Studies on the Mechanism of Drug-Induced Microsomal Enzyme Activities

V. Phenobarbital Stimulation of Endogenous Messenger RNA and Polyuridylic Acid-Directed L-[^{14}C]-Phenylalanine Incorporation

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SUMMARY

The *in vivo* administration of phenobarbital increases amino acid incorporation in rat liver microsomes *in vitro*.

1. The increased incorporation is observed with different amino acids and is not due to changes in cofactor level, amino acid concentration, or factors present in the cell sap.

2. Microsomes from phenobarbital-treated rats are more active in L-[^{14}C]-phenylalanine incorporation in the absence of polyuridylic acid. However, L-[^{14}C]-phenylalanine incorporation is stimulated to the same extent by polyuridylic acid in microsomes from control and phenobarbital-treated rats. In contrast, after the removal of endogenous messenger RNA the microsomes from phenobarbital-treated rats are more than twice as sensitive as control microsomes to polyuridylic acid-directed L-[^{14}C]-phenylalanine incorporation. This appears due to a phenobarbital-induced increase in both the endogenous microsomal messenger RNA content and the number of microsomal binding sites for messenger RNA. No difference in amino acid incorporation is observed in ribosomes from control and phenobarbital-treated rats, indicating that deoxycholate-soluble factors, presumably components of the endoplasmic reticulum, are important in protein synthesis and are altered by phenobarbital.

3. Microsomes from phenobarbital-treated rats contain a relatively greater proportion of membrane-bound ribosomes compared to free ribosomes.

INTRODUCTION

The administration of phenobarbital increases the activities of a number of rat liver microsomal enzyme systems (1–3). These changes are accompanied by morphological alterations characterized by an increase in the amount of agranular endoplasmic reticulum (4–6). A number of studies suggest that the phenobarbital-induced increases in enzyme activity are due to enzyme synthesis, although there is no direct evidence demonstrating an actual increase in enzyme protein. For example, the stimulatory effect of phenobarbital on some of these enzymes is prevented by ethionine or puromycin (7, 8), and chronic pheno-

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barbital administration increases rat liver microsomal protein content. In addition, liver microsomes from phenobarbital-treated rats show greater amino acid-incorporating activity when measured *in vivo* (9) or in cell-free preparations (10) and exhibit a greater sensitivity to stimulation by polyuridylic acid (11).

In this paper we investigated the mechanism of the phenobarbital stimulation of protein synthesis. We examined the activity of isolated microsomes and ribosomes in the incorporation of phenylalanine under conditions where incorporation was directed by endogenous messenger ribonucleic acid (mRNA). We also examined the optimal cofactor requirements for this activity in both phenobarbital-treated and control rats. In addition, we present further data on the effect of phenobarbital on the sensitivity of microsomes and ribosomes to synthetic messenger RNA, polyuridylic acid. This activity was measured under conditions where L-[^{14}C]-phenylalanine incorporation was directed solely by the added messenger RNA. In other experiments we examined the effect of phenobarbital on the distribution of free and membrane-bound ribosomes and on the yield of rat liver smooth endoplasmic reticulum.

MATERIALS AND METHODS

Materials. Sigma Chemical Company supplied the ATP, reduced glutathione, and phosphocreatine. California Corporation for Biochemical Research supplied the creatine phosphokinase (EC 2.7.3.2) and GTP. ^{14}C-labeled amino acids were obtained from the New England Nuclear Corporation. The source of polyuridylic acid was Miles Laboratories, Elkhart, Indiana.

Methods. Groups of four Sprague-Dawley female rats weighing 160–170 *g* were injected intraperitoneally with 80 mg/kg of sodium phenobarbital in 0.9% sodium chloride at 42 and 18 hr prior to decapitation. Controls were given saline only. Rats were fasted 42 hr before decapitation. The livers were removed, homogenized in a loose-fitting Potter-Elvehjem type homogenizer in 5 volumes of 0.25 M sucrose, and centrifuged for 10 min at 12,000 *g* to sediment the nuclear and mitochondrial fractions. The supernatant was centrifuged at 105,000 *g* for 1 hr and the microsome pellet was suspended in 0.25 M sucrose at protein concentration of 17.5 mg/ml.

The ribosomes were isolated by the method of Korner (12). The mitochondrial supernatant was treated with one-ninth its volume of 5% deoxycholic acid in 0.03 M Tris-HCl buffer, pH 7.5, 1.5×10^{-3} M $MgCl_2$ and 5.0×10^{-2} M KCl and was centrifuged for 2 hr at 105,000 *g*. The pellets were resuspended in 0.03 M Tris-HCl buffer pH 7.5, 1.5×10^{-3} M $MgCl_2$. The standard incubations contained the following in a volume of 1.7 ml: 20 μmoles potassium phosphate pH 7.4, 2.5 μmoles ATP, 0.5 μmole GTP, 10 μmoles $MgCl_2$, 40 μmoles phosphocreatine, 0.25 mg creatine phosphokinase (EC 2.7.3.2), 100 μmoles reduced glutathione (GSH), neutralized with KOH, 150 μmoles sucrose, 0.085 μmole uniformly labeled L-[^{14}C]-phenylalanine (specific activity 9.13 μC/μmole), 0.2 ml (containing 2 mg protein) supernatant fluid from control rats, and either 0.4 ml (containing 7.0 mg protein) microsomes or 0.4 ml (containing 3.5 mg protein) ribosomes. The incubations were made in duplicate at 37° for 15 min as described earlier (10).

Where indicated, preincubations of the microsomes or ribosomes were performed for 12 min at 37° in the presence of all the ingredients listed above but in the absence of L-[^{14}C]-phenylalanine. After preincubation, the flasks were chilled in ice, additional creatine phosphate (40 μmoles), creatine phosphokinase (0.25 mg), L-[^{14}C]-phenylalanine, and polyuridylic acid (300 μg) were added. The flasks were then incubated in the manner indicated above.

The reactions were stopped with 1.7 ml of cold 10% trichloroacetic acid (TCA) containing 0.1% L-[^{12}C]-phenylalanine. The precipitated proteins were washed and counted as previously described (13). Zero time counts were usually less than 10 cpm per milligram proteins. These values were subtracted from the values obtained with the incubated proteins. Protein estimations were done by the Folin-Ciocalteu method

of Lowry *et al.* (14). RNA was estimated by the orcinol method of Mejbaum (15). Sucrose density gradient centrifugations to separate free from bound ribosomes in the microsomal fractions of rat liver were carried out by the method of Henshaw *et al.* (16). For the preparation of membrane fraction from the microsomes we used the method of Dallner *et al.* (17).

