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The Effect of Phenobarbital on the Synthesis of Nascent Protein on Free and Membrane-Bound Polyribosomes of Normal and Regenerating Liver

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SUMMARY

The synthesis of nascent protein on free and membrane-bound polyribosomes or normal and regenerating liver was measured following a pulse injection of [^{3}H]leucine into the portal vein of rats. Neither the pool size nor specific activity of leucine was markedly affected by the various experimental conditions. A single dose of phenobarbital (100 mg/kg) elevated maximally (2–2.3-fold) the synthesis of nascent peptide on membrane-bound polyribosomes 3–14 hr after treatment with the inducer, whereas formation of protein on free polyribosomes increased 1.6-fold only 5 hr after the barbiturate. Partial hepatectomy caused a 2-fold increase in the synthesis of nascent protein on membrane-bound polyribosomes as early as 1 hr after the operation. The rate of peptide synthesis on membrane-bound polyribosomes increased up to 3.5-fold over sham-operated controls 12 hr posthepatectomy. The formation of protein on free polyribosomes following partial hepatectomy was elevated approximately 2-fold 1–12 hr after the operation. When partially hepatectomized animals were treated 1 hr before surgery with a single dose of phenobarbital, no further increase in the synthesis of nascent protein occurred on either population of polyribosomes, suggesting (a) maximal translation in response to the stress of partial hepatectomy and (b) the competitive nature of hyperplasia and the functional process of protein synthesis on membrane-bound polyribosomes.

Immunoprecipitation of pulse-labeled nascent protein on free and membrane-bound polyribosomes with an antiglobulin to rat liver NADPH–cytochrome *c* reductase revealed a 2-fold elevation in the amount of reductase synthesized on membrane-bound polyribosomes 3–5 hr after phenobarbital administration; no concomitant change in the synthesis of nascent enzyme was noted on free polyribosomes. Although NADPH–cytochrome *c* reductase was equally distributed between the two populations of polyribosomes, the total concentration of enzyme was 20 times higher on membrane-bound polyribosomes than on free polyribosomes.

INTRODUCTION

In the relatively early period following partial hepatectomy of rats, the proliferative requirement for DNA synthesis in liver predominates over the ability of the tissue to respond functionally to the presence of

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phenobarbital by the induced formation of drug-metabolizing enzymes. Thus partial hepatectomy causes a delay in the onset of appearance of barbiturate-induced mixed-function oxidase activity (1–4) and a premature termination of induction of NADPH–cytochrome *c* reductase (5). The factors involved in the metabolic regulation of cell proliferation and function in this system are unknown.

A number of reports have provided evidence that a differential function may exist for the two species of polyribosomes in the liver. Membrane-bound polyribosomes are believed to be involved primarily in the synthesis of serum proteins (6–8), whereas free polyribosomes synthesize predominantly intracellular proteins (9, 10). Since both the induction of drug-metabolizing enzymes and partial hepatectomy are associated with important reorganizations of endoplasmic reticulum, it is conceivable that regulatory mechanisms for these processes are associated with a distinct population of polyribosomes. To investigate this possibility the effects of the inducer phenobarbital, and of liver hyperplasia induced by partial hepatectomy, on the synthesis *in situ* of nascent protein on free and membrane-bound polyribosomes were measured.

MATERIALS AND METHODS

Materials. L-[4,5-^3H]Leucine (55 Ci/mmole) was purchased from New England Nuclear Corporation, and phenobarbital sodium, from Merck & Company.

Preparation of animals. Male Sprague-Dawley rats (Charles River Laboratories) weighing 175–225 g were employed in all experiments. Animals were housed over corncob bedding, and alternating periods of 12 hr dark and 12 hr light were maintained. Purina rat chow and water were available ad libitum.

Partial hepatectomies and sham operations were performed under ether anesthesia between 6:00 and 8:00 a.m. according to the method of Higgins and Anderson (11).

