Properties of the Inactive Ribosomal Components in Rat Liver and Hepatoma

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1. The relative concentrations of the inactive ribosomal components were compared in normal and regenerating rat liver and in two transplantable rat hepatomas (hepatomas 7800 and 5123D). 2. The size of the ribosomal-subunit pools in normal liver was not significantly affected by partial hepatectomy or neoplasia although, as shown previously, significant changes do occur in the monomer pool. 3. Further, the subunit pools in both liver and hepatoma were not significantly influenced by several treatments that caused dramatic changes in the size of the ribosomal monomer (and dimer) pools. 4. The high concentration of inactive monomers and dimers in the hepatomas appears to arise from limitations at the translational level, since they can be incorporated into pre-existing polyribosomes under the influence of cycloheximide.

A proportion of the ribosomal components in mammalian cells are non-functional *in vivo* and appear to serve as a reserve pool of ribosomes for the formation of polyribosomes. The pool size of the non-functional monomeric (80s) ribosomes in rat liver can be varied through interference with the normal functioning of the polyribosomes by using inhibitors of protein biosynthesis (Staehelin, Wettstein & Noll, 1963; Villa-Trevino, Farber, Staehelin, Wettstein & Noll, 1964; Stewart & Farber, 1967) or by partial hepatectomy (Webb, Blobel & Potter, 1966; Rizzo & Webb, 1968), or by other forms of physiological stress (Webb *et al.* 1966; Drysdale & Munro, 1967). The pool of non-functional ribosomes (e.g. monomers and dimers) is abnormally large in the solid transplantable hepatomas carried intramuscularly (Webb, Blobel, Potter & Morris, 1965; Kwan, Webb & Morris, 1968); a large pool of non-functional monomeric ribosomes has also been observed in other tumours (Penman, Scherrer, Becker & Darnell, 1963; Kuff & Roberts, 1967). The tendency of monomeric ribosomes to dimerize during isolation appears to be a species-specific phenomenon (Reader & Stanners, 1967). Ribosomal subunits with sedimentation constants of approx. 60s and 45s have also been identified in the cytoplasm of mammalian cells (Henshaw, Revel & Hiatt, 1965; Girard,

Latham, Penman & Darnell, 1965; Hogan & Korner, 1968).

The full physiological significance of the monomers and subunits in the mammalian system remains to be established. Translation appears to be initiated in the bacterial system through the sequential addition of the subunits to the messenger RNA (Mangiarotti & Schlessinger, 1967; Nomura & Lowry, 1967; Kaempfer, 1968); both monomers (Baliga, Pronczuk & Munro, 1968) and subunits (Vaughan, Warner & Darnell, 1967; Colombo, Vesco & Baglioni, 1968; Hogan & Korner, 1968) have been proposed as initiators in the mammalian system. Studies *in vivo* (Vaughan *et al.* 1967) suggest that the ribosomal components of the polyribosomes are cycled through the monomer and subunit pools.

Information is lacking about the relative size of the ribosomal-subunit pools in resting, normal proliferating and neoplastic tissue. The rat liver system is well adapted to such a study. In the present investigation a comparative study was made of the size of the inactive pools of ribosomal components (including the subunit pools) in normal, regenerating and neoplastic rat liver; the effect of various inhibitors of protein biosynthesis on these pools was also investigated. These studies were carried out in an attempt to obtain information on

the physiological significance of these pools in mammalian cells and their relationship, if any, to the neoplastic transformation.

MATERIALS AND METHODS

Animals. Normal liver was obtained from male rats (250g.) of the Sprague-Dawley strain. The rats received food and water *ad libitum*; food was withheld approximately 16 hr. before the start of the experiment. Lighting was controlled from 6 a.m. to 6 p.m. The regenerating liver was obtained from rats 22 hr. after partial hepatectomy (Higgins & Anderson, 1931); food was withheld after the operation. Hepatomas 7800 (gen. 40-42) and 5123D (gen. 71-72) were carried intramuscularly in rats of the Buffalo strain (Morris, 1965; Morris & Wagner, 1968).

Cycloheximide and puromycin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) and actinomycin D (Merck, Sharpe and Dohme, Montreal, P.Q., Canada) were administered intraperitoneally. Carbon tetrachloride was given by intubation in an equal volume of mineral oil (Webb *et al.* 1966).

