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The Effect of Phenobarbitone on Protein Synthesis by Liver Polyribosomes in Fed and Starved Rats

By GIOVANNI RAGNOTTI and M. GIOVANNA ALETTI
Institute of General Pathology, Centre for Cellular Pathology, C.N.R., University of Milan, 20133 Milan, Italy

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1. The effect of phenobarbitone on the rate of protein synthesis and on the sedimentation patterns of various liver subcellular fractions containing ribosomes was studied in rats. 2. Phenobarbitone treatment increased the incorporation of [^{14}C]leucine into protein by all preparations, provided they had not been subjected to preliminary treatment with Sephadex G-25. The phenobarbitone-induced effect on incorporation was associated with a gain in liver weight and a higher degree of polyribosomal aggregation. 3. Preparations that were treated with Sephadex G-25 incorporated more radioactivity into protein, but did not show the response to phenobarbitone treatment. 4. When the influence of starvation and phenobarbitone was studied separately on membrane-bound and membrane-free polyribosomes, it was shown that whereas both classes of polyribosomes were affected by starvation, apparently only the former class was susceptible to phenobarbitone stimulation of protein synthesis. 5. The decreased capacity for protein synthesis of polyribosomes from starved rats was independent of their association with the membranes of the endoplasmic reticulum, but resulted from polyribosomal disaggregation, from an intrinsic defect of the polyribosomes themselves and from changes in composition of the cell sap. 6. The results are discussed in relation to the problem of the control of protein biosynthesis and of the functional separation of membrane-bound and membrane-free polyribosomes.

The structural and functional integrity of a cell depends, among other factors, on a balance between the rate of protein synthesis and degradation. Any condition affecting its ability to maintain a normal rate of protein biosynthesis will also lead to a progressive impairment of function.

Starvation in the rat is accompanied by loss in liver weight (Schultz, 1949; Thomson *et al.*, 1953), degranulation of endoplasmic reticulum (Henshaw *et al.*, 1963), fragmentation of polyribosomes (Wittman *et al.*, 1969; Richardson *et al.*, 1971; Arora & De Lamirande, 1971) and a decreased rate of protein biosynthesis (Wagle, 1963; Sox & Hoagland, 1966; Richardson *et al.*, 1971). In contrast, phenobarbitone, a potent inducer of many liver microsomal enzyme systems (Conney & Burns, 1962; Gillette, 1963), increases the amount of the smooth endoplasmic reticulum (Orrenius *et al.*, 1965) and the rate of protein synthesis in rat liver both *in vivo* (Kato *et al.*, 1965*a*; Jick & Shuster, 1966; Glazer & Sartorelli, 1972) and *in vitro* (Gelboin & Sokoloff, 1961; Kato *et al.*, 1965*b*, 1966).

As part of an investigation being carried out in this laboratory on the regulation of the rate of protein synthesis in normal (Ragnotti, 1971) and injured liver cells (Ragnotti *et al.*, 1970; Cajone *et al.*, 1971), the effects of phenobarbitone have been studied in both fed and starved rats. The results show that the starvation-induced effects are almost completely prevented by phenobarbitone administration. Both membrane-bound and membrane-free polyribosomes exhibited impaired function in the starved state, but apparently only the former class of polyribosomes responded to phenobarbitone administration.

The results are discussed in relation to the problem of the control of protein biosynthesis and of the functional separation of membrane-bound and membrane-free polyribosomes.

Materials and Methods

Materials

Chemicals. ATP, GTP, phosphoenolpyruvate and pyruvate kinase [EC 2.7.1.40; specific activity approx. 150 units (μmol/min)/mg] were purchased from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, West Germany). Phenobarbitone (disodium salt of phenylethylbarbituric acid, Merck A.G., Darmstadt, West Germany) was a gift from Bracco S.p.A. (Milan, Italy). Other chemicals were of A.R. grade and were obtained from either E. Merck A.G. or from British Drug Houses Ltd. (Poole, Dorset, U.K.). Organic

solvents were purchased from Carlo Erba S.p.A. (Milan, Italy). L-[U-^{14}C]Leucine (specific radioactivity 311mCi/mmol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). The scintillator (Omnifluor) was a product of New England Nuclear Chemicals G.m.b.H. (Frankfurt am Main, West Germany).

Animals. Male albino rats (Wistar strain) weighing approx. 200g were maintained on a diet of laboratory chow (Piccioni, Brescia, Italy) and water *ad libitum*. They were used either fed or starved for 48 or 96h. Phenobarbitone (20mg/ml in 0.154M-NaCl) was administered by intraperitoneal injection once daily at a dose of 80mg/kg body weight (Kato *et al.*, 1966). Thus animals treated for 48 and 96h received two or four injections respectively. Those starved for 96h and treated with phenobarbitone for 48h received their injections 48 and 24h before being killed. Control animals, subjected to the same housing and feeding schedule as the experimental animals, were injected with appropriate amounts of 0.154M-NaCl adjusted to the same pH as the phenobarbitone solution with NaOH; the animals were killed by cervical dislocation and their livers quickly removed and transferred to ice-cold medium A. All subsequent operations were performed at a temperature between 0° and 4°C.

