The Distribution of Ribosomes between Different Functional States in Livers of Fed and Starved Mice

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1. To investigate the role of ribosome function in regulating protein synthesis, the activity, distribution and functional states of ribosomal particles were investigated in livers of mice fed *ad libitum* or starved overnight. 2. The distribution of protein-synthesizing activity between polyribosomes of different sizes was analysed after incorporation of radioactive leucine, and the quantitative distribution of ribosomes as native subunits, monomers and polyribosomes was analysed after incorporation of orotic acid. Precursors labelled with ^3H or ^{14}C were given separately to fed and starved mice, so that livers from the two groups of animals were processed together. 3. The former experiments showed that starvation has little effect on the distribution of protein-synthesizing activity across polyribosome sedimentation patterns, though the latter experiments showed that the proportion of ribosomes existing as monomers increased from 9.5% to 15.2%, whereas the proportion existing as polyribosomes decreased from 81.4% to 75.6%. Starvation had a negligible effect on the proportion of native subunits, which accounted for 9.1% and 9.2% of the ribosomes in fed and starved mice respectively. 4. The monomeric ribosome fraction was isolated and subjected to ionic conditions which selectively dissociate single ribosomes. Starvation increased the proportion of monomers that dissociated from 59% to 72%, so the monomers that accumulate in livers of starved animals are single ribosomes and not monoribosomes resulting from degradation of polyribosomes. 5. The fate of newly formed ribosomal particles was studied by measuring the specific radioactivity of native subunits, monomers and polyribosomes at different times after injection of radioactively labelled orotic acid. Starvation did not appear to affect equilibration between newly formed particles and polyribosomes, and the radioactivity of polyribosomes in both groups of mice reached about 90% of that in native subunits after 4h. The radioactive labelling of monomers proceeded at a slower rate, especially after starvation. At 4h, the radioactivity of monomers was 64% and 55% that of native subunits in fed and starved mice respectively.

A decreased rate of protein synthesis in bacteria (Davis, 1971) and cultured mammalian cells (Hogan & Korner, 1968a) caused by a deficiency of carbon source or amino acids is accompanied by transfer of ribosomes from the active polyribosome pool to a pool of single ribosomes which are not actively synthesizing protein, but the proportion of native subunits is unaltered.† In these conditions the number of

'dissociation-factor' molecules available for splitting unprogrammed ribosomes into native subunits probably remains constant, so providing optimum flow of ribosomes through the cycle of polyribosomes and native subunits. The excess of particles which accumulate then form a pool of single ribosomes (Davis, 1971).

The responses of ribosomes in intact animals have not been so clearly defined. Numerous studies have shown that starvation causes 'breakdown' of polyribosomes to monomers and oligomers in mammalian liver (Munro, 1970). Changes that might occur in the proportion of native subunits have not been measured directly, though differences in amounts of 'postmicrosomal RNA' led Wunner *et al.* (1966) to conclude that the number of subunits is increased by starvation. Since the monomers that accumulate in livers of starved animals have not been characterized,

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† In this paper, ribosomes in different function states are referred to as follows: 'monoribosomes and polyribosomes', ribosomal structures with one or more ribosomes attached to mRNA; 'single ribosomes', those inactive ('run-off' or 'unprogrammed') ribosomes that are not attached to mRNA; 'monomers', the unfractionated '80S' group of particles, which consists of both monoribosomes and single ribosomes.

these particles might not be unprogrammed single ribosomes, but rather monoribosomes produced by breakdown of polyribosomes. Reports of decreased amounts of RNAase* inhibitor in livers of starved or malnourished animals (Onishi, 1970; Enwonwu & Sreebny, 1971) suggest that enzymic degradation of polyribosomes might occur during isolation from starved animals.

In the present paper, experiments designed to allow quantitative measurement of the proportions of ribosomal particles that exist as native subunits, single ribosomes, monoribosomes and polyribosomes in livers of mice fed *ad libitum* or starved overnight are described. In previous investigations, interference from glycogen has necessitated comparison of polyribosomes in livers of starved animals with polyribosomes in starved animals after refeeding, but use of an enzymic method for degrading glycogen in homogenized livers (Gamulin *et al.*, 1972) has permitted us to make a direct comparison between ribosomal particles in livers of mice fed *ad libitum* or starved.