Homogenization of tissues. The normal or regenerating livers, or hepatomas 5123D or 7800, were homogenized in 0.25M-sucrose (1g./2ml.) in buffer (0.05M-tris-HCl-0.025M-KCl-0.005M-MgCl₂, pH 7.5) with a Potter-Elvehjem homogenizer fitted with a Teflon pestle; a clearance between the pestle and the homogenizing vessel was selected to give approx. 85% cell breakage (Kwan *et al.* 1968). The concentration of the cytoplasmic proteins in the post-microsomal supernatant (i.e. after centrifuging at 100000g for 90 min.) was also determined to confirm that the cell breakage and cytoplasmic volume of the three different types of tissue were similar. The cytoplasmic proteins were precipitated with 0.5M-trichloroacetic acid, then extracted consecutively with aq. 95% (w/v) ethanol and ethanol-ether (1:1, v/v) at 40°, before the measurement of their concentration by the biuret reaction (Gornall, Bardawill & David, 1949), with serum albumin as standard. The concentrations of cytoplasmic protein/g. of normal liver, regenerating liver and hepatoma 5123D and 7800 were measured to be 94.2, 90.3, 87.5 and 89.2mg. respectively.

Pool size of ribosomal components. The liver or hepatoma was chilled immediately after removal, then homogenized as described above. A sample of the postmitochondrial supernatant (i.e. after centrifugation at 10000g for 10 min.) was treated with 1.25% sodium deoxycholate (0.2 vol. of 6.25% stock solution) to obtain the 'total' ribosomal components, or with 0.2 vol. of buffer to obtain only the 'membrane-free' ribosomal components. The relative pool sizes of the inactive ribosomal components (including subunits) were obtained by layering samples of the postmitochondrial supernatant over exponential gradients of 7-37% (w/v) sucrose in the buffer described above, then centrifuging for 18.5 hr. at 50000g_{av} in an SW25 rotor of a Beckman centrifuge; under these conditions most of the polyribosomes sedimented to the bottom of the gradient tube. When it was desired to observe the relative size distribution of the polyribosomes, monomers and dimers, the deoxycholate-treated postmitochondrial supernatant was layered over exponential 10-35% (w/v) sucrose gradients, then centrifuged for 4.5 hr. in an SW25 rotor at 63000g_{av}. The gradients were monitored at 260nm.,

as described by Webb (1967). Duplicate gradients containing equivalent samples of postmitochondrial supernatant from normal and regenerating liver were monitored at 320nm. to obtain an estimate of the contribution of ferritin to the extinction profile (Kwan *et al.* 1968; Rizzo & Webb, 1968). It was unnecessary to apply similar corrections to the corresponding profiles from hepatomas 5123D and 7800 since the concentration of ferritin in these tumours was low. The E_{320} was multiplied by the factor 1.57 to obtain the equivalent E_{260} value (Wilson & Hoagland, 1965).

Labelling of soluble proteins. The degree of inhibition of protein biosynthesis by cycloheximide was measured by measuring the incorporation of L-[¹⁴C]leucine into the soluble cell proteins of hepatomas from control and cycloheximide-treated rats. The L-[¹⁴C]leucine (20 μCi/250g.) was injected intraperitoneally 10 min. before removal of the tumour. Samples of the postmicrosomal supernatant were treated with 0.5M-NaOH for 1 hr. at 25°, then the protein was precipitated with 1.0M-trichloroacetic acid. The pellet was extracted consecutively with 95% ethanol and ethanol-ether (1:1, v/v) at 40°. The residual protein from one sample was dissolved in NCS solubilizer (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.), then counted in toluene-2,5-diphenyloxazole scintillant at an efficiency of 85%. The protein content of the residue from a second sample was measured by the biuret reaction (Gornall *et al.* 1949).

RESULTS

Normal and regenerating rat liver. The size-distribution patterns in Fig. 1 give an estimate of the relative pool sizes of the inactive ribosomal components in (a) normal adult liver and (b) the 22 hr.-regenerating liver. The identity of the peaks was confirmed by addition of purified ribosomes, before and after dissociation with EDTA, to the deoxycholate-treated postmitochondrial supernatant before separation according to size on density gradients. A comparison of the profiles obtained before and after treatment with deoxycholate suggests that most of the subunits are unattached to the endoplasmic reticulum in normal and regenerating liver. This relationship, which also applies to the hepatomas (see below), greatly simplifies the estimation of the relative concentration of the ribosomal subunits, since the free ribosomal components can be quantitatively extracted from the tissue; a proportion of the membrane-bound ribosomal components may be lost during the preparation of the postmitochondrial supernatant (Blobel & Potter, 1967). As shown previously (Rizzo & Webb, 1968), approx. 70% of the monomers are membrane-free in normal and regenerating rat liver. The monomer peaks in the profiles of the untreated preparations in Fig. 1 are higher than normal, since the monomers are located near the bottom of the gradient after centrifugation for 18.5 hr. and overlap membrane components of the endoplasmic reticulum. The latter account for the higher total extinction in the

