Physiology of Rat-Liver Polysomes

THE STABILITY OF MESSENGER RIBONUCLEIC ACID AND RIBOSOMES

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Starvation of rats for several days led to marked decrease in cytoplasmic polysomes and accumulation of breakdown products having *S* values less than 200S. Re-feeding of the starved animals induced a rapid reassembly of polysomes. These newly formed polysomes, in the presence of actinomycin D, decayed in a biphasic fashion: about two-thirds decayed with an apparent half-life of 3–3½ hr. but the other one-third were much more stable. Evidence that polysome decay is an accurate reflexion of messenger RNA stability is presented, and it is concluded that in the presence of large doses of actinomycin D, rat-liver cytoplasm contains messenger RNA classes of widely varying stability, the more stable class having a half-life of at least 80 hr. The half-life of liver ribosomes was also determined and was found to be 110–127 hr.

The adult rat liver is a vigorous protein-synthesizing machine, producing protein both for internal use and for export. This divided function makes it a particularly useful tissue for the study of polysome function. Experiments presented in this paper have made use of starvation followed by re-feeding as a means of producing a synchronized population of cytoplasmic polysomes, whose fate and synthetic specialization may be examined. In the present paper we discuss ribosome and polysome stability. In the following paper (Wilson, Hill & Hoagland, 1967) the qualitative aspects of protein synthesis by polysomes are considered.

MATERIALS

[6-¹⁴C]Orotic acid hydrate and L-[¹⁴C]leucine were obtained from New England Nuclear Corp., Boston, Mass., U.S.A.; inorganic [³²P]orthophosphate was from IsoServe Inc., Boston, Mass., U.S.A. Labelled compounds were introduced by intraperitoneal injection. Female Sprague-Dawley rats weighing 150–300 g. were obtained from the Charles River Breeding Laboratories, Wilmington, Mass., U.S.A. They were maintained on Purina Laboratory Chow and water *ad libitum* unless otherwise indicated. Sodium deoxycholate and sodium dodecyl sulphate (from Mann Research Laboratories Inc., New York, N.Y., U.S.A., and Merck, Sharp and Dohme, Rahway, N.J., U.S.A., respectively) were freshly dissolved in water before each procedure. Actidione (cycloheximide) was a gift from Dr M. Lubin. Puromycin hydrochloride and [¹⁴C]polyuridylic acid were gifts from Dr J. Davies. Recrystallized enzymes were purchased from the Worthington Biochemical Corp., Freehold, N.J., U.S.A. Actinomycin D was a gift from Dr

E. Modest. It was dissolved in 95% (v/v) ethanol–0.9% NaCl (1:1, v/v) at a concentration of 0.5 mg./ml. shortly before intraperitoneal injection.

METHODS

Chemical analysis. RNA was estimated by modifications of the Schmidt & Tannhauser (1945) and Scott, Fraccastore & Taft (1956) procedures suggested by Munro and co-workers (Hallinan, Fleck & Munro, 1963; Fleck & Munro, 1962; Hutchinson, Downie & Munro, 1962). Samples of tissue fractions adjusted to contain less than 1 mg. of RNA were precipitated with cold 5% (w/v) trichloroacetic acid or 0.5 N-HClO₄ and immediately centrifuged. The precipitates were washed twice with the same concentration of acid and the final pellet was carefully drained of acid and hydrolysed in 2.5 ml. of 0.5 N-KOH for 1 hr. at 37°. Protein, DNA and other acid-insoluble material were then precipitated with 0.5 ml. of 3 N-HCl. After centrifugation at 15000g for 30 min. at 0° the supernatant was diluted and assayed for ultraviolet absorption as usual. The *E*₂₆₀/*E*₂₈₀ ratio of the supernatant was 1.3–1.35 and absorption at 320 mμ was negligible. An extinction coefficient of 34.2 *E* units/mg./ml./cm. was used for hydrolysed RNA. According to this procedure our best estimates of total liver RNA are approximately 7.6 mg./g. wet wt. of liver, in contrast with the value of 9.0 ± 0.5 mg./g. that we previously reported (Wilson & Hoagland, 1965). The false high reading was due to turbidity of acid-insoluble material remaining in the supernatant after short periods of centrifugation, and to the excessive solubilization of protein material by N-KOH (Fleck & Munro, 1962). Estimates of total RNA in ribosomes were not altered by the above modifications.

DNA was estimated by the diphenylamine reaction by the method of Burton (1956) except that N-HClO₄ was used to hydrolyse DNA in acid-washed samples. Extraction was

carried out twice at 70° for 20 min. and calf-thymus DNA served as standard. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as standard. Phospholipid was extracted by the procedure of Marinetti, Erbland & Stotz (1958). Phosphate was estimated by the procedure of Fiske & Subbarow (1925).

