Changes in Free and Membrane-Bound Ribosomes During the Development of Chick Liver

A NEW CELL-FRACTIONATION APPROACH

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A major difficulty in studying quantitative changes in free and membrane-bound ribosomes in a tissue under different physiological conditions is that membrane-bound ribosomes are not usually recovered quantitatively in a conventional microsomal fraction. This problem was resolved for developing chick liver by determining the conditions for the isolation of a microsomal fraction containing the highest practicable yield of rough vesicles, and then separating it into free-ribosome- and rough-vesicle-containing fractions. With the aid of a marker enzyme for the microsomal membranes and the RNA content of the recovered membrane-bound ribosomes, it was possible to correct for the recovery of rough vesicles and hence to determine the concentration of membrane-bound ribosomes in the homogenate. Despite the fact that morphological studies have suggested that most of the cellular ribosomes are not bound to membrane in chick liver cells at the earliest developmental age studied (6 days of egg incubation), 49% of the total ribosomes were found to be membrane-bound by using the new fractionation technique. This fraction increased (to 66%) during development. The discrepancy between the cell-fractionation and morphological approaches could not be attributed to artifacts of the separation method but rather to difficulties inherent in the morphological approach.

A considerable body of morphological evidence suggests that, in most animal cells, ribosomes exist in two topographical states, either bound to membranes of the endoplasmic reticulum (membrane-bound ribosomes) or scattered freely in the cytoplasmic matrix (free ribosomes) (Palade, 1955; Porter, 1961). Free and membrane-bound ribosomes have also been isolated from cell or tissue homogenates by a variety of biochemical methods (Henshaw *et al.*, 1963; Bloemendal *et al.*, 1964; Blobel & Potter, 1967; Chauveau *et al.*, 1962; Hallinan & Munro, 1965; Goldberg & Green, 1967; Attardi *et al.*, 1969).

At present, the significance of free ribosomes in animal cells has not been clearly established (see Campbell, 1970). In an attempt to examine this problem further, the distribution of ribosomes between the free and membrane-bound states during the development of chick liver was studied by means of cell fractionation. Great care was taken to ensure that the separation of free and membrane-bound ribosomes was complete and that the membrane-bound ribosomal content of the tissue homogenates was not underestimated.

Materials

Animals

Fertile eggs obtained from the New South Wales Government Experimental Station (Seven Hills, N.S.W., Australia) were incubated at $38 \pm 0.5^\circ\text{C}$ with relative humidity of 60%. The average stage of embryonic development (see Hamilton, 1952) corresponded closely to the incubation age. Post-hatched chickens and roosters were fed on 17% Growing Mash (Crago Stockfeeds Pty. Ltd., Sydney, Australia) containing 0.05% Zoamix [Dow Chemical (Australia) Ltd., Sydney, N.S.W., Australia] and were used unstarved for the experiments.

Chemicals

The sucrose used was either analytical-reagent grade (Univar) or RNAase*-free sucrose (Mann Research Laboratories Inc., New York, N.Y., U.S.A.). Glucose 6-phosphate and bovine serum albumin were obtained from Sigma Chemical Co.,

* Abbreviation: RNAase, ribonuclease.

St. Louis, Mo., U.S.A. All other chemicals were analytical-reagent grade quality where obtainable.

Solutions

TKM buffer contained 25mM-KCl, 5mM-MgCl₂, 50mM-Tris-HCl, pH7.3-7.6 (3°C); 0.25M-sucrose-11TKM buffer contained 0.25M-sucrose, 275mM-KCl, 55mM-MgCl₂, 550mM-Tris-HCl, final pH7.3-7.6 (3°C) when diluted 11-fold with 0.25M-sucrose.

Methods

Preparation of subcellular fractions

Embryonated eggs, incubated for the desired period, were opened and the embryos transferred to ice-cold 0.25M-sucrose or 0.25M-sucrose-TKM buffer (see below). Post-hatched animals were killed by decapitation.

In most experiments, liver homogenates were prepared in 0.25M-sucrose-TKM buffer by using the procedures described by Pollak & Ward (1967). However, it was necessary to homogenize 6-8-day chick-embryo liver in an homogenizer with a more tightly fitting Teflon pestle rotating at 2000-3000 rev./min to obtain complete (>98%) cell breakage. Moreover, in most experiments with 6-8-day chick-embryo liver (but not older liver), homogenization was performed in 0.25M-sucrose without added cations (see the Results section).

A summary of the subsequent fractionation procedure is depicted in Scheme 1.

The homogenate was centrifuged at 10000g_{av}. for 10min (SW 39 rotor; Spinco ultracentrifuge) to give a supernatant (S fraction) and a nuclear-mitochondrial pellet. The pellet was resuspended in 0.25M-sucrose without added cations and was washed three times by centrifugation and resuspension by using the 296 angle rotor of the PR2 centrifuge (International Equipment Co., Boston, Mass., U.S.A.) with a centrifugal field of 5000g_{av}. for 10min per wash. The combined supernatants (R fraction) were then mixed with 0.1 vol. of 0.25M-sucrose-11TKM buffer and centrifuged at 26000g_{av}. for 20min (50Ti rotor; Spinco ultracentrifuge) to give a pellet of purified rough microsomes (RP) and a supernatant containing free ribosomes and smooth vesicles. The latter were collected by centrifugation at 133000g_{av}. for 105-120min (RPS fraction, Scheme 1).

Free and membrane-bound ribosomes were separated from the S fraction by using a modification of the procedure of Bloemendal *et al.* (1967). After the addition of 0.1 vol. of 0.25M-sucrose-11TKM buffer if necessary, the S fraction (2.5ml) was layered on a discontinuous gradient consisting of 1.25ml of 2M-sucrose-TKM buffer overlaid with 1.25ml of 1.5M-sucrose-TKM buffer. The system was centrifuged at 165000g_{av}. for 16-24h or at 131000g_{av}.