Methods

Cellular fractionation. The following media were used: medium A consisting of 0.25M-sucrose in TKM 1 buffer [50mM-Tris–HCl (pH7.8 at 20°C)–25mM-KCl–5mM-$MgSO_4$,$7H_2O$]; medium B consisting of 0.15M-sucrose in TKM 2 buffer [35mM-Tris–HCl (pH7.8 at 20°C)–25mM-KCl–10mM-$MgSO_4$, $7H_2O$]. The livers were passed through a tissue press [made to the design of Porterfield (1960)]. The liver mince was homogenized in 2.0vol. of medium A for the preparation of the postmitochondrial supernatant and membrane-free and membrane-bound polyribosomes, and in 2.5 vol. of medium A for the preparation of the detergent-treated polyribosomes (Wettstein *et al.*, 1963). The preparation of the subcellular fractions was that previously described (Ragnotti, 1971) with the following modifications.

(a) Postmitochondrial supernatant. This was prepared by centrifuging the homogenate for 20min at 13000*g* in the 870 rotor ($r_{av.}$ 6.9cm) of the International B20 refrigerated centrifuge. After discarding the fatty layer at the top, the upper two-thirds of the supernatant were collected and a portion (5ml) was passed through a column (1cm × 20cm) of Sephadex G-25 (coarse grade) equilibrated with medium A, the first 2.5ml being collected.

(b) Cell-sap fraction. The postmitochondrial supernatant was centrifuged at 65000rev./min for 150min in the FAR 65 rotor ($r_{av.}$ 5.7cm) of the L2-65 B Spinco ultracentrifuge. After removal of the fatty layer, the upper two-thirds of the supernatant (cell sap) were collected. A 5ml portion was loaded on to a column of Sephadex G-25 (course grade) (1cm × 20cm) equilibrated with medium B, the first 2.5ml being collected. The remainder of the cell sap was retained without further treatment.

(c) Membrane-bound and membrane-free polyribosomes. These were isolated as previously described (Ragnotti, 1971) with only minor modifications. In particular, centrifugation of the postmitochondrial supernatant on a discontinuous gradient of 0.5–1.6M-sucrose in TKM 1 buffer, for 150min at 65000rev./min in a FAR 65 rotor ($r_{av.}$ 5.7cm) of the Spinco L2 65 B ultracentrifuge, yielded the membrane-bound polyribosomes at the interface and the free polyribosomes at the bottom of the centrifuge tube. Membrane-bound polyribosomes, resuspended in cell sap, were sedimented on a 1 ml cushion of 2M-sucrose in TKM 2 buffer by centrifugation at 65000 rev./min for 30min in a Spinco FAR 65 rotor. The supernatant was discarded and the tube rinsed with 5ml of medium B. The membrane-bound polyribosomes, together with cushion, were resuspended in medium B by gentle homogenization with a loose-fitting Teflon pestle to half the original volume of the postmitochondrial supernatant. The suspensions were centrifuged in a cooled swinging-bucket rotor ($r_{av.}$ 9.5cm) at 3500*g* for 5min to remove aggregated material. A portion of the supernatants was diluted with medium B to an RNA concentration of 1mg/ml for the determination of the protein-synthesizing activity.

(d) Detergent-treated membrane-bound polyribosomes. These were prepared by resuspending the membrane-bound polyribosomes, which had sedimented at the interface of the 0.5–1.6M-sucrose, to the original postmitochondrial volume with concentrated cell sap (obtained from a 1:1 homogenization) to inhibit ribonuclease (Lawford *et al.*, 1966; Blobel & Potter, 1966). The suspension was gently homogenized, mixed with 0.1vol. of sodium deoxycholate (1.3% final concn.) and centrifuged on a discontinuous gradient of 0.5–2.0M-sucrose in TKM 1 buffer in the Spinco FAR 65 rotor for 150min. The polyribosomes that sedimented at the bottom of the centrifuge tube were resuspended in TKM 2 buffer and centrifuged at low speed to remove aggregated material. A portion of the supernatant was diluted with medium B to an RNA concentration of 1mg/ml for amino acid incorporation experiments.

Amino acid incorporation by subcellular fractions. The incubation mixture (volume 0.1ml) contained 0.01ml of postmitochondrial supernatant (before or after Sephadex treatment), 2mM-ATP, 0.25mM-GTP, 10mM-phosphoenolpyruvate, 0.31μCi of [^{14}C]leucine, 50μg of pyruvate kinase, 6.4mM-$MgSO_4$,$7H_2O$, 131mM-sucrose, 18.5mM-KCl and 28.9mM-Tris–HCl

buffer, pH7.8 at 20°C. Other incubations contained the same volume of membrane-free, membrane-bound or detergent-treated membrane-bound polyribosomes. In these cases the mixture also contained cell sap (0.02ml) at the protein concentration specified. Since the protein-synthetic activity of the isolated polyribosomes plus cell sap depends on the ratio rRNA incubated/cell-sap protein (Ragnotti, 1971; Lowe & Hallinan, 1973), care was taken to keep this ratio constant and above the limiting value of 50 (Munro *et al.*, 1964). After 30min at 37°C without shaking, the incorporation process was stopped by the addition of 1.4ml of an ice-cold solution, containing 10mM-L-leucine, 10mM-EDTA (disodium salt) and 154mM-NaCl. After adjustment to pH7.0 with 4M-NaOH (Ragnotti *et al.*, 1970), the incorporation of radioactivity was determined as previously described (Ragnotti *et al.*, 1969), with the modification that Omnifluor (4g/litre of toluene) was used as scintillant. Radioactivity was counted with an efficiency of 80% (background 16c.p.m.) in a Tri-Carb liquid-scintillation spectrometer (model 3365, Packard Instruments Co. Inc.); the standard error was 5% or better. The magnitude of the quenching, measured by the channels-ratio method, was found to be similar for all incubation mixtures. Incorporation values were corrected by subtraction of a control kept at 0°C for 30min (Staehelin *et al.*, 1963). The radioactivity of the controls (about 45c.p.m. above background) was independent of the amount of protein, RNA, or of the time elapsed before the addition of the leucine–EDTA solution. It reflected the radioactivity trapped on the filter.