Fractionation of tissue. Rats were killed by decapitation and their livers were quickly excised and chilled. All subsequent procedures were carried out as rapidly as possible at 0–2°. The livers were weighed in medium X [0.15 M-sucrose–25 mM-KCl–0.1 M-tris buffer (pH 7.3 at 2°)–8 mM-MgCl₂], minced and very gently homogenized in 2 vol. of medium X as described by Wilson & Hoagland (1965). The homogenate was centrifuged at 15000g for 4.5 min. to sediment unbroken cells, nuclei, cell debris and some mitochondria. Polysomes were isolated from both the 15000g supernatant fraction and 15000g pellet by treatment with 1.5% (w/v) sodium deoxycholate followed by zone centrifugation (Staehelin, Wettstein & Noll, 1963).

RNA was estimated on the resulting pellet and supernatant fractions as described above. All chemical analyses were performed on polysome suspensions after a brief clearing centrifugation. In all of our experiments polysomes were capable of incorporating amino acids *in vitro* (Hoagland, Scornik & Pfefferkorn, 1964) and were ribonuclease-sensitive, i.e. they broke down to disomes and monosomes on treatment with 5 µg. of ribonuclease/ml. at 25° for 3 min. The RNA/non-ferritin protein ratio of the polysome suspensions was consistently 1.0–1.3, and the phospholipid content was 10% by weight.

Isolation of ribosomal RNA and measurement of RNA radioactivity were carried out as described by Wilson & Hoagland (1965). Sucrose-density-gradient sedimentation values were estimated as described by Martin & Ames (1961) with ferritin as an internal sedimentation marker. Estimations were verified by analytical ultracentrifugal analysis, which was performed by Dr K. Schmid. Since we were primarily interested in quantitation of heavy-polysome material throughout these experiments, we did not design our gradient technique for resolution of monosome, disome or trisome peaks. Thus our use of relatively brief, linear gradient centrifugation of large quantities of polysome material (1–2 mg.) coupled with extinction measurements on separate gradient fractions precluded complete resolution of monosomes, disomes and trisomes. When resolution of these smaller units was desired, centrifugation was extended to 2.5–6 hr. with exponential sucrose gradients. In such experiments 0.25–0.5 mg. of polysome material was layered on gradients and the extinction was measured with a Gilford continuous-flow spectrophotometer.

Isolation of 50s and 30s ribosomal sub-units. Ribosome material (2 mg.) suspended in medium X was sedimented by centrifugation at 165000 g_{av} for 2 hr. at 0° in a Spinco model L-2 ultracentrifuge (50 rotor). The pellet was taken up in 1 ml. of 0.15 M-sucrose–10 mM-EDTA–0.1 M-tris (pH 7.8 at 0°)–25 mM-KCl and kept at 0° for 1 hr. The suspension was then layered over a linear 15–35% (w/v) sucrose gradient. The gradient solutions contained in addition to sucrose 5 mM-EDTA, 0.1 M-tris (pH 7.8) and 25 mM-KCl. Centrifugation was carried out in a Spinco model L-2 ultracentrifuge (SW 25.1 rotor) at 25000 rev./min. (53500 g_{av} for 6 hr. at 2°. Extinction measurement and assay of radioactivity were carried out as described by Wilson & Hoagland (1965).

Estimation of protein-synthetic capacity in vivo. Rats were injected with L-[¹⁴C]leucine as described below. Their livers were excised and thoroughly homogenized in 2 vol. of medium X with a tight-fitting Potter–Elvehjem homogenizer (clearance about 0.1 mm.). The 15000g supernatant fraction was prepared as usual. Microsomes were isolated from the 15000g supernatant fraction by centrifugation at 105000 g_{av} for 4 hr. Samples of the 105000g supernatant and microsomal fractions were assayed for content of [¹⁴C]polypeptide by precipitation and washing with 5% trichloroacetic acid. The precipitates were then heated in 5% trichloroacetic acid at 90° for 30 min., cooled and transferred to Millipore filters. The filters were washed with 30 ml. of 5% trichloroacetic acid and 5 ml. of ethanol–ether (3:1, v/v) and were dried. Radioactivity was determined in a Nuclear–Chicago scintillation counter.

Determination of the specific radioactivity of intracellular amino acid pools was carried out with the generous assistance of Dr S. C. Hartman. A 10 ml. sample of 105000g supernatant fraction derived from 5 g. of liver was made 10% (w/v) with respect to trichloroacetic acid. After being stirred briefly at room temperature the mixture was centrifuged at 20000g for 10 min. The supernatant was then extracted ten times with an equal volume of ethyl ether. The resulting aqueous solution was placed under a steady stream of nitrogen for 2 hr. before being diluted with 1 vol. of sodium citrate buffer, pH 2.2, at 22°. Then 3 ml. of the resulting solution was analysed for amino acid content and radioactivity according to the procedure of Moore, Spackman & Stein (1958) in a Beckman model 120 B amino acid analyser fitted with a Nuclear–Chicago continuous-flow scintillation counter. Anthracene was used as scintillator. In our experiments aspartic acid, glutamic acid, serine, glycine and alanine, as well as leucine and isoleucine, were appreciably labelled.

