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IN VITRO INDUCTION BY PHENOBARBITAL OF DRUG MONOOXYGENASE ACTIVITY IN MOUSE ISOLATED SMALL INTESTINE*

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Abstract—Induction of drug monooxygenase activity by phenobarbital was studied in the mouse isolated small intestine. Incubation for 2 hours in the presence of 5×10^{-4} M phenobarbital resulted in a 2–3 fold increase of the *O*-dealkylation activity for 7-ethoxycoumarin based on the protein content of the mucosal cell homogenate. The highest activity was located in the jejunum between 4 and 12 cm distal to the pylorus. The induction started after a lag phase of about 30 min and could be prevented by the simultaneous addition of $1{\cdot}5 \times 10^{-5}$ M puromycin or 5×10^{-7} M actinomycin D. Puromycin decreased the basal rate of *O*-dealkylation, but actinomycin D at this concentration did not. Actinomycin D, 2×10^{-5} M, caused a "superinduction", i.e. stimulation of the induced *O*-dealkylation activity 30 per cent greater than that with phenobarbital alone. Incorporation of ^{14}C-leucine into mucosal proteins was doubled in the presence of phenobarbital but the effect was abolished by inhibitors of protein synthesis. Spectroscopic measurements did not indicate major changes in the content of cytochrome b_5 and cytochrome P-450. However, the activity of NADPH-cytochrome *c* reductase increased in parallel with the *O*-dealkylation activity. Hence, the induction can probably be attributed to a more rapid turnover of the monooxygenase cycle.

THE PHENOMENON of drug tolerance stimulated numerous investigations of the induction mechanism of hepatic drug monooxygenase systems.[1] Apart from its significance in the pharmacological field, this induction process is one of the few that can be studied as a model for the regulation of enzyme synthesis in higher organisms. A large number of observations has led to the belief that the activity of the monooxygenase system is directly regulated by the levels of its various substrates.[2–5] Upon pretreatment with barbiturates,[6] polycyclic hydrocarbons,[7] or aliphatic compounds[5] the liver and some other organs respond by increasing the content of cytochrome P-450. Generally a direct correlation between cytochrome P-450 concentration and monooxygenase activity for a certain drug or substrate cannot be obtained since various cytochrome P-450 species with different but overlapping substrate specificities have been reported.[8–11] If, however, in special cases, as with cyclohexane, the active cytochrome P-450–substrate complex could be determined, the increase in hydroxylation activity of the microsomal fraction after pretreatment with phenobarbital could be related directly to the quantity of the cytochrome P-450–cyclohexane comples.[12] It is also possible that an increase in cytochrome P-450 reductase activity will enhance the monooxygenation rate. The induction of this reductase by spironolactone can be taken as an example.[13]

* Part of the thesis of R. Scharf.

The mechanisms by which both components of the monooxygenase system increase are difficult to elucidate by *in vivo* studies and more progress has come from *in vitro* investigations on cell cultures. Using benz[a]anthracene as an inducer and cultured cells derived from whole fetal hamsters, Nebert and Gelboin[13] showed that the activity of the aromatic hydrocarbon hydroxylase increased after a lag phase of 30 min. The content of cytochrome P-450 increased simultaneously[14] and both effects could be abolished by prior addition of puromycin, cycloheximide, or actinomycin D.[15] Similar lag times and responses of the hydroxylase induction to inhibitors of RNA and protein synthesis were found in cultured fetal rat hepatocytes exposed to aromatic hydrocarbons, phenobarbital, *p,p'*-DDT[15] and various biogenic amines.[16] It was concluded that these inducers stimulate aryl hydroxylase activity by two mechanisms: induction at the transcriptional level and a secondary posttranscriptional effect.

During studies on the monooxygenase activity of small intestine we observed a dramatic increase in the *O*-dealkylation activity for 7-ethoxycoumarin after oral administration of phenobarbital to mice.[17] This prompted us to develop an *in vitro* model using small intestine. In a preliminary communication,[18] we reported that incubation of small intestine with phenobarbital for only 2 hr caused a 2–3 fold increase in the *O*-dealkylation activity of the jejunum. Because similar experiments on perfused liver have not succeeded so far, we have examined further the mechanism of the induction process using the isolated jejunum.