Density-gradient analysis of polyribosome profiles. These were performed as described previously (Ragnotti, 1971).

Measurement of RNA and protein in the subcellular fractions. The RNA content of the isolated polyribosomal fractions to be assayed for protein synthesis was adjusted to a concentration of 1mg/ml by determining E_{260} and converting the value obtained into orcinol RNA by a method previously described (Ragnotti, 1971). The RNA content of the postmitochondrial supernatants and of the diluted ribosomal suspensions assayed for protein synthesis was determined by the orcinol method (Mejbaum, 1939) and cell sap protein by the biuret method (Layne, 1957).

Statistical treatment. Except when otherwise stated, the differences of the means were tested for statistical significance by the analysis of variance.

Results

Effect of phenobarbitone on liver weight

Phenobarbitone significantly increases rat liver weight over that of the controls without affecting the body weight or the water content of the livers (Table 1).

Influence of phenobarbitone on the amount and distribution of RNA in various liver subcellular fractions

Phenobarbitone treatment (Table 2) increases total liver RNA by 6%. Fractionation of the postmitochondrial supernatant into membrane-bound and membrane-free polyribosomes shows that the administration of the drug is associated with a redistribution of rRNA in the two polyribosomal fractions. The increase in polyribosomal RNA was 30% and this was associated with a shift in the ratio free/membrane-bound plus membrane-free polyribosomes from 59 to 32% in the phenobarbitone-treated group. Since this latter value is similar to that in normal livers (Webb *et al.*, 1965; Blobel & Potter, 1967;

Table 1. *Effect of phenobarbitone administration on rat liver weight*

For the details of the phenobarbitone treatment see the Materials and Methods section. The 'relative liver weight' is defined as g of liver/100g body wt. The values represent the mean ±S.E.M. of twelve experiments. All animals weighed 185–210g at the start of the experiment and all livers contained 68–70% water.

Animals	Phenobarbitone treatment	Variation of body wt. during treatment (g)	Relative liver wt. at death	Increment over the untreated control (%)
Fed for 48h	−	+ 9 ± 1.15	4.57 ± 0.08	—
	+	+10 ± 1.00	5.19 ± 0.09*	+14
Fed for 96h	−	+19 ± 1.61	4.37 ± 0.17	—
	+	+20 ± 1.64	5.80 ± 0.12*	+33
Starved for 48h	−	−26 ± 1.10	3.08 ± 0.07	—
	+	−28 ± 0.76	4.30 ± 0.09*	+40
Starved for 96h	−	−52 ± 2.09	2.97 ± 0.04	—
	+ (96h)	−53 ± 2.72	4.75 ± 0.06*	+60
	+ (48h)	−46 ± 2.34	4.08 ± 0.10*	+37

* Value significantly different from control, $P < 0.01$

Table 2. *Effect of phenobarbitone on RNA distribution in the homogenate and in various subcellular fractions isolated from the livers of rats starved for 48 h*

For the details of phenobarbitone treatment and the preparation of the fractions see the Materials and Methods section. The values are the mean ±S.E.M. of four experiments.

	RNA in subcellular fractions (mg/g of fresh liver)		% Variation over the control
	−Phenobarbitone	+Phenobarbitone	
Homogenate	12.63 ± 0.19	13.32 ± 0.16	+6
Postmitochondrial supernatant	6.08 ± 0.11	6.97 ± 0.21	+15
Pellet	6.51 ± 0.08	6.32 ± 0.10	+6
Postmitochondrial supernatant + pellet	12.59	13.29	
% Recovery over homogenate	100	100	
Unfractionated microsomal fraction	5.15 ± 0.08	5.86 ± 0.12	+14
Cell sap	0.59 + 0.08	0.60 ± 0.01	—
Membrane-bound polyribosomes	1.48 ± 0.04	3.23 ± 0.09	+118
Membrane-free polyribosomes	2.16 ± 0.03	1.51 ± 0.11	−30
Membrane-bound + membrane-free polyribosomes	3.64	4.74	+30
$\frac{\text{Membrane-free polyribosomes}}{\text{Membrane-free + membrane-bound polyribosomes}} \times 100$	59	32	

Ragnotti *et al.*, 1970; Venkatesan & Steele, 1972) the administration of the drug protects the integrity of the membrane–ribosome complex.

Relative efficiency of different subcellular fractions in incorporating [^{14}C]leucine into protein

Passage of the postmitochondrial supernatant or cell-sap fractions through a column of Sephadex G-25 increases the specific radioactivity of the synthesized protein when the particulate fraction is incubated with radioactive amino acid (Munro *et al.*, 1964). Since the effect of phenobarbitone on protein synthesis might be mediated by changes in the low-molecular-weight components that are removed by Sephadex treatment, all incorporation experiments were done with fractions before and after Sephadex treatment.