RESULTS

Effect of Phenobarbital-Treatment on the Content of Protein and RNA of Liver Microsomes

Phenobarbital administered to rats increases their average liver weight by 16%. The microsomal pellet obtained from these rats appears larger than that of the controls. The average yield of microsomal protein isolated from phenobarbital-treated rats is approximately 30% greater than the yield from controls. The average yield of microsomal RNA isolated from phenobarbital-treated rats is increased by about 10%. Thus, there is a disproportionately greater content of protein and hence a slight decrease in the RNA to protein ratio of microsomes from phenobarbital-treated rats.

The Effect of in Vivo Phenobarbital Treatment on in Vitro Microsomal Amino Acid Incorporation

Figure 1 shows the *in vitro* incorporation of ^{14}C-labeled L-arginine, L-leucine, L-lysine, L-phenylalanine, and L-valine in microsomes from phenobarbital-treated and control rats. Using 85 mμmoles of ^{14}C amino acid precursor in the system, there was at least a twofold increase in the incorporating activity of phenobarbital microsomes. With the exception of L-[^{14}C]-phenylalanine the increased incorporation ranged within the relatively narrow limits of 108–134%. With L-[^{14}C]-phenylalanine, however, the microsomes from phenobarbital-treated rats incorporated over 268% greater amounts than did the control microsomes. This may be due to a relatively

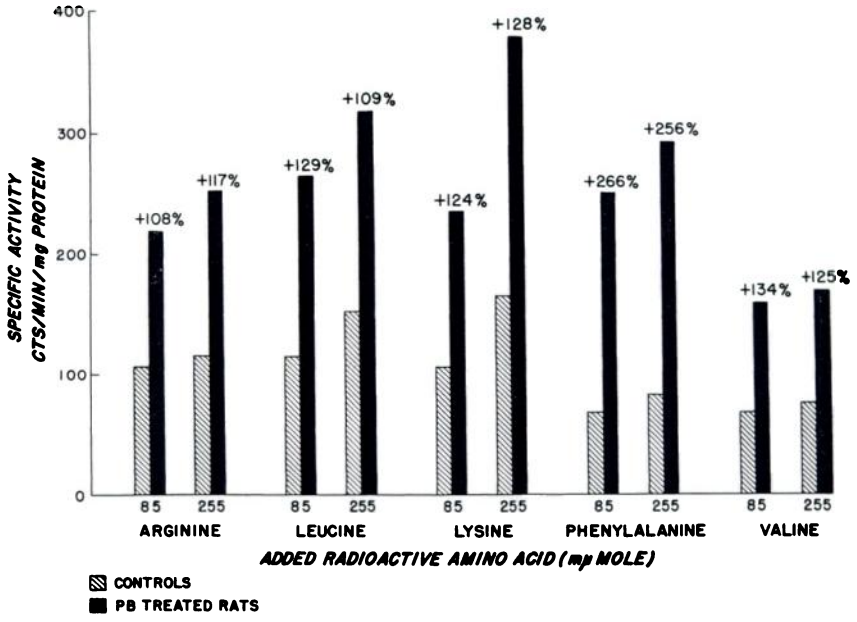

FIG. 1. *In vitro amino acid incorporation by microsomes from control and phenobarbital-treated rats*

Incubations were as indicated in Methods. The specific acivities of each L-amino acid were as follows: arginine (S. A. 9.23 mC/mmole), leucine (S.A. 5.7 mC/mmole), lysine (S. A. 7.1 mC/mmole), phenylalanine (S. A. 9.13 mC/mmole), valine (S. A. 4.8 mC/mmole).

greater content of L-[^{14}C]-phenylalanine in the newly synthesized proteins of phenobarbital-treated rats. Figure 1 also shows amino acid incorporation in the same preparations when 255 mμmoles, or a threefold greater concentration, of amino acid was used. The similar results obtained with this level of amino acid indicate that the observed differences are not due to altered levels of endogenous amino acids.

Effect of Phenobarbital on the Rate of L-[^{14}C]-Phenylalanine Incorporation into Protein

Figure 2 demonstrates the rate of incorporation over a 30-min period for the microsomes from normal and phenobarbital-treated rats. Both the initial rate of incorporation as well as the total amount of amino acid incorporated at 30 min is higher in the preparation from phenobarbital-treated rats. If the effect of phenobarbital was to increase the stability of the microsomal incorporating system, one might expect similar initial rates. The results shown in Fig. 2 suggest that the greater incorporation in microsomes from phenobarbital-treated animals is independent of any preservative effect of phenobarbital on the active life of the incorporating system.

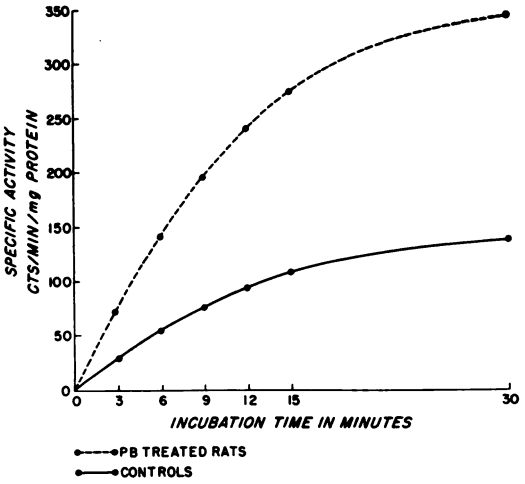

FIG. 2. *The rate of* L-[^{14}C]-*phenylalanine incorporation in microsomes from normal and phenobarbital-treated rats*

Incubations were as indicated in Methods.

We also found that the increased L-[^{14}C]-phenylalanine incorporation of phenobarbital-treated microsomal preparations relative to the control is maintained over an eight-fold variation in the level of L-[^{14}C]-phenylalanine added to the system. This suggests that the stimulation of L-[^{14}C]-phenylalanine into protein by phenobarbital is not due to a decrease in the endogenous L-[^{12}C]-phenylalanine levels which might have decreased the dilution of L-[^{14}C]-phenylalanine added to the system and hence yielded a higher specific activity of the L-[^{14}C]-phenylalanine precursor pool.