METHODS

Materials. Cytochrome *c*, NADH and NADPH were purchased from Boehringer Mannheim GmbH, Mannheim, Germany. ^{14}C-labeled L-leucine (specific activity 59 mCi/m-mole) was obtained from Amersham-Buchler, Frankfurt/Main, Germany. Soluene® (sample solubilizer) and Dimilune® (chemiluminescence inhibitor) were products of Packard Instrument Company, Inc. Zürich, Switzerland. Actinomycin D, puromycin (from *Streptomyces alba-niger*) and amino acid hydrolysate (prepared from casein) were obtained from Serva GmbH, Heidelberg, Germany. Trypsin inhibitor (chromatographically purified from soya beans) and sodium phenobarbital were purchased from E. Merck AG, Darmstadt, Germany.

The preparation of 7-ethoxycoumarin from 7-hydroxycoumarin (umbelliferone) and ethyl iodide has been described previously.[19]

All other chemicals were commercially available and were analytical grade.

Preparation and incubation of small intestines. Male mice (strain NMRI/Han 20) of 25–30 g body wt were used in all experiments. The animals received diet (Altromin®), and water *ad lib.* After killing the mice by decapitation, the jejunum was prepared according to a modification of the method of Lehrmann *et al.*[17] Segments located 3 cm distal to the pylorus were used for all experiments. The small intestine was perfused *in situ* with Tyrode solution pH 8·2 and 15 cm of the jejunum was excised, purged of all intestinal contents, perfused once again and immediately mounted on syringes of the incubation apparatus (Fig. 1). The time for the preparation was less than 3 min.

The device shown in Fig. 1 allows the incubation of the isolated small intestine by simultaneous gassing with Carbogen® and perfusion with Tyrode solution of the lumen. This is achieved by the special construction of the syringe top. The incubation

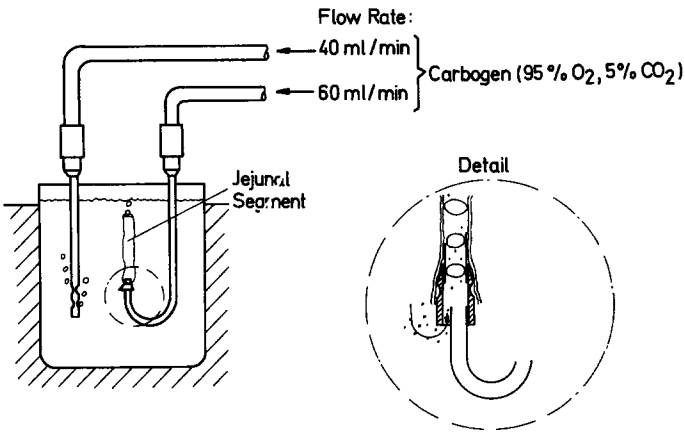

FIG. 1. Experimental device for the *in vitro* incubation of mouse small intestine. 700 ml of Tyrode solution pH 8·2 was kept at 37° in a 800 ml beaker. During the time of incubation the solution was gassed with carbogen at a rate of 40 ml/min through a perforated glass tubing. 15 cm of the small intestine was mounted on top of a specially built syringe needle (see inset in Fig. 1) which allowed simultaneous perfusion with the medium and gassing with carbogen. At the end of the experiment the pH had dropped to 7·3. Eighteen experiments were run at a time.

mixture containing 600 ml of Tyrode solution (pH 8·2 at 37°) and amino acids was supplemented with phenobarbital and inhibitors of protein biosynthesis depending on the experiment.