(1) *Protein synthesis in postmitochondrial supernatant* (*Table* 3). In every experiment the fractions incorporated more radioactivity after Sephadex chromatography than before. There are, however, clear differences in the response to phenobarbitone treatment. In the fed animals 48 h of treatment with phenobarbitone significantly stimulated incorporation provided the fractions had not been subjected to Sephadex chromatography. Continuation of drug treatment for another 48 h did not further increase the stimulation. No phenobarbitone effect could be demonstrated in fractions incubated after Sephadex chromatography. In starved animals, the incorporation of radioactivity into protein fell progressively with the duration of the starvation period, the effect being seen in fractions before and after Sephadex treatment. After a 48 h starvation period, phenobarbitone completely prevented the impairment of protein synthesis exhibited by the control fraction not treated with Sephadex, the amount of radioactivity incorporated being equal to that of the fed animals treated with phenobarbitone for 48 or 96 h. The effect was smaller and statistically not significant after Sephadex treatment.

In the group starved for 96 h the effect of phenobarbitone was evident in both Sephadex-treated and untreated fractions, the stimulation being statistically significant in animals receiving the drug for either the whole period of starvation or only during the latter 48 h.

The polyribosome profiles of the postmitochondrial supernatants from the experiments in Table 3 are shown in Fig. 1. Simple inspection of the profiles shows that starvation progressively decreases the amount of heavy polyribosomes with a concomitant increase in the lighter forms. Phenobarbitone administration, except perhaps for the fed animals, decreased the proportion of monomers and dimers, with the result that most of the rRNA was present in the form of heavy polyribosomes. There was no significant difference between the polyribosomal profiles from the livers of rats starved for 96 h and receiving phenobarbitone throughout and those treated only during the last 48 h.

When postmitochondrial supernatants from the livers of fed, 48 h-starved and starved phenobarbitone-treated animals, were subjected to Sephadex chromatography, they did not differ significantly in their

Table 3. *Effect of phenobarbitone on the protein-synthesizing activity of postmitochondrial supernatant fractions*

The animals, either fed or starved for 48 or 96h, were treated with phenobarbitone and the fractions were prepared and incubated as specified in the Materials and Methods section. Radioactivity incorporated into protein is the mean ±S.E.M. of six (48h) or three (96h) experiments. The mean amount of rRNA incubated (mg/ml) was 0.203, 0.179 for fed rats and 0.234, 0.199 for starved rats, before and after Sephadex chromatography respectively. Phenobarbitone increased these amounts by less than 10%.

(a) Fed animals

Treatment ...	−Phenobarbitone		+Phenobarbitone (48h)		+Phenobarbitone (96h)	
Postmitochondrial supernatant	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex
Radioactivity incorporated (c.p.m./mg of RNA)	54150 ±3000	200250 ±8250	73300* ±4350	209650‡ ±9800	71400* ±2700	197800‡ ±3450

(b) Starved animals

Length of starvation period ...	48h				96h					
Treatment ...	−Phenobarbitone		+Phenobarbitone		−Phenobarbitone		+Phenobarbitone (96h)		+Phenobarbitone (48h)	
Postmitochondrial supernatant ...	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex
Radioactivity incorporated (c.p.m./mg of RNA)	44700 ±2750	172400 ±8150	72700* ±3200	190950‡ ±10100	17150 ±4000	105950 ±8700	41050† ±4650	151100† ±10000	39200† ±2100	166750† ±15600

* $P < 0.01$.
† $P < 0.05$.
‡ $P > 0.05$.

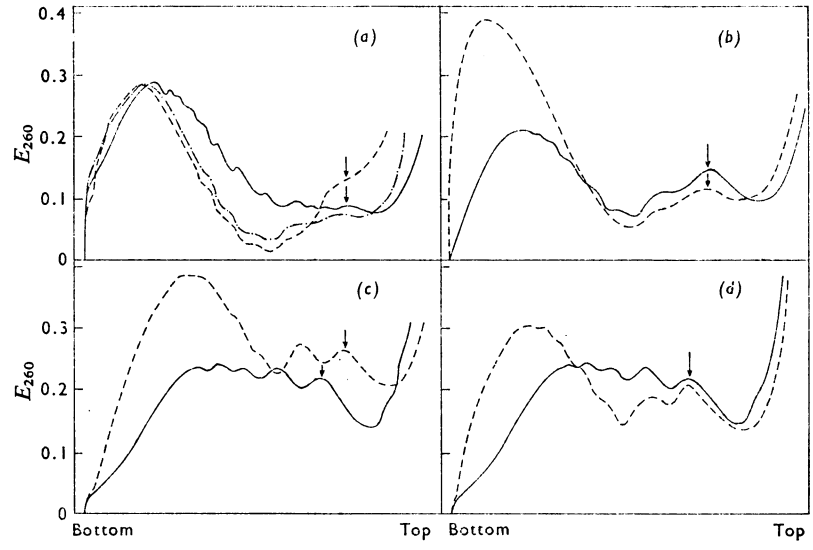

Fig. 1. *Polyribosome profiles of the postmitochondrial supernatants assayed for protein biosynthesis* (*Table* 2)

Suspensions (0.5ml), prepared as specified in the Materials and Methods section, were layered over a 12ml linear gradient of sucrose (15–50%) in TKM 1 buffer and centrifuged for 150min at 40000rev./min in a Spinco SW 40 rotor ($r_{av.}$ 11.1cm) or in an International SB 283 rotor ($r_{av.}$ 10.3cm) at 0°C. Arrows indicate the position of monomers. (*a*) ——, Fed; ----, 48h-fed phenobarbitone-treated; -·-·-·-, 96h-fed phenobarbitone-treated animals (0.63, 0.64 and 0.65mg of RNA respectively). (*b*) ——, 48h-starved; ----, 48h-starved phenobarbitone-treated animals (0.57 and 0.91mg of RNA respectively). (*c*) ——, 96h-starved; ----, 96h-starved phenobarbitone-treated animals (0.74 and 0.92mg of RNA respectively). (*d*) ——, 96h-starved; ----, 96h-starved 48h-phenobarbitone-treated animals (0.74 and 0.91mg of RNA respectively).