Fig. 1. Appearance of discontinuous gradient after prolonged ultracentrifugation (chick liver)

The fractions F1-F6 are defined in the text.

for 20h in the SW50 rotor. A pellet (fraction F6) beneath a faintly opalescent 2M-sucrose layer (fraction 5), and a strongly opalescent 1.5M-sucrose layer were discernible (Fig. 1). Generally four membranous main bands (fractions F1-F4) could be seen above the 2M-sucrose layer.

The following fractions were collected: the entire upper layer, containing fractions F1 and F2 and the overlying 0.25M-sucrose-TKM buffer layer; fractions F3 and F4 (combined); fraction F5; fraction F6. The surface of the pellet (fraction F6) was gently washed with TKM buffer and the washes were added to fraction F5.

The combined rough-membrane suspension fractions F3+F4, from one gradient was diluted to 5ml with TKM buffer and 1.0ml portions were each layered on to 4ml linear continuous sucrose gradients (0.44-0.88M) prepared in TKM buffer. The tubes were centrifuged at 165000g_{av}. for 50min or 131000g_{av}. for 60min in the SW 50 rotor at 0-4°C. The supernatants of these gradients were removed by decantation and the pellets (purified rough microsomes) were rinsed with TKM buffer. The supernatants plus rinses were diluted and centrifuged at 133000g_{av}. for 105-120min (50Ti rotor) as were the other fractions (F1+F2 and F5). The pellet derived from the 0.44-0.88M-sucrose-gradient supernatants ('interface free-ribosome fraction') contained the free ribosomes contaminating the rough membranes present in the 1.5M-sucrose layer and the 1.5M/2M-sucrose-interface fractions.

In some experiments, the first nuclear-mitochondrial pellet wash was performed with the homogenization medium and the supernatant was combined with the first postmitochondrial supernatant to constitute the S fraction. In this case, the R

Scheme 1. Summary of procedure for the fractionation of homogenates of developing chick liver

The procedure was carried out as described in the Methods section. The fractions are defined in the text. NM pellet, nuclear-mitochondrial pellet.

fraction consisted of the next three 0.25M-sucrose-wash supernatants combined.

Continuous sucrose gradients

Linear continuous sucrose gradients in TKM buffer were prepared by forming stepped discontinuous gradients (five to ten steps) and storing these at room temperature for 20–30 min and then in the cold (2°C) for 1–5 h. The linearity of the gradients was sometimes checked by puncturing a tube with a needle, collecting drop fractions and determining their sucrose content by using an Abbe refractometer.

Electron microscopy

Microsomal and other fractions were either centrifuged (e.g. at 165 000g_{av} for 80 min, SW 50 rotor) to form a pellet which was subsequently covered with 1–3% (w/v) glutaraldehyde in 0.1–0.2M-sodium phosphate buffer, pH 7.4, or they were fixed in suspension by the addition of Karnovsky's fluid (Karnovsky, 1965) or glutaraldehyde-sodium phosphate buffer (to a final glutaraldehyde concentration of 1–3% and buffer concentration of 0.04–0.15M) and then sedimented. The fixation time varied from 2 to 96 h. The blocks were post-fixed in 1–2% (w/v) OsO₄-sodium phosphate buffer, dehydrated in graded aq. acetone and then acetone (15 min per dehydration) and embedded in Araldite.

Liver tissue was fixed as described by Karnovsky (1965) followed by staining for 1–4 h in 1–2% OsO₄ in sodium phosphate buffer (pH 7.4) or directly in 1–2% OsO₄-cold sodium phosphate buffer for 1½–6 h. The blocks were dehydrated and embedded as described above.

Analytical methods

Protein was determined by the method of Lowry *et al.* (1951), phospholipid and DNA were determined by the method of Volkin & Cohn (1954), and RNA was measured by the method of Munro & Fleck (1966). Glucose 6-phosphatase (EC 3.1.3.9) activity was measured at pH 6.5 in maleate buffer as described by Swanson (1955) except that the incubation temperature used was 31–32°C. Phosphate was determined by the assay of Chen *et al.* (1956).

The phospholipid present in a fraction was calculated by multiplying the phospholipid phosphorus content by 25.

Results

Note on terminology

For purposes of brevity, the terms 'rough microsomes' and 'rough-microsome fraction' will be used to describe a fraction containing rough and smooth vesicles but no free ribosomes. The RNA

content of rough-microsome fractions is taken as a direct measure of the ribosomal content.

Isolation of microsomal fractions

The present microsomal isolation procedure is a modification of the procedure of Oates (1970), which is a variation of the procedure described by Hers *et al.* (1951) for rat liver. For chick (embryo) liver, homogenization in 0.25M-sucrose-TKM buffer proved to be essential if excessive contamination of the subsequent 0.25M-sucrose-wash fractions with nuclear fragments was to be avoided. However, this precaution was not necessary for 6–8-day chick-embryo liver, and this could be homogenized in 0.25M-sucrose or 0.25M-sucrose-TKM buffer.

The aim of this aspect of the fractionation was to recover as much microsomal material, minimally contaminated by mitochondria, nuclear fragments and other cell structures, as possible. It did not permit complete recovery of microsomal elements, however, since some glucose 6-phosphatase, an enzyme localized on the endoplasmic reticulum and the microsomal fraction of chick and rat liver (Hers *et al.*, 1951; Pollak & Shorey, 1967; Oates, 1970; Tice & Barnett, 1962) was found in the final nuclear-mitochondrial pellet (Table 1).

Electron microscopy of the S and R fractions revealed that they consisted primarily of rough and smooth vesicles and free ribosomes (see Plate 1a). Mitochondria and lysosomes were present to a relatively small extent.

Separation of free and membrane-bound ribosomes from the S fraction

After centrifugation of the discontinuous gradient four fractions were collected as described in the Methods section (cf. Bloemendal *et al.*, 1967; see Scheme 1 and Fig. 1).

