Physiology of Rat-Liver Polysomes

PROTEIN SYNTHESIS BY STABLE POLYSOMES

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Certain qualitative aspects of protein synthesis in the livers of starved, starved-re-fed and actinomycin D-treated rats have been examined by polyacrylamide-gel electrophoresis. Animals were exposed to a mixture of ^{14}C -labelled acids for 18–20 min. and killed, and an ultrasonic extract of newly formed protein in microsomal vesicles was prepared and examined by gel electrophoresis. In normal and starved-re-fed animals, 27% of the newly synthesized protein was albumin. During starvation, when RNA synthesis was decreased, the percentage of newly formed protein as albumin rose. After actinomycin D treatment of starved-re-fed rats, when only stable messenger RNA persisted in the cytoplasm, albumin synthesis increased to 63% of the total. This finding suggested that albumin was the primary protein synthesized on stable messenger RNA.

In the preceding paper (Wilson & Hoagland, 1967) evidence was presented that rat liver contains polysomes that differ greatly in stability. In the presence of actinomycin D one group of polysomes, representing about one-third of the total, was relatively long-lived, decaying with a half-life of about 80 hr. In the present paper we give evidence that a predominant protein product of this class of polysomes is albumin.

MATERIALS AND METHODS

Female Sprague-Dawley rats weighing 160–165 g. were maintained as described in the preceding paper (Wilson & Hoagland, 1967). The ^{14}C -labelled amino acid mixture and ^{14}C -labelled algal-protein hydrolysate were obtained from New England Nuclear Corp., Boston, Mass., U.S.A., and neutralized with NaOH before intraperitoneal injection. 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.

Preparation of an ultrasonic extract of microsomes containing labelled protein. Experimental animals were injected with ^{14}C -labelled algal-protein hydrolysate or ^{14}C -labelled amino acid mixture as indicated in the text. Then 18–20 min. later they were killed by decapitation and their livers quickly excised and chilled. All subsequent procedures were carried out at 0–4°. The livers were weighed, minced and homogenized in 1 vol. of medium X [0.15 M-sucrose–0.1 M-tris buffer (pH 7.3 at 2°)–25 mM-KCl–8 mM-MgCl₂]. Homogenization was carried out in a loose-fitting (clearance about 0.25 mm.) Potter-Elvehjem homogenizer, with 6 strokes at 450 rev./min. The homogenate was centrifuged at 15000 g for 4.5 min.

Microsomes were isolated from the 15000 g supernatant

fraction by centrifugation at 105000 g for 4 hr. The pellet was suspended in 1 ml. of medium X with gentle homogenization and treated for 5 min. in a Raytheon model DF101 ultrasonic oscillator set at 150 μA . The resulting suspension was then centrifuged at 105000 g for 4 hr. to sediment fragmented microsomes and debris. The supernatant was decanted and stored frozen, until used for gel electrophoresis.

Essentially no radioactivity was incorporated into hot-acid-insoluble material when animals were exposed to cycloheximide (300 mg./kg.) 25 min. before injection of ^{14}C -labelled amino acid. As indicated in the previous paper (Wilson & Hoagland, 1967) the distribution of amino acids in the free intracellular amino acid pool and their relative specific radioactivities were the same in starved, re-fed and actinomycin D-treated rats, although, as also noted, the total amino acid specific radioactivity varied in the different conditions. Thus it is unlikely that qualitative differences in protein-synthetic activity described below were due to lack of some particular amino acid.

Gel electrophoresis. Disk electrophoresis in polyacrylamide gel was carried out as described by Ornstein (1964) and Davis (1964). The apparatus was obtained from E-C Apparatus Corp., Philadelphia, Pa., U.S.A. It was adapted to hold glass cylinders having an inside diameter of 5 mm. and overall length of 6 cm. Acrylamide, *NN'*-methylenebisacrylamide (Bis) and *NNN'*-tetramethylethylenediamine (Temed) were obtained from the Eastman Chemical Co., Rochester, N.Y., U.S.A. Supernatant fractions containing between 200 and 500 μg . of protein were subjected to electrophoresis for 1.5–2 hr. at 4°. The pH of the tris-glycine buffer system was 9.5. The voltage was held constant throughout at 300 v while amperage fell from about 35 to 20 mA. Migration of extract protein was towards the anode with less than 5% of the radioactivity migrating towards the cathode.