Fig. 1. Size distribution of the inactive ribosomal components in the untreated (—) and deoxycholate-treated (---) postmitochondrial supernatant of rat liver. (a) Profiles of components from normal liver; (b) profiles of components from the 22hr.-regenerating liver. The top of the gradient is to the right of the figure. The supernatant was diluted 1.5-fold and 1.0ml. was layered on the gradients. Fractions (1.0ml.) were collected. D, M, S_1 and S_2 indicate the position of the dimer peak, monomer peak, and the 60s and 45s subunit peaks respectively. The distribution profile of ferritin within the gradient is shown in the insert. The size-distribution profiles of the ribosomal components were corrected for the E_{260} of ferritin as described in the Materials and Methods section.

untreated preparation, since the solubilized membrane components remain near the top of the gradient in the deoxycholate-treated preparation. It was shown by Rizzo & Webb (1968) that a proportion of the monomers in the postmitochondrial supernatant are unattached to membranes. This is not apparent from the present patterns, owing to the extensive overlap of the monomer peak by membrane components sedimenting near the bottom of the gradient in the untreated preparation; the profile of the deoxycholate-treated supernatant gives a more reliable estimate of the concentration of monomers in the postmitochondrial supernatant.

A comparison of the patterns in Figs. 1(a) and 1(b) confirms the decrease in the size of the monomer peak, observed by Rizzo & Webb (1968) to occur shortly after partial hepatectomy. However, the results further suggest that there is little or no change in the size of the subunit pools after partial hepatectomy. Although the patterns shown in Fig. 1(b) represent the relative size distribution in

the 22hr.-regenerating liver, no significant change was observed 12hr. or 43hr. after partial hepatectomy.

Hepatomas 7800 and 5123D. Fig. 2 shows the size-distribution patterns of the inactive ribosomal components in the postmitochondrial supernatant of (a) hepatoma 7800 and (b) hepatoma 5123D, before and after treatment of the postmitochondrial supernatant with deoxycholate. The size-distribution patterns of these transplantable hepatomas are characterized by abnormally large pools of monomers and dimers; these components are unattached to membranes and are non-functional *in vivo* (Kwan *et al.* 1968). The estimation of the concentration of inactive ribosomal components in the hepatomas is complicated by the tendency of a proportion of the monomers to complex with one another to form dimers, or with subunits to form complexes that sediment at rates intermediate between those of the monomers and dimers. The complexes between the monomers and the 60s and 45s subunits sediment to give shoulders on the

Fig. 2. Size distribution of the inactive ribosomal components in the untreated (—) and deoxycholate-treated (---) postmitochondrial supernatant of rat hepatomas. (a) Profiles of components from hepatoma 7800; (b) profiles of components from hepatoma 5123D. The corrected heights of the 60s subunit peaks are indicated by bars. A 1.0ml. sample of the diluted supernatant was layered, as for Fig. 1. Fraction size, 1.0ml.

receding limb of the dimer peak and the leading limb of the monomer peak respectively. Only the former peak is prominent in the patterns shown in Fig. 2. When the component that sediments as a shoulder on the trailing edge of the dimer peak was collected, incubated at 37° and then resedimented by the procedure previously used to study the properties of the dimers (Webb & Potter, 1966; Kwan & Webb, 1967), the breakdown products appeared predominantly as monomers and 60s subunits (T. E. Webb, unpublished observations). It seems reasonable to assume that the second complex, which sediments as a shoulder on the monomer peak and which appears less frequently, is composed of a monomer and a 40s subunit. Since a significant proportion of the 60s subunits are complexed with the monomers it was necessary to apply a correction to the height of the 60s peak. This correction was obtained by dividing the area (or height) of the intermediate peak, after correction for the extensive overlap by the adjoining monomer and dimer peaks, by a factor of 2.5. The corrected heights are indicated by bars on the size-distribution patterns. The monomers and dimers

in the postmitochondrial supernatant of the hepatomas were shown by Webb & Potter (1966) and Kwan *et al.* (1968) to be unattached to membranes. The present results also suggest that most of the subunits are unattached. Furthermore, the size of the subunit pools in the two hepatocellular carcinomas are of the same order as in the normal and regenerating liver.