Examination of each of the fractions by electron microscopy permitted the following identifications to be made: F6 fraction, free polyribosomes (Plate 1b); F5 fraction, free monoribosomes and polyribosomes; 'interface free-ribosome fraction' (IFR), free monoribosomes (or subunits) and some amorphous material. The membrane fractions (F1–F4) appeared to be similar to the corresponding rat liver fractions (see Bloemendal *et al.*, 1967), except that significant numbers of paucigranular vesicles could be found in the 'smooth'-vesicle fraction (F1 + F2).

Separation of free and membrane-bound ribosomes from the R fraction

Examination of the first nuclear-mitochondrial pellet with the electron microscope suggested that free ribosomes were present to some extent. Although these could sometimes be separated by

Table 1. *Glucose 6-phosphatase content of the nuclear-mitochondrial pellets remaining after isolation of the total-microsome fraction*

A total-microsome fraction was isolated as described in the Methods section, the homogenization medium being 0.25M-sucrose or 0.25M-sucrose-TKM buffer. The age of embryos refers to the period of egg incubation and the age of post-hatched animals is given in days after hatching. The number of experiments is given in parentheses.

Age of chick (days)	Homogenization medium	Glucose 6-phosphatase in final nuclear-mitochondrial pellet (% of homogenate value)
Embryo		
6-8	Sucrose	13.5-22.6 (6)
12	Sucrose-TKM buffer	12.5 (1)
17	Sucrose-TKM buffer	18-27 (5)
Post-hatched		
0-+2 (perinatal)	Sucrose-TKM buffer	13-15 (3)
+100-+400 (adult)	Sucrose-TKM buffer	19-29 (4)

Table 2. *Separation of free-ribosome fractions from R fractions by using different procedures*

Free-ribosome fractions were isolated from R fractions by differential centrifugation (to give the RP and RPS fractions) or by using the discontinuous-gradient method normally used to subfractionate fraction S. The homogenization medium was 0.25M-sucrose or 0.25M-sucrose-TKM buffer.

Expt. no.	Age of chick (days)	Medium used for washing	RNA in free-ribosome fraction (as % of RNA in homogenate) from R fraction by		RNA in free ribosomes from R fraction	
			Differential centrifugation	Discontinuous gradient method	(% of sedimentable RNA in R fraction)	(% of total free ribosomes in homogenate)
Embryo						
1	6	Sucrose-TKM buffer	4.1	—	17	11
2	6	Sucrose	3.2	—	22	8
3-5	7	Sucrose	1.8-2.5	—	12-20	6*
6	7½	Sucrose	—	1.9	16	5
7	8½	Sucrose-TKM buffer	—	4.4	19	11
8-9	8	Sucrose	3.6-4.6	—	18-22	10-14
10-11	10	Sucrose-TKM buffer	5.5-6.0	—	19-20	15-16
12	12	Sucrose-TKM buffer	—	6.2	20	20
13	12½	Sucrose-TKM buffer	—	3.6	21	9
14-18	17	Sucrose-TKM buffer	5.1-6.7	—	23-24	19-21
Post-hatched						
19-22	0-+2	Sucrose-TKM buffer	4.2-4.8	—	23-24	12.4-12.5†
23	Adult‡	Sucrose-TKM buffer	4.6	5.7	17	20§
24-28	Adult	Sucrose-TKM buffer	4.7-6.2	—	17-25	18-25

* Only one of the three experiments.

† Only two experiments.

‡ S fraction = postmitochondrial supernatant + one-wash supernatant of first nuclear-mitochondrial pellet (in 0.25M-sucrose-TKM buffer).

§ Refers to RPS fraction in this case.

using the discontinuous-gradient procedure (see Scheme 1 and Table 2) the R fraction from 6-7-day embryonic liver was usually too dilute. Advantage was taken of the effect of added TKM buffer on the state of aggregation of the microsomal vesicles in the R fraction. The aggregates that formed were so large (they were visible as slightly dense structures

under the phase-contrast microscope) that they could be separated effectively by a single differential-centrifugation step (Scheme 1).

The data in Table 2 demonstrate that the differential-centrifugation and discontinuous-gradient techniques give a similar estimate of the free ribosomal RNA content of the R fraction. About 80% (75-88%)

of the sedimentable RNA present was attributable to rough microsomes. This conclusion is substantially in agreement with that of Blobel & Potter (1967), namely that membrane-bound ribosomes sediment preferentially during preparation of a conventional postmitochondrial supernatant.

Cellular complement and proportion of ribosomes bound to membrane during the development of chick liver

To determine the cellular complement of membrane-bound ribosomes it was therefore necessary to determine the extent of contamination of the final nuclear-mitochondrial pellet with membrane-bound ribosomes.

Although glucose 6-phosphatase is probably present in both rough and smooth vesicles isolated from rat liver (Dallner, 1963; Pollak & Shorey, 1967; Cassidy, 1971) and is therefore not a marker for rough vesicles, a number of observations suggest that its activity does, under the conditions used, parallel membrane-bound ribosomal RNA sufficiently to permit an approximate estimate of the unrecovered membrane-bound ribosomal RNA to be made.

It was noted that the glucose 6-phosphatase and RNA contents of the final nuclear-mitochondrial pellet were directly correlated. For example, for 6-7-day embryos final pellets containing 11.5, 12.8, 14.6 and 17.1 % of the cellular RNA were associated with 13.5, 15.6, 19.0 and 22.6 % of the homogenate glucose 6-phosphatase activity respectively (four separate experiments). This conclusion was confirmed and the relationship between the two quantities demonstrated more clearly when the amount of RNA and glucose 6-phosphatase activity in nuclear-mitochondrial pellets from the same homogenate were determined at different stages of the microsomal recovery procedure.

The glucose 6-phosphatase content of the nuclear-mitochondrial pellet during the wash procedure was observed to parallel its RNA content (Fig. 2) and the recovery of enzyme in all fractions was essentially quantitative (99%; vertical lines of Fig. 2). Moreover, as the sedimentable RNA in the wash fractions is largely attributable to membrane-bound ribosomes (see Table 2), and since no more than 5-10 % of the RNA in the R fraction was non-sedimentable (K. O'Toole, unpublished work), it follows that the glucose 6-phosphatase activity recovered in the wash fraction tends to parallel the membrane-bound ribosomal RNA content of these fractions.