Effect of ATP, $MgCl_2$, Creatine Phosphate, and Creatine Phosphokinase, GTP, and GSH on the Incorporation of L-[^{14}C]-Phenylalanine

Figure 3 shows the effect of varying amounts of ATP, $MgCl_2$, and creatine phosphate plus creatine phosphokinase on L-[^{14}C]-phenylalanine incorporation in microsomes isolated from phenobarbital-treated and control rats. The greater activity of microsomes from phenobarbital-treated rats is observed both at optimal and suboptimal cofactor concentrations. Both microsomes from phenobarbital-treated and control rats exhibited maximal incorporation at the same levels of cofactors added to the system. This indicates that the increased L-[^{14}C]-phenylalanine incorporation *in vitro* in the microsomal preparation from phenobarbital-treated rats is not due to a possible phenobarbital-induced variation in the endogenous levels of ATP, $MgCl_2$, or creatine phosphate and creatine phosphokinase.

The effects of various concentrations of GTP and GSH on L-[^{14}C]-phenylalanine incorporation are shown in Fig. 4. The greater incorporating activity of the microsomes from phenobarbital-treated rats was observed over a wide range of concentrations of added GTP and GSH. The similar shapes of the curves of incorporating activity versus cofactor concentration indicates that microsomes from control and

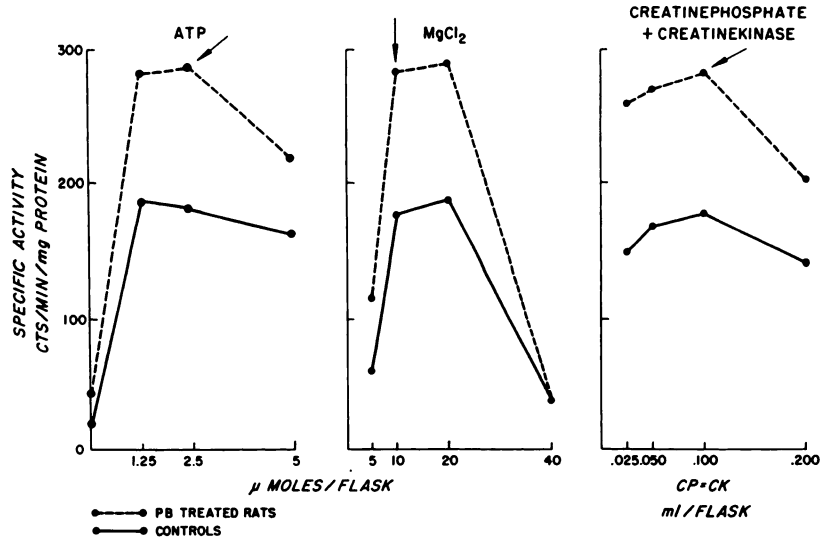

FIG. 3. *Effect of ATP, $MgCl_2$, and creatine phosphate and creatine phosphokinase on* L-[^{14}C]-*phenylalanine incorporation in microsomes from control and phenobarbital-treated rats*

The arrow indicates the amount of added cofactors in the standard incubation. Experimental conditions are described in Methods. Concentrations of CP and CK were 400 μmoles/ml and 2.5 mg/ml, respectively.

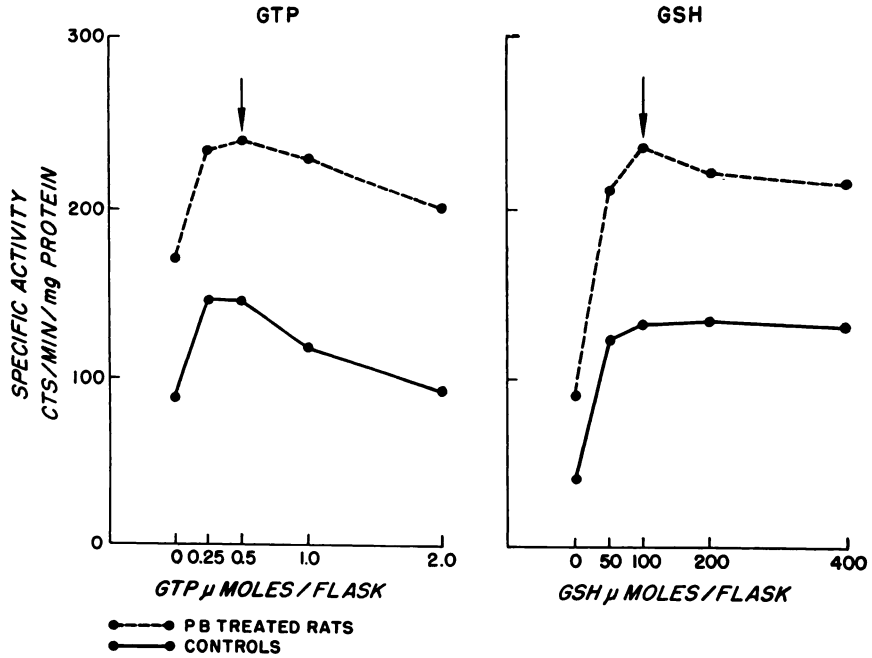

FIG. 4. *Effect of GTP and GSH on* L-[^{14}C]-*phenylalanine incorporation by the microsomes from control and phenobarbital-treated rats*

The arrow indicates the amount of added cofactors in the standard incubation. Experimental conditions are described in Methods.

phenobarbital-treated rats have similar if not identical requirements for amino acid incorporation. The identical requirements for GTP are of particular interest in the light of the report by Hoagland *et al.* (18) of a microsomal inhibitor of amino acid incorporation whose action is antagonized by GTP. Our data indicate that the effect of phenobarbital is not mediated through an alteration of this factor. If this were the case, no increased incorporation would be observed with high levels of GTP and the shape of the GTP requirement curve would be altered.

Effect of Microsome and Ribosome Concentration on the L-[^{14}C]-*Phenylalanine Incorporating Activity in Preparations from Control and Phenobarbital-Treated Rats*

Figures 5A and 5B show the results of experiments in which various amounts of microsomes and ribosomes were incubated in the standard incubation mixture. The input of microsomal protein varied from 3.5 to 10.5 mg protein and the input of ribosomes varied from 2.67 to 5.25 mg protein. The specific activity of protein was determined and then corrected for the amount of RNA added to each flask. Over a threefold range of added microsomes the L-[^{14}C]-phenylalanine incorporating activity into protein of the microsomes from phenobarbital-treated rats was more than 250–350% more active than control preparations (Fig. 5A). On the other hand, ribosomes prepared from the same phenobarbital-treated rats were only 20–40% more active than control ribosomes (Fig. 5B). Thus, in the case of microsomes the increased activity is maintained over a wide range of microsomal concentrations indicating that the effect is not due to a relative lack of microsomal enzymes such as aminoacyl transferases which might be relatively deficient in the control preparation. The increased activity may be due to either a greater content of messenger RNA or a more rapid reading of the messenger RNA present. With ribosomes prepared by