RNA base-ratio analysis. Rats that had been starved for 5 days were injected with actinomycin D (0.45 mg./kg. or 0.50 mg./kg.). Then 3 hr. later they were injected with 1 mc of [³²P]orthophosphate. After 8–10 hr. of re-feeding the rats were killed and liver polysomes isolated as described above. Polysomal RNA was prepared and submitted to sucrose-density-gradient analysis as described. Fractions from the gradients containing ³²P-labelled RNA to be analysed for base ratio were passed through a Sephadex G-25 (fine grade) column to exclude medium X components and contaminating material. The RNA was collected in a volume of 3 ml. and slowly evaporated to dryness. It was then hydrolysed and prepared for chromatography as described by Davidson & Smellie (1952).

Two-dimensional thin-layer chromatography was carried out as described by Randerath & Randerath (1966) with 0.9 M-LiCl followed by 1.35 M-sodium formate buffer, pH 3.4. Mononucleotide spots were cut out and eluted in 0.7 M-MgCl₂–0.02 M-tris buffer (pH 7.0).

RESULTS

Cytoplasmic ribosome content and turnover of ribosomal RNA. Since the studies on polysome structure and function described below involved the use of starvation, re-feeding and actinomycin D treatment of rats, we have determined the effects of these manipulations on the cytoplasmic ribosome

Table 1. *Liver RNA and DNA content after starvation, re-feeding and actinomycin D treatment*

Each value represents the mean of at least ten separate determinations. DNA content/liver remained essentially constant in all rats. Rats were starved for 5 days where indicated and actinomycin D treatment was as follows: 1 mg./kg. first dose, followed by a second dose of 0.9 mg./kg. 15 hr. later. Animals were killed 27 hr. after the first dose.

Source of liver	RNA recovered as		DNA (mg./g. wet wt. of liver)	Ribosome RNA/DNA ratio	Total RNA/DNA ratio	% of total RNA as ribosomes
	Total RNA (mg./g. wet wt. of liver)	ribosomes (mg./g. wet wt. of liver)				
Normal rats	7.6	3.2	3.2	1.0	2.38	42
Rats starved	7.75	3.9	7.0	0.55	1.1	50
Rats starved-re-fed 10-12 hr.	6.65	4.0	3.6	1.1	1.85	60
Rats starved-re-fed 24 hr.	7.5	3.6	3.4	1.03	2.21	48
Rats starved-re-fed 10-12 hr., then actinomycin D-treated	6.9	4.4	5.8	0.76	1.20	64

content of the liver. The results of such analyses are given in Table 1. We have noted previously (Wilson & Hoagland, 1965) the low value for percentage of total RNA in normal liver recovered in ribosomes (column 2). Starvation for 5 days decreased both 'ribosomal' RNA and 'non-ribosomal' RNA (per unit DNA) by about 50% (Table 1, columns 4 and 5), although RNA synthesis was not completely inhibited, as shown by incorporation of radioactive precursors.

On re-feeding, ribosomal RNA (i.e. total RNA recovered from ribosomes) was rapidly restored, but 'non-ribosomal' RNA was replenished more slowly.

Actinomycin D treatment of starved-re-fed rats not only prevented RNA synthesis but resulted in the disappearance of a portion of the RNA synthesized during re-feeding (Table 1). These findings, demonstrating net ribosome loss in the absence of substantial ribosome synthesis, suggested that rat-liver ribosomes turn over at a relatively rapid rate.

Experiments to determine the steady-state half-life of ribosomal RNA were carried out by injection of [^{14}C]orotic acid into normal rats followed by 'chase' injections of unlabelled orotic acid. Ribosomes were then isolated at various times from the 15000g supernatant fraction, and radioactivity of their 50s sub-unit was measured (Fig. 1). This method was more convenient and economical than isolating ribosomal RNA for each time point. Control experiments showed that essentially all the radioactivity in the 50s particle resided in its RNA and that the specific radioactivity of the 30s particle decayed in parallel with the specific radioactivity of the 50s ribosomal particle.

Fig. 1. Decay of specific radioactivity of the 50s ribosomal sub-unit after injection of $5\mu\text{C}$ of [^{14}C]orotic acid ($5\mu\text{C}/\mu\text{mole}$) into 160g. normal rats. Animals were given a 'chase' injection of $25\mu\text{moles}$ of unlabelled orotic acid 2.5 hr. later. Total liver ribosome content changed little during the experiment. Therefore results were plotted as specific radioactivity of the 50s sub-unit. Each point represents the average of two rats. This Figure depicts one of several similar experiments.

Fig. 1 shows that 50s-ribosome specific radioactivity decayed according to first-order kinetics with a half-life of 110-127 hr. It might be argued that this value reflects the turnover of only a minor ribosomal component and that the bulk of the ribosome is much more stable. However, in a similar experiment with starved rats that were re-fed and given [^{14}C]orotic acid concurrently, a half-life of 90-100 hr. was obtained. In this case total liver RNA was labelled to a much greater extent yet the same half-life was obtained.

We therefore conclude that rat-liver ribosomes turn over rapidly in relation to cell generation time, which has been variously reported to be 191–453 days (MacDonald, 1961). This conclusion is in agreement with the work of Loeb, Howell & Tompkins (1965) that appeared after our experiments were largely completed. C. Hirsch & H. Hiatt (personal communication), using a somewhat different approach, have also obtained a value of about 120 hr. for the half-life of the rat-liver ribosome.