The RNA/phospholipid ratio of the fraction-S- and fraction-R-derived rough microsomes was similar in experiments with embryonic and perinatal liver (Table 3), suggesting that microsomes of similar composition (proportion of rough to smooth vesicles)

Fig. 2. RNA and glucose 6-phosphatase in nuclear-mitochondrial pellets isolated by differential centrifugation (21-day embryonic-chick liver)

Two nuclear-mitochondrial pellets were isolated by centrifuging portions of a liver homogenate, prepared in 0.25M-sucrose-TKM buffer, at 20000g_{av} for 15 min and 8000g_{av} for 10 min (SW 39 rotor). The 8000g_{av} pellets were washed 0, 1, 2 and 3 times in 0.25M-sucrose at 5000g_{av} for 10 min as described in the Methods section. Each of the five pellets was resuspended in 0.25M-sucrose-TKM buffer and assayed for RNA and glucose 6-phosphatase. The absolute values for the homogenate were 7.93 mg/g and 14.5 μmol of P_i/min per g respectively. NM, nuclear-mitochondrial.

were being recovered in the postmitochondrial supernatant and wash fractions from this age group.

Although in the adult liver the RNA/phospholipid ratio of the fraction-S-derived rough microsomes tended to be significantly lower than that of the fraction-R-derived rough microsomes (Table 3), this may not affect the results appreciably (see below).

Although as little as 12.5 % of the homogenate glucose 6-phosphatase was present in the final nuclear-mitochondrial pellet in some instances, significant numbers of rough vesicles could always be detected by electron microscopy (Plate 1c).

The RNA/phospholipid data (Table 3) suggest that in embryonic and perinatal chick the average proportion of rough to smooth vesicles may not

Table 3. *Chemical composition of rough microsomes separated from S and R fractions*

Results are given as the means $\pm$ S.E.M. for more than two experiments. The number of experiments is given in parentheses.

Age of chick (days)	RNA/phospholipid ratio of fraction-S-derived rough microsome fraction	RNA/phospholipid ratio (S fraction) RNA/phospholipid ratio (R fraction)	
		Range	Mean
Embryo			
7	0.57	0.96–1.19	1.08 (2)
8	0.43	—	0.86 (1)
12	0.39	—	0.93 (1)
17	0.33	1.00–1.10	1.05 (2)
Post-hatched			
0–+2	0.26	0.81–0.93	0.87 (2)
Adult	0.21	0.55–0.63	0.58 $\pm$ 0.04 (4)

Table 4. *RNA content of final nuclear-mitochondrial pellets after subtraction of the membrane-bound ribosomal RNA assessed by using the glucose 6-phosphatase correction*

The number of experiments is given in parentheses. Results are given as means, $\pm$ S.D. for more than two experiments.

Age of chick (days)	RNA in microsome-free pellet [as % of RNA in homogenate (calc.)]	
	Range	Mean
Embryo		
6–7	6.46–7.90	7.17 $\pm$ 0.66 (4)
8	4.78–7.40	6.1 (2)
12	—	5.4 (1)
17	10.64–12.50	11.6 (2)
Post-hatched		
0–+2	8.80–9.11	9.0 (2)
Adult	8.20–10.20	9.55 $\pm$ 0.92 (4)

be greatly different in the S and R fractions. Thus, glucose 6-phosphatase activity parallels the recovery of a fraction of similar composition and hence it parallels the membrane-bound ribosomal RNA content of these fractions.

To calculate the unrecovered membrane-bound ribosomal RNA present in the final nuclear-mitochondrial pellet for embryonic and perinatal animals, it is thus simply a matter of equating the recovery of glucose 6-phosphatase with the recovery of membrane-bound ribosomes.

By using this correction, the RNA in the final nuclear-mitochondrial pellet that is not attributed to rough microsomes can be calculated (Table 4). For embryonic and perinatal liver, the amount of RNA is low (5–12% of the homogenate RNA) but always more than zero. Since nuclear RNA represents less than 11% of the cellular RNA for developing chick liver (O'Toole, 1972), this amount of RNA must be close to that expected for nuclear RNA. As a corollary, it is unlikely that the above glucose 6-phosphatase correction will lead to a significant

underestimate of the membrane-bound RNA present in the homogenate.

As was noted earlier, there may be some tendency to recover smooth (or light rough) vesicles preferentially in the adult chick liver postmitochondrial supernatant fraction (Table 3). Since smooth vesicles contain virtually no RNA (Oates, 1970), but possibly some glucose 6-phosphatase, a preferential recovery of smooth vesicles could mean that the glucose 6-phosphatase recovery is higher than the recovery of membrane-bound ribosomes, i.e. that the above correction method would underestimate the membrane-bound ribosomal RNA content of the homogenate. The magnitude of this error will depend on the total glucose 6-phosphatase content of smooth vesicles relative to that of rough vesicles in the homogenate and on the recovery of glucose 6-phosphatase in the isolated microsome fraction.

In Table 4, the RNA in the microsome-free nuclear-mitochondrial pellet for the adult chicken is calculated by using the glucose 6-phosphatase method. It is apparent from the above argument that the value obtained, only about 10% of the homogenate RNA (Table 4), may be an overestimate of the RNA which would actually be present in the microsome-free pellet. By using the glucose 6-phosphatase correction method, 50% of the cellular RNA can be attributed to rough microsomes in the adult (Table 5, see below). However, the lower limit for RNA in the microsome-free pellet is the sum of RNA in nuclear fragments and mitochondria and this must be fairly close to the calculated value in Table 4. The difference between the calculated pellet RNA and this lower limit must therefore be small in relation to the total membrane-bound ribosomal RNA (cf. 6–21-day embryos). In other words, the glucose 6-phosphatase correction must be valid in the adult also.