Phenobarbital was dissolved in 0.9% NaCl and injected intraperitoneally 1 hr before partial hepatectomy or sham operation at a dose of 100 mg/kg; control animals received an equivalent volume (approximately 1.0 ml) of 0.9% NaCl.

L-[4,5-^3H]Leucine was diluted in 0.9% NaCl (50 μCi/ml) and injected into the portal vein at a dose of 100 μCi/kg after laparotomy under ether anesthesia.

Preparation of hepatic polyribosomes. After [^3H]leucine had been allowed to incorporate for 3 min, free and membrane-bound polyribosomes were prepared on a discontinuous sucrose (ribonuclease-free) gradient without the use of deoxycholate by the method of Blobel and Potter (12). Details of this procedure have been reported previously (13). Protein samples were solubilized by heating at 60° for 20 min with 1 ml of Protosol (New England Nuclear), and radioactivity was determined after addition of 10 ml of Liquifluor scintillation fluid in a Packard Tri-Carb liquid scintillation spectrometer with a counting efficiency of 30%.

Protein was determined by the method of Lowry *et al.* (14).

Electron microscopy. Pellets of free or membrane-bound polyribosomes were fixed for 2 hr in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.4. The samples were washed in buffer and postfixed in 1% osmium tetroxide, pH 7.4. The pellets were dehydrated in a series of graded ethanol concentrations and treated with propylene oxide before embedment in Vestopal W (Henley Company). Sample blocks were sectioned on a Porter-Blum MT-2 ultramicrotome, and the sections were stained in uranyl acetate and lead citrate before viewing in a Zeiss EM-9S electron microscope.

Pool size of leucine. Homogenates prepared from livers perfused with ice-cold 0.9% NaCl from animals previously pulsed for 3 min with 100 μCi/kg of [^3H]leucine were treated with 0.5 N perchloric acid and centrifuged at 2500 × *g*. The acid-soluble supernatant fractions were neutralized with 6 N K_2CO_3, and the concentration of leucine was determined with a Beckman automatic amino acid analyzer.

Immunoprecipitations. Antireductase globulin against rat liver NADPH–cytochrome *c* reductase (specific activity, 35 μmoles/min/mg of protein) was prepared as previously described (15).