ability to incorporate [^{14}C]leucine into protein. This could be the consequence of (*a*) the preferential breakdown of polyribosomes from phenobarbitone-treated rats during Sephadex treatment, (*b*) the removal of a component necessary for the full expression of the protein-synthesizing capacity of polyribosomes after phenobarbitone treatment, or (*c*) the removal of low-molecular-weight inhibitors present in the control fractions but not in those from phenobarbitone-treated rats. These three possibilities are considered in turn.

(*a*) The polyribosomal profiles of control and phenobarbitone-treated postmitochondrial supernatants were analysed before and after Sephadex treatment. Sephadex chromatography did not have any influence on the physical state of the polyribosomes.

(*b*) If this hypothesis was correct, the addition of an extra amount of cell sap (0.01ml/0.1ml of incubation mixture not treated with Sephadex and obtained from the liver of a 12h-starved animal) to the Sephadex-treated fractions should increase the amount of radioactivity incorporated by fractions from phenobarbitone-treated rats but not by those from controls. The results showed that after cell-sap addition, the specific radioactivities of the protein synthesized by fractions from control and phenobarbitone-treated animals were decreased to practically the same extent (−21, −68% for controls and −27, −57% for experimental animals, before and after Sephadex chromatography respectively).

This, together with the results obtained in Table 3, which demonstrate that (i) the phenobarbitone stimulation is consistently greater in the fractions not treated with Sephadex and (ii) Sephadex treatment increases the activity of the fractions from the controls more than these from phenobarbitone-treated animals, favours hypothesis (*c*), which suggests that the reduction of phenobarbitone stimulation after Sephadex treatment occurs as a consequence of an increase in the activity of controls rather than a decrease in the activity of the polyribosomes from phenobarbitone-treated rats. From the above experiments it is, however, clear that the routine use of Sephadex may mask important effects in this system.

(2) *Protein synthesis in membrane-bound and membrane-free polyribosomes.* The postmitochondrial supernatant has several advantages over other amino acid-incorporating systems used in this type of study (Richardson *et al.*, 1971) but, because of its relative complexity, it does not permit the exact definition of the subcellular structures which are responding to phenobarbitone. The postmitochondrial supernatant

Table 4. *Protein-synthesizing activity of membrane-bound and membrane-free polyribosomes from the livers of control and phenobarbitone-treated rats*

The animals, starved for 48h or 96h, were treated with phenobarbitone as specified in the Materials and Methods section. Within the treatment, membrane-bound and membrane-free polyribosomes, isolated from the same pooled livers, were incubated as specified in the Materials and Methods section. Radioactivity incorporated into protein is the mean ±S.E.M. of six (48h) or three (96h) experiments. The mean concentration of rRNA incubated (mg/ml) was 0.098 for membrane-bound and 0.096 for membrane-free polyribosomes. The same cell sap, homologous with the ribosomal suspensions, was used in corresponding incubations of membrane-bound and membrane-free polyribosomes and its mean protein concentration (mg/ml of incubation mixture) was 6.38, 5.63 for controls and 5.57, 4.70 for samples from phenobarbitone-treated rats, before and after Sephadex chromatography respectively.

Length of starvation period ...	48h				96h					
Treatment ...	−Phenobarbitone		+Phenobarbitone		−Phenobarbitone		+Phenobarbitone (96h)		+Phenobarbitone (48h)	
Cell sap ...	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex
(a) Membrane-bound polyribosomes										
Radioactivity incorporated (c.p.m./mg of RNA)	14300 ±650	160400 ±6900	25850* ±1150	271500* ±19400	10600 ±850	83550 ±2300	21850† ±2650	216000* ±13100	19100† ±750	218750* ±7900
(b) Membrane-free polyribosomes										
Radioactivity incorporated (c.p.m./mg of RNA)	12000 ±1050	162200 ±14250	13200‡ ±1950	163950‡ ±14250	4550 ±1550	87850 ±13300	6450‡ ±1200	147800† ±9750	11600† ±1100	190000† ±1150

* $P < 0.01$.
† $P < 0.05$.
‡ $P > 0.05$.

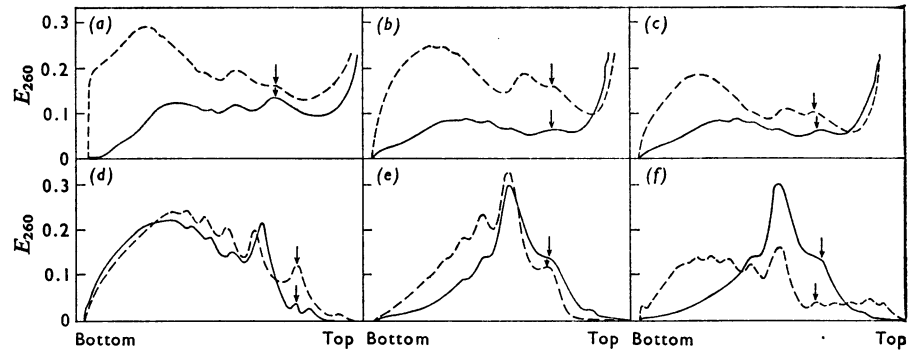

Fig. 2. *Sedimentation patterns of the membrane-bound and membrane-free polyribosomes assayed for protein biosynthesis (Table 4)*