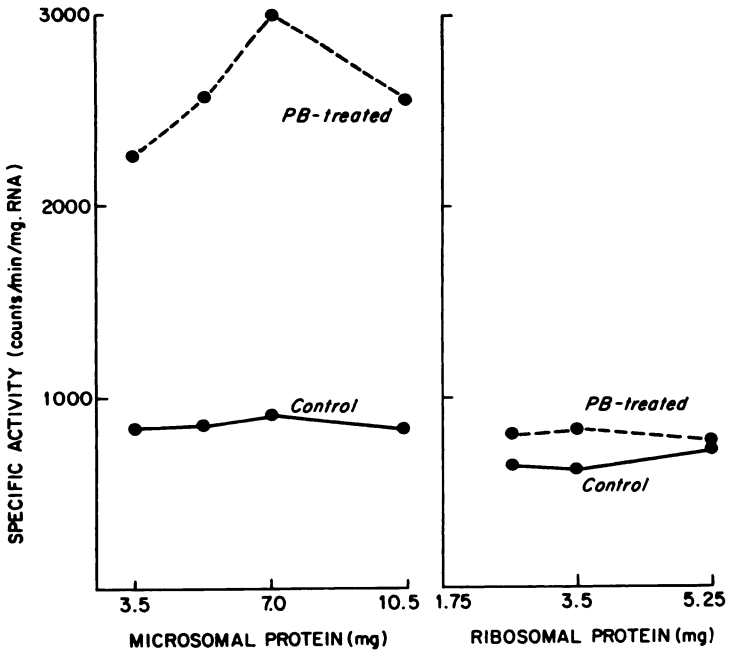

FIG. 5. *Effect of microsome and ribosome concentration on* L-[^{14}C]-*phenylalanine incorporation in control and phenobarbital-treated systems*

A, left; B, right.

deoxycholate treatment, the large differences in activity between the preparations are not observed. Thus deoxycholate treatment removes those factors that cause increased incorporating activity in the microsomes from phenobarbital-treated rats.

Effect of Cell Sap on L-[^{14}C]-*Phenylalanine Incorporation by Microsomes from Control and Phenobarbital-Treated Rats*

The microsomes from phenobarbital-treated rats are more active than those from the controls over the range of 0.5–4 mg of supernatant protein (Fig. 6). In all standard incubations, saturating levels of control soluble fractions are used. Thus, the increased incorporation is not due to factors which are normally present in cell sap. Figure 6 also shows that the microsomes from phenobarbital-treated rats require twice the amount of cell sap (1.0 mg cell sap protein) to attain maximum activity than do the control microsomes (0.5 mg cell sap protein).

L-[^{14}C]-*Phenylalanine Incorporation in Phenobarbital-Treated and Control Adrenalectomized Rats*

The administration of cortisone to rats increases liver weight and stimulates both RNA synthesis and the incorporation of amino acids into protein (19, 20). Furthermore, the administration of phenobarbital causes an increased release of ACTH from the hypophysis and elevates the concentration of plasma corticosterone in rats. Hence, one possible mechanism for the phenobarbital-induced stimulation of L-[^{14}C]-phenylalanine incorporation is indirect, i.e., through the release of ACTH resulting in corticosteroid stimulation of protein synthesis. This appears not to be the mechanism of phenobarbital stimulation. Thus, Fig. 7 shows that phenobarbital treatment stimulates microsomal L-[^{14}C]-phenylalanine-incorporating activity in adrenalectomized rats. Another possible effect of corticosteroid was an effect on free amino acid pool size in the liver. We, therefore, compared the microsomal preparation from control adrenalectomized and phenobarbital-treated adrenalectomized rats over

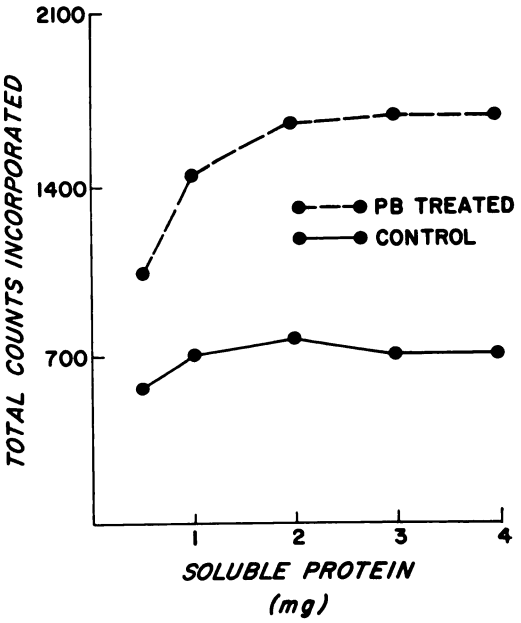

FIG. 6. *Effect of soluble fraction on* L-[^{14}C]-*phenylalanine incorporation by microsomes from control and phenobarbital-treated rats*

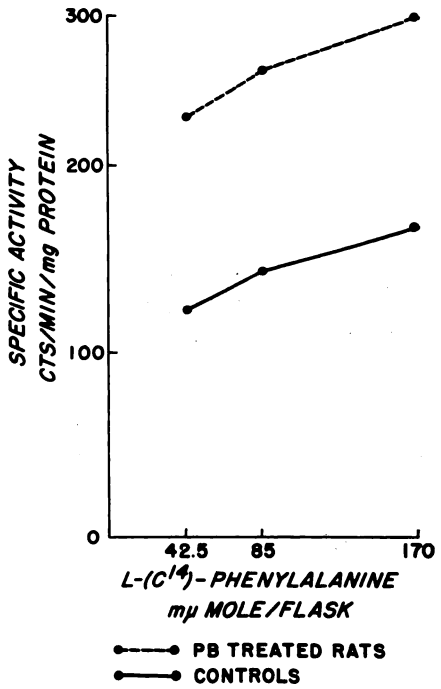

FIG. 7. *Effect of phenobarbital treatment of adrenalectomized rats on the ability of microsomes to incorporate* L-[^{14}C]-*phenylalanine*

a fourfold difference in L-[^{14}C]-phenylalanine concentration. The greater incorporation in the preparation from phenobarbital-treated rats was observed over the entire range of amino acid concentration tested. This indicates that the action of phenobarbital is not mediated through an effect on adrenal metabolism.