Effect of starvation and re-feeding on polyribosome size. Starvation of rats for a short period resulted in a 75–80% decrease in the quantity of heavy-polyosome material (greater than 183s) extractable with deoxycholate. A similar loss of polysomes after prolonged starvation has been noted by Webb, Blobel & Potter (1964). Sucrose density gradients of polysomes from animals starved for various periods are shown in Fig. 2. The broad peak of material resulting from polysome disaggregation, seen in Fig. 2(b), sedimented at approx. 150s. The peak was resolved by further gradient centrifugation and analytical ultracentrifuge analysis into 15% of 183s tetrasomes, 30% of 155s trisomes, 35% of 123s disomes, 5% of 109s particles (Pfuderer, Cammarano, Holladay & Novelli, 1965) and 10% of 83s monosomes. Of this group the tetrasomes and trisomes were ribonuclease-sensitive, i.e. broke down to disomes and monosomes on treatment with 5 μ g. of ribonuclease/ml. for 3 min. at 25°.

Re-feeding of animals starved for 4–5 days led to gradual replenishment of cytoplasmic polysome aggregates in 8–12 hr. (Fig. 2). In this period total liver r-RNA* increased twofold (Table 1) and the quantity of polysomes (i.e. material larger than 183s) increased four- to six-fold. Polysome re-formation did not occur when rats were fed after being treated with actinomycin D (1 mg./kg.), puromycin (250 mg./kg.) or cycloheximide (250 mg./kg.). These findings suggest that both RNA synthesis and protein synthesis were necessary for polysome re-formation.

Polyacrylamide-gel electrophoretic patterns of pulse-labelled proteins (Wilson *et al.* 1967) and assays of total protein-synthetic capacity of livers of starved-re-fed rats closely resembled those of normal rats. Thus, in terms of total and gross qualitative protein-synthetic capacity as well as polysome and ribosome content per cell, the liver of starved-re-fed rats resembled normal liver.

During the period from 10–40 hr. of continuous re-feeding *ad libitum* there was little change in the gradient profiles of cytoplasmic ribosomes.

This type of 'step-down-step-up' experiment thus permitted the synchronization of a complement

* Abbreviations: r-RNA, ribosomal RNA; s-RNA, 'soluble' RNA; m-RNA, messenger RNA.

Fig. 2. Alterations in sucrose-density-gradient profiles of rat-liver polysomes (ribosomes) as a result of starvation and subsequent re-feeding. Ribosomes were isolated from the 15000g supernatant fractions as described in the Methods section. Sucrose-density-gradient (linear 17–34%) centrifugation was carried out for 1.5 hr. at 25000 rev./min. in the Spinco model L-2 ultracentrifuge (SW 25.1 rotor). Corrected E_{260} was obtained from the following formula:

$$\text{Corrected } E_{260} = \text{total } E_{260} - 1.59 E_{320}$$

Extinction of sucrose and medium components was corrected for as described by Wilson & Hoagland (1965). (a) Normal; (b) starved 40 hr.; (c) starved 120 hr.; (d) starved 120 hr., re-fed 12 hr.; (e) starved 120 hr., re-fed 30 hr.

of new cytoplasmic polysomes whose fate could subsequently be followed. Such a condition would theoretically eliminate m-RNA age as a significant variable in studies of polysome stability.

Stability of cytoplasmic polysomes. Rats were

Fig. 3. Mortality of starved-re-fed rats after a single dose of actinomycin D. Rats were starved for 5 days and then fed for 10-12 hr. Actinomycin D was then given in the indicated dosage. Each point for 300g. rats represents the average of three animals. Each point for the smaller rats represents at least 10 animals. The average range for each point was about 15 hr. Body wt. of rats: $\circ$, 150-160g.; $\bullet$, 180-190g.; $\triangle$, 300g.

starved for 5 days, then fed for 10-12 hr. to synchronize cytoplasmic polysome reassembly. Actinomycin D was given. Dosage was adjusted to provide adequate longevity of the animals while stopping essentially all DNA-dependent RNA synthesis (and/or transport to the cytoplasm). Experimental animals were usually selected from larger groups of rats treated with actinomycin D. Accumulated results on the mortality of rats after only a single dose of actinomycin D are shown in Fig. 3. To verify the absence of new m-RNA in polysomes, [^{14}C]orotic acid or $\text{Na}_2\text{H}^{32}\text{PO}_4$ was injected 3.5-4 hr. after actinomycin D treatment was begun, concurrently with subsequent actinomycin D treatment and also 3 hr. before the animals were killed. Harbers & Muller (1962) have shown that conversion of orotic acid into RNA precursors is not affected by actinomycin D. Polysomes isolated from actinomycin D-treated rats contained less than 25% of total radioactivity of control starved-re-fed animals and all of this radioactivity was confined to 4s RNA, as has been noted by others (Moulé & Landin, 1965; Revel & Hiatt, 1964b).

Fig. 4 shows the accumulated results from a large number of experiments on the kinetics of polysome breakdown in the presence of actinomycin D. In these experiments polysome breakdown gave rise to 83s and 123s particles almost exclusively. Thus actinomycin D did not promote shifts from large polysomes to smaller ones, as is the case during starvation, but rather promoted complete conversion of large polysomes into free ribosomes and Mg^{2+} -dependent disomes.