Experiments were designed to determine whether most of the inactive monomers and dimers in the hepatomas were competent for protein synthesis *in vivo*. Attempts were made to force the monomers and dimers in the hepatoma on to messenger RNA by use of cycloheximide. Low concentrations of this drug have been shown to promote the attachment of inactive ribosomes in hamster embryo cells to messenger RNA (Stanners, 1966). Fig. 3(a) shows the size-distribution pattern of inactive ribosomal components of hepatoma 7800, 30min. and 150min. after the administration of 1.0mg. of cycloheximide/kg. A comparison of these patterns with the control patterns in Fig. 2(a) indicates that there is a marked decrease in the size of the monomer and dimer peaks, with the greatest effect at 30min.;

Fig. 3. Size distribution of the inactive ribosomal components in the deoxycholate-treated postmitochondrial supernatant. (a) Profiles of components from hepatoma 7800 after treatment of the rats with 1 mg. of cycloheximide/kg. for 30 min. (—) and for 150 min. (---); (b) profiles of components from hepatoma 5123D after treatment of the rats with 1 mg. of cycloheximide/kg. for 15 min. (---), and after pretreatment for 5 min. with actinomycin D (1 mg./kg.) followed by treatment for 30 min. with 1 mg. of cycloheximide/kg. (—). Other conditions were as for Fig. 1. Bars indicate corrected heights of subunit peaks.

the concentration of the monomers has begun to increase after treatment for 60 min. The two small peaks that sediment as shoulders on the monomer and dimer peaks represent complexes between the monomers and the 45s and 60s subunits respectively. It appears that a decrease in the size of the monomer and dimer pools results in a corresponding breakdown of these complexes. However, no marked change occurred in the absolute concentration of the subunits during cycloheximide treatment.

It was shown in separate experiments (see the Materials and Methods section) that 1.0 mg. of cycloheximide/kg. inhibited the incorporation of L-[^{14}C]leucine into the soluble proteins of the tumours by 90–95% within 5 min. after administration; this degree of inhibition was maintained for several hours. Thus cycloheximide reaches effective concentrations in the tissues very shortly after administration. On the other hand, the decrease in size of the monomer–dimer pool in the tumours is clearly a time-dependent process. The size-distribution patterns shown in Fig. 3(b) indicate

that there is only a small decrease in the concentration of monomers and dimers in hepatoma 5123D 15 min. after the administration of 1 mg. of cycloheximide/kg. (cf. Fig. 2b). Similarly there was no observable decrease in the concentration of monomers and dimers in hepatoma 7800 10 min. after the administration of the drug (T. E. Webb, unpublished results). There appears to be a maximum decrease in the size of the monomer and dimer pools of hepatoma 5123D approx. 30 min. after the administration of cycloheximide.

The results in Fig. 3(b) show that the depression of the monomer–dimer pool size by cycloheximide does not require DNA-dependent RNA synthesis, since it occurs in rats pretreated for 5 min. with 1 mg. of actinomycin D/kg. This concentration of actinomycin D has been shown to cause almost complete inhibition of RNA synthesis in rat liver immediately after administration (Goldblatt, Sullivan & Farber, 1969). Therefore the phenomenon does not require new messenger RNA synthesis. That cycloheximide causes the incorporation of at least a proportion of the ribosomes

in the monomer-dimer pool into pre-existing polyribosome structures is suggested by the results in Fig. 4. Within 45 min. after the administration of 1.0 mg. of cycloheximide/kg. there is a shift in the mean polyribosome-size distribution to the heavier species, which is concurrent with the reduction in the size of the monomer-dimer pool.

Table 1 presents a summary of the effects of various treatments *in vivo* and *in vitro* on the size of the monomer-dimer and 60s and 45s subunit pools in normal and neoplastic rat liver. The

marked increases in the monomer-dimer pool size in these tissues after the administration of carbon tetrachloride or puromycin is known to result from polyribosome breakdown (Villa-Trevino *et al.* 1964; Webb *et al.* 1966). However, none of the treatments tried, with the exception of carbon tetrachloride, caused significant changes in the size of the subunit pools. It appears that the sizes of the latter pools remain constant under a number of physiological conditions.

DISCUSSION

It seems probable that the non-functional ribosomal components serve as a reserve pool from which ribosomes or subunits may be withdrawn for translation of messenger RNA, and into which the same components may enter on termination of translation (see the introduction). Although such a scheme is compatible with the structure and function of the membrane-free polyribosomes, it seems inapplicable to the bound polyribosomes, since the ribosome appears to be fixed to the membrane (Sabatini, Tashiro & Palade, 1966).