By use of double-labelling techniques, possible interference from a number of artifacts has been minimized. When livers from the two groups of mice are pooled, polyribosomes are isolated under identical conditions and there is little possibility of differential breakdown by RNAase after the animals have been killed.

Experimental

Materials

L-[4,5-³H]Leucine (1–22 Ci/mmol), L-[1-¹⁴C]leucine (62 mCi/mmol), [³H]orotic acid (17–27 Ci/mmol) and [6-¹⁴C]orotic acid (61 mCi/mmol) were all obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Scintillation chemicals were obtained from Packard Instruments Ltd., Caversham, Berks., U.K.

α -Amylase (637–790 international units/mg of protein) was supplied by Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Millipore filters (HAWP 02500) were obtained from Millipore (U.K.) Ltd., London N.W.10, U.K.

Analytical-grade sucrose (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) was treated with charcoal to remove RNAase (Steele & Busch, 1967) and stored as a stock 50% (w/w) solution.

Methods

Treatment of animals. Male albino mice weighing 25–30 g were maintained on an Oxoid 41B diet and water *ad libitum*. When mice were starved, food was withdrawn for 18 h commencing at 16:00 h, and they were housed in special cages to prevent coprophagia.

* Abbreviation: RNAase, ribonuclease.

Radioactively labelled polypeptide and RNA precursors were injected intraperitoneally in 0.2 ml of 0.9% NaCl solution.

[¹⁴C]Leucine (10 μ Ci/mouse) and [³H]leucine (100 μ Ci/mouse) were injected 6 min before the mice were killed. This time-interval was chosen because Cox & Mathias (1969) showed that labelling of nascent polypeptides was maximal after 6–8 min.

[¹⁴C]Orotic acid (10 μ Ci/mouse) and [³H]orotic acid (100 μ Ci/mouse) were injected 24 h before the mice were killed when ribosome distribution was to be studied. Labelling of mouse liver ribosomal RNA has reached a plateau 24 h after injection of a single dose of radioactive orotic acid (Avdalović, 1970). For measuring the specific radioactivity of ribosomal particles, 50 μ Ci of [³H]orotic acid was injected when a 24 h incorporation period was used, but 100 μ Ci of [³H]orotic acid were used for the shorter time-intervals.

Preparation and analysis of hepatic polyribosomes. To analyse hepatic polyribosomes, a group of six mice (three fed and three starved) was used for each experiment. Fed and starved animals were killed alternately and their livers pooled immediately. For each g of tissue, 2 ml of ice-cold 0.25 M-sucrose in TKM buffer (5 mM-magnesium acetate, 25 mM-KCl and 50 mM-Tris-HCl, pH 7.6 at 25°C) was added before the livers were homogenized with a Potter-Elvehjem Teflon-glass homogenizer. After centrifugation of the homogenate at 1°C for 10 min at 15000g (r_{av} , 7.9 cm), the resulting 'postmitochondrial' supernatant was incubated with 40 units of α -amylase/ml for 15 min at 4°C to degrade glycogen (Gamulin *et al.*, 1972). Immediately before introduction into an MSE BXIV zonal rotor, 8 ml of the postmitochondrial supernatant was mixed with an equal volume of 2% (v/v) Triton X-100 in TKM buffer containing 0.25 M-sucrose. The rotor had previously been loaded with a 15–45% (w/w) sucrose gradient in TKM buffer, and the sample was overlaid with 100 ml of 0.125 M-sucrose in TKM buffer.

After centrifugation for 3 h 20 min at 7°C and 30000 rev./min ($\omega^2 t = 11.8 \times 10^{10}$), the rotor was unloaded by displacing the gradient through an LKB Uvicord II spectrophotometer, allowing continuous recording of optical transmittance at 254 nm. Fractions of 10 ml were collected for measuring radioactivity.