It is unlikely that the final nuclear-mitochondrial pellet contains significant amounts of free-ribosomal material for the following reasons:

(1) free ribosomes were recovered mainly in the S

Table 5. *Changes in membrane-bound ribosomes during development*

Membrane-bound ribosomal-RNA yields are corrected for recovery by using the glucose 6-phosphatase method. The numbers of experiments are given in parentheses. When only two experiments were done, the individual values are given in brackets. Results are expressed as means, $\pm$ S.E.M. when the mean is derived from two or more experiments.

Age of chick (days)	RNA in total rough microsomes (mg/g)	RNA in total rough microsomes (% of RNA in homogenate)	RNA/DNA ratio of homogenate	RNA in total rough microsomes per unit of homogenate DNA
Embryo				
6-7	4.22 [4.50, 3.94]	38.4 $\pm$ 1.0 (5)	3.54 $\pm$ 0.06 (4)	1.36
8	4.02 $\pm$ 0.38 (3)	38.8 $\pm$ 2.3 (4)	3.62 [3.55, 3.69]	1.40
12	4.49 [4.09, 4.89]	43.9 [45.6, 42.1]	3.44 (1)	1.51
17	3.74 [3.56, 3.91]	48.0 [48.8, 47.2]	2.73 $\pm$ 0.13 (3)	1.31
Post-hatched				
0-+2	2.94 [3.24, 2.64]	38.2 [37.1, 39.3]	2.75 $\pm$ 0.15 (5)	1.05
Adult	3.62 $\pm$ 0.36 (4)	49.3 $\pm$ 1.9 (4)	2.63 $\pm$ 0.28 (6)	1.30

Table 6. *Proportion of ribosomes bound to membrane during development*

Mean values are given, $\pm$ S.E.M. for more than two experiments.

Age of chick (days)	Percentage of cellular ribosomes bound to membrane	
	Range	Mean
Embryo		
6	47.1-51.0	49.2 $\pm$ 1.1 (3)
7	—	48.2 (1)
8	44.0-56.0	49.1 $\pm$ 2.4 (4)
12	51.5-59.2	55.3 (2)
17	61.2-63.0	62.1 (2)
Post-hatched		
0-+2	52.2-52.3	52.3 (2)
Adult	64.0-66.9	65.7 $\pm$ 0.6 (4)

fraction and only to a small extent in the R fraction (Table 2). This indicates a tendency toward complete recovery early in the wash procedure; (2) nearly all the RNA in the final nuclear-mitochondrial pellet after the glucose 6-phosphatase correction can be accounted for by remnants of nuclei, cytoplasmic blebs, mitochondrial RNA and other 'debris'. This leaves little RNA that could be derived from free ribosomes.

Thus the method outlined in this paper gives a valid estimate of the free and membrane-bound ribosomal RNA content of the homogenate if (a) the recovered free-ribosomal RNA is equated with the total cellular free-ribosomal RNA; (b) the recovered rough-microsomal RNA is corrected for incomplete recovery (glucose 6-phosphatase method).

Table 5 shows that the concentration of membrane-

bound ribosomes per g of tissue does not change markedly during chick liver development. Since the non-parenchymal cellular volume (mainly blood volume) is probably highest in the 6-8-day chick liver (Romanoff, 1960; Brauer *et al.*, 1963) and the nuclear volume, as a fraction of the cell volume, is also highest at this stage (Sandström & Westman, 1971), and since the average DNA content per g of tissue does not change appreciably during development (this can be shown by recalculating some of the data of Table 5) these results suggest that the concentration of membrane-bound ribosomes per unit of cytoplasmic volume is probably at least as high in the parenchymal cells of the 6-8-day animals as it is in those of perinatal animals of the adult.

The results also indicate that the amount of rough-microsomal RNA per mg of DNA in liver homogenates is as high in the 6-7-day chick embryo as it is in the adult. This does not mean, however, that the number of membrane-bound ribosomes per average 6-7-day chick-embryo parenchymal cell is equal to the number present in an adult cell because (1) by comparison with the 6-day embryonic liver, adult liver may contain larger numbers of other cells in addition to parenchymal cells (e.g. erythrocytes, littoral cells) and these will contribute significantly to the DNA (but not to the RNA; see below) content of the homogenate; (2) the RNA/DNA ratio of whole homogenates of adult liver, unlike that of whole homogenates of 6-7-day embryonic liver, tended to be highly variable (Table 5). Thus it is probable that other factors may be involved in this case, e.g. age and sex, which have not been considered.

The fraction of ribosomes bound to membrane is high in the 6-8-day average chick liver cell (49%;

EXPLANATION OF PLATE I

Subcellular fractions from developing chick liver

(a) R fraction from 21-day embryos, obtained by washing the first nuclear-mitochondrial pellet three times in 0.25 M-sucrose and combining the wash supernatants. See the text for details. Final magnification is $\times 16480$. (b) Membrane-poor region in a pellet of fraction F6 (13-day embryos). Structures identified as membrane contaminants are indicated by arrows. Final magnification is $\times 24480$. (c) Rough vesicles in a final nuclear-mitochondrial pellet (12-day embryos). Structures identified as rough vesicles are indicated by arrows. In this experiment, the final nuclear-mitochondrial pellet contained only 12.5% of the glucose 6-phosphatase of the homogenate. Final magnification is $\times 24480$. Pellets were fixed with glutaraldehyde, post-fixed with OsO_4 and stained with uranyl acetate. RV, rough vesicle; SV, smooth vesicle; M, mitochondrion or presumed mitochondrial fragment. The bars represent $1\mu\text{m}$.