The resulting gel columns were stained with Amido Black, in 50% (v/v) methanol in 10% (v/v) acetic acid (Matheson,

Coleman and Bell, East Rutherford, N.J., U.S.A.) for at least 1 hr., then destained in an E-C Apparatus Co. destainer. In contrast with the Ornstein and Davis destaining procedures, our apparatus passed current at right angles to the direction of the original run. After being destained, each gel column was placed in a glass tube, extruded and sectioned into 1 mm. disks.

Samples were prepared for radioactivity determination by liquid-scintillation counting after dissolving each disk in 0.05 ml. of 30% (w/v) H_2O_2 at 37° for 1.5–2 hr. The digests were then counted in a Nuclear-Chicago scintillation counter with 10 ml. of 10 parts of toluene scintillator [4 g. of 2,5-diphenyloxazole and 0.5 g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l. of toluene] to 3 parts of ethylene glycol monomethyl ether as scintillator. The efficiency of counting was approx. 55%.

Recovery of original trichloroacetic acid-insoluble radioactive material in gel fractions varied from 75 to 100%. When significant loss of radioactivity did occur, usually owing to excessive peroxide treatment, the resulting gel radioactivity profiles did not change.

In control experiments (H. Z. Hill & M. B. Hoagland, unpublished work), it was shown that gel radioactivity was not due to free amino acids, charged s-RNA* or lipid material. Different electrophoretic runs of supernatant fractions and radioactivity profiles from different experimental animals were readily reproducible.

RESULTS

In the preceding paper (Wilson & Hoagland, 1967) it was shown that when starved-re-fed rats were treated with actinomycin D they lost about 60% of their newly formed liver polysomes over a period of 10 hr., until there remained a complement of much more stable polysomes. This made it possible to determine the nature of proteins made by stable polysomes, by gel electrophoresis. Our electrophoretic analyses were performed on an ultrasonic extract of microsomes that had been found to be representative of most newly formed protein of the liver (H. Z. Hill & M. B. Hoagland, unpublished work). The gel-electrophoresis radioactivity profile of this fraction, from the livers of starved-re-fed rats, is shown in Fig. 1. A variety of protein peaks are resolved. The most prominent among these is albumin. Similar profiles were obtained from normal and regenerating-liver extracts (H. Z. Hill & M. B. Hoagland, unpublished work).

We have demonstrated that the major peak is albumin: (1) by its co-electrophoresis with a rat serum albumin marker; (2) by its solubility, in contrast with the other protein peaks, in acidic ethanol; (3) by its specific co-precipitation with a rat serum albumin-antiserum complex (H. Z. Hill & M. B. Hoagland, unpublished work).

The area under the albumin radioactivity peak,

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

relative to the area of total radioactivity, was determined by planimetry. The results of such estimations on starved, re-fed and actinomycin D-treated animals are shown in Table 1. After 5 days of starvation, when liver protein synthesis per unit of DNA had decreased by about 70% (Wilson & Hoagland, 1967), the percentage of labelled protein

Fig. 1. Characteristic gel electrophoretic profile of the microsomal ultrasonic extract isolated from livers of starved-re-fed rats. Animals were starved for 4–5 days, then fed for 10 hr. before intraperitoneal injection of 100 μ C of 14 C-labelled amino acid mixture. After 18–20 min. animals were killed and the microsomal ultrasonic extract was isolated and assayed as described in the Materials and Methods section. Recovery of original hot-acid-insoluble radioactive material in gel fractions was essentially quantitative.

Table 1. *Relative contribution of albumin to gel profiles of newly formed protein in microsomal ultrasonic extracts from livers of various experimental animals*

Source of experimental liver	% of gel radioactivity in albumin peak	
	Mean	S.D.
Normal rats	26	2.1
Rats starved 5 days	47	0.94
Rats starved 5 days, re-fed 12 hr.	28	2.5
Rats starved 5 days, re-fed 12 hr., and actinomycin D-treated 26 hr.*	63	3.2

* Actinomycin D treatment and exposure to 14 C-labelled amino acids was as described in Fig. 2.