In experiments designed specifically to study alterations in the native subunit and monomer fractions, groups of eight animals (four fed and four starved) were used for each experiment. A 10 ml sample of postmitochondrial supernatant was treated as described above. The zonal rotor was loaded with 380 ml of a 15–35% (w/w) sucrose gradient and a 250 ml cushion of 50% (w/w) sucrose to prevent heavy polyribosomes from reaching the outer wall of the

rotor during the centrifugation, which was for 6 h at 7°C and 30000 rev./min ($\omega^2 t = 20.1 \times 10^{10}$).

Dissociation of single ribosomes into subunits. Selective dissociation of single ribosomes into subunits was performed by a method based on that of Lawford (1969) and modified by Von der Decken *et al.* (1970).

Monomers were recovered from appropriate fractions of the sucrose gradient after centrifugation of postmitochondrial fractions for 6 h in the BXIV zonal rotor. Portions (18 ml) of the combined monomer fractions were layered over 2 ml of 2M-sucrose in TKM buffer and centrifuged for 12 h at 185000g (r_{av} , 6.9 cm) in an MSE 8 × 25 ml titanium angle-head rotor at 2°C. The pelleted ribosomes were resuspended in 0.5 ml of TKM buffer containing 0.25M-sucrose, and the suspension was clarified by centrifugation at 10000g (r_{av} , 7.9 cm) for 10 min at 2°C.

To confirm that the particles isolated were monomeric ribosomes, a sample (0.05 ml) of the resus-

pended monomers was diluted to 0.2 ml with 0.25M-sucrose in TKM buffer and layered over 12 ml of a 15–26% (w/w) isokinetic gradient (Noll, 1967). The particles were centrifuged at 4°C for 14 h at 25000 rev./min ($\omega^2 t = 35 \times 10^{10}$) in an MSE 6 × 15 ml swing-out rotor. The remainder of the sample was diluted to 4 ml with 0.25M-sucrose in TKM buffer, and then 6 ml of 0.25M-sucrose solution containing 150 mM-KCl and 50 mM-Tris-HCl (pH 7.8 at 25°C) was added to give a final ionic concentration of 2 mM-Mg²⁺ and 100 mM-K⁺.

The resulting suspension of monomers (10 ml, containing approx. 0.5 mg of RNA) was centrifuged through a 15–45% (w/w) sucrose gradient in the BXIV zonal rotor. The sucrose gradient contained 1 mM-MgSO₄, 150 mM-KCl and 50 mM-Tris-HCl (pH 7.8 at 25°C), and the sample was overlaid with 100 ml of 0.125M-sucrose in the same buffer. Centrifugation was for 8 h at 7°C and 30000 rev./min ($\omega^2 t = 28 \times 10^{10}$).

Fig. 1. Separation of ribosomal particles for measurement of specific radioactivity of RNA in native subunits, monomers and polyribosomes

Postmitochondrial supernatant fractions prepared from livers of (a) fed and (b) starved mice were centrifuged through concave 14–50% (w/w) sucrose gradients as described in the Experimental section. Hatched areas indicate fractions collected for analysis of native subunits (nS), monomers (M) and polyribosomes (P).

Measurement of radioactivity in double-isotope experiments. Ribosomes were collected by filtration through Millipore filters (HAWP 02500). The filters were washed three times with buffer of the composition used in the gradients from which the particles had been taken, and then the damp filters were dissolved in 10 ml of Bray's (1960) scintillation medium. ^3H and ^{14}C radioactivity were measured simultaneously by using two channels of a Packard Tri-Carb model 3375 liquid-scintillation counter. In one channel, ^{14}C was counted with an efficiency of 50%, and in the other channel ^3H was counted with an efficiency of 35% and ^{14}C with an efficiency of 30%. A Fortran program written for a C.D.C. 6600 computer was used to calculate d.p.m. for each radioisotope.

Measurement of the specific radioactivity of RNA in native subunits, monomers and polyribosomes after injection of [^3H]orotic acid. The method used for measuring the specific radioactivity of RNA in different types of ribosome was similar in two series of experiments.