The possibility that the inactive monomers are also involved in the control of ribosome synthesis has been supported by the results of studies on the bacterial (Morris & DeMoss, 1966) and mammalian (Rizzo & Webb, 1968) systems. With the latter, a linear and inverse relationship was established between the size of the monomer pool and the rate of ribosome synthesis in the regenerating rat liver. It was concluded that the two parameters were directly or indirectly coupled. However, the possibility that a similar relationship exists between

Fig. 4. Size-distribution pattern of the ribosomal components of hepatoma 7800 before (----) and after (—) treatment of the rats with 1 mg. of cycloheximide/kg. 45 min. before removal of the tumour. A 0.6 ml. sample of the postmitochondrial supernatant was layered over the gradients. Other conditions were as for Fig. 1.

Table 1. *Effect of various treatments on the pool size of the inactive ribosomal components in liver and hepatoma*

The results are based on liver from normal rats and on hepatoma 7800. Cycloheximide was administered intraperitoneally at a dose of 1 mg./kg. Puromycin (100 mg./kg.) was injected intraperitoneally 30 min. before the animals were killed. Carbon tetrachloride in mineral oil (1:1, v/v) was incubated at a dose of 1.2 ml. of the mixture/100 g. 1 hr. before the animals were killed. For the incubations *in vitro*, either the tissue was kept at 4° for 15 min. before homogenization, or the postmitochondrial supernatant (PMS) was incubated at 37° for 3 min. in the absence of an added energy source. Similar results were obtained whether the corrected areas or the heights of the peaks were used as a basis for calculation. M-D refers to the monomer-dimer peak.

Treatment	Ribosomes.....	Liver (% of control)			Hepatoma (% of control)		
		M-D	60s	45s	M-D	60s	45s
<i>In vivo</i>							
Cycloheximide							
10 min.		98	99	99	90	90	100
30 min.		—	—	—	47	98	112
Puromycin		250	108	115	235	106	100
Carbon tetrachloride		310	200	220	180	188	210
<i>In vitro</i>							
Incubation of excised tissue at 4°		93	112	88	87	130	78
Incubation of PMS at 37°		103	100	88	106	104	106

the size of the ribosomal subunit pools and the rate of ribosome synthesis was not investigated. Possible involvement of the subunits in the feedback control of ribosome synthesis appears unlikely since the results of the present study indicate that the sizes of the subunit pools are not significantly affected by several treatments known to cause marked changes in the rate of ribosome synthesis (Rizzo & Webb, 1968). However, the possibility that only minor fluctuations in the subunit pools are sufficient to serve as a signal for feedback control cannot be ruled out. This apparent constancy of the sizes of the subunit pools appears to apply to other cells. For example, the number of ribosomal subunits in Landschutz ascites cells remains constant during dramatic changes in the relative proportions of monomers and polyribosomes in response to changes in the availability of amino acids (Hogan & Korner, 1968). The concentration of ribosomal subunits also remains constant in *Azotobacter vinelandii* during exponential growth and during the shift to stationary phase (Oppenheim, Scheinkbuks, Biava & Marcus, 1968).

The observation that the abnormally high concentration of inactive monomers and dimers are present in the cell extracts of the solid transplantable hepatomas from animals treated with cycloheximide for 10 min. rules out the possibility that these components arise by ribosomal run-off during preparation. Since protein synthesis is inhibited within 5 min., the polyribosomes would be 'frozen' in these cells (Colombo, Felicetti & Baglioni, 1966). The results further indicate, however, that these monomers and dimers are normal and are not derived from dying cells insofar as they will attach to messenger RNA (apparently already in polyribosome structures) after longer treatment with cycloheximide. The insensitivity to actinomycin D of this change confirms that new messenger RNA synthesis is not involved. The ability of cycloheximide to transfer the inactive ribosomal components of Syrian hamster embryo cells into polyribosomes was studied by Stanners (1966). This investigator concluded that the shift was due to a decrease in the rate of movement of the ribosome along the messenger RNA. Such a mechanism probably accounts for the present results.

The concentration of inactive ribosomes in certain ascites-tumour cells is low when they are grown in highly nutrient medium (Hogan & Korner, 1968; Plagemann, 1968) whereas the concentration of these components is relatively high when these tumours are carried in the animal (Webb & Potter, 1966; Hogan & Korner, 1968). The present results suggest that this difference is due to a decreased efficiency of translation of

messenger RNA, rather than to a lack of messenger RNA or to the presence of defective ribosomes in these cells. A deficiency of a factor or factors required for the initiation of translation in the solid hepatomas might be a consequence of a limited blood supply, or of the fact that a large proportion of the cells are at a particular phase of the cell cycle.

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