The suspensions (0.5ml), prepared as specified in the Materials and Methods section, were layered over a 12ml linear gradient of sucrose (15–50%) in TKM 1 buffer and centrifuged for 150min at 40000rev./min in a Spinco SW 40 ($r_{av.}$ 11.1cm) or in an International SB 283 rotor ($r_{av.}$ 10.3cm). Arrows indicate the position of monomers. (*a*), (*b*), (*c*) membrane-bound and (*d*), (*e*), (*f*) membrane-free polyribosome profiles from livers from control (——) and phenobarbitone-treated (– – – –) rats. (*a*) 48h-starved and 48h-starved phenobarbitone-treated animals (0.26 and 0.62mg of RNA respectively); (*b*) 96h-starved and 96h-starved phenobarbitone-treated animals (0.19 and 0.56mg of RNA respectively); (*c*) 96h-starved and 96h-starved, 48h-phenobarbitone-treated animals (0.19 and 0.38mg of RNA respectively); (*d*) 48h-starved and 48h-starved phenobarbitone-treated animals (0.51 and 0.55mg of RNA respectively); (*e*) 96h-starved and 96h-starved phenobarbitone-treated animals (0.49 and 0.48 mg of RNA respectively); (*f*) 96h-starved and 96h-starved 48h-phenobarbitone-treated animals (0.49 and 0.46mg of RNA respectively).

was thus fractionated into membrane-bound and membrane-free polyribosomes and the influence of phenobarbitone on the activity and on the physical properties of the two classes of polyribosomes was studied. These experiments were performed only on starved animals, since the high glycogen content of the livers from fed rats makes polyribosome preparation difficult (Lowe *et al.*, 1970).

The results (Table 4) show that, in every situation tested, membrane-bound polyribosomes consistently exhibited increased incorporation in the phenobarbitone-treated groups. At variance with the results obtained for postmitochondrial supernatant, the effect was demonstrable also in the presence of Sephadex-treated cell sap.

In contrast, the membrane-free polyribosomes exhibited no response to phenobarbitone treatment in the 48h-starved group. When the starvation period was prolonged to 96h the decrease in the incorporation was about 50% for the control and phenobarbitone-treated groups, except for the polyribosomes from the phenobarbitone-treated group incubated with Sephadex-treated cell sap, where the fall was only 10%. This suggests that an inhibitor of small molecular weight was in part responsible for the decreased activity of free polyribosomes from the phenobarbitone-treated group. The phenobarbitone effect was significant, independently of the Sephadex treatment, in the group starved for 96h and receiving phenobarbitone for the latter 48h.

The polyribosome profiles of the fractions reported in Table 4 are shown in Fig. 2. Starvation for either 48 or 96h causes a marked distortion in the profile of membrane-bound polyribosomes in that a high monomer component was present. Phenobarbitone greatly increases the degree of aggregation and the profiles are eventually similar to those seen in the normal fed rat (Fig. 1*a*). Phenobarbitone treatment did not influence the profile of the free polyribosomes from rats starved for 48 or 96h (in which extensive degradation to monomers was seen), except for rats receiving phenobarbitone for the latter 48h of a 96h starvation period, where a significant increase in the polyribosome peak was observed (Fig. 2*f*).

Effect of heterologous cell sap on the incorporation of [^{14}C]leucine into protein by detergent-treated membrane-bound polyribosomes

Although the changes in degree of aggregation of membrane-bound polyribosomes appeared to account for the phenobarbitone effect, other possibilities could not be excluded, for example the influence of cell sap and membranes of the endoplasmic reticulum. To test these possibilities, membrane-bound polyribosomes from 48h-starved and starved phenobarbitone-treated animal were freed from membranes as described in the Materials and Methods section. This preparation is referred to as 'detergent-treated membrane-bound polyribosomes'.

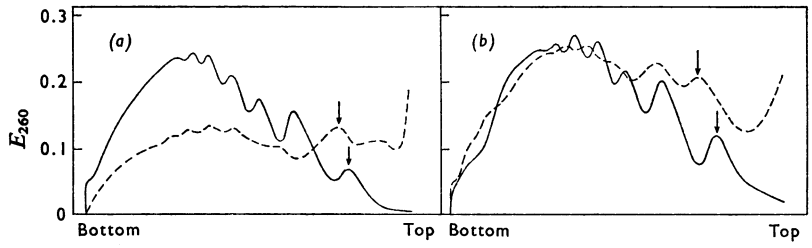

Fig. 3. *Sedimentation patterns of membrane-bound polyribosomes: effect of detergent treatment*

The ribosomal suspensions (0.5ml), prepared from the same pooled livers as specified in the Materials and Methods section, were layered over a 12ml linear gradient of sucrose (15–50%) in TKM 1 buffer, and centrifuged for 150min at 40000rev./min in a Spinco SW 40 rotor ($r_{av.}$ 11.1cm). Arrows indicate the position of monomers. (*a*) membrane-bound (----) and detergent-treated membrane-bound (——) polyribosomes from 48h-starved animals (0.28 and 0.43mg of RNA respectively); (*b*) membrane-bound (----) and detergent-treated membrane-bound (——) polyribosomes from 48h-starved phenobarbitone-treated animals (0.61 and 0.48mg of RNA respectively).