The first series of experiments was designed to measure specific radioactivity of RNA in fed and starved mice 24 h after injection of precursor. Six groups, each consisting of three mice, were injected with [^3H]orotic acid at 10:00 h and then starved for 18 h, commencing at 16:00 h. Another six groups, each consisting of three mice, were also injected with [^3H]orotic acid, but then fed *ad libitum*. The mice were killed 24 h after injection of orotic acid and the livers from animals in each group were pooled.

A second series of experiments was performed to investigate the effects of starvation on the rate of orotic acid incorporation into ribosomes. Food was withdrawn at 16:00 h from the mice which were to be starved, whereas the control groups were fed *ad libitum*. The animals were injected with orotic acid at 10:00 h the next day, and groups of six mice (three fed and three starved) were killed 1, 2, 4 or 6 h later.

Postmitochondrial supernatant fractions were prepared and treated with α -amylase as described above. Triton X-100 (10%, v/v, in TKM buffer) was added to portions (0.4 ml) of these fractions to a final concentration of 1%, and they were then layered over 10.8 ml of 14–50% (w/w) concave sucrose gradients. The gradients were formed over 0.5 ml of a cushion of 50% (w/w) sucrose by running TKM buffer into a mixing chamber containing 8.5 ml of 50% (w/w) sucrose in TKM buffer. These concave gradients were designed specifically to separate native subunits from 'soluble' proteins without, at the same time, causing the larger polyribosomes to reach the ends of the tubes. After centrifugation for 6 h 30 min at 4°C in an MSE 6×15 ml titanium swing-out rotor at 35000 rev./min ($\omega^2 t = 32 \times 10^{10}$), optical transmittance at 254 nm was measured as the gradients were displaced upwards through an LKB Uvicord II spectrophotometer.

Fractions (1–3 ml) were collected which contained native subunits, monomers and polyribosomes as shown in Fig. 1. Portions (0.9 ml) of each fraction were then taken for measurement of RNA by a method based on that of Scott *et al.* (1956). The RNA was precipitated, washed with 0.2 M-HClO₄ and then hydrolysed to nucleotides by incubation in 2.5 ml of 1 M-KOH at 37°C for 1 h. Proteins were then precipitated by addition of 0.5 ml of 6 M-HCl and the E_{260} of the supernatant was measured. A portion (1 ml) of this supernatant was mixed with Hyamine hydroxide (0.2 ml). After addition of 15 ml of Bray's (1960) scintillation medium, radioactivity of the nucleotides was measured.

Results

Population of hepatic ribosomes in livers of fed and starved mice

Amino acid incorporation by polyribosomes. The distribution of radioactive nascent polypeptides attached to hepatic polyribosomes is not grossly altered when mice are starved overnight (Fig. 2a). In both fed and starved mice the amount of radioactivity incorporated increases with the size of polyribosome, but the heavy polyribosomes of starved animals incorporate relatively more radioactivity than do those from animals fed *ad libitum*. The differences are slight, but consistent in all fractions containing heavy polyribosomes. The relative incorporation of radioactive leucine by monomers is the same in both groups of animals.

Size distribution of polyribosomes. The distribution of radioactively labelled RNA in hepatic polyribosomes shows a relative accumulation of monomers and a decreased proportion of heavy polyribosomes in livers of starved mice (Fig. 2b). The proportion of lighter polyribosomes is the same in both groups of animals. After centrifugation for 200 min, the resolution between native subunits and monomers is not sufficient to show clearly whether or not there is an increase in the number of native subunits in starved animals.

Comparison of these two experiments, each of which gave similar results when the isotopic labels were reversed, indicates that the increased proportion of monomers in starved mice results from accumulation of single ribosomes that are free of nascent polypeptide. These results make it most unlikely that the changes observed could be produced by the action of RNAase.