It is clear from Fig. 4 that polysome decay was

Fig. 4. Cellular polysome content of 160g. rats that were starved for 5 days, fed for 10-12 hr. and then treated with actinomycin D. Dosage for points from 1 to 15 hr. was 1.0-1.2 mg./kg. For points from 15 to 30 hr. 1 mg./kg. was given followed at 12-17 hr. by another injection of 0.9 mg./kg. For points beyond 30 hr. 0.9 mg./kg. was given at 12 hr. intervals. Average mortality time for this last group was 30-36 hr. The m-RNA renewal in polysomes extracted from each animal was excluded as described in the text. After single injections of 1 mg. of actinomycin D/kg., new m-RNA appeared in polysomes at 16-22 hr. (Schwartz, Sodergren, Garofalo & Sternberg, 1965) of treatment. The number of animals included in each point is indicated in the Figure. Polysomes from both the 15000g supernatant and pellet fractions were analysed as in Fig. 2. The area of the polysome portion of gradient profiles, arbitrarily defined as the bottom 40% of the gradient, was determined quantitatively by planimetry. This excluded contribution of extinction from polysome breakdown material. The same number of DNA equivalents of extract was placed on each gradient. Polysome breakdown occurred in parallel in the 15000g supernatant and pellet.

biphasic in character. The results best fit a theoretical curve as shown in Fig. 5. This curve is based on the assumption that there were two classes of polysomes, both decaying with first-order kinetics, one (36% of the total) having a half-life of 80 hr., the other (64%) a half-life of 3-3½ hr.

At doses of actinomycin D sufficient to inhibit m-RNA renewal in polysomes it was impossible to obtain points beyond 40 hr. of treatment, because of drug toxicity. Therefore this experiment does not

exclude the existence of a small class of polysomes having a half-life greater than 80 hr.

Relationship between polysome stability and messenger stability. The interpretation of the above experiments depends critically on demonstrating that polysome breakdown after actinomycin D treatment is at least a reasonable reflexion of m-RNA stability. We approached this question in several ways. Polysome breakdown after initiation of

actinomycin D treatment might be due to activation of random cytoplasmic nuclease activity. To test this a small amount of normal liver (10% of wet wt.) containing radioactive polysomes was added, before homogenization, to livers from starved rats and to livers of actinomycin D-treated rats. Extraction of polysomes in the usual manner followed by gradient analysis and measurement of radioactivity showed that the radioactivity profiles were in good agreement with the extinction profile of normal polysomes. This result suggested that random nuclease activity played no role in the observed decay of polysomes.

In another experiment the protein-synthetic capacity of the apparently decaying polysomes was studied *in vivo* by determining the radioactivity of protein in the 15 000g supernatant fraction isolated shortly after injection of L-[¹⁴C]leucine. Such a direct measurement revealed a 40% decrease in amino acid incorporation at 12 hr. of actinomycin D treatment, but only a slight further decrease from 14 to 28 hr. (Table 2). Table 2 also shows that actinomycin D treatment markedly increased the specific radioactivity of the free amino acid pool. If protein radioactivity were corrected for the increase in amino acid pool specific radioactivity, the decrease in protein-synthetic capacity would roughly parallel the decrease in cytoplasmic polysome content.

Such correction for labelling of amino acid pools is important particularly when low doses of ¹⁴C-labelled amino acid are used, as has often been the case in the literature (Revel & Hiatt, 1964a; Guidice & Novelli, 1963). Actinomycin D-treated

Fig. 5. Correspondence of the data presented in Fig. 4 with a theoretical curve (----) derived from an exponentially decaying mixture of 36% of stable polysomes (half-life 80 hr.) and 64% of unstable polysomes (half-life 3.5 hr.) (solid lines).

Table 2. *Protein-synthetic activity in vivo and free amino acid pool specific radioactivity in livers from starved, starved-re-fed and actinomycin D-treated rats*

Rats weighing 130 g. were injected with 2.5 μ C of L-[¹⁴C]leucine (131 μ C/ μ moles)/100 g. at 50 and 35 min. before being killed. Subsequently at 18–20 min. before being killed they were injected with an additional 5 μ C of L-[¹⁴C]leucine/100 g. Assay of trichloroacetic acid-precipitable radioactivity and amino acid pool specific radioactivity were carried out as described in the Methods section. Where indicated animals were starved for 5 days and re-fed for 10–12 hr. Each value represents the mean of the number of experiments shown in parenthesis.

Source of liver	Radioactivity in hot trichloroacetic acid-insoluble material derived from 15 000g supernatant fraction (counts/min./mg. of microsomal RNA)	Specific radioactivity of amino acids in 105 000g supernatant fraction	
		(counts/min./ μ mole of leucine)	(counts/min./ μ mole of total labelled amino acids)
Rats starved	50 350 (3)	1900 (1)	603 (1)
Rats starved-re-fed	38 100 (4)	1046 (1)	346 (1)
Rats starved-re-fed, and actino- mycin D-treated for:			
12 hr.	25 050 (1)		
14.5 hr.	23 906 (3)	1403 (1)	1685 (1)
25 hr.	24 300 (2)		
28 hr.	21 100 (2)		

rats showed no apparent loss of protein-synthetic activity *in vivo* at doses of L-[^{14}C]leucine below $5\mu\text{C}/100\text{g}$. The probable explanation for this is that actinomycin D influences the labelling of the intracellular amino acid pool.