Fig. 2. Gel-electrophoretic profiles of starved (a) and actinomycin D-treated (b) rats. The profile shown in (a) was obtained from the liver microsomal ultrasonic extract of a rat that had been starved for 5 days before intraperitoneal injection of $100\mu\text{C}$ of ^{14}C -labelled amino acid mixture and then killed. The profile shown in (b) is representative of the liver microsomal ultrasonic extract of a starved-re-fed rat that had been treated with actinomycin D for 26 hr. Animals were starved for 5 days, then fed for 10–12 hr. Actinomycin D (1 mg./kg.) was then injected, followed 12 hr. later by a second dose of actinomycin D (0.9 mg./kg.). Rats were killed at various intervals after initial treatment. They were given an injection of $100\mu\text{C}$ of ^{14}C -labelled amino acid mixture 18–20 min. before being killed. Ultrasonic extracts were isolated and analysed as described in the Materials and Methods section.

in the microsomal ultrasonic extract represented by albumin had increased from the normal value of 26% to 47% (Table 1 and Fig. 2a). On re-feeding, radioactivity profiles were rapidly restored to normal (Fig. 1). Actinomycin D treatment of starved-re-fed rats produced a rapid rise in the percentage of counts in the albumin peak (Table 1 and Fig. 2b). After 26 hr. of exposure to actinomycin D, when protein synthesis per unit of DNA had fallen 70–75%, approx. 60% of total ultrasonic-extract radioactive protein was albumin.

As demonstrated by Schwartz, Sodergren, Garofalo & Sternberg (1965) and by Wilson & Hoagland (1967), inhibition of m-RNA renewal in the cytoplasm after a single (1 mg./kg.) injection of actinomycin D persists for only 16–22 hr. On the appearance of new m-RNA in the cytoplasm, marked changes occur both in total protein synthesis and in the appearance of the gel pattern of newly formed protein. A typical electrophoretic run

is shown in Fig. 3. Total protein synthesis recovered to 55% of untreated starved-re-fed animals by 26 hr. (Fig. 3a) and the quantity of new albumin relative to other newly formed proteins fell far below the normal level (Fig. 3b). Quantitative determination of the response by planimetry revealed that, though the percentage of radioactivity in albumin had risen to 60% 17 hr. after a single dose of actinomycin D, this value fell to 16% in the ensuing 10 hr. as cytoplasmic m-RNA was replenished. As we have already noted (Fig. 2), the administration of a second dose of actinomycin D 12 hr. after the first dose resulted in a continued rise in the proportion of newly formed protein in albumin.

This experiment suggests that with reversal of actinomycin D inhibition the m-RNA species that code for many of the more slowly migrating proteins were more rapidly restored in the cytoplasm than m-RNA coding for albumin.

The significance of the results described above

Fig. 3. Changes in total (a) and qualitative (b) protein-synthetic activity of the liver on renewal of m-RNA in the cytoplasm after actinomycin D treatment. In (a) rats were starved for 5 days, then fed for 12 hr. They were then treated with actinomycin D (1 mg./kg.) and 12 hr. later one group was given an additional dose of actinomycin D (0.9 mg./kg.) (O—O) and another group was injected with 0.9% NaCl (●—●). Total protein synthetic activity was assayed as described by Wilson & Hoagland (1967). The specific radioactivity of free amino acid pools of starved and actinomycin D-treated rats were taken to be 2 and 1.5 times that of starved-re-fed rats for all time points. Two rats were used for each point and the Figure represents one of two similar experiments. In (b) the gel-electrophoretic profile is given of the microsomal ultrasonic extract isolated from rats given only one dose of actinomycin D (1 mg./kg.) 26.5 hr. before being killed.

depends largely on whether the gel pattern of newly formed protein in ultrasonic extracts of microsomes accurately reflects the distribution of total newly formed protein of the liver. Since this extract contains only about 10% of the total radioactivity of the liver, it is conceivable that metabolic changes, or exposure to actinomycin D, might cause shifts of certain labelled proteins from one cell fraction to another. As indicated, work in this Laboratory has shown that in normal liver the ultrasonic extract of microsomes is in fact representative of total liver protein synthesis (H. Z. Hill & M. B. Hoagland, unpublished work). In those experiments livers were fractionated to obtain about 70% of total liver hot-acid-insoluble radioactivity in five supernatant fractions as outlined in Scheme 1. In the present experiments similar fractionations of radioactive proteins were performed on livers of starved, re-fed and actinomycin D-treated rats. Table 2 indicates that changes in the proportion of albumin to total protein in all fractions occurs in parallel.

Similar results were obtained when the pulse-labelling period was decreased from 18–20 min. to 5 min., thus suggesting that actinomycin D did not cause increased turnover of the more slowly migrating protein in the experiment shown in Table 2.

