous gradient. Inclusion of high-speed supernatant into the discontinuous-gradient buffers did not alter polyribosomal profiles.

Difficulties arise in the analysis of several incubated samples in terms of the requirement for simultaneous centrifuge spins and in running each sample on different continuous-gradient spins. Freezing and storage of the ribosome pellet (before resuspension)

Fig. 4. Effect of storage of ribosome pellets on the quality of polyribosome aggregation

Hepatocytes from fed-rat liver were used to prepare ribosome pellets as described in Fig. 1. Homogenization was in undiluted rat liver high-speed supernatant. The ribosome pellet obtained after the discontinuous gradient step was either analysed immediately ($—$) or frozen and stored in liquid N_2 and analysed the following morning ($\cdots$).

in liquid nitrogen resulted in a small degree of disaggregation (Fig. 4). However, this treatment meant that profiles could be examined for time points as little as 2 h apart by using only one ultracentrifuge and rotor, and an experimental set of samples could be analysed by identical procedures. The small change in polyribosome aggregation state was deemed acceptable for the advantages offered.

The final basic procedure for the isolation of polyribosomes is outlined in Scheme 1. It bears similarities to the 'classical' method for polyribosome isolation (e.g. Blobel & Potter, 1967), but has been adapted to analyse the polyribosome profile of small amounts of tissue. The major difference in the methodology is the necessity for inclusion of detergents during the homogenization step, rather than their addition directly to the postmitochondrial supernatant. The principle of detergent lysis has also been used to isolate polyribosomes from a variety of cultured mammalian cell lines (Daskal *et al.*, 1976; Thomas *et al.*, 1977).

Typical polyribosome profiles of hepatocytes immediately after isolation

Polyribosome profiles were analysed for hepatocytes from both fed and 48 h-starved rats, and representative profiles of 10–12 separate preparations are given in Fig. 5. If perfusion was performed with medium containing amino acids, the aggregated

Scheme 1. Finalized method for polyribosome isolation

Details of buffers and other conditions are given in the Materials and Methods section.

- (1) Tissue (400mg wet wt.) was homogenized (30 passes) in 3 ml of high-speed supernatant containing Triton X-100 (1.33%) and sodium deoxycholate (0.44%).
- (2) The homogenate was centrifuged (12000 g, 5 min) at 4°C to produce a postmitochondrial-supernatant fraction.
- (3) Then 2.5 ml of the postmitochondrial supernatant was layered over 1.3 ml of buffer B containing 0.4M-sucrose and 0.7 ml of buffer B containing 1.0M-sucrose.
- (4) This discontinuous gradient was centrifuged at 160000 g for 90 min.
- (5) The buffer was removed by aspiration and the inside of the tube dried. Then the entire tube with the polyribosome pellet was frozen in liquid N₂.
- (6) The pellet was resuspended in 250 µl of buffer C and the suspension clarified by centrifugation at 200 g for 3 min.
- (7) Portions of the clarified supernatant were then further processed as follows:
 - (a) 100 µl was layered over 10–40% sucrose gradients and, after centrifugation at 160000 g for 30 min, the sedimentation profile was scanned at 260 nm;
 - (b) 10 µl was diluted with 1 ml of buffer C and used to measure A_{260}/A_{280} ratios;
 - (c) 50 µl was analysed for total RNA content.

Fig. 5. Characterization of the initial polyribosome aggregation state of hepatocytes isolated from fed rats

Hepatocytes were isolated either after perfusion with Krebs-Henseleit buffer containing 10× normal concn. of rat plasma amino acids and 10 nM-insulin (—) or with Krebs-Henseleit buffer alone (····). The patterns are representative of a number of similar experiments.

Fig. 6. Polyribosome profiles of intact liver from fed and 48 h-starved rats

The methods were as detailed in Fig. 5. The traces are scans of material from approx. 100mg wet wt. of tissue. Liver pieces fed (—) and 48 h-starved (····) were processed in parallel.

profiles were obtained for cells from both fed and starved rats. With Krebs-Henseleit buffer alone in the perfusion the profiles were of a more disaggregated nature (Fig. 5). The extent of disaggregation was similar for either nutritional state, although the percentage of monomers and dimers is slightly greater for 48h-starved-rat hepatocytes (Table 2). It was noted that incubation of polyribosomes of good aggregation state with 0.5mM-EDTA for 2min at 37°C resulted in a profile containing almost only monomers and dimers. The requirement for amino

acid inclusion in the perfusate of isolated liver preparations has previously been noted as essential for maintenance of aggregated polyribosomes (Jefferson & Korner, 1969). The results obtained here bear out those findings.