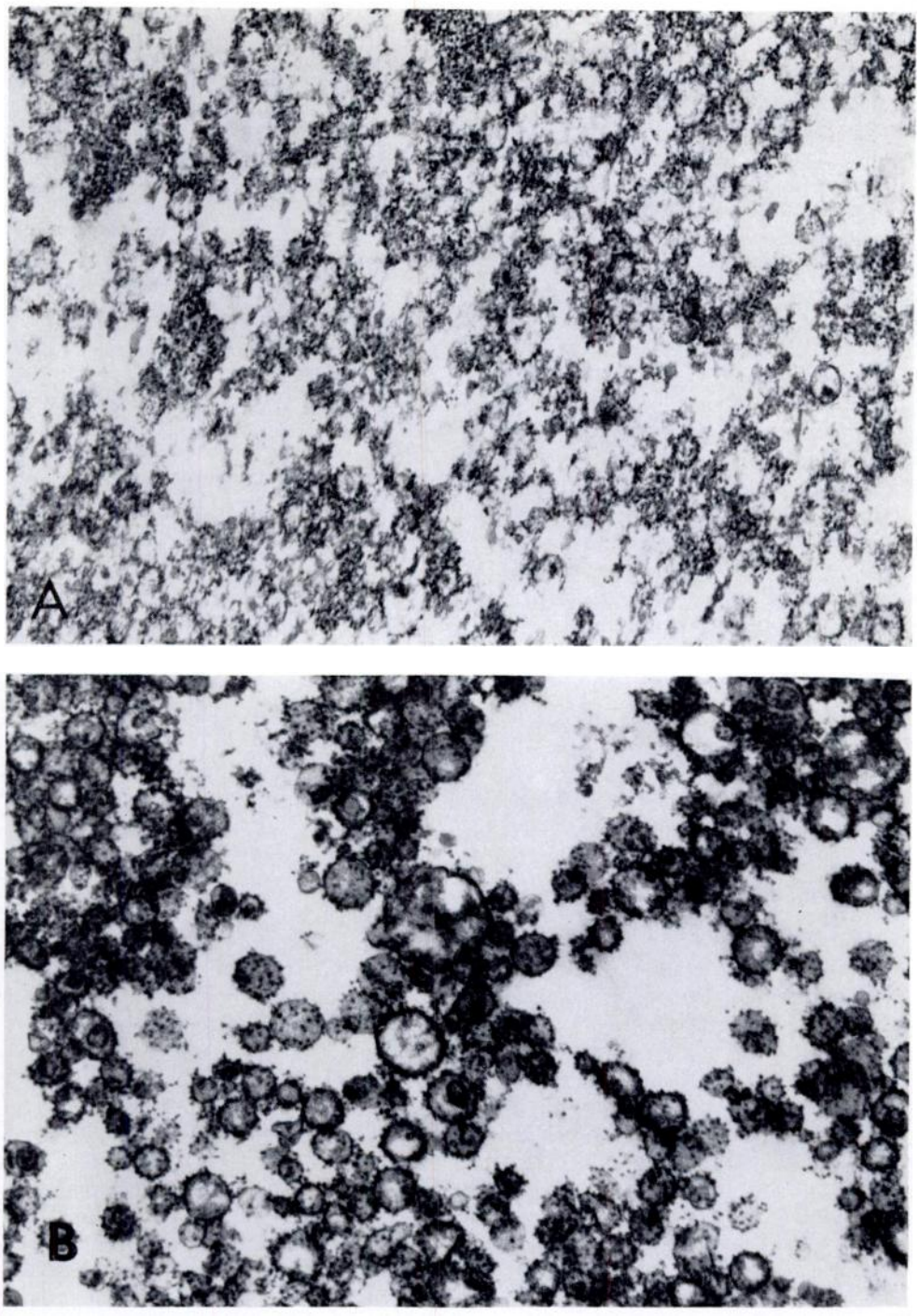

FIG. 1. *Electron micrographs of free and membrane-bound polyribosomes of normal liver*
Samples for electron microscopy were prepared as described under MATERIALS AND METHODS. × 33,000.
A. Free polyribosomes. B. Membrane-bound polyribosomes.

Precipitation of nascent NADPH–cytochrome *c* reductase by antireductase globulin was carried out by incubation of free (0.2 mg of protein) or bound (1.5 mg of protein) polyribosomes labeled with [^3H]-leucine with antireductase globulin or normal γ-globulin (5 mg of protein for free polyribosomes; 10 mg of protein for bound polyribosomes) and excess purified NADPH–cytochrome *c* reductase as carrier enzyme (50 μg protein) in 0.14 M NaCl–20 mM phosphate buffer, pH 7.4. Incubations were conducted for 1 hr at 37°, followed by 20 hr at 4°. After centrifugation at 2000 × *g* for 30 min, antigen-antibody precipitates were washed three times each with 10 ml of 0.9% NaCl. After solubilization at 60° for 20 min, 10 ml of Liquifluor were added, and radioactivity was determined. Net precipitated radioactivity by antireductase globulin was calculated as the counts per minute remaining after subtraction of background radioactivity obtained with normal γ-globulin.

RESULTS

Preparation of free and membrane-bound polyribosomes without the use of deoxycholate necessitated evaluation of the purity of the two fractions. Electron micrographs indicated that each population of polyribosomes was relatively free of the other (Fig. 1) and that the radioactivity associated with each fraction represented a purity of at least 96% (Table 1).