Preparation of jejunal homogenate and microsomal fraction. Mucosal cells were scraped off the incubated jejunum and homogenized as described by Lehrmann *et al.*[17] The small intestines from three separate incubations were pooled for one preparation. For subsequent differential centrifugation the cells were homogenized in ice-cold 0·25 M sucrose without adding trypsin inhibitor which would have changed the sedimentation pattern completely.[17] After an initial centrifugation at 8000 *g* for 15 min the microsomal fraction was spun down at 105,000 *g* for 50 min. The pellet was resuspended in a KCl–sucrose buffer (1·15 m KCl, 0·25 M sucrose and 0·05 M Tris–HCl buffer pH 7·5). Protein was determined by the biuret method of Gornall.[20]

^{14}C-Leucine incorporation experiments. ^{14}C-Radioactivity in the mucosal proteins after incubation with labelled leucine was determined by a simplified procedure of Negeshi and Omura.[21] The homogenate was treated with concentrated trichloroacetic acid (TCA) to give a final concentration of 10% TCA. The precipitate was extracted with an ethanol–ether mixture (3:1 v/v) to remove RNA and lipids and was then washed four times with 5% TCA. The pellet was solubilized by treatment with Soluene® (1 ml/20 mg of protein) at 50° for about 5 hr. Scintillation fluid (15 ml) was added to the clear solution. The samples were counted in a Tracer-lab liquid scintillation counter.

Fluorometric and spectroscopic measurements. The monooxygenase activity of mucosal homogenates was followed by a direct fluorometric test which we have described recently.[19] The reaction is based on the *O*-dealkylation of 7-ethoxycoumarin to the highly fluorescent umbelliferone. 3·0 ml of the assay mixture were placed in a 10 mm fluorescence cuvette; after addition of NADPH the fluorescence increase at 455 nm (excitation at 380 nm) was followed in a Zeiss PMQ 2 fluorometer.

The activity of NADPH-cytochrome *c* reductase[22] in the homogenate was assayed by recording the absorbance increase of yeast cytochrome *c* at 550 vs 540 nm in an Aminco DW-2 spectrophotometer. The same instrument was used in the split beam mode to record difference spectra for oxidized minus reduced cytochrome b_5 and the cytochrome P-450—carbon monoxide complex.[12] Concentrations and experimental details are given in the legends to the figures and tables.

RESULTS

Optimizing the induction process. Addition of NADPH to a mucosal cell homogenate supplemented with 7-ethoxycoumarin results in a linear increase of fluorescence with time. The reaction slows down and finally stops when oxygen and NADPH become depleted. Readmission of the cosubstrates restores the activity which may proceed for as long as 80 min at a constant rate. NADH does not stimulate further the *O*-dealkylation[17] in contrast to the liver microsomal monooxygenase system.[23–26] Extraction of the assay mixture at the end of the incubation period allows the isolation of umbelliferone, the main product of the reaction.

Homogenates obtained from isolated jejunum previously incubated in Tyrode solution supplemented with phenobarbital showed a threefold increase in fluorescence compared to a control incubated in a phenobarbital-free medium (Fig. 2).

Carbon monoxide inhibits this reaction indicating the involvement of cytochrome P-450 as reported earlier in the *in vivo* induction of small intestine.[16] In the same paper we demonstrated an uneven distribution of the monooxygenase activity with a maximum in the jejunum and no activity in the ileum and large intestine. It could not be excluded, however, that this distribution was due to the varying con-