Tables 2. *Polyribosome size distribution of intact liver and hepatocyte suspensions immediately after isolation*

Details of procedures for isolation of hepatocytes are given in the Materials and Methods section. Polyribosomes were isolated by the procedures given in Scheme 1. Values are means $\pm$ S.E.M. for the numbers of independent observations given in parentheses.

	Percentage of total ribosomes as		
	Monomers	Dimers	Polyribosomes
Isolated hepatocytes			
Fed rat: perfusion with amino acids (11)	11.9 $\pm$ 0.9	12.9 $\pm$ 1.4	76.1 $\pm$ 2.0
Fed rat: perfusion with Krebs–Henseleit buffer alone (2)	10.6	26.8	62.6
48h-starved rat: perfusion with amino acids (1)	6.7	7.8	85.5
48h-starved rat: perfusion with Krebs–Henseleit buffer alone (1)	14.7	33.1	51.2
Intact liver			
Fed rat (6)	10.2 $\pm$ 1.5	10.0 $\pm$ 1.9	79.8 $\pm$ 3.3
48h-starved rat (2)	22.1	21.0	56.9

Table 3. *Recovery of RNA at various stages of polyribosome isolation from intact liver and isolated hepatocytes*

Details of procedures are given in the Materials and Methods section. The recovery of RNA at each stage of the polyribosome isolation was not influenced by either perfusion conditions or nutritional state of the donor animal. Thus all values, whether from isolated hepatocytes or intact liver, were pooled for calculation and the values are means $\pm$ S.E.M. for the numbers of independent observations given in parentheses.

	Isolated hepatocytes	Intact liver	
		Fed	48h-starved
Tissue RNA content (mg of RNA/g wet wt. of tissue)	7.84 $\pm$ 0.23 (9)	11.78 $\pm$ 0.45 (4)	14.22 (2)
RNA recovery into postmitochondrial supernatant (% of total cell content)	98.7 $\pm$ 4.9 (9)	83.7 $\pm$ 2.6 (6)	
RNA recovery into ribosome pellet (% of postmitochondrial-supernatant content)	35.0 $\pm$ 4.7 (9)	26.3 $\pm$ 0.16 (6)	
RNA content of ribosomal pellet (mg of RNA/g wet wt. of tissue)	2.67 $\pm$ 0.30 (9)	2.45 $\pm$ 0.15 (6)	

Polyribosome profiles of intact liver

Polyribosomes from fed-rat liver were highly aggregated; this was dependent on the time of preparation (Fig. 6). The diurnal rhythm of polyribosome aggregation has been shown previously (Fishman *et al.*, 1969), but it should be noted here that all isolated-cell experiments were carried out at 10:00h, and the extent of aggregation is approximately maximal at this time. We have found that the quality of perfusion is important for obtaining aggregated polyribosome profiles from isolated liver cells. In a few experiments with cellular ATP contents below the normal range, the preparations gave polyribosome profiles with increased proportions of monomers and dimers.

The profile from starved-rat liver showed an increased content of monomers and dimers when compared with that from fed-rat liver (Fig. 6) and was similar to that obtained for both fed- and starved-rat liver cells obtained after perfusion with Krebs–Henseleit buffer alone (Fig. 5).

A comparison of the actual percentages of ribosomes as monomers, dimers and polyribosomes (Table 2) indicates the similarity between fed-rat liver and isolated cells perfused with the amino acid medium, and that between starved-rat liver and isolated liver cells prepared with Krebs–Henseleit buffer alone. Apart from the lower initial RNA content of isolated liver cells compared with intact liver, the extraction of RNA at each stage of the method is approximately similar for both isolated cells and whole liver (Table 3), and the amount of RNA contained in the ribosomal pellet is similar to that reported by others (Clemens & Pain, 1974).

This method has permitted the definition of polyribosomes containing 11 or 12 ribosomes per mRNA molecule; the peak aggregation state obtained by this method with intact liver is approx. 11 ribosomes/mRNA molecule (Fig. 1). For isolated cells peak aggregation state ranges from 8 to 11 ribosomes/mRNA molecule with amino acids present in the perfusion medium (Figs. 3, 4 and 5).