Analysis of the pool size and specific activity of leucine in the livers of control and phenobarbital-treated, sham-operated, and partially hepatectomized animals showed that there was no marked alteration in the pool size of leucine among the various groups at each time point (Table 2). Furthermore, changes in the specific activity of leucine were minimal and did not appear to exhibit any correlation with the alterations in the synthesis of nascent protein produced either by treatment with phenobarbital or by partial hepatectomy.

That [^3H]leucine is incorporated into nascent protein and not into intrinsic proteins of polyribosomes was suggested by a relatively short linear incorporation time of 3 min for both free and bound polyribosomes

TABLE 1

Cross-contamination of free and membrane-bound polyribosomes

Animals were pulse-labeled with 100 μCi/kg of [^{3}H]leucine for 3 min via the portal vein, and free and membrane-bound polyribosomes were isolated as described under MATERIALS AND METHODS. Each labeled fraction from the initial centrifugation was subsequently recentrifuged on a separate discontinuous sucrose gradient, and the areas of each gradient corresponding to free and membrane-bound polyribosomes were removed and counted.

Experiment	Labeled fraction from first centrifugation	Labeled fraction from recentrifugation	Radioactivity	
			Total	Percentage
			cpm	%
I	Free	Free	450	100
		Bound	20	4
	Bound	Bound	19,000	100
		Free	30	0.2
II	Free	Free	480	100
		Bound	15	3
	Bound	Bound	18,000	100
		Free	60	0.3

(Fig. 2). In addition, injection of puromycin, as well as a variety of other inhibitors of protein synthesis, into the portal vein 1 min before pulse-labeling with [^3H]leucine for 3 min resulted in marked inhibition of incorporation of labeled amino acid onto free and membrane-bound polyribosomes (13).

Sham-operated animals given a single injection of phenobarbital exhibited a pronounced increase in the labeling of nascent protein synthesized on membrane-bound polyribosomes 3–14 hr later; however, the synthesis of protein on free polyribosomes was not significantly enhanced by phenobarbital except at 5 hr after administration of the inducer (Fig. 3).

Partial hepatectomy requires the synthesis of a variety of proteins for replication, and this need was reflected in a pronounced stimulation of the rate of synthesis of peptide, with a 3.5-fold maximal elevation occurring on membrane-bound polyribosomes at 14 hr and a 60–70% increase in the formation of nascent peptide taking place on free

TABLE 2

Pool size and specific activity of leucine after partial hepatectomy and treatment with phenobarbital

The pool sizes and specific activities of leucine of perchloric acid-soluble extracts prepared from liver were determined as described under MATERIALS AND METHODS. Animals were pulse-labeled for 3 min via the portal vein with 50 μCi/kg of [^{3}H]leucine at the indicated times after sham operation or partial hepatectomy. Animals were administered 100 mg/kg of phenobarbital intraperitoneally 1 hr before operation. Each value represents one determination from acid-soluble supernates pooled from the perfused livers of three animals.

Treatment	Time after operation	Leucine content		Specific activity	
		Total	Percentage[a]	Total	Percentage
	hr	*μmole/g (wet wt)*	%	*cpm/μmole leucine*	%
NaCl, sham	1	0.138	100	36,900	100
Phenobarbital, sham	1	0.128	93	38,900	93
NaCl, PH[b]	1	0.140	100	54,100	100
Phenobarbital, PH	1	0.106	76	76,100	141
NaCl, sham	3	0.110	100	39,900	100
Phenobarbital, sham	3	0.099	90	36,000	90
NaCl, PH	3	0.088	100	55,700	100
Phenobarbital, PH	3	0.084	95	65,300	117
NaCl, sham	14	0.082	100	50,500	100
Phenobarbital, sham	14	0.068	83	70,400	139
NaCl, PH	14	0.069	100	86,700	100
Phenobarbital, PH	14	0.094	115	76,400	88

[a] The percentage of values for phenobarbital-treated animals in comparison with their respective NaCl-treated controls is taken as 100.