Proportions of native subunits and monomers. By prolonging the time of centrifugation to a total of 6 h, a clear separation was obtained between native subunits and monomers (Fig. 3). A cushion of 50% (w/w) sucrose was used to prevent loss of heavy polyribo-

Fig. 2. Sucrose-density-gradient analysis showing the distribution of (a) radioactively labelled nascent polypeptides attached to polyribosomes and (b) radioactively labelled RNA of ribosomes

Rate-zonal centrifugation of postmitochondrial supernatant fractions was for 3 h 20 min at 30000 rev./min in a BXIV zonal rotor, as described in the Experimental section. (a) Fed mice injected with [^3H]leucine ($\circ$); starved mice injected with [^{14}C]leucine ($\bullet$). (b) Fed mice injected with [^3H]orotic acid ($\circ$); starved mice injected with [^{14}C]orotic acid ($\bullet$). Individual points are presented as a percentage of total d.p.m. in the gradients, which were: (a) ^3H , 65 376, ^{14}C , 9359; and (b) ^3H , 904 015, ^{14}C , 92 435. nS, Native subunits; M, monomers and P, polyribosomes. ----, % transmittance at 254 nm.

somes on the outer wall of the rotor, complete recovery of all polyribosomes being essential for calculating the percentage of particles present in each class. Results similar to those shown in Fig. 3 were obtained in three separate experiments, in one of which the isotopic labels were reversed. These experiments show that although starvation provokes accumulation of monomers the proportion of native subunits is unaltered.

Composition of the monomer fraction in livers of fed and starved mice

Although single ribosomes and monoribosomes have not been resolved in the experiments described, it should be possible to distinguish these particles by selectively dissociating the single ribosome component of the monomer fraction by use of appropriate ionic conditions. Lawford (1969) has shown that the 'run-off' ribosomes that accumulate in the presence

Fig. 3. Sucrose-density-gradient centrifugation for separation of the monomer fraction from native subunits and polyribosomes

Rate-zonal centrifugation of a postmitochondrial supernatant fraction was for 6 h at 30000 rev./min in a BXIV zonal rotor, as described in the Experimental section. Fed mice were injected with [^3H]orotic acid (○), starved mice with [^{14}C]orotic acid (●). Individual points are presented as a percentage of total d.p.m. in the gradient, which were: ^3H , 65376; ^{14}C , 9359. nS, native subunits; M, monomers and P, polyribosomes. ----, % transmittance at 254 nm.

of puromycin can be split into derived subunits by low concentrations of Mg^{2+} , but the monoribosomes obtained by treating polyribosomes with RNAase are resistant to this treatment. To determine whether such preferential dissociation of single ribosomes could be used to determine the composition of monomer fractions, four fed mice were injected with [^3H]leucine and four starved mice were injected with [^{14}C]leucine to label nascent polypeptides. Livers from the two groups were pooled, and monomers were collected from appropriate fractions after centrifugation of the postmitochondrial supernatant fraction for 6 h (as in Fig. 3). Analysis of the isolated monomers by centrifugation in an isokinetic gradient (see the Experimental section) showed that these particles sedimented in 5 mM- Mg^{2+} as a single peak with a sedimentation coefficient of 80S. When the monomers were resuspended and centrifuged through a sucrose gradient containing 1 mM- Mg^{2+} and 150 mM- K^+ , however, approx. 70% of the particles dissociated. At least 80% of the ^3H and ^{14}C radioactivity remained attached to undissociated particles (Fig. 4a), so the treatment employed causes preferential dissociation of single ribosomes that are free of nascent polypeptides, and the monomer fractions from both groups of mice include monoribosomes

that are resistant to dissociation. Monomers were then isolated from portions of the appropriate fractions in the double-labelling experiments designed to measure proportions of monomers and native subunits (Fig. 3). In three experiments, treatment of the monomers as described resulted in a mean of 59% of the monomers derived from fed animals and 72% of the monomers derived from starved mice dissociating into subunits. One of these experiments is shown in Fig. 4(b). These results are in accord with the prediction that the increased number of monomers in livers of starved mice results from accumulation of single ribosomes.