Other reports for peak aggregation states for whole liver show a great deal of variation between 7 and 18 ribosomes per mRNA molecule (Blobel & Potter, 1967; Clemens & Pain, 1974; Ramsey & Steele, 1977). As freezing of the ribosome pellet may shift the maximum aggregation state in our method by 1–2 ribosomes (Fig. 4), we feel confident that the patterns reported here for isolated hepatocytes and whole liver compare favourably with other published results for intact rat liver.

The detrimental effect of freezing may be due to contaminants in the ribosomal pellets. Before being clarified (resulting in A_{260}/A_{280} of approx. 1.60–1.75), ribosome resuspensions for both intact liver and isolated cells have A_{260}/A_{280} ratios of 1.35–1.45. As purified ribosome suspensions are reported to have ratios of approx. 1.8–1.9 (e.g. Clemens & Pain, 1974), the contaminants present may cause some disaggregation during warming at 37°C for pellet resuspension.

Changes in polyribosomes during incubation of isolated hepatocytes

During incubation of hepatocytes in normal Eagle's medium with foetal calf serum, two basic patterns are observed. When the pattern is initially well aggregated (amino acid perfusion), there is some dissociation within 2 h (Fig. 7a). The pattern is then similar to that observed as a result of perfusion with Krebs–Henseleit buffer alone (Fig. 7b); incubation after perfusion with only Krebs–Henseleit buffer results in little alteration of profile, but there is a decreased amount of material on the gradient. Little change occurs in either case after a further 4 h incubation. In terms of distribution of ribosomal aggregation, incubation results in a marked loss of polyribosomes and build-up of monomers and dimers during the first 2 h of incubation of cells initially exhibiting well-aggregated profiles, and only small further changes (Table 4). Starved-rat liver cells from perfusion with Krebs–Henseleit buffer alone show no alteration in aggregation state throughout the entire 6 h incubation, whereas fed-rat liver cells with Krebs–Henseleit buffer alone give patterns intermediate between the other two types (Table 4). During the incubation period the RNA content of cells decreased, and this is mirrored by decreased RNA in the postmitochondrial supernatant (Table 5). However, the RNA content of the ribosomal pellet decreased much more rapidly; this indicates that, especially during the initial 2 h of incubation, a proportion of the ribosomes dissociate into subunits, which are retained at the top of the discontinuous gradient (Bloemendal *et al.*, 1974). This largely explains the rapid initial loss of RNA in the scanned profiles and the subsequent small decrease (Table 5). Thus the overall effect is a loss of both the largest

Fig. 7. Alteration in polyribosome aggregation state during incubation in normal Eagle's medium plus 10% foetal calf serum

Polyribosomes were extracted from hepatocytes immediately after cell isolation (—) or after incubations for 2 h (---) or 6 h (····). The methods were as described in the text and in Fig. 5. (a) Profile of polyribosomes derived from fed-rat liver cells isolated after perfusion with Krebs–Henseleit buffer supplemented with amino acids (10× normal concn. in rat plasma) and 10nM-insulin. (b) Profile of polyribosomes derived from fed-rat liver cells isolated after perfusion with Krebs–Henseleit buffer alone.

polyribosome aggregates and total complete ribosomes during incubation of rat hepatocytes.

Protein synthesis in isolated hepatocytes during incubation

The protein-synthetic capacity of fed-rat hepatocytes isolated after perfusion with the amino acid medium assessed as an indication of the effect of changes in the polyribosome aggregation state. (For the results described here, alterations in polyribosome profile were similar to those in Fig. 7a.) The incorporation of [3 H]leucine into albumin and total

Table 4. *Alteration of polyribosomal aggregation state during incubation of hepatocytes from fed and 48 h-starved rats in normal Eagle's medium plus 10% foetal calf serum*

Details of conditions are given in the text. Values are means $\pm$ S.E.M. for the numbers of independent observations given in parentheses.