[b] PH, partial hepatectomy.

polyribosomes at 14–24 hr after the operation (Fig. 4). The increase in specific radioactivity of nascent protein on the membrane-bound fraction increased as early as 1 hr after operation, while a significant elevation in the labeling of peptide synthesized on free polyribosomes occurred 14 hr postoperation. Injection of phenobarbital 1 hr before partial hepatectomy did not result in any additional increase in the rate of incorporation of [^3H]leucine into protein over partial hepatectomy alone on either class of polyribosomes (Fig. 4, inset), suggesting that translational processes were maximally elevated by the operation.

The enhanced synthesis of protein on membrane-bound polyribosomes by phenobarbital was dramatically reflected in an increase in the ratio of bound to free polyribosomes synthesizing nascent peptide (Fig. 5A). In contrast, the increased ratio of specific activities of bound to free polyribosomes resulting after partial hepatectomy was not further influenced by administration of phenobarbital (Fig. 5B).

Immunoprecipitation of nascent protein by an antiglobulin to rat liver NADPH–cytochrome *c* reductase was carried out in 0.14 M NaCl in 20 mM phosphate buffer, pH 7.4, to minimize nonspecific precipitation by polyribosomes (16). Under these conditions background precipitation with γ-globulin averaged 29% for free polyribosomes and 8% for membrane-bound polyribosomes (Table 3). Incubations were performed with an excess of antibody as determined from antigen-antibody titrations (Fig. 6). Animals given a single intraperitoneal dose of 100 mg/kg of phenobarbital exhibited a 2-fold elevation in immunoreactive radioactivity on membrane-bound polyribosomes 3–5 hr after treatment with the inducer (Table 3); however, no alteration in precipitable labeled protein by antireductase

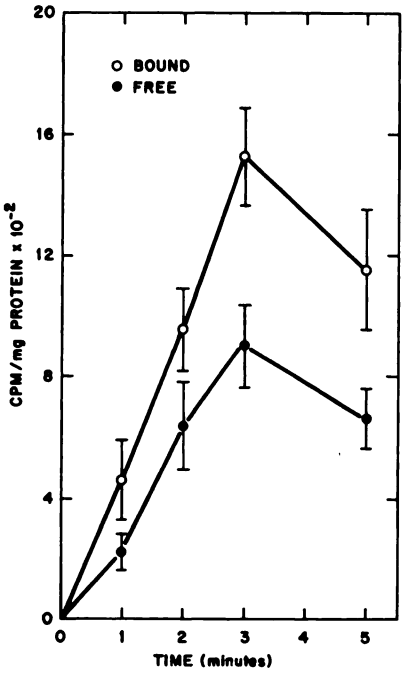

FIG. 2. *Incorporation of [³H]leucine into nascent protein of free and membrane-bound polyribosomes of normal liver*

Each point represents the mean ± standard error of separate determinations on four animals. Rats were anesthetized with ether and pulse-labeled with [³H]leucine via the portal vein after laparotomy. At the indicated time intervals the livers were excised and free and membrane-bound polyribosomes were prepared as described previously (13).

globulin was exhibited with free polyribosomes. Although the immunoprecipitated protein was equally distributed between both populations of polyribosomes from control and phenobarbital-treated animals, the total amount of antigen to antireductase globulin was 20 times greater on membrane-bound polyribosomes than on the free fraction.

DISCUSSION

A number of reports have indicated the requirement for protein synthesis in the induction of drug-metabolizing enzymes by phenobarbital. Thus the incorporation of labeled amino acids into ribosomal protein both *in vivo* and *in vitro* was stimulated by phenobarbital, and inhibition by puromycin of the induction of aminoazo dye demethylation by phenobarbital has been observed (17–20).