Effect of Preincubation of Microsomes on L-[^{14}C]-Phenylalanine Incorporation in the Absence and Presence of Polyuridylic Acid

Figure 8 shows the effect of time of preincubation of microsomes from control and phenobarbital-treated rats on subsequent L-[^{14}C]-phenylalanine incorporation. After the indicated preincubation period, additional creatine phosphokinase, creatine phosphate, and radioactive phenylalanine were added; the reaction mixture was then incubated for 15 min in the presence or absence of polyuridylic acid (300 μg). The absolute loss of L-[^{14}C]-phenylalanine-incorporating activity in the microsomes from phenobarbital-treated rats is twice as great as in the initially less active microsomes from control rats. However, the rate of loss of L-[^{14}C]-phenylalanine-incorporating activity appears to be similar in both preparations. Thus, the greater incorporating activity of microsomes from phenobarbital-treated rats appears not to be due to a relatively greater stability of the endogenous messenger RNA under our incubation conditions. There is a complete loss of L-[^{14}C]-phenylalanine-incorporating activity in both preparations after 15 min preincubation. This loss of activity is dependent on the presence of each of the cofactors required for maximum L-[^{14}C]-phenylalanine incorporating-activity, i.e., Mg^{2+} ion, ATP, GTP, GSH, and creatine phosphate-

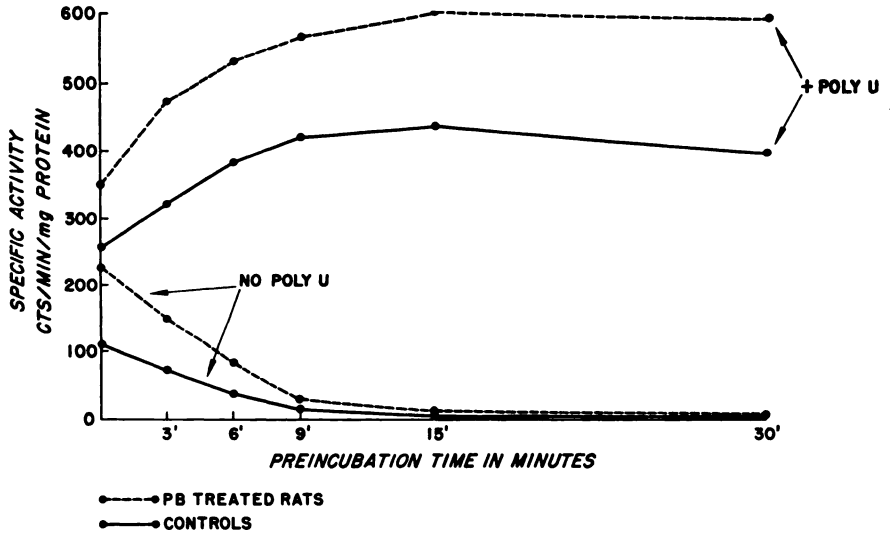

FIG. 8. *Effect of preincubation time on the polyuridylic acid-directed L-[^{14}C]-phenylalanine incorporation by microsomes from control and phenobarbital-treated rats*

Details of conditions are given in Methods.

creatine phosphokinase (23). The loss of activity is likely due to a removal or inactivation of messenger RNA since exogenous messenger RNA reactivates the system. Figure 8 also shows an inverse relationship between the loss of endogenous L-[^{14}C]-phenylalanine-incorporating activity during the preincubation and the stimulatory activity of polyuridylic acid. Thus, polyuridylic acid-directed L-[^{14}C]-phenylalanine incorporation increases per milligram protein from 150 cpm in the control preparation with no preincubation to 390

cpm in the same preparation that has been preincubated for 9 min. Similarly, in microsomes from phenobarbital-treated rats the difference between incorporation in the presence and absence of polyuridylic acid increases per milligram protein from 130 cpm with no preincubation to 517 cpm after 9 min of preincubation. Thus, the preparation from phenobarbital-treated rats shows a greater endogenous messenger RNA activity and after preincubation, a greater sensitivity to exogenous messenger RNA or polyuridylic acid. In both preparations the sensitivity to polyuridylic acid increases as endogenous messenger RNA is removed, but in microsomes from phenobarbital-treated rats this increase is much greater. Figure 9 shows that low levels of synthesis. Liver microsomes are also known to contain nucleases which are capable of degrading polyuridylic acid. The addition of larger amounts of polyuridylic acid (100–200 μg) stimulates L-[^{14}C]-phenylalanine incorporation to approximately the same extent in both control and phenobarbital nonpreincubated microsomes. Thus, the addition of 200 μg of polyuridylic acid increases the specific activity of proteins in preparations from both control and phenobarbital-treated rats by about 150 cpm per milligram protein. This suggests that the number of sites available for polyuridylic acid binding in the nonpreincubated preparations are similar in both the control and phenobarbital microsomes. In other experiments, we found (22)

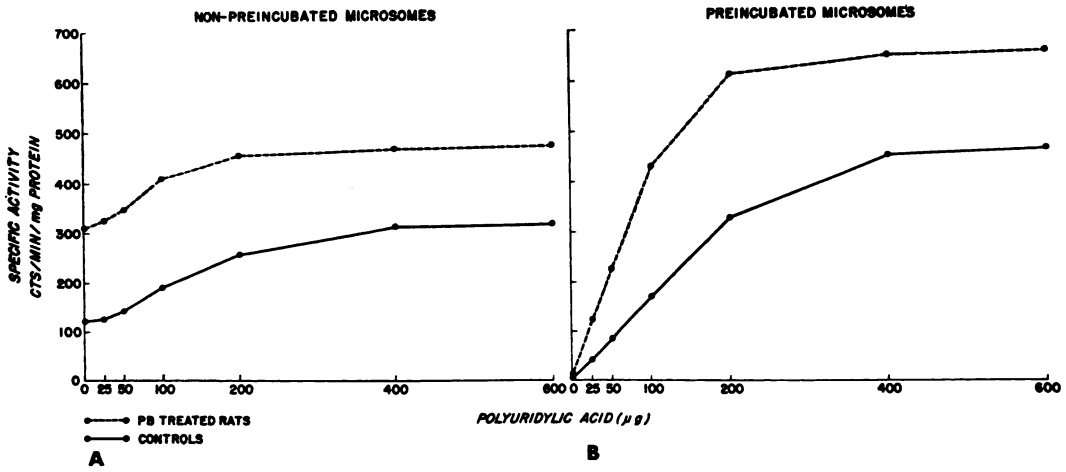

FIG. 9. *Effect of various amounts of polyuridylic acid on* L-[^{14}C]*-phenylalanine incorporation by nonpreincubated (A) and by preincubated (B) microsomes from control and phenobarbital-treated rats*