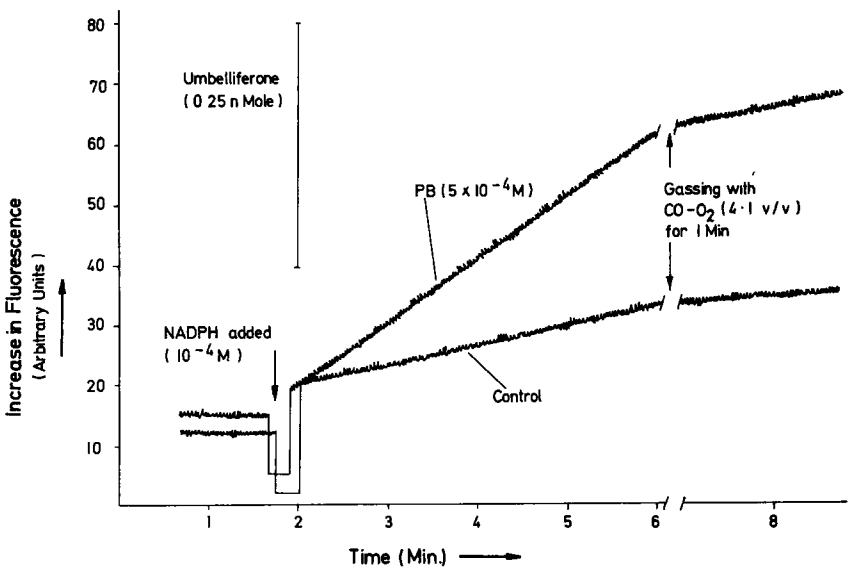

FIG 2 Fluorometric measurement of the *O*-dealkylation of 7-ethoxycoumarin in small intestinal homogenates (reproduction of original traces). The assay mixtures contained in a total volume of 3 ml: 1 mg of protein of a homogenate prepared from 3 small intestines incubated for 120 min, 5×10^{-4} M of 7-ethoxycoumarin, 10^{-4} M NADPH and 0·1 M Tris buffer pH 79. Gassing for 1 min with the CO–O_2 mixture was performed by insertion of a thin polyethylene tube into the cuvette.

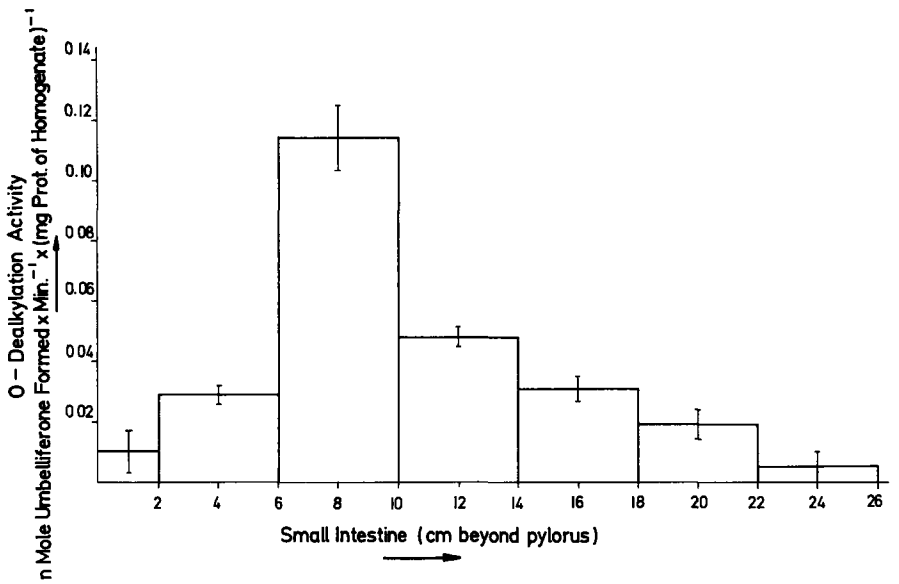

FIG. 3. Localization of the *O*-dealkylation activity in mouse small intestine The whole small intestine was incubated for 120 min in the presence of 5×10^{-4} M phenobarbital. Pieces 4 cm long (indicated by the horizontal bars) were homogenized and assayed for activity as described in the legend to Fig. 2. The vertical bars give the standard deviation from five preparations.

centrations of phenobarbital during oral induction. Therefore, the distribution was studied again by *in vitro* incubation with phenobarbital where the same concentration of the drug could act on all parts of the small intestine.

Figure 3 shows that under these conditions the *O*-dealkylation activity was again highest in the jejunum, between 4 and 12 cm distal to the pylorus. This section was therefore used in all further experiments. Little activity was inducible in the duodenum and ileum. No activity was found in the stomach or large intestine.

Further experiments revealed the optimal conditions for the induction process (Fig. 4). Increasing the phenobarbital concentration to 5×10^{-4} M further stimulates the monooxygenase activity but above that level a decrease is observed. Addition of an amino acid hydrolysate did not significantly alter the activity of the controls but further enhances the phenobarbital-mediated increase in activity. Under these conditions a specific activity of $65 \pm 11{\cdot}1$ pmoles of umbelliferone formed per min and mg protein in the homogenate was obtained which corresponded to an average induction factor of 3.