EXPLANATION OF PLATE 2

Embryonic liver tissue

(a) Liver from 6-day-old-chick fixed with 1% OsO₄ in 0.1M-sodium phosphate buffer, pH7.4. Rough endoplasmic reticulum is present mainly as large vesicles or swollen cisternae. Final magnification is $\times 15600$. (b) 6-day-old-rat liver fixed with Karnovsky's fluid (Karnovsky, 1965) and post-fixed with OsO₄. Rough endoplasmic reticulum is present mainly as flat cisternae. Final magnification is $\times 20400$. (c) 11-day-old-rat liver fixed with Karnovsky's fluid and post-fixed with OsO₄. Ordered configurations of ribosomes (OC) identified as membrane-bound ribosomes where the membrane has been sectioned obliquely. Regions showing background material and linear arrays of ribosomes continuous with identifiable rough endoplasmic reticulum are shown at J. Final magnification is $\times 32400$. RER, rough endoplasmic reticulum; M, mitochondrion; N, nucleus. All sections are stained with uranyl acetate. The bars represent 1 μ m.

Table 7. *Recovery of free and membrane-bound ribosomes in a conventional microsome fraction*

Groups of 'standardized' experiments are indicated by horizontal dotted lines. 'Conventional microsomes' in this case refers to those in the S fraction. The homogenization medium was that used by many authors, i.e. 0.25 M-sucrose-TKM buffer. A = postmitochondrial supernatant; B = postmitochondrial supernatant + wash supernatant of first nuclear-mitochondrial pellet. The wash was performed by resuspension in 0.25 M-sucrose-TKM buffer and re-centrifugation under the same conditions as were used to prepare the postmitochondrial S supernatant.

Expt. no.	Age of chick (days)	Homo-genizer*	Nature of S fraction	Recovery of ribosomes in S fraction (as % ribosomes in homogenate†)		Ratio of recovery of free ribosomes to recovery of membrane-bound ribosomes
				Free	Bound	
Embryo						
1	6	No. 1	A	89	18	4.9
2 (i)	12	No. 1	A	84	44	1.9
3	6	No. 1	B	83	20	4.2
4	8	No. 1	B	89	27	3.3
2 (ii)	12	No. 1	B	91	56	1.6
5	12	No. 2	A	80	23	3.5
6	17	No. 2	A	81	26	3.1
7	21	No. 2	A	88	51	1.7
Post-hatched						
8	+2	No. 2	A	88	48	1.8
9	Adult	No. 2	A	75	40	1.9
10	Adult	No. 2	A	82	25	3.3

* Homogenizer no. 1 produced somewhat higher shear forces than homogenizer no. 2.

† Free ribosomes in homogenate taken as 100%; membrane-bound ribosomes in homogenate taken as 100%.

Table 6). Although the results of Table 6 appear to indicate that this fraction may increase during embryonic development and decrease at hatching, this pattern was not always observed (O'Toole & Pollak, 1971), suggesting that there is only a tendency toward a greater proportion of membrane-bound ribosomes in the late embryonic period.

A different situation exists for adult liver. In this case, the proportion of ribosomes bound to membrane is reproducibly higher than in the 6-8-day embryonic liver. On no occasion has any overlap been observed between the 6-8-day embryo value and the adult value.

These analytical studies were made on whole liver, not on isolated liver parenchymal cells. However, since chick-embryo liver consists on a weight basis predominantly of parenchymal cells (Benzo & Nemeth, 1971), since it is known that most of the increase in liver mass that occurs in the chick during development is a result of parenchymal cell proliferation (Benzo & Nemeth, 1971) and since the ribosomal and endoplasmic reticulum content of chick (embryo) liver appears to be largely confined to the parenchymal cells (K.O'Toole, unpublished work), it follows that essentially all the ribosomal material present in chick (embryo) liver homogenates will be derived from the parenchymal cells.

Recovery of membrane-bound ribosomes by means of a conventional microsome fraction

It could be argued that these changes during

development will be reflected in a conventional microsomal fraction, the only difference being that the absolute values would be different owing to a loss of some rough vesicles into the nuclear-mitochondrial pellet. That this may not be the case is illustrated in Table 7. When 'standardized' conditions are used, the relative recovery of free and membrane-bound ribosomal RNA in an S fraction may change during development.

Electron microscopy of whole liver tissue

When 5-7-day chick-embryo liver was fixed with OsO₄, the subcellular morphology appeared to be rather disorganized. In particular, the rough endoplasmic reticulum was mainly in the form of large vesicles or swollen cisternae (giving the impression of smooth vesicles at low magnification; Plate 2a). Other authors have made similar observations (Pollak & Shorey, 1967; Stephens & Bils, 1967; Benzo & Nemeth, 1971).

To determine whether this appearance could be modified by using a different fixative, 5-7-day embryonic liver was also fixed with Karnovsky's fluid, a formaldehyde-glutaraldehyde fixative designed to decrease mechanical damage to the cells during preparation of soft tissues for fixation (Karnovsky, 1965). A somewhat different appearance was now obtained. The cytoplasm appeared more ordered, and the rough endoplasmic reticulum was present mainly as (flat) cisternae (Plate 2b). With either

fixative, however, 'free' ribosomes (but see the Discussion section) were abundant and the rough endoplasmic reticulum appeared to be moderately well developed.

The reason for the discrepancy between the two fixatives is not clear, but possibly the formaldehyde component of Karnovsky's fluid, having a considerably higher diffusion constant than OsO_4 (Baker, 1963), penetrates the tissue and reaches a fairly high concentration in the innermost cells more quickly, thereby minimizing toxic side effects, for example, vesiculation of the endoplasmic reticulum (see Porter, 1961; Karnovsky, 1965).

If this is the explanation, then Karnovsky-fixed liver parenchymal cells may more closely resemble the situation *in vivo*. In any case, one might suspect that the more ordered arrangement of the cytoplasm of Karnovsky-fixed cells is more probable *in vivo* than the disordered arrangement suggested by OsO_4 fixation (see also Sandström & Westman, 1971).