Experimental conditions are as described in Methods.

polyuridylic acid (25–50 μg) have very little stimulatory effect on L-[^{14}C]-phenylalanine incorporation in either nonpreincubated preparation. This is likely due to the rapid degradation of low levels of polyuridylic acid by endogenous nucleases. Barondes and Nirenberg (21) have shown that much of the polyuridylic acid added to *E. coli* extracts was degraded within a few minutes. This degradation was not dependent on polyphenylalanine that preincubation of microsomes renders them completely devoid of L-[^{14}C]-phenylalanine-incorporating activity but increasingly sensitive to polyuridylic acid-directed L-[^{14}C]-phenylalanine incorporation. Figure 9B shows the effect of polyuridylic acid on L-[^{14}C]-phenylalanine incorporation in preincubated microsomes from control and phenobarbital-treated rats. Here both preparations are sensitive to low levels of polyuridylic acid which

are not effective in the nonpreincubated preparations. This is likely due to a greater potential for polyuridylic acid attachment after endogenous messenger RNA has been removed. This explanation is further supported by the data in Fig. 8, which show that the effect of polyuridylic acid increases as endogenous L-[^{14}C]-phenylalanine-incorporating activity decreases. Figure 9B shows that over a wide range of polyuridylic acid concentration (25–200 μg) the preincubated microsomes from phenobarbital-treated rats are approximately twice as active as control preparations. Thus, under conditions where L-[^{14}C]-phenylalanine incorporation is directed solely by exogenous messenger RNA, i.e., polyuridylic acid, the microsomes from phenobarbital-treated rats are about twice as active as control microsomes. This is in contrast to the nonpreincubated microsomes where the effect of polyuridylic acid was identical in preparations from both control and phenobarbital-treated rats. The most likely explanation for the relatively greater sensitivity of preincubated microsomes from phenobarbital-treated rats is that these microsomes have both more endogenous messenger RNA and more messenger RNA binding sites. The excess sites of the microsomes from phenobarbital-treated rats are bound with messenger RNA and are not available to polyuridylic acid in the nonpreincubated preparations but

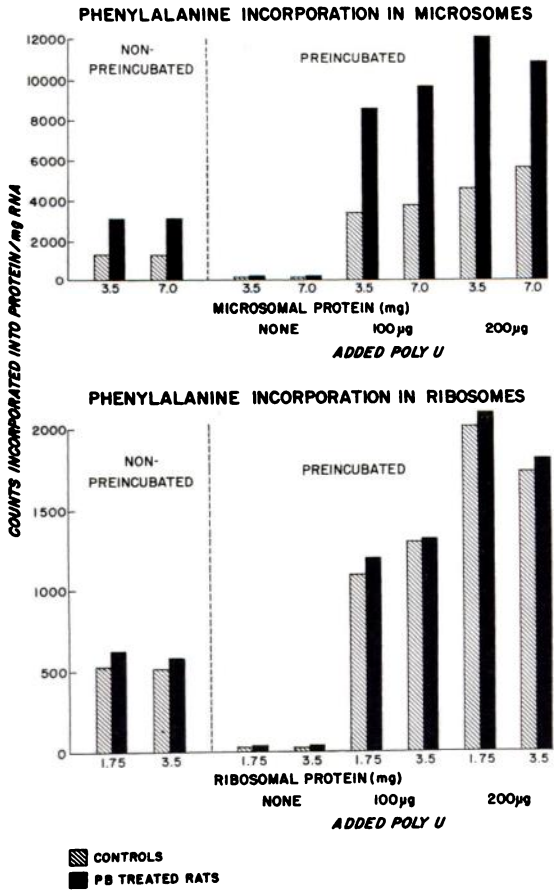

FIG. 10. L-[^{14}C]-*Phenylalanine incorporation in nonpreincubated microsomal and ribosomal preparations from control and phenobarbital-treated rats*

Experimental details as in Methods.

become available during the preincubation upon removal of endogenous messenger RNA. This suggests that the greater endogenous incorporating activity of microsomes from phenobarbital-treated rats is due to an increase in the microsomal content of messenger RNA and the greater sensitivity of preincubated microsomes from phenobarbital-treated rats to polyuridylic acid is due to an increased number of available binding sites for polyuridylic acid. The greater sensitivity of phenobarbital microsomes to polyuridylic acid is not due to a possible decrease in the endogenous ribonuclease activity since the increased sensitivity is observed with greater than saturating levels of polyuridylic acid (400–600 μg).

L-[^{14}C]-*Phenylalanine Incorporation in Nonpreincubated and Preincubated Microsomal and Ribosomal Preparations from Control and Phenobarbital-Treated Rats*

The top of Fig. 10 shows the results of an experiment similar to that shown in Fig. 9. In this experiment incorporation was measured with two levels of microsomal input and the results were calculated on the basis of total incorporation per milligram of microsomal RNA in the flask. With nonpreincubated microsomes, L-[^{14}C]-phenylalanine incorporation remains constant at two levels of added microsomes, and in each case the microsomes from phenobarbital-treated rats are about twice as active as controls. After preincubation, however, both control and phenobarbital microsomes are inactive unless polyuridylic acid is added to the system. With the addition of either 100 μg or 200 μg of polyuridylic acid the phenobarbital microsomes are about twice as active as the control preparations. The lower half of the chart shows a similar experiment performed in ribosomes isolated from the same group of rats. Although there are marked differences in microsomal L-[^{14}C]-phenylalanine incorporation there is only a negligible difference between ribosomes from control and phenobarbital-treated rats. This is true when L-[^{14}C]-phenylalanine incorporation is directed either by endogenous messenger RNA or by polyuridylic acid. Hence, the factors responsible for the stimulatory activity of phenobarbital are removed by deoxycholate treatment during the preparations of the ribosomes and are likely components of the endoplasmic reticulum.