Calculation of the percentage distribution of ribosomal particles in livers of fed and starved mice

By calculating the total amount of radioactively labelled RNA in different regions of gradients (Fig. 3), the percentage of native subunits, monomers and polyribosomes in livers of fed and starved mice may be measured (Table 1). From the percentage of monomers (Table 1) and the percentage dissociation of this fraction (assuming that 1 mM- Mg^{2+} and 150 mM- K^+ provoke 100% dissociation of single ribosomes, without affecting the integrity of mono-

Fig. 4. Sucrose-density-gradient analysis of monomer fractions subjected to 1 mM- Mg^{2+} and 150 mM- K^+ , showing the distribution of (a) radioactively labelled nascent polypeptides, and (b) radioactively labelled RNA of ribosomes

Rate-zonal centrifugation of monomer fractions was for 14 h at 30000 rev./min, as described in the Experimental section. Monomers were isolated from mice (a) after fed mice had been injected with [3H]leucine (○), starved mice with [^{14}C]leucine (●), and (b) after fed mice had been injected with [3H]orotic acid (○), starved mice with [^{14}C]orotic acid (●). Individual points are presented as a percentage of total d.p.m. in the gradients, which were: (a) 3H , 2865; ^{14}C , 455; and (b) 3H , 46817; ^{14}C , 19827. dS, derived subunits; M, monomers. ----, % transmittance at 254 nm.

Table 1. *Composition of the ribosomal population in livers of mice starved or fed ad libitum*

Results are the means ($\pm$ S.E.M.) of percentages obtained in three separate experiments of the type shown in Fig. 3. Values in parentheses are percentages after correction for differences in specific radioactivity between the three types of ribosomal particle (Table 3). *P* indicates the degree of statistically significant difference between percentages of particles in fed and starved mice.

Ribosomal particles	Fed mice (%)	Starved mice (%)
Native subunits	9.1 $\pm$ 0.6 (11.2)	9.2 $\pm$ 0.6 (10.4)
Monomers	9.5 $\pm$ 0.9 (12.0)	15.2 $\pm$ 0.1 (17.1) (<i>P</i> < 0.01)
Polyribosomes	81.4 $\pm$ 1.5 (76.8)	75.6 $\pm$ 0.6 (72.5) (<i>P</i> < 0.05)

Table 2. *Percentage of single ribosomes and monoribosomes in ribosomal populations derived from livers of mice starved or fed ad libitum*

Results are the means ($\pm$ S.E.M.) of percentages obtained in three separate experiments of the type shown in Figs. 3 and 4. Values in parentheses are percentages after correction for differences in specific radioactivity between different types of ribosomal particle (Table 3), assuming that the specific radioactivity of monoribosomes is equal to that of polyribosomes. *P* indicates the degree of statistically significant difference between percentages of particles in fed and starved mice.

Ribosomal particles	Fed mice (%)	Starved mice (%)
Single ribosomes	5.8 $\pm$ 0.6 (8.3)	10.9 $\pm$ 0.5 (13.2) (<i>P</i> < 0.01)
Monoribosomes	3.7 $\pm$ 0.6 (3.7)	4.3 $\pm$ 0.6 (4.0)

Table 3. *Specific radioactivity of RNA in ribosomal particles of fed and starved mice 24h after administration of [³H]orotic acid*

Results are means ($\pm$ S.E.M.) from six experiments in which the pooled livers of three mice were used.

Ribosomal particles	10 ⁻³ $\times$ Specific radioactivity (d.p.m./mg. of RNA)	
	Fed mice	Starved mice
Native subunits	397.8 $\pm$ 75.0	443.4 $\pm$ 90.0
Monomers	407.1 $\pm$ 89.4	431.4 $\pm$ 83.4
Polyribosomes	531.9 $\pm$ 104.4	530.1 $\pm$ 110.4

ribosomes), the percentages of single ribosomes and monoribosomes in livers of fed and starved mice may be measured (Table 2), and it is apparent that the increased proportion of monomers in starved animals may be accounted for entirely by an accumulation of single ribosomes.

These measurements, however, depend on the assumption that the specific radioactivity of all ribosomes is equal. Measurement of specific radioactivity 24h after injection of [³H]orotic acid (Table 3) showed that in both groups of animals, the specific radioactivity of native subunits was similar to that of monomers, but the polyribosomes had a higher specific radioactivity. These differences could be caused by incomplete equilibration of single ribo-

somes with the active ribosome cycle, even after 24h, and by dilution of the native subunits with newly formed particles that have not incorporated radioactivity. Percentages of ribosomes after correction for differences in specific radioactivity are shown in parentheses in Tables 1 and 2. The percentages shown in Table 2 were corrected on the assumption that monoribosomes had the same specific radioactivity as other polyribosomes. Although the percentages of polyribosomes are decreased in both groups of animals after correction, the general conclusions to be drawn from these experiments are not affected. It is possible, however, that there is actually a slight decrease in the proportion of native subunits after starvation.