Nutritional state	Perfusion	Time (h)	Percentage of total ribosomes as		
			Monomers	Dimers	Polyribosomes
Fed	Amino acids (6)	0	12.8 $\pm$ 0.7	15.0 $\pm$ 0.7	73.2 $\pm$ 2.2
		2	24.2 $\pm$ 2.2	24.0 $\pm$ 2.5	51.8 $\pm$ 4.3
		6	29.8 $\pm$ 2.2	25.7 $\pm$ 2.9	44.5 $\pm$ 4.7
Fed	Krebs-Henseleit buffer only (2)	0	10.6	28.6	61.8
		2	21.0	26.9	52.1
		6	25.5	26.5	47.6
Starved	Amino acids (1)	0	6.7	7.8	85.5
		2	21.1	20.6	58.3
		6	32.8	20.5	46.7
Starved	Krebs-Henseleit buffer only (1)	0	14.7	33.1	51.2
		2	15.3	29.4	55.3
		6	17.0	29.9	53.1

Table 5. *Alteration in RNA content of hepatocytes during incubation in normal Eagle's medium plus 10% foetal calf serum*
Hepatocytes were isolated by perfusion with either Krebs-Henseleit buffer alone or this buffer supplemented with 10 $\times$ normal rat plasma concentration of amino acids (East *et al.*, 1973). Values are the means $\pm$ S.E.M. for nine separate experiments.

	Time (h)	0	2	6
Total cell RNA (mg of RNA/g wet wt. of cells)		7.84 $\pm$ 0.23	6.85 $\pm$ 0.29	5.46 $\pm$ 0.29
RNA in postmitochondrial supernatant (mg of RNA/g wet wt. of cells)		7.47 $\pm$ 0.47	6.09 $\pm$ 0.38	4.48 $\pm$ 0.36
RNA in ribosome pellet (mg of RNA/g wet wt. of cells)		2.67 $\pm$ 0.30	1.67 $\pm$ 0.32	1.15 $\pm$ 0.30
RNA recovery into ribosome pellet (% of postmitochondrial-supernatant content)		35.0 $\pm$ 4.7	22.8 $\pm$ 3.4	19.9 $\pm$ 2.9

proteins decreased over the incubation period 2–6 h, with low concentrations of leucine in the extra-cellular medium. This was especially dramatic with high concentrations of cells. As suggested by Khairallah & Mortimore (1976) and Seglen & Solheim (1978), 5 mM-leucine was included in the incubation buffer; this largely counteracted the decrease in leucine incorporation. Total protein synthesis was linear with time for the first 2 h, then decreased over the next two 2 h periods to 73 and 60% of the initial rate (Fig. 8). After an initial lag of 20 min in albumin synthesis, the rate of synthesis is roughly linear with time for the period 1–4 h, then decreases to 50% of that rate for the next 2 h of incubation (Fig. 8). A decrease in total protein synthesis might correlate with the decrease in ribosomal aggregation (Table 4). The effect of incubation time on albumin synthesis was not, however, significantly different from that on total synthesis; the observed decrease in the amount of ribosomal material in the albumin-synthesizing-polyribosome region (18–19 ribosomes/mRNA molecule) throughout the incubations has not therefore selectively influenced the rate of albumin synthesis.

Fig. 8. *Synthesis of albumin and total proteins by hepatocytes isolated from fed rat livers*

Hepatocytes were isolated from fed rats after perfusion with Krebs-Henseleit buffer supplemented with 10 $\times$ normal concn. of rat plasma amino acids and 10 M-insulin. Results are from six preparations, and the values are means $\pm$ S.E.M. for albumin (■) and total protein (●) synthesis.

Incorporation of [^3H]leucine into albumin was 10.5, 13.3 and 13.2% of that into total proteins after 2, 4 and 6 h respectively. These values agree with those published for rat liver *in vivo* and *in vitro* (Peavy *et al.*, 1977; Feldhoff *et al.*, 1977; Schreiber & Urban, 1978), and also further emphasize that the ability to synthesize albumin is not preferentially lost.

From the leucine content of albumin (Morgan & Peters, 1971) and the specific radioactivity of leucine used, the absolute rate of albumin synthesis may be calculated. The preparations in the present study (over the 1–4 h period) synthesized $0.823 \pm 0.081 \mu\text{g}$ of albumin/h per mg dry wt. of cells. Given the variation in specific radioactivities, media and incubation conditions, this value is comparable with that reported for other isolated-cell studies (Grant & Black, 1974; Jeejeebhoy *et al.*, 1975; Crane & Miller, 1977; Feldhoff *et al.*, 1977).

Preliminary studies on the synthesis of an intracellular protein (tyrosine aminotransferase) have shown that its synthesis is also continuous throughout the incubation period of 6 h (F. A. O. Marston, A. J. Dickson, & C. I. Pogson, unpublished work).