Effect of Mg^{2+} Ion Concentration on Polyuridylic Acid Stimulation of L-[^{14}C]-*Phenylalanine Incorporation in Preincubated Microsomes and Ribosomes*

In determining the cofactor requirements for L-[^{14}C]-phenylalanine incorporation we observed that the optimal Mg^{2+} concentration required for L-[^{14}C]-phenylalanine incorporation directed by endogenous messenger RNA, i.e., in the nonpreincubated system, was different than the optimum Mg^{2+} concentration required for polyuridylic acid-directed L-[^{14}C]-phenylalanine incorporation. Thus, with endogenous messenger RNA-directed L-[^{14}C]-phenylalanine incorporation there is a broad optimum of Mg^{2+} concentration ranging from 6 to 12 mM whereas with polyuridylic acid-directed L-[^{14}C]-phenylalanine incorporation there is a sharp Mg^{2+} ion optimal concentration of 12 mM. Thus, it seemed that one possible explanation for the greater sensitivity of preincubated phenobarbital microsomes for polyuridylic acid-directed L-[^{14}C]-phenylalanine incorporation might be that it was due to an increased affinity of the phenobarbital microsomes for Mg^{2+} ion. Figure 11 shows, however, that over a wide range of suboptimal to optimal Mg^{2+} ion concentrations the microsomes from phenobarbital-treated rats were more active. The ribosomes from control and phenobarbital-treated rats, however, show similar polyuridylic acid-directed L-[^{14}C]-phenylalanine incorporation over a wide range of Mg^{2+} ion concentrations. Also in other experiments we found that both the endogenous Mg^{2+} content and the ability to bind Mg^{2+} was identical in microsomes from control and phenobarbital-treated rats.

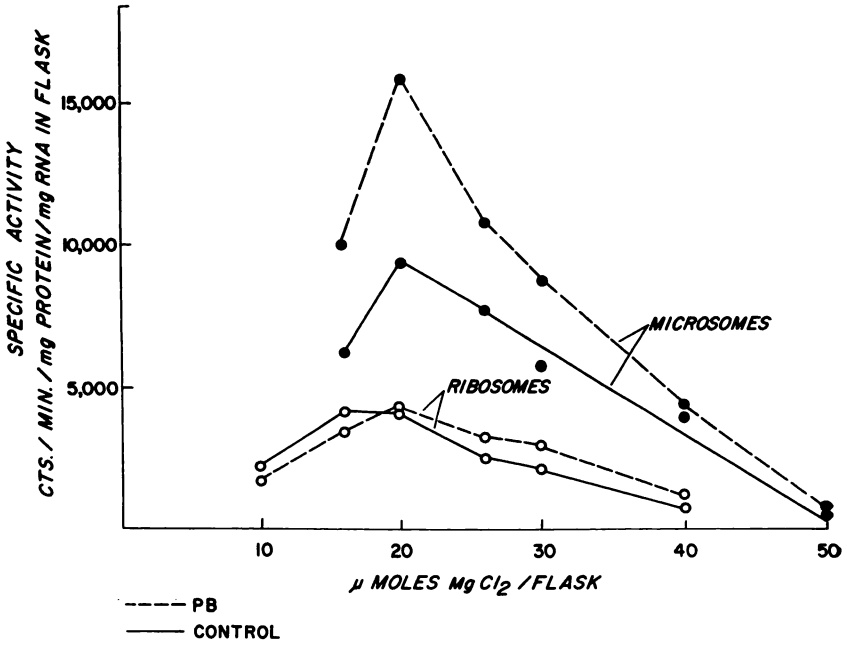

FIG. 11. *Effect of Mg^{2+} on polyuridylic acid stimulation of* L-[^{14}C]*-phenylalanine incorporation of preincubated microsomes and ribosomes*

Effect of Phenobarbital Pretreatment on the Distribution of Membrane-Bound Ribosomes to Free Ribosomes in Rat Liver Microsomes

Henshaw *et al.* (16) have used sucrose density gradient centrifugation to show the distribution of free ribosomes and those bound to the endoplasmic reticulum. We applied this technique to microsomes obtained from control and phenobarbital-treated rats. Figure 12 shows the distribution of the microsomal fraction sedimented for 2 hr in a linear sucrose gradient of 5–20% layered over a 50% sucrose solution. The microsomes from phenobarbital-treated rats have a greater proportion of more rapidly sedimenting components; these represent ribosomes attached to endoplasmic reticulum, as compared to the less rapidly sedimenting polysomes and ribosomes that are not attached to large membrane components. These results indicate that the phenobarbital microsomes contain a greater proportion of ribosomes attached to membranes than do control microsomes. Table 1 shows the results of four sucrose density gradient experiments in which the distribution of microsomes from control and phenobarbital rats was determined. In each case the ratio of heavy to light fraction is greater in the phenobarbital than in the control microsomes. Thus, phenobarbital-induced increases in this ratio ranged from 28 to 99% in the four experiments. Henshaw *et al.* (16) have suggested that the bound microsomes are the more active species in amino acid incorporation. If this is so, then at least part of the increased L-[^{14}C]-phenylalanine incorporation activity may be explained by a shift in the distribution of ribosomes from the free to the membrane-bound state.

Effect of Phenobarbital Pretreatment on the Yield of Agranular Endoplasmic Reticulum

Several investigators (4–6) have shown by electron microscopy that phenobarbital increases the amount of agranular endoplasmic reticulum. We isolated this subcellular fraction by the method of Dallner (17) and found an increased yield of this

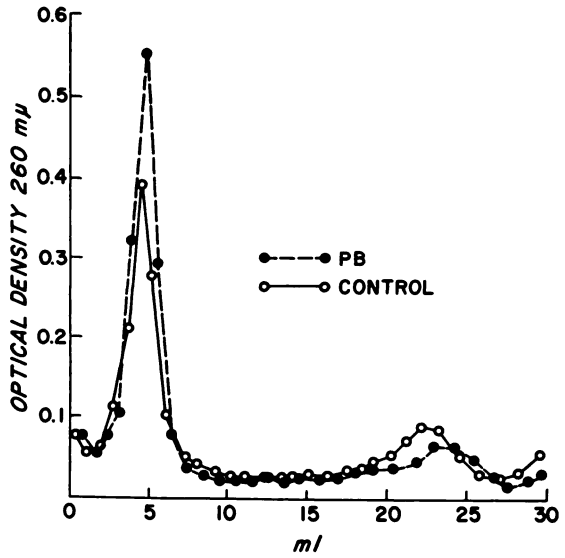

FIG. 12. *Sucrose density gradient analysis of microsomal fractions of liver from phenobarbital-treated and control rats*

TABLE 1

Effect of phenobarbital pretreatment on the ratio of bound to free ribosomes in the microsomal fraction of rat liver

Expt. no.	Sample	Heavy[a]	Inter-mediate[a]	Light[a]	Ratio heavy:light	Per cent increase
1	Control	11.04	3.08	6.22	1.78	—
	Phenobarbital	14.13	1.44	5.60	2.53	+42
2	Control	9.39	2.16	3.63	2.6	—
	Phenobarbital	11.63	2.00	2.59	4.9	+76
3	Control	9.55	1.21	5.22	1.88	—
	Phenobarbital	11.53	1.51	3.05	3.75	+99
4	Control	11.44	2.36	5.49	2.1	—
	Phenobarbital	13.13	1.70	4.49	2.7	+28

[a] Figures refer to total absorption at 260 mμ for the areas eluted from the sucrose density gradient. "Heavy" refers to tubes 2–9; "intermediate" to tubes 9–19; and "light" to tubes 20–25.