We may conclude from this experiment that there was considerably less translation of m-RNA per cell in actinomycin D-treated rats than in control starved-re-fed rats. Taken together with the observed decrease in polysomes after actinomycin D treatment the results are certainly consistent with (but do not prove) the interpretation that inhibition of m-RNA synthesis results in the net loss of a substantial portion of cytoplasmic m-RNA.

It was further considered that the decay of polysomes in the presence of actinomycin D might be the result of dissociation of ribosomes and messenger without accompanying breakdown of the latter. This possibility is particularly important to consider in the light of recent work in several Laboratories suggesting that spacing of ribosomes on m-RNA might vary under different metabolic conditions (Noll, 1965; Sidransky, Staehelin, & Verney, 1964; Conconi, Bank & Marks, 1966). To investigate this problem we have isolated polysomes containing

radioactive m-RNA and determined the fate of the latter in actinomycin D-treated animals.

It has been shown by Georgiev, Samarina, Lerman, Smirnov & Severtzov (1963), Harel, Harel, Boer, Imbenotte & Carpeni (1964), Muramatsu, Hodnett & Busch (1964) and others that actinomycin D inhibits r-RNA synthesis to a much greater extent than m-RNA or s-RNA synthesis. Thus by administering an intermediate dose of actinomycin D one can inhibit ribosome synthesis without appreciably affecting messenger synthesis. We therefore generated polysomes containing radioactive m-RNA by re-feeding starved rats in the presence of [^{14}C]orotic acid and an intermediate dose of actinomycin D. These conditions allowed complete re-formation of polysomes without concomitant r-RNA synthesis, as measured by the absence of labelled 29s r-RNA (Fig. 6). The only peak of radioactivity other than the 4s peak was found at approx. 17s. When starved rats were fed in the presence of the usual dose of actinomycin D, which is inhibitory to m-RNA synthesis as well as r-RNA synthesis, polysomes did not re-form and the 17s radioactivity peak did not appear.

This was presumptive evidence that the 17s peak was m-RNA. Further characterization of the 17s RNA was carried out by treating polysomes containing radioactive 17s RNA, as prepared above, with ribonuclease ($0.1\mu\text{g}/\text{ml}$). After a short incubation at 37° almost complete breakdown of these polysomes occurred, and on gradient analysis of the RNA of the polysome breakdown material no radioactive 17s RNA was found.

The radioactive 17s RNA was also characterized as m-RNA by base-ratio analysis as described in the Methods section. For this purpose, dosage of actinomycin D was adjusted to allow synthesis of a small quantity of r-RNA as well as 17s m-RNA, as shown in Fig. 7. This was done to permit verification of our technique for analysing m-RNA and r-RNA base ratios. The results of the analysis of 29s and 17s radioactive RNA are shown in Table 3. The base ratio of the ^{32}P -labelled 29s RNA was essentially identical with that of the bulk 29s r-RNA. The base ratio of the ^{32}P -labelled 17s RNA, however, was markedly different from the bulk r-RNA having a $(\text{GMP} + \text{CMP})/(\text{AMP} + \text{UMP})$ ratio approaching that of DNA. When the base ratio of the ^{32}P -labelled 17s RNA was corrected for contamination by ^{32}P -labelled 18s r-RNA, the $(\text{GMP} + \text{CMP})/(\text{AMP} + \text{UMP})$ ratio was almost identical with that of rat-liver DNA. In two other experiments where synthesis of 29s r-RNA was completely inhibited (presumably 18s r-RNA synthesis was completely inhibited also) by actinomycin D the $(\text{GMP} + \text{CMP})/(\text{AMP} + \text{UMP})$ ratio of 17s m-RNA was 0.79 and 0.83.

We may summarize our characterization of the

Fig. 6. Sucrose-density-gradient profile of polysomal RNA isolated from a 150g. starved rat that had been treated with an intermediate dose of actinomycin D ($0.55\text{mg}/\text{kg}$.) 3hr. before re-feeding in the presence of $5\mu\text{C}$ of [^{14}C]orotic acid ($5\mu\text{C}/\mu\text{mole}$). After 8hr. of re-feeding polysomes were isolated from the 15000g supernatant fractions as usual. Polysomes were then taken up in medium R (0.5% sodium dodecyl sulphate in 0.01M -tris buffer, $\text{pH}7.4$ at 37°), incubated at 37° for 4min. and layered directly on a 17–34% linear sucrose gradient that was 0.1M with respect to tris buffer, $\text{pH}7.3$ at 3° . Centrifugation was conducted at $53500g_{\text{av}}$ for 52hr. at 3° . Extinction (○—○) and radioactivity (●—●) were assayed as described in the Methods section.

radioactive 17s RNA as follows: it is a component of cytoplasmic polysomes formed in the presence of an intermediate dose of actinomycin D; it is preferentially attacked by low concentrations of ribonuclease; and it has a base ratio closely resembling that of rat-liver DNA. In addition to these criteria we may also note that DiGirolamo, Henshaw & Hiatt (1964) and Brawerman, Biezunski & Eisenstadt (1965) have shown that cytoplasmic RNA sedimenting at about 18s is more active than

other cytoplasmic RNA fractions in stimulating protein synthesis *in vitro*. We consider that these characteristics are sufficient to characterize the 17s RNA as messenger.