Effects of additions to minimal essential medium on polyribosome disaggregation during incubation

Although Eagle's minimal essential medium plus foetal calf serum contains a complex mixture of amino acids and vitamins and has been shown to be a medium which prolongs the viability of isolated hepatocytes in suspension (Dickson & Pogson, 1977), it does not prevent disaggregation of polyribosomes during incubations. A variety of factors shown to cause polyribosome aggregation in the intact rat and in isolated perfused liver were added to incubations of hepatocytes isolated from fed rats after perfusion with Krebs–Henseleit buffer containing ten times the normal rat plasma concentrations of amino acids plus 20 mM-glucose and 10 nM-insulin. Inclusion of amino acids, glucose and insulin in the perfusion medium results in a highly aggregated polyribosome profile immediately after isolation (Fig. 5), but these constituents (at the same final concentration) in the incubation with Eagles' medium and 10% foetal calf serum resulted in the same rate of polyribosome disaggregation as in controls. Thus, although the presence of these factors slowed the loss of polyribosomes during hepatocyte isolation, the effect was temporary. Tryptophan administered to intact rats or isolated perfused rat liver caused increased aggregation of polyribosomes (Sidransky *et al.*, 1971; Swan *et al.*, 1971; Majumdar & Jørgensen, 1976). As the mechanism for stimulation of disaggregation of polyribosomes in starvation might be similar to the events occurring during incubation of isolated hepatocytes, we added tryptophan (final concn. 2.5 mM) to incubations, but this had no effect on polyribosome loss.

Glucagon and glucocorticoids stimulate specific protein synthesis (tyrosine aminotransferase) in isolated hepatocyte suspensions (Ernest *et al.*, 1977). A stimulation of protein synthesis might be expected to preserve aggregated polyribosome profiles, but addition of glucagon (0.1 μM final concn.) and triamcinolone (0.1 μM final concn.) did not prevent disaggregation.

NH_4Cl is a potent inhibitor of protein degradation and nitrogen loss by isolated hepatocytes (Seglen, 1975), but we found that its addition (5 mM final concn.) did not prevent polyribosome loss.

General considerations of polyribosomes in isolated rat hepatocytes

Under optimal conditions, the polyribosome profile of isolated hepatocytes immediately after isolation is identical with that of intact liver. Other authors, using other conditions, found there to be little more than monomers and dimers in isolated hepatocyte suspensions (Grant & Black, 1974; Tanaka *et al.*, 1978). We also found the extent of subsequent disaggregation during incubation to be less than that reported by others (Grant & Black, 1974). The cause of polyribosome loss is unclear, but does not seem to be associated with a requirement for increased amino acid supply, as has been observed with the isolated perfused rat liver (Jefferson & Korner, 1969). The disaggregation occurs rapidly, within the first 2 h of incubation (Table 4), and is associated mainly with an increased percentage of subunits and free ribosomes. The content of subunits and free ribosomes in the hepatic ribosomal population increases in abnormal nutritional and hormonal states in the intact rat, as in diabetes (Korner & Gumbley, 1966; Stirewalt *et al.*, 1967; Clemens & Pain, 1974). A common factor may be responsible for the altered ribosomal aggregation state in the intact animal and after hepatocyte isolation. This cause is presently a matter for speculation alone, but it is noteworthy that isolated rat hepatocytes cultured for 1–5 days recover from initial polyribosome disaggregation (Tanaka *et al.*, 1978).

The potential use of isolated hepatocyte suspensions to investigate protein synthesis has attracted much attention. Although isolated hepatocytes show selective loss of large polyribosomes during incubation (Table 4), there does not appear to be preferential loss of the ability to synthesize the proteins requiring many ribosomes per mRNA molecule, as is the case for albumin (Fig. 8). A loss of large polyribosomes need not greatly influence the capacity to synthesize albumin. Taylor *et al.* (1978), using immunoprecipitation *in vitro*, have shown that after polyribosome disaggregation by mild ribonuclease action, albumin continues to be synthesized at a high rate, but by polyribosomes of only 4–5 ribosomes per mRNA molecule.

Although the observed polyribosome loss need not greatly impair protein synthesis by isolated rat hepatocytes, most data stress that the rates of protein synthesis by isolated cells are within the 'physiological range'. However, the absolute rates are always less than those obtained from isolated perfused liver or *in vivo* (e.g. Feldhoff *et al.*, 1977). The effects on hepatocyte ribosomes during isolation and incubation procedures may result in decreased protein-synthetic capacity and suggests that caution should be taken before extrapolating results directly to the situation *in vivo*.

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