It should be noted that reproducible results require special care in mounting the small intestine to avoid mechanical damage, and proper control of pH and flow rate. The monooxygenase activity in control mice was periodically elevated, probably due to environmental conditions in the animal house or to the diet. In these animals the maximal activity attained after induction remained unchanged so that lower induction factors were observed.

The time course of the induction is shown in Fig. 5. After a lag phase of 25 min the activity reaches its maximum at about 120 min of incubation and then decreases again, probably as a consequence of structural lesions of the mucosa.

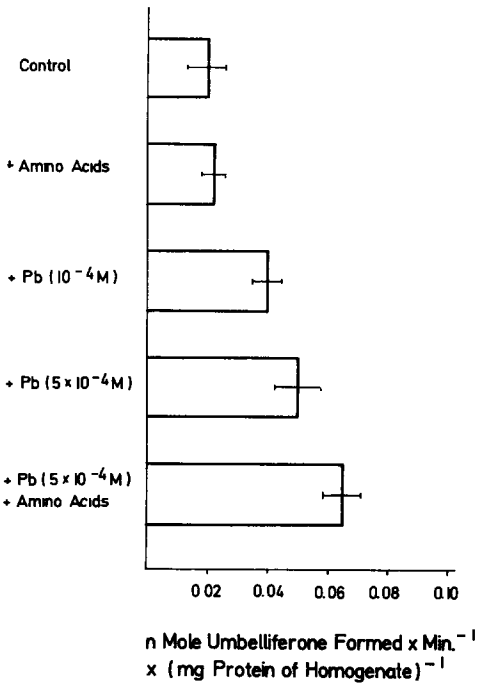

FIG. 4. Effects of amino acids and phenobarbital concentration on the induction of the *O*-dealkylation activity. Amino acids were added as a protein hydrolysate at a concentration of 0·1 mg/ml. The values represent means ± standard deviation for 3–10 experiments.

Studies on the mechanism of induction. When the isolated small intestine was incubated under optimum conditions in the presence of $1{\cdot}5 \times 10^{-5}$ M puromycin the stimulating effect of phenobarbital was completely suppressed. Even the basal rate of *O*-dealkylation in controls was diminished by puromycin (Fig. 6).

Actinomycin D in concentration of 5×10^{-7} M also abolished the induction, although it had no effect on the basal rate. A surprising result was obtained when

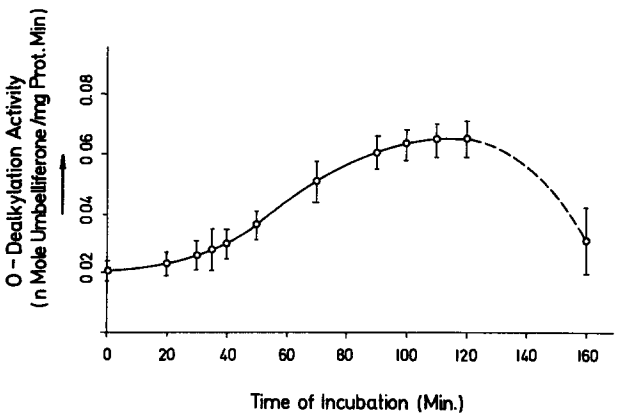

FIG. 5. Dependence of the induction by phenobarbital on time. The incubation medium contained 5×10^{-4} M phenobarbital and 0·1 mg/ml of the protein hydrolysate. The bars indicate the standard deviation from triplicates of three experiments.