The fate of labelled 17s RNA in rats treated with a full dose of actinomycin D was investigated as follows: rats that had been starved for 5 days were first given an intermediate dose of actinomycin D (0.5mg./kg.). Then 3hr. later they were fed and given [^{14}C]orotic acid. After 8hr. of polysome re-formation, the rats were given a full dose of actinomycin D (sufficient to inhibit further m-RNA synthesis). Then 8hr. after this last injection the rats were killed. The gradient distribution of extinction and radioactivity in liver extracts from these animals, and from animals similarly prepared but not given the final full dose of actinomycin D, is shown in Fig. 8. The specific radioactivity of the larger polysomal material is the same in the two sets of animals, whereas the breakdown material in the actinomycin D-treated animals is substantially lower. Extraction and sucrose-density-gradient analysis of the RNA derived from the breakdown material (83–183s) and the polysome region revealed the profiles shown in Fig. 9. RNA extracted from the polysome region showed the characteristic 17s peak (Fig. 9b), and its specific radioactivity was identical with that of 17s RNA extracted from the control animals not treated with the full inhibitory dose of actinomycin D. RNA extracted from the breakdown material showed no intact 17s RNA, and the specific radioactivity of this material was about 20% that of the heavy-polysomal RNA.

Finally, in the above experiment gradient analysis of the RNA in the cell supernatant fraction (after sedimentation of ribosomes) yielded primarily 4s RNA (Munro & Korner, 1964), indicating that intact 17s m-RNA had not been completely dissociated from ribosomes and preserved in the cytoplasm under the influence of actinomycin D.

Thus, excluding the possibility of extensive ribonuclease action during our preparations of

Fig. 7. Sucrose-density-gradient profile of ^{32}P -labelled polysomal RNA used for base-ratio analysis. Starved rats weighing 150g. were injected with 0.45mg. of actinomycin D/kg. and 2.5hr. later with 1mc of [^{32}P]orthophosphate. Then after 110min. they were re-fed and given an additional 1mc of [^{32}P]orthophosphate. Then 10hr. later they were killed and polysomal RNA was isolated and submitted to gradient analysis as in Fig. 6 (○—○, extinction; ●—●, radioactivity), except that centrifugation was for 37hr. Fractions 5 and 13 were used for base-ratio analysis (Table 3).

Table 3. Base composition of bulk and ^{32}P -labelled rat-liver polysomal RNA of Fig. 7

Type of nucleic acid	Proportions of 2'- or 3'-ribo-nucleotides (moles/100 moles)				GMP + CMP AMP + UMP
	GMP	CMP	AMP	UMP	
Bulk 29s RNA	32.55	35.30	17.0	15.15	2.11
^{32}P -labelled 29s RNA	34.7	32.3	17.0	16.0	2.03
Bulk 18s RNA	31.9	26.5	20.6	21.0	1.415
^{32}P -labelled 17s RNA	26.8	20.3	28.1	24.8	0.892
Corrected ^{32}P -labelled 17s RNA	24.6	20.0	29.6	25.6	0.812
DNA*	23.7	20.7	28.4	26.4	0.817

* Taken from Woodhouse (1954); TMP in DNA is assumed to be equivalent to UMP in RNA.

Fig. 8. Representative sucrose-density-gradient profiles of 15000 g_{av} supernatant polysomes isolated from livers of control (a) and actinomycin D-treated (b) rats containing radioactive m-RNA. Liver polysomes containing radioactive m-RNA were generated as described in Fig. 6. One group of rats were then given actinomycin D (1 mg./kg.) and the other group was injected with 0.1 ml. of 0.9% NaCl. Then 8 hr. later polysomes were isolated from both groups according to the usual procedure. Gradient analysis was conducted as in Fig. 2 (O—O, extinction; ●—●, radioactivity). The areas designated as heavy polysomes and breakdown material were used in the experiments shown in Fig. 9.

RNA, it seems reasonable to conclude that polysome decay after actinomycin D treatment is a reflexion of m-RNA degradation. Whether m-RNA degradation after actinomycin D treatment reflects the catabolic portion of natural turnover is, of course, uncertain.

DISCUSSION

In our experiments ribosomes were characterized, in part, as particles sedimentable through a zone of m-sucrose after exposure to 1.3% sodium deoxycholate. The possibility of r-RNA degradation during this procedure has been reasonably well (but not entirely) excluded (Wilson & Hoagland, 1965; Kuff & Zeigel, 1960; Chauveau, Moulé, Rouiller & Schneebeli, 1962). Under these conditions the portion of normal liver RNA recoverable in ribosomes was consistently less than 55%. The remaining fraction of total RNA was isolated from the supernatant, after deoxycholate treatment, primarily as 4s material.