DISCUSSION

The experiments described in this paper indicate that albumin synthesis by the liver is more refractory to conditions that decrease RNA synthesis than is synthesis of most other liver proteins. Thus both starvation, which produces a decrease in RNA synthesis, and actinomycin D treatment, which shuts off virtually all RNA synthesis, lead to a decrease in polysomes, decrease in total protein synthesis and increase in the synthesis of albumin relative to other proteins. We therefore conclude that albumin synthesis is mediated by relatively stable m-RNA.

In the preceding paper (Wilson & Hoagland, 1967) it was concluded that about 36% of liver polysomes contain relatively stable m-RNA. Albumin represented 63% of the total protein synthesized when only stable polysomes were present in the liver. Thus, if polysome quantity and protein synthesis were proportional in our experiments, albumin synthesis should represent about 23% (63% of 36%) of total liver protein synthesis. This value agrees quite well with the value of 25–30% arrived at from our studies on total protein synthesis in starved-re-fed and normal liver and the value of 30% from the work of Peters (1962).

Scheme 1. Fractionation of total newly formed protein of rat liver. Sodium deoxycholate was used as described by Littlefield, Keller, Gross & Zamecnik (1955).

Under conditions of starvation, when RNA synthesis was decreased but not entirely inhibited, albumin synthesis relative to synthesis of other proteins was increased. This finding again suggests that the liver is somewhat frozen in the production of albumin and does not rapidly decrease its synthesis in response to environmental stimuli.

The nature of the other proteins synthesized by stable polysomes has not yet been investigated. Gordon & Humphrey (1960) found that albumin represented about 64% of the total liver export protein. Therefore it is tempting to speculate that most of the proteins synthesized by stable polysomes in normal liver are destined for export. Such a possibility was envisaged by Hiatt (1962).

The synthesis in liver of large quantities of

specialized proteins by m-RNA stable in the presence of actinomycin D resembles the situation in rabbit reticulocytes, where globin synthesis is directed by a stable m-RNA. These highly specialized tissues seem to represent end stages in evolutionary development where the capacity of rapid response to environmental stimuli has, in part, been sacrificed in the interest of economy.

The possibility that both stable and unstable m-RNA exist within the cytoplasm of the same hepatic cell leads to speculation about the mechanism of selective messenger breakdown. Aronson (1965) has presented evidence that a fraction of bacterial-spore m-RNA is relatively stable and is found in association with cytoplasmic membrane. Yudkin & Davis (1965) have also presented evidence

Table 2. *Distribution of labelled protein and albumin in various supernatant fractions of liver obtained as outlined in Scheme 1*

In these experiments normal female rats weighing 150–200 g. were used. Where indicated, rats were starved for 5 days and re-fed for 12 hr. Groups of starved re-fed rats were given 1 mg. of actinomycin D/kg. and 12 hr. later half the animals were given an additional dose of 0.9 mg. of actinomycin D/kg. while the other half were given a placebo injection of 0.1 ml. of 0.9% NaCl–95% ethanol (1:1, v/v). Then 13.5 hr. later the rats were injected with 100 μ C of 14 C-labelled amino acids and processed as described in the Materials and Methods section.

Source of experimental liver	Supernatant fraction	% of total liver hot-acid-precipitable radioactivity				
		I	II	III	IV	V
Rats starved–re-fed (normal about same)		21	11.5	12.5	11	12
Rats starved		24	9.5	14.5	11	11
Rats starved–re-fed, actinomycin D-treated (two doses)		14	12	14	13	14
Rats starved–re-fed, actinomycin D-treated (one dose)		28	11.5	11.5	12	14
	Supernatant fraction	% of total hot-acid-precipitable radioactivity in gel found in albumin peak				
		I	II	III	IV	V
Rats starved–re-fed		3	28 (2.5*)	27	24.5	25
Rats starved		11 (2.3)	47 (0.94*)	46	43	46
Rats starved–re-fed, actinomycin D-treated (two doses)		16	63 (3.2*)	63	60	64
Rats starved–re-fed, actinomycin D-treated (one dose)		1	16 (0.56*)	16	15	17

* S.D. based on at least five determinations.

that a fraction of *Bacillus megaterium* m-RNA is relatively resistant to actinomycin D and confined to membrane. As suggested by Pitot (1964), stable messenger in liver might indeed be intimately associated with the endoplasmic reticulum in a manner that protects it from breakdown, possibly by impeding transcription or nuclease action or both. In this light it would be interesting to examine experimental systems such as the newborn rat (Campbell, Serck-Hanson & Lowe, 1965), where correlation between development of the rough endoplasmic reticulum and development of stable m-RNA could be made.

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