The sedimentation profiles of these and of the original membrane-bound polyribosomal suspensions are shown in Fig. 3. Confirming previous results (Webb *et al.*, 1964; Munro, 1968), centrifugation of the polyribosomal fractions through the dense sucrose layer resulted in a loss of the lighter species, with preferential recovery of the heavier polyribosomes. Since the loss was not proportional to the amount in the original suspensions, the detergent-treated membrane-bound polyribosomes from control and phenobarbitone-treated rats showed identical sedimentation profiles. Their protein-synthetic activity (Table 5) is thus independent of the degree of aggregation and depends only on the intrinsic properties of the polyribosomes. Detergent-treated membrane-bound polyribosomes incubated in the presence of homologous cell sap, whether or not treated with Sephadex, incorporated significantly less radioactive leucine into protein than did the phenobarbitone-treated counterpart. When control detergent-treated membrane-bound polyribosomes were incubated in the presence of heterologous cell sap, the incorporation was significantly higher than that in the presence of homologous cell sap, only when cell sap not treated with Sephadex was used. Conversely, control cell sap significantly lowered the activity of experimental polyribosomes only if cell sap not treated with Sephadex was used. The source of cell sap had no influence on the protein-synthetic activity of the free polyribosomes.

Discussion

The following points deserve discussion: (i) the influence of starvation and of phenobarbitone on the physical and functional properties of rat liver polyribosomes, and (ii) the relevance of the results obtained to the problem of the control of protein biosynthesis in the intact cell and of the functional separation of membrane-bound and membrane-free polyribosomes.

As a consequence of starvation the rate of protein synthesis in rat liver is decreased. The results of the experiments presented in this report demonstrate: (i) that detergent-treated membrane-bound polyribosomes of comparable size from control and experimental livers retain the same differences in protein-synthesizing capacity as the cruder preparations from which they were derived, (ii) that cell sap from phenobarbitone-treated rats does not restore the activity for protein synthesis of the starved polyribosomes, and (iii) the presence in the cell sap from starved rats of soluble low-molecular-weight inhibitors. Starvation seems then to exert direct and indirect effects on the activity of the polyribosomes, directly, by decreasing their amount of aggregation and intrinsic ability to synthesize protein, and indirectly, by promoting the build-up in the cytosol of soluble low-molecular-weight inhibitors. The membranes of the endoplasmic reticulum do not seem to exert any influence on the activity of the associated polyribosomes. Phenobarbitone administered to well-fed or starved animals results in a significant increase in the amount of [^{14}C]-leucine incorporated into protein. The increased rate of protein synthesis *in vitro* is associated with the maintenance of a normal degree of aggregation of the membrane-bound polyribosomes and with an increase in liver weight. Since phenobarbitone does not significantly increase the degree of polyribosomal aggregration in fed animals, the enhanced rate of protein synthesis which followed the administration of the drug implies that in the normal liver a portion of the polyribosomal population, though aggregated, is inactive with respect to protein synthesis. After phenobarbitone administration, following the demand for an increased rate of protein biosynthesis, the dormant polyribosomes are brought into action. This conclusion implies the existence in the normal liver of a 'ready for use' functional reserve. A similar hypothesis was proposed by Scornik (1969) for regenerating rat liver. Since the increased protein-synthesizing activity of the polyribosomes from pheno-

Table 5. *Protein-synthesizing activity of detergent-treated membrane-bound and membrane-free polyribosomes from livers from control and phenobarbitone-treated rats: effect of heterologous cell sap*

The animals, starved for 48 h, were treated with phenobarbitone as specified in the Materials and Methods section. Within the treatment, detergent-treated membrane-bound and membrane-free polyribosomes were isolated from the same pooled livers and incubated as specified in the Materials and Methods section. Radioactivity incorporated into protein is the mean ±S.E.M. of four experiments. The mean amount of rRNA incubated (mg/ml) was 0.104 for detergent-treated membrane-bound and 0.100 for membrane-free polyribosomes. The same cell sap, homologous with the ribosomal suspensions, was used in corresponding incubations of membrane-bound and membrane-free polyribosomes, and its mean protein concentration (mg/ml of incubation mixture) was 6.69 and 4.63 for control and 6.28 and 4.70 for samples from phenobarbitone-treated rats, before and after Sephadex chromatography respectively. The statistical significance of the differences was tested by Student's *t* test for paired variates.

Source of cell sap ...	−Phenobarbitone		+Phenobarbitone		−Phenobarbitone		+Phenobarbitone	
Cell sap ...	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex
(a) Detergent-treated membrane-bound polyribosomes								
Treatment ...	−Phenobarbitone				+Phenobarbitone			
Radioactivity incorporated (c.p.m./mg of RNA)	22850 ±1400	176600 ±10050	25650* ±1850	197000† ±14500	25150 ±1900	199600 ±14750	33050* ±3100	236150† ±7800
(b) Membrane-free polyribosomes								
Treatment ...	−Phenobarbitone				+Phenobarbitone			
Radioactivity incorporated (c.p.m./mg of RNA)	15350 ±1950	163700 ±16650	16850† ±1550	163550† ±18900	16850 ±2350	179600 ±21900	20500† ±1200	196300† ±29200

* $P < 0.05$.
† $P > 0.05$.

barbitone-treated rats is associated with an increase in liver weight, in agreement with the conclusions of Kuriyama *et al.* (1969) for a single membrane protein, it must be concluded that the administration of the inducer not only results in an enhancement of the overall rate of protein biosynthesis but also in a decreased rate of protein degradation. This conclusion is of particular interest not only for understanding the mechanism involved in the process of cellular hypertrophy, but because it implies that phenobarbitone administration preserves the integrity of the hepatocyte under conditions that would normally lead to its atrophy.