Both Stephens & Bils (1967) and Pollak & Shorey (1967) noted whirls, 'tortuous loops' and linear arrays of uranophilic granules only in 5–6-day embryonic liver (Pollak & Shorey, 1967) which were 'apparently free in the cytoplasm' (Stephens & Bils, 1967). Stephens & Bils (1967) suggested that these represented ordered arrays of ribosomes engaged in specialized protein synthesis, whereas Pollak & Shorey (1967) implied that they represented non-ribosomal membrane-precursor granules in the process of being assembled into membranes. We now believe that, since the organization of these particles closely resembles that of particles present on the surface of some rough membranes, they may be membrane-bound ribosomes (see Palade, 1955, 1964). It should be pointed out that background material (obliquely sectioned membrane?) was evident in micrographs presented by both Pollak & Shorey (1967) and Stephens & Bils (1967). In the present work, these particle configurations were often clearly evident in 6–12-day embryo liver cells fixed with either OsO_4 or Karnovsky's fluid (Plate 2c). However, the background material was frequently seen to be continuous with clearly identifiable rough endoplasmic reticulum (Plate 2c). Further, micrographs of homogenates, microsome or rough-microsome fractions sometimes showed rough vesicles cut obliquely or contained in the section where the organization of the membrane-bound ribosomes on the membrane closely resembled the particle configurations described by Stephens & Bils (1967) *in situ*.

Discussion

In preliminary experiments it was noted that if homogenates of untreated 6–8-day chick-embryo

liver were centrifuged to precipitate nuclei and mitochondria, a large part (40–50%) of the total RNA also sedimented. Since nuclear RNA was found to represent no more than 11% of the cellular RNA (O'Toole, 1972) and since it was believed that most of the free ribosomes would be recovered preferentially in the postmitochondrial-supernatant fraction (Blobel & Potter, 1967), this observation suggested that in 6–8-day chick liver cells, a large fraction of the cellular ribosomes are membrane-bound. This conclusion, however, was not in agreement with that of Pollak & Shorey (1967) and Karrer (1960) whose observations suggested that most of the ribosomes are free in these cells. The validity of the assumption that the non-nuclear RNA present in the initial nuclear-mitochondrial pellet was largely attributable to fragments of rough endoplasmic reticulum was thus rendered questionable in this case.

To resolve this problem and to determine whether, in fact, 6-day embryonic-liver parenchymal cells are endowed with a large complement of membrane-bound relative to free ribosomes, the present study was undertaken. Three main approaches have been or are currently being used by other authors to assess the membrane-bound ribosomal RNA content of a given tissue or cell type. The first approach assumes that complete recovery of the membrane-bound ribosomes can be achieved in the supernatant fraction by centrifuging the homogenate, prepared in an ion-supplemented medium, under controlled conditions. However, it is known that large losses of ribosomes into discarded pellets may result, even when very low centrifugal forces, e.g. 800g, are used (Howell *et al.*, 1964; Blobel & Potter, 1967). In the second approach, cell nuclei and free ribosomes are separated from the homogenate by density-gradient procedures and a fraction designated 'bound ribosomes' is isolated from the residual homogenate by using detergents (Blobel & Potter, 1967). In the third approach, the homogenate is centrifuged so as to sediment essentially all the membrane-bound ribosomes into the nuclear-mitochondrial pellet, leaving the free ribosomes in the supernatant (e.g. Loeb *et al.*, 1967; Boshes, 1970; Fridlender & Wettstein, 1970; Venkatesan & Steele, 1972). The centrifugal conditions are usually determined empirically (e.g. Loeb *et al.*, 1967; Venkatesan & Steele, 1972) but some authors apparently define the free ribosomes as those that do not sediment with the nuclei and mitochondria (e.g. Boshes, 1970).

These approaches proved to be inapplicable to developing chick liver. The first approach (controlled centrifugation of the homogenate) was found to be unsatisfactory because the preparation of post-nuclear and postmitochondrial supernatants resulted in nuclear or nuclear-mitochondrial pellets significantly contaminated with rough vesicles (O'Toole, 1972). The second and third approaches could not

be used because the identity of the 'bound'-ribosomal fraction isolated by these methods would be in doubt, particularly in the case of 6-day chick-embryo liver (see above). In fact, homogenates and nuclear-mitochondrial pellets from 6-day embryonic liver were found to contain (by electron microscopy) many large closed vesicles (containing apparently free ribosomes and soluble protein) apparently derived from whole cells by a pinching-off process (compare with Plate 1 of Burghouts *et al.*, 1970). These would possibly also contaminate postnuclear supernatants prepared by the method of Blobel & Potter (1967).

To overcome these difficulties, it was therefore necessary to derive a new method which did not rely on the assumption that the non-nuclear RNA present in the initial nuclear-mitochondrial pellet was attributable to membrane-bound ribosomes. This was achieved by: (1) decreasing the RNA in this nuclear-mitochondrial pellet to as low a value as possible by means of differential centrifugation while still recovering only rough and smooth vesicles and free ribosomes in the wash fluids; (2) correcting for the unrecovered membrane-bound ribosomes by using glucose 6-phosphatase as marker enzyme for the endoplasmic reticulum in this tissue.

It must be stressed that this approach does not depend on the close parallel between the recovery of rough-microsomal RNA and that of glucose 6-phosphatase which was observed in experiments with embryonic or perinatal liver (Fig. 2), although this phenomenon is clearly helpful. The present results indicate that the recovery of glucose 6-phosphatase from adult liver, namely 70–80% (Table 1), is sufficiently high to permit a reliable estimate to be made of the amount of membrane-bound ribosomes.

Changes in free and membrane-bound ribosomes during development

Although developing chick liver has been studied extensively by electron microscopy, few authors have specifically commented on the changes in the proportion of free to membrane-bound ribosomes (changes in the rough endoplasmic membranes do not necessarily reflect changes in the concentration of membrane-bound ribosomes). Moreover, different reports appear to conflict.

Thus Stephens & Bils (1967) and Sandström & Westman (1971) observed a high and roughly constant concentration of free ribosomes throughout embryonic development, whereas Pollak & Shorey (1967) and Benzo & Nemeth (1971) observed a substantial decrease of free ribosomes during development. Stephens & Bils (1967) found that the concentration of rough endoplasmic reticulum increased only between days 3 and 4 and between days 8 and 10, but Pollak & Shorey (1967) and Sandström

& Westman (1971) noted a continual increase during embryonic development. Benzo & Nemeth (1971) observed a large increase in rough endoplasmic reticulum between days 5 and 8, followed by some decrease to day 11.