The present report suggests that differences in the pool size and specific activity of the administered isotope, leucine, play a minor if not insignificant role in the increased synthesis of nascent protein produced by phenobarbital or partial hepatectomy. The possibility of an enhancement in the rate of protein synthesis due to preferential stabilization by phenobarbital of a particular population of polyribosomes has been suggested by a decrease in the activity of microsomal ribonuclease 12–24 hr following

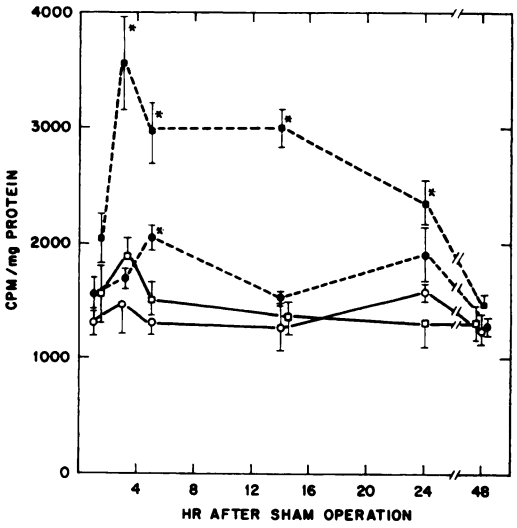

FIG. 3. *Effect of phenobarbital on incorporation of [³H]leucine into nascent protein on hepatic free and membrane-bound polyribosomes of sham-operated animals*

Each point represents the mean ± standard error of separate determinations on eight animals. Animals were treated 1 hr before sham operation with 0.9% NaCl or phenobarbital (100 mg/kg), and nascent protein was pulse-labeled with [³H]-leucine for 3 min at the indicated times after operation, as described in Fig. 2. See the text for additional experimental details. O——O, free polyribosomes, NaCl-treated; □——□, bound polyribosomes, NaCl-treated; ●- - -●, free polyribosomes, phenobarbital-treated; ■- - -■, bound polyribosomes, phenobarbital treated. Asterisks (*) indicate statistical significance ($p < 0.05$) compared with NaCl-treated animals.

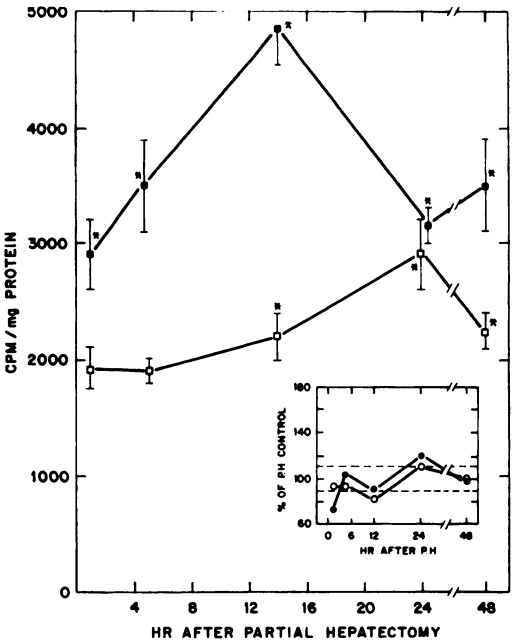

FIG. 4. *Effect of partial hepatectomy and phenobarbital on incorporation of [³H]leucine into nascent protein on free and membrane-bound polyribosomes*

Each point represents the mean ± standard error of separate determinations on six animals. Animals were treated 1 hr before partial hepatectomy with 0.9% NaCl and pulse-labeled with [³H]leucine for 3 min at the indicated times after operation, as described in Fig. 2. The inset indicates the response of partially hepatectomized (P.H.) animals to a single dose (100 mg/kg) of phenobarbital administered 1 hr before operation, with data expressed as a percentage of NaCl-treated, partially hepatectomized controls. □, free polyribosomes NaCl-treated; ■, bound polyribosomes, NaCl-treated; ○, free polyribosomes, phenobarbital-treated; ●, bound polyribosomes, phenobarbital-treated. Asterisks (*) indicate statistical significance ($p < 0.05$) compared with NaCl-treated, sham operated animals in Fig. 3.