It is noteworthy that much of the deoxycholate-supernatant RNA comes from fractions rich in membrane. In fact, large quantities of RNA can be extracted from endoplasmic reticular and plasma membranes without the use of detergents (D. Wallach & P. Ziegler, personal communication). This RNA has been the subject of much investigation (Chauveau *et al.* 1962; Moulé & DelHumeau de Ongay, 1964; Moulé, Bouvet & Chauveau, 1963; Moulé, Rouiller & Chauveau, 1960; Shapot & Pitot, 1966), yet little has been learned of its significance.

A significant finding in these studies is that starvation for 5 days decreased liver r-RNA and 'non-ribosomal' RNA to half their normal value (Table 1). On re-feeding the animals for as short a period as 10–12 hr. r-RNA reached the normal level, whereas 'non-ribosomal' RNA was still 55% below normal. This observation suggests that the synthesis of these two kinds of RNA is separately controlled, as has been shown in bacteria (Kjeldgaard & Kurland, 1963; Rosset, Julien & Monier, 1966).

Fig. 9. Sucrose-density-gradient profiles of RNA isolated from polysome gradient fractions as indicated in Fig. 8: (a) heavy polysomes from control rats; (b) heavy polysomes from actinomycin D-treated rats; (c) breakdown material from actinomycin D-treated rats. Gradient fractions were pooled and centrifuged at 165000 g_{av} for 5 hr. Pellets were taken up in medium R and treated as in Fig. 6 (O—O, extinction; ●—● radioactivity), except that sucrose-density-gradient centrifugation was extended for only 25 hr.

The nature of events leading to the disappearance and reappearance of polysomes during starvation and subsequent re-feeding is obscure. Particularly noteworthy is the sluggish reappearance of large polysomes after re-feeding, in view of the quantity and rapidity of RNA synthesized. The possibility of increased nuclease activity has not been ruled out. Also, alterations in attachment mechanisms of ribosomes to m-RNA could cause a lag in formation of large polysomes. The starvation-re-feeding cycle was originally used in these experiments to permit study of the fate of a synchronized population of polysomes, thereby eliminating m-RNA age as a significant variable. In retrospect, having seen the

nature of the polysome decay curve (Fig. 5), it is clear that similar results would be obtained from studies on normal actinomycin D-treated animals where m-RNA of varying age would be present in polysomes.

The possibility of stable liver m-RNA was introduced by Hiatt (1962) and subsequently supported by Revel & Hiatt (1964b) with a series of experiments demonstrating that in actinomycin D-treated rats: (1) protein synthesis in the liver persisted *in vivo*; (2) extracted microsomes retained amino acid-incorporating ability; (3) RNA capable of stimulating amino acid incorporation *in vitro* could be extracted. Though these experiments are certainly suggestive of stable m-RNA they are not conclusive since liver r-RNA might possess stimulatory activity *in vitro* and since correction for possible differences in amino acid pool specific radioactivity were not carried out along with their protein-synthesis experiments *in vivo*.

Studies of rat-liver polysome stability after actinomycin D treatment have also been conducted by Staehelin *et al.* (1963) and Munro, Jackson, & Korner (1964). However, these experiments give no definite indication of stable m-RNA since a precise kinetic evaluation of polysome breakdown was not undertaken.

Relatively-long-lived rat-liver m-RNA has also been suggested by Pitot, Perainco, Lamar & Kennan (1965) from a somewhat different approach. Conversely, most reports seem to indicate a much shorter half-life, 1.75–4 hr., for m-RNA in rat liver (Staehelin *et al.* 1963; Villa-Trevino, Farber, Staehelin, Wettstein & Noll, 1964; Trakatellis, Axelrod & Montjar, 1964; Staehelin, Wettstein, Oura & Noll, 1964). Even though the discrepancy in these reports is difficult to explain, it is likely that all are correct in that there is a wide range of stability among liver m-RNA species. Our work suggests, however, that most liver m-RNA species fall into two large classes with respect to stability in the presence of large doses of actinomycin D.

Our value for the half-life of stable polysomes in the presence of actinomycin D (80 hr.) may be taken as a minimum estimate of the rate of decay of stable m-RNA since the half-life of the ribosome itself (127 hr.) might contribute to the loss of polysomes. If polysome-bound ribosomes and free ribosomes decay at equal rates then m-RNA in the stable polysomes would have a much longer half-life than 80 hr.

Finally, it is of some interest that m-RNA, as characterized in these experiments, sedimented rather homogeneously at about 17S. A similar phenomenon has been observed in [^{14}C]orotic acid pulse-labelling experiments, leading to the suggestion that m-RNA specifically binds to 18S r-RNA (Henshaw, Revel & Hiatt, 1965). However, as

shown in Fig. 6, prolonged centrifugation separated m-RNA from bulk 18s r-RNA. It remains obscure why most m-RNA was found in an apparent single peak. Perhaps this finding is related to a major liver function (albumin synthesis), the messenger of which might theoretically sediment at approx. 17s.

Evidence that qualitatively different proteins are synthesized by polysomes of different life span is presented in the following paper (Wilson *et al.* 1967).

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