Fig. 5. Incorporation of [³H]orotic acid into (a) monomers and (b) polyribosomes

Fed (○) and starved (●) mice injected with [³H]orotic acid were killed at different times, and the specific radioactivities of native subunits, monomers and polyribosomes were measured as described in the Experimental section. Incorporation into monomers and polyribosomes is expressed as a percentage of incorporation into native subunits (both d.p.m./mg of RNA). The means (±S.E.M.) of specific radioactivities ($10^{-3} \times$ d.p.m./mg of RNA) of native subunits for groups of six animals were:

Time (h)	Fed mice	Starved mice
1	128.7 ± 34.6	155.7 ± 13.8
2	177.3 ± 30.8	180.2 ± 20.9
4	271.3 ± 77.8	238.3 ± 51.0
6	303.9 ± 71.0	297.5 ± 25.7

Fate of newly formed ribosomes in livers of fed and starved mice

Experiments with cultured mammalian cells (Girard *et al.*, 1965; Joklik & Becker, 1965; Hogan & Korner, 1968b) and chick embryo muscle (Kabat & Rich, 1969) have shown that newly formed ribosomes enter the cytoplasm as native subunits, which then become attached to polyribosomes. The newly formed particles appear in the monomer fraction more slowly, suggesting that the single ribosome component of the latter fraction does not form part of the active ribosome cycle. If starvation increases the proportion of single ribosomes, it might be possible to detect differences in the rate at which newly formed particles equilibrate with polyribosomes and monomers. The specific radioactivity of RNA in native subunits, monomers and polyribosomes of mouse liver was measured at intervals after injection of [³H]orotic acid. In Fig. 5, the specific radioactivities

of monomers and polyribosomes are expressed as a percentage of the specific radioactivity of native subunits, and it is clear that newly formed particles equilibrate with monomers more slowly (Fig. 5a) than they do with polyribosomes (Fig. 5b). Starvation apparently decreases the rate at which newly formed subunits equilibrate with monomers, but it has no effect on their equilibration with polyribosomes.

Discussion

An increased proportion of monomers and lighter polyribosomes, accompanied by a corresponding loss of large polyribosomes, is a feature generally observed with polyribosome sedimentation patterns derived from livers of starved animals (Munro, 1970). These changes may be accounted for by a decreased rate of initiation relative to the rate of elongation (Henshaw *et al.*, 1971), but polyribosome patterns of this type

could equally well be produced by enzymic breakdown of polyribosomes or by a shift in the size distribution of available mRNA to smaller species. It is not possible to distinguish between these possibilities by a simple analysis of polyribosome patterns; an analysis of the accumulating monomers is necessary. If the changes produced by starvation can be shown to result from a decreased rate of initiation, then direct measurement of any changes in the proportion of native subunits should indicate whether it is the supply of these particles that limits the rate of initiation.

Use of the double-labelling techniques described in the present paper should minimize the influence of artifacts, so allowing a more precise comparison of polyribosome sedimentation patterns. These experiments were facilitated by the use of a BXIV zonal rotor, which allowed increased resolution of particles, as well as providing sufficient quantities of material for accurate measurement of radioactivity and further study of particular fractions. The most important advantage of performing experiments in this way is that polyribosomes from two groups of animals may be prepared and analysed under identical conditions, and it is probable that the 'polyribosome breakdown' that is frequently observed in livers of starved animals when the polyribosomes from starved and re-fed animals are prepared separately (Webb *et al.*, 1966; Wunner *et al.*, 1966; Wilson & Hoagland, 1967)

results from degradation of the particles during isolation.