a single dose of phenobarbital (21, 22) and by the greater stability of hepatic polyribosomes after multiple doses of the barbiturate (23). However, in agreement with previous reports (23, 24), we have not been able to detect any ribonuclease activity associated with either free or membrane-bound polyribosomes of 0.9% NaCl- or phenobarbital-treated rats.[2] These results suggest the unimportance of increased hepatic polyribosome stability and possibly an elevation in nascent peptide synthesis as a result of a diminution of microsomal ribonuclease activity, particularly at the early time periods examined.

The functional significance of the difference in the relative rates of synthesis of nascent protein by the two populations of polyribosomes in the inductive processes initiated by phenobarbital remains to be elucidated. Previous histological and biochemical studies have indicated that separate functional roles may exist for free and bound polyribosomes of liver (6, 7, 9, 10, 25–27). Serum proteins, such as albumin, which are exported from the hepatic cell, are synthesized predominantly on polyribosomes bound to the endoplasmic reticulum (6, 7, 9, 10), whereas proteins for intracellular use, such as ferritin, are fabricated mainly on free polyribosomes (9, 10). Our experiments indicate that the percentages of immunoprecipitable NADPH–cytochrome *c* reductase distributed on both free and bound polyribosomes are similar. These results are in agreement with the study by Ragnotti *et al.* (28), in which the synthesis of NADPH–cytochrome *c* reductase was measured *in vitro* by a nonimmunochemical procedure. The present study indicates further that a single dose of phenobarbital accelerates the synthesis of nascent NADPH–cytochrome *c* reductase only on membrane-bound polyribosomes of rat liver and that a greater total amount of this enzyme is associated predominantly with this fraction. These data indicate that phenobarbital may specifically augment the synthesis of proteins required for mixed-function oxidase activity, on membrane associated polyribosomes. This is further supported by the work of Glaumann (20), who demonstrated that phenobarbital-stimulated incorporation of amino acids into the rough endoplasmic reticulum of the liver was not associated with increased synthesis of albumin, a protein normally fabricated exclusively by membrane-bound polyribosomes.

[2] Unpublished observations.

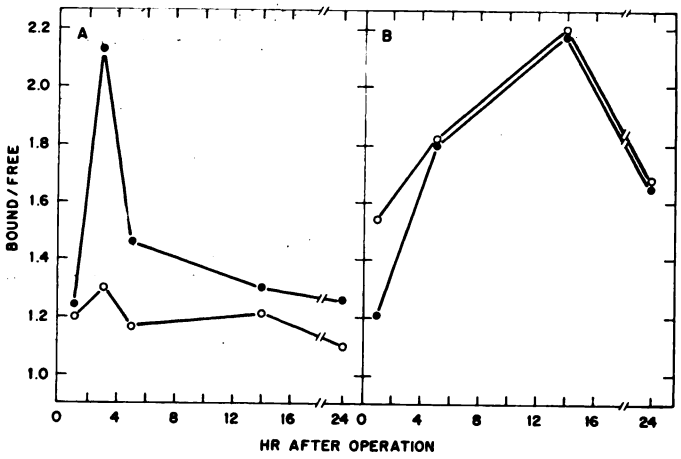

FIG. 5. *Ratio of nascent protein synthesis on membrane-bound and free polyribosomes after phenobarbital treatment 1 hr before sham operation or partial hepatectomy*

Each point presents the mean of six to eight animals, with a standard error of 10–15%. A. Ratios of membrane-bound to free polyribosomes for 0.9% NaCl-treated (○) and phenobarbital-treated (●), sham-operated animals. B. Ratios of membrane-bound to free polyribosomes for NaCl-treated (○), and phenobarbital-treated (●), partially hepatectomized animals. Ratios were derived from the specific activities of nascent protein synthesized on each fraction of polyribosomes.