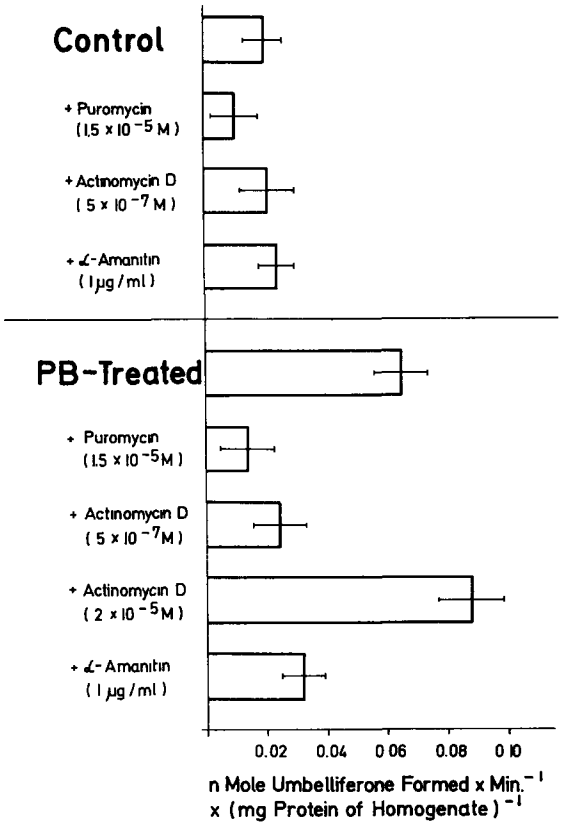

FIG. 6. Effect of inhibitors of protein biosynthesis on the *O*-dealkylation activity. Tyrode solution was supplemented as indicated. Values represent the means of three experiments. Puromycin was dissolved according to Gorski *et al.*[27] 4·95 mg of puromycin, suspended in 0·5 ml of buffered saline (0·04 M Na_2HPO_4—NaH_2PO_4 pH 7·4 in 0·9% NaCl) was added to 700 ml of Tyrode solution to give a concentration of $1·5 \times 10^{-5}$ M.

the actinomycin concentration was raised to 2×10^{-5} M. Instead of inhibiting the induction, a "superinduction" occurred with activities about 30 per cent higher than with phenobarbital alone. This paradoxical effect of actinomycin has also been reported for the induction of the aminotransferase,[28] tryptophan dioxygenase[29] and various other enzymes.[30]

Since puromycin is known to block only the translation process we have tried to remove the inhibitor after 60 and 90 min of incubation with phenobarbital and to continue the incubation with Tyrode solution and amino acids alone (Fig. 7). The results show that after changing the medium the activity increased rapidly again without an appreciable lag phase indicating the transcriptional part of the induction process had already occurred.

It could be argued that enough phenobarbital had remained in the mucosal cells to cause this rapid increase after the change of medium. In a control experiment however, where phenobarbital was applied for a time of 3 min, sufficient for equilibration, only a 10 per cent increase in activity was observed. This suggests that phenobarbital does not accumulate intracellularly and that it readily equilibrates with the medium.

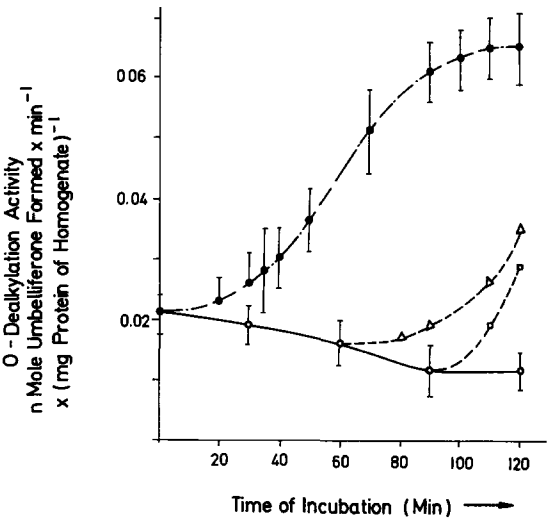

FIG. 7. Effect of preincubation with phenobarbital and puromycin on *O*-dealkylation activity. Solid circles indicate the time course of induction with 5×10^{-4} M phenobarbital and amino acids (0·1 mg/ml). Open circles correspond to the activities obtained in the presence of $1{\cdot}5 \times 10^{-5}$ M puromycin. When the medium was changed after 60 min or 90 min to pure Tyrode solution without phenobarbital and puromycin, the activity increased as shown by the open triangles and open squares, respectively.