TABLE 2

Yield of smooth-membrane fraction from control and phenobarbital-treated rat liver

Expt. no.	Sample	RNA (mg/g liver)[a]	Protein (mg/g liver)[a]	Per cent of control (protein content)	RNA: protein
1	Control	0.100	1.6	—	0.06
	Phenobarbital	0.085	2.1	131	0.04
2	Control	0.102	1.5	—	0.07
	Phenobarbital	0.150	4.7	313	0.03
3	Control	0.026	0.6	—	0.04
	Phenobarbital	0.052	1.2	200	0.04

[a] Figures represent yields of material from 1 g liver.

fraction when the animals had had prior treatment with phenobarbital. Thus, in three experiments (Table 2) the phenobarbital pretreatment increased the yield of this fraction by 31, 213, and 100%. This result confirms the report of Orrenius *et al.* (6), who found a phenobarbital-induced increase in both the granular endoplasmic reticulum and the agranular endoplasmic reticulum.

DISCUSSION

Phenobarbital represents one of a large number of drugs which increase the activities of a number of liver microsomal enzyme systems (1–3). In addition to pharmacologically useful drugs, carcinogens such as 3-methylcholanthrene and 9,10-benz[*a*]pyrene, increase the activities of certain microsomal enzyme systems. One of the enzyme systems enhanced to a large extent by both phenobarbital and 3-methylcholanthrene is the system that hydroxylates aromatic rings. Thus, the microsomal hydroxylation of hexobarbital, aniline, and the polycyclic aromatic hydrocarbons is increased manyfold. In addition, enzymic reactions involving *N*-demethylation, *S*-demethylation, and *O*-demethylation, nitro reduction, and glucuronide formation as well as NADPH cytochrome reductase and NADPH oxidase are increased by prior treatment of the animal with "inducing" drugs (1, 2). Other microsomal enzymes such as glucose 6-phosphatase, NAD^+-cytochrome C reductase, and otherwise inducible enzymes of the cell sap such as tryptophan pyrrolase and arginase (23) are either depressed or unaffected by the administration of phenobarbital (5, 22). There appears to be a broad range of activity both with respect to the type of molecule which is able to elicit the stimulation and with respect to the enzyme activities that are stimulated. Thus, a single inducing drug will stimulate a number of microsomal enzyme systems and any given enzyme activity can be stimulated by drugs of considerably varied structure. On the other hand, the stimulation is not general in all cases. For example, 3-methylcholanthrene exhibits a relatively limited range of stimulatory activity, does not affect microsomal protein content nor affect gross morphologic changes in the agranular endoplasmic reticulum. On the other hand, phenobarbital causes increases in a number of different activities, increases microsomal protein content, and alters the morphology of the agranular endoplasmic reticulum.

In various studies (6, 7, 24) simultaneous ethionine, puromycin, or actinomycin treatment have been found to prevent the drug-induced increases in microsomal enzyme activity. The prevention or reduction of these increases by drugs inhibiting protein or RNA synthesis suggests that the increased enzyme activities are the result of increased messenger RNA and protein synthesis. 3-Methylcholanthrene has been found to cause an increase in both the RNA:DNA ratio and the messenger RNA content of rat liver nuclei (25). This agent also increases microsomal amino acid incorporation (22). Other studies have shown that phenobarbital causes a microsome specific increase in *in vivo* amino acid incorporation (9). If phenobarbital increases the rate of synthesis of a sufficiently high proportion of the total proteins synthesized, one would expect the overall rate of amino acid incorporating activity to be increased. In our experiments we found that the extent of stimulation of L-[^{14}C]-phenylalanine incorporation by polyuridylic acid was identical in microsomes from control and phenobarbital-treated rats. This was true, however, only if the polyuridylic acid was added to microsomes that contained endogenous messenger RNA. This suggests that the enzymes and cofactors required for polyuridylic acid-directed L-[^{14}C]-phenylalanine incorporation are present in saturating amounts in each incubation. Since microsomal L-[^{14}C]-phenylalanine incorporation was identical with respect to exogenous messenger RNA, the greater incorporation by nonpreincubated control microsomes is likely due to a greater content of endogenous messenger RNA. Furthermore, when the endogenous messenger RNA is removed by a preincubation, the

microsomal system is no longer equal in sensitivity to polyuridylic acid. After removal of endogenous messenger RNA by preincubation, the phenobarbital microsomes display greater L-[^{14}C]-phenylalanine-incorporating activity in the presence of either subsaturating or saturating levels of polyuridylic acid. This suggests that preincubation removed larger amounts of endogenous messenger RNA from microsomes of phenobarbital-treated rats and thereby yielded a greater number of sites for polyuridylic acid attachment in this preparation. Thus, the microsomes from phenobarbital-treated rats devoid of their greater endogenous messenger RNA content contain a greater number of sites for messenger RNA attachment and are thereby more sensitive to the addition of an exogenous messenger RNA such as polyuridylic acid.

Both the greater L-[^{14}C]-phenylalanine-incorporating activity of nonpreincubated phenobarbital microsomes and the greater sensitivity to added polyuridylic acid are characteristic of microsomes from phenobarbital-treated rats, but not of ribosomes from the same animals. Thus, the sodium deoxycholate treatment which solubilizes the lipoprotein components of the endoplasmic reticulum, and thereby renders the ribosomes free from membrane, appears to remove the increased amount of messenger RNA as well as the increased number of attachment sites induced by phenobarbital treatment. These results suggest that the membrane or deoxycholate soluble microsomal components play an important role in protein synthesis, perhaps by properly aligning messenger RNA and ribosomes. Campbell *et al.* (26) have found that microsomes but not ribosomes from regenerating liver have a higher *in vitro* amino acid-incorporating activity. In this respect, their results with liver regeneration are similar to those reported here.

Henshaw *et al.* (16) have suggested that the endoplasmic reticulum plays a role in the protein-synthesizing activity of microsomes. This conclusion was reached by the findings that the ribosomes attached to membrane components were more active in protein synthesis than free ribosomes. Our studies indicate that a small part of the greater activity of microsomes from phenobarbital-treated rats may be due to the observed shift to a greater proportion of membrane-bound ribosomes.

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