TABLE 3

Precipitation with antireductase globulin of free and membrane-bound polyribosomes prepared from 0.9% NaCl- and phenobarbital-treated normal rats

Incubations of free and membrane-bound polyribosomes were carried out as described in Fig. 6. Control values are given as the means ± standard errors of duplicate determinations of preparations from 10 animals. Values from phenobarbital-treated animals represent the means of duplicate determinations on preparations from two or three rats. The number of hours in parentheses represents the time after treatment with phenobarbital.

Treatment	Polyribosome fraction	Radioactivity added	Radioactivity + γ-globulin (A)	Radioactivity + antireductase globulin (B)	A − B	[(A − B)/ cpm added] × 100
		cpm	*cpm*	*cpm*	*cpm*	
NaCl	Free	80 ± 10	20 ± 2	50 ± 7	30	37
	Bound	3090 ± 400	240 ± 30	1070 ± 130	830	27
Phenobarbital (1 hr)	Free	100	50	80	30	30
	Bound	3250	310	1170	860	26
Phenobarbital (3 hr)	Free	100	15	45	30	30
	Bound	4640	190	1980	1790	39
Phenobarbital (5 hr)	Free	90	30	70	40	44
	Bound	4970	450	1770	1310	26
Phenobarbital (15 hr)	Free	120	50	90	40	33
	Bound	4740	340	1200	860	18
Phenobarbital (24 hr)	Free	120	30	60	30	25
	Bound	3760	380	1120	740	20

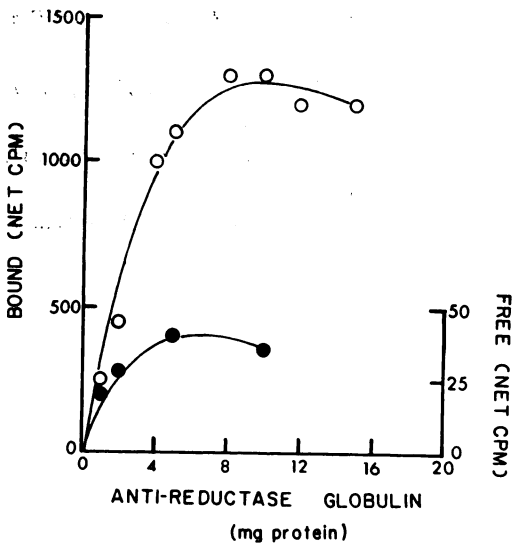

FIG. 6. *Precipitation of free and membrane-bound polyribosomes with antireductase globulin*

Free and membrane-bound polyribosomes were prepared from normal rats which had been pulse-labeled for 3 min with 100 μCi/kg of [^{3}H]leucine. Incubations of labeled polyribosomes with antireductase globulin were carried out as described under MATERIALS AND METHODS. The concentration of free polyribosomes (●——●) was 0.2 mg of protein, and that of membrane-bound polyribosomes (○——○) was 1.5 mg of protein. "Net cpm" represents the counts per minute present in the antigen-antibody precipitate after subtraction of the counts per minute in the γ-globulin precipitate blank.

Imposition of the stress of partial hepatectomy upon animals previously treated with phenobarbital did not result in an elevation of the rate of synthesis of nascent protein on free and membrane-bound polyribosomes over that caused by partial hepatectomy alone. These findings are in agreement with the concepts of previous reports from this laboratory showing the competitive effects of hepatic cellular proliferation upon the ability of the liver to respond to the functional demands of an inducer such as phenobarbital (1–3, 5). Since the regenerating liver is in a state of hyperplasia, the increased requirement for proteins involved in replication results, as shown in the present report, in an elevated rate of synthesis of nascent peptide on both free and membrane-bound polyribosomes. Phenobarbital treatment did not further increase the rate of new peptide synthesis, suggesting that the system was translating at maximal rates and that the competitive processes of growth and cell function may reside, at least in part, at the level of peptide synthesis.

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