However, all the above studies do suggest that most ribosomes are not bound to membranes in 5–7-day embryonic-chick liver cells. This would be consistent with the (apparently) widely held view that rapidly proliferating embryonic cells are associated with little endoplasmic reticulum, whereas free ribosomes are abundant (Rosbash & Penman, 1971). The present cell-fractionation data are in complete disagreement with this, as 49% of the ribosomes were found to be membrane-bound in 6-day liver parenchymal cells (Table 6).

In the present investigation it was found that 5–7 day embryonic-chick liver showed a moderately well-developed rough endoplasmic reticulum. Although the appearance of liver cells differed when two different fixatives (OsO_4 and Karnovsky's fluid) were used, superficial examination suggested that most (80%) of the ribosomes were nevertheless unassociated with membranes, irrespective of the fixative used.

Since this was only an impression, attempts were made to determine quantitatively the proportion of free and membrane-bound ribosomes by means of morphometric methods. This approach proved to be abortive, since it appeared to be virtually impossible to distinguish unequivocally between free ribosomes and ribosomes bound to obliquely sectioned membranes (see Loud, 1967). In fact, the unequivocal identification of ribosomes in the cell cytoplasm was also often difficult (see Wibó *et al.*, 1971).

Consequently we have concluded that the subjective impressions obtained by the perusal of micrographs, at least as far as free and membrane-bound ribosomes are concerned, must be treated with considerable caution. On the other hand, no evidence could be found to indicate that incomplete separation of free ribosomes had occurred in the cell-fractionation procedure (O'Toole, 1972). In fact, all attempts were made to ensure that free ribosomes were quantitatively recovered from chick liver homogenates. For example, it was not assumed that free ribosomes could be completely sedimented by a prolonged centrifugation of the microsomal fraction over a 2M-sucrose layer as suggested by Blobel & Potter (1967). Free ribosomes in the 2M-sucrose layer (see Sarma *et al.*, 1972) and those contaminating the membrane fraction above the 2M-sucrose layer (K. O'Toole & J. K. Pollak, unpublished work; Vernie *et al.*, 1971) were taken into account (see Scheme 1) and it was tentatively concluded that the results of the cell-fractionation procedure were valid.

Many authors apparently believe that in rapidly proliferating undifferentiated cells most of the cytoplasmic ribosomes are not bound to membrane

and that differentiation is accompanied by an increase in the fraction of ribosomes which are membrane-bound (Porter, 1954, 1961; Goldberg & Green, 1967; Arnstein, 1969; Rosbash & Penman, 1971; see also Webb *et al.*, 1965). The present results may only partially support these contentions. Although 6-day chick-embryo liver cells are rapidly dividing and apparently synthesize little protein for export (Duck-Chong, 1963; Kaminski & Durieux, 1954, 1956; Nace & Schechtman, 1948; Pickering & Gladstone, 1925; Schjeide *et al.*, 1960), a large fraction of the cellular ribosomes were found to be membrane-bound (Table 6). Further, although a high concentration of free ribosomes was observed in 6-day liver cells, a corollary of these findings is that an equally high concentration of membrane-bound ribosomes is also present (Table 6).

On the other hand, the contention that the fraction of ribosomes bound to membrane increases during differentiation is borne out by the present experiments (Table 6).

Comparison of the present results with other cell-fractionation studies of developing tissues is difficult. Free and membrane-bound ribosomes have usually been separated from conventional microsomal or postnuclear fractions and the recovery of membrane-bound ribosomes has not been determined (e.g. Andrews & Tata, 1971; La Via & Prater, 1971; Payne & Boulter, 1969; Priestley & Malt, 1968; Tata, 1967; Webb *et al.*, 1965). It could be argued therefore that developmental changes observed during these investigations may also have been due to changes in the relative recovery of membrane-bound ribosomes in the postmitochondrial or postnuclear fraction (cf. Table 7). For this reason, the conclusions of Webb *et al.* (1965) and Priestley & Malt (1968), that all of the ribosomes of foetal-rat liver and foetal-mouse kidney are free in the cytoplasm and that there is an increase in the fraction of membrane-bound ribosomes during development, must be taken with some reservation. An investigation that cannot be criticized for this reason is that of Gaye & Denamur (1969). These authors used the cell-fractionation method of Blobel & Potter (1967) to determine the proportion of membrane-bound ribosomes in mammary-gland homogenates at intervals after injection of the animal with prolactin. Unfortunately they did not recover the free ribosomes present in the 2M-sucrose layer of the discontinuous gradient (Sarma *et al.*, 1972), so that even these changes must be treated with some reservation.

Kimmel (1969) has suggested that the fraction of cytoplasmic ribosomes bound to membrane is directly correlated with the capacity of the cell to synthesize protein destined for secretion. In fact, it has been shown that membrane-bound ribosomes are mainly involved in the synthesis of secreted proteins

whereas free ribosomes mainly synthesize intracellular proteins (Goldberg & Green, 1967; Ganoza & Williams, 1969; Hicks *et al.*, 1969; Redman, 1969; Gaye & Denamur, 1970; Glaumann, 1970; Daille *et al.*, 1971; Takagi & Ogata, 1971; Nihei, 1971; Permutt & Kipnis, 1972; Pryme *et al.*, 1973). Since, however, it is known that membrane-bound ribosomes are also involved in the synthesis of membrane and possibly some other intracellular proteins (Dallner *et al.*, 1966; Ragnotti *et al.*, 1969; Pitot *et al.*, 1969; Chuah & Oliver, 1971), the high content of membrane-bound ribosomes in 6-day embryonic-liver cells (Tables 5 and 6) may be explained. These cells are dividing rapidly and the synthesis of both membrane and soluble protein must be occurring at a high rate. The higher proportion of membrane-bound ribosomes in the corresponding adult cells may therefore reflect the observed greater emphasis on the synthesis of secreted protein at this stage.

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