An important criticism of the double-labelling techniques used to analyse ribosome distribution is that the specific radioactivity of RNA is not necessarily equal in each functional group of ribosomes, so that any differences must be taken into account when changes in ribosome distribution are measured. Similar criticisms apply to analysis of polyribosome sedimentation patterns from E_{260} measurements, however, since a number of substances other than RNA are known to contribute to absorption at this wavelength.

The experiments described in this paper clearly show that starvation causes a specific increase in the number of monomers relative to polyribosomes (Fig. 3). Since the accumulating monomers do not incorporate amino acids (Fig. 2a), and since there is an increased proportion of monomers which dissociate when the concentration of Mg^{2+} is decreased (Fig. 4b), these particles are most probably free of peptidyl-tRNA and mRNA, i.e. they are single ribosomes rather than monoribosomes. This conclusion is also supported by a decrease in the rate at which the monomer fraction equilibrates with newly formed ribosomes (Fig. 5b).

Enzymic breakdown of polyribosomes is inevitably accompanied by an increased proportion of monoribosomes, so the absence of any significant increase

Scheme 1. *Functional distribution of ribosomes*

Active ribosomal particles translating mRNA flow through initiation (1) and termination (2) stages, with dissociation factor probably being released during initiation. In conditions of decreased protein synthesis, active single ribosomes might be transferred to a pool of inactivated single ribosomes (3), though this process could be reversed (4) whenever an increased rate of protein synthesis is required.

in this fraction in starved mice (Table 2) makes it most unlikely that the changes observed could result from degradation of polyribosomes during analysis. This observation also argues against the proposed involvement of RNAase and its inhibitor in regulating the integrity of polyribosomes (Kraft & Shortman, 1970; Gavard & de Lamirande, 1972), at least during starvation.

Munro and co-workers (Munro, 1970; Clifford *et al.*, 1972) have postulated that RNAase activity associated with native subunits is responsible for an increased degradation of RNA, as these particles accumulate in conditions of decreased protein synthesis. The results presented in the present paper do not support such a mechanism, since no increase was detected in the native subunit fraction (Fig. 3). Further, assuming that the effects of starvation result from a decreased rate of initiation, the absence of any significant decrease in the proportion of native subunits implies that they are not the rate-limiting factors for initiation.

The reaggregation of hepatic polyribosomes that occurs when starved animals are re-fed is not prevented by actinomycin (Fleck *et al.*, 1965; Staehelin *et al.*, 1967), suggesting that RNA synthesis is not necessary for reaggregation, mRNA having been preserved intact.

The changes observed could most simply be explained by a process of the type that is thought to occur in bacteria (Davis, 1971) and cultured mammalian cells (Hogan & Korner, 1968b) deprived of amino acids or carbon source, as depicted in Scheme 1. A decrease in the rate of initiation (1) relative to the rate of translation would result in release of ribosomal particles (2) exceeding the number of molecules of dissociation factor available for production of native subunits. There would then be no change in the proportion of native subunits, but the excess of particles would accumulate as single ribosomes. In eukaryotic cells, excess of single ribosomes may be inactivated in some way (3), possibly by phosphorylation of ribosomal proteins (Kabat, 1972). When required, these ribosomes could be reactivated (4), and so return to the active ribosome cycle. Although the results shown in Fig. 5 are compatible with the inactivation of some single ribosomes, the lack of any significant change in the proportion of native subunits would suggest that starvation inhibits initiation primarily by decreasing the activity or supply of some other factor required in the process.

If the above interpretation is correct, the spacing between those ribosomes that do remain attached to mRNA in starved animals should be increased, unless some molecules of mRNA are reversibly inactivated. According to Kuff & Roberts (1967), the average length of nascent polypeptide per polyribosomal ribosome should be directly proportional to polyribosome size and to the spacing between ribo-

somes on the mRNA. The relatively higher degree of amino acid incorporation by larger polyribosomes from starved mice (Fig. 2a) could possibly be explained by an increased separation between ribosomes translating some large molecules of mRNA in starved animals. A large increase in spacing of ribosomes on all molecules of mRNA should cause a general shift in the polyribosome pattern to smaller aggregates. Such changes were not observed (Figs. 2b and 3), but this may simply reflect the rather short duration of starvation.

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