The increase in activity of the *O*-dealkylation system may be due to increased levels of cytochrome P-450. We therefore determined its content in homogenates of controls and phenobarbital-treated small intestines. The levels of cytochrome b_5 were also measured in the same preparations (Table 1). Surprisingly, cytochrome P-450 was induced only slightly and cytochrome b_5 was even lower than in controls.

Since the monooxygenase system, in addition to cytochrome P-450, contains also the flavoprotein NADPH-cytochrome P-450 reductase, this enzyme was determined by its cytochrome *c* reductase activity. It was mentioned in the introduction that both components may be differently influenced by the induction process. Figure 8 shows the time course of the induction of NADPH-cytochrome *c* reductase activity

TABLE 1. EFFECT OF PHENOBARBITAL ON THE CYTOCHROME P-450 AND CYTOCHROME b_5 CONTENT OF SMALL INTESTINE HOMOGENATES

Mucosal cell homogenate of incubated jejunum	Cytochrome P-450 (pmoles/mg protein)	Cytochrome b_5 (pmoles/mg protein)
Control	6 ± 2	24 ± 2
Phenobarbital-treated (5×10^{-4} M)	8 ± 2	21 ± 3
Phenobarbital-treated (5×10^{-4} M) + Actinomycin D (5×10^{-7} M)	6 ± 2	14 ± 4

Six ml of a homogenate suspension (mg protein/ml) were equally divided into two cuvettes and the baseline was recorded.

Cytochrome b_5 was determined from the absorbance difference between 424 and 409 nm ($\Delta\epsilon_{mM} = 170$) after addition of 10^{-5} M NADH to the sample cuvette.

Subsequent bubbling (20 sec) of carbon monoxide through the sample cuvette and reduction of both cuvettes by sodium dithionite (about 1 mg) yielded a difference spectrum with a peak at 450 nm. Cytochrome P-450 was calculated from the difference in absorbance between 450 nm and 490 nm ($\Delta\epsilon_{mM} = 91$). n = 4 for all experiments.

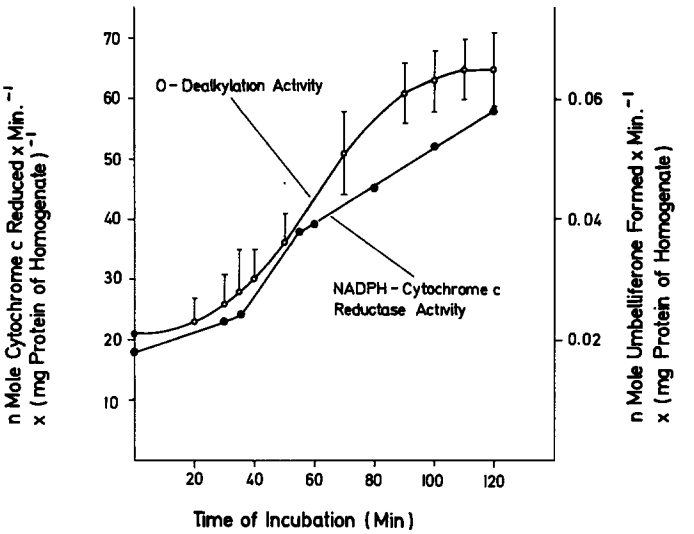

FIG. 8. Effect of incubation with phenobarbital on NADPH-cytochrome *c* reductase activity of the isolated jejunum. The small intestine was incubated for 120 min in Tyrode solution supplemented with amino acid hydrolysate (0·1 mg/ml) and phenobarbital (5×10^{-4} M). The assay mixture for cytochrome *c* reductase activity contained in a total volume of 3 ml the following concentrations: 1 mg of protein of the jejunal homogenate, 10^{-5} M cytochrome *c*, 3×10^{-5} M NADPH, 10^{-3} M KCN, 0·1 M Tris buffer pH 7·5. The curve represents a typical experiment.

compared with the increase in *O*-dealkylation activity. It can be seen that an almost parallel increase of both activities occurs. After 120 min of