Re-feeding of the animal restores the normal degree of polyribosomal aggregation and the rate of protein synthesis in rat liver (Sox & Hoagland, 1966). Phenobarbitone and the diet have thus a similar effect on the functional capacity of rat liver polyribosomes. This suggests that phenobarbitone and the diet influence the rate of protein synthesis by acting through a common physiological effector and suggests also the possibility that this or related molecules may be used to ameliorate a damaged liver function.

Though the present experiments do not provide direct experimental evidence for the nature of the mechanism involved, there is indirect support to the hypothesis that this mechanism may involve the redox state of the cell. From the analysis of the results obtained in various laboratories (Williamson *et al.*, 1967; Brosnan *et al.*, 1970; Gaja *et al.*, 1973; Sox & Hoagland, 1966; Pilkis & Korner, 1971; Smuckler & Trump, 1968; Ragnotti *et al.*, 1970; Cajone *et al.*, 1971; Nolan & Hoagland, 1971) it appears that an increased reduction of the cytoplasm is constantly accompanied by polyribosomal disaggregation and by a decreased rate of protein biosynthesis and that these alterations are promptly reversed on normalization of the redox state. Further, preliminary experiments performed in our laboratory have demonstrated that, in agreement with the findings of Murthy (1966), the addition of NADPH or NADH to a cell-free system inhibits protein synthesis in a concentration-dependent fashion.

Phenobarbitone administration, by increasing the activity of the hepatic 'drug-metabolizing system' (Conney *et al.*, 1960), increases the removal of reducing equivalents (Holtzman *et al.*, 1968), hence maintaining the cytoplasm in an oxidized state.

In this respect the results obtained by Rawat & Kuriyama (1973) are relevant. They demonstrated that the fall in the NAD^+/NADH ratio, which follows ethanol administration, is prevented by phenobarbitone.

Hence phenobarbitone should enhance the rate of protein synthesis by maintaning the cytoplasm in an oxidized state. The lowered phenobarbitone stimulation of protein synthesis after Sephadex chromatography may be interpreted in the sense that this treatment removes the excess of reducing equivalents from the control fractions.

When the activity and the polyribosomal patterns of membrane-bound and membrane-free polyribosomes from starved and starved phenobarbitone-treated animals were examined, it was found that whereas phenobarbitone had a great effect on the activity and the sedimentation profiles of the membrane-bound polyribosomes, it was ineffective with respect to the free polyribosomes, except when administered for the latter half of a 96h-starvation period. Since the ratio of membrane-free to membrane-bound polyribosomes, altered by starvation, is restored to normal after phenobarbitone administration and since both classes of polyribosomes respond to phenobarbitone administration with an increased rate of synthesis of a specific membrane protein (NADPH–cytochrome *c* reductase; Glazer & Sartorelli, 1972), the results can be interpreted as follows. After 48h of starvation without phenobarbitone treatment, a segment of the membrane-bound polyribosomal population detaches from the membranes and becomes free in the cytoplasm. The increased proportion of free polyribosomes without any major change in the total amount (Table 2), and the enlargement of the free ribosomal pool after starvation (Henshaw *et al.*, 1963), are in agreement with this interpretation. After this period of starvation, the free ribosomal population would then consist in control animals of two distinct classes of polyribosomes, one originally free in the cytoplasm and one originally bound to the membranes of the endoplasmic reticulum, whereas only the former is represented in the experimental livers. Thus phenobarbitone preserves the integrity of the membrane–ribosome interaction.

When the starvation period was increased to 96h, free polyribosomes from both control and experimental groups were equally and extensively degraded to monomeric ribosomes, but more free polyribosomes were recovered in the pellet from the control than from the phenobarbitone-treated group (G. Ragnotti & M. G. Aletti, unpublished work). The group starved for 96h and receiving phenobarbitone for the latter half of the period yielded the same polyribosome profile as those starved and phenobarbitone-treated for 48h, although the amount of free polyribosomes recovered was intermediate between the starved controls and those starved for 96h and treated throughout. These observations seem to indicate that all free polyribosomes are degraded to monomers in the control group, but only that part of the polyribosome population that is not responsive to phenobarbitone do so in the experimental group. The remaining polyribosomes, which are responsive to phenobarbitone, remain aggregated and may eventually bind to the membrane of the endoplasmic reticulum.

This interpretation receives experimental support from recent results obtained by Lowe & Hallinan (1973) on the biosynthesis *in vitro* of the phenobarbitone-inducible (Kuriyama *et al.*, 1969) membrane protein cytochrome *c* reductase. These authors have demonstrated that this enzyme is actively synthesized by free polyribosomes and that its synthesis is greatly enhanced soon after phenobarbitone administration.

In agreement with previous reports (Loeb *et al.*, 1967; Talal & Kaltreider, 1968; Ragnotti *et al.*, 1969; Tanaka *et al.*, 1970; Lee *et al.*, 1971; Venkatesan & Steele, 1972; Mishra *et al.*, 1972), our results suggest first that the two classes of polyribosomes present within the liver cell, though morphologically distinct, are not functionally separate and secondly that the membrane–ribosome interaction is a dynamic relationship that shifts as the functional requirements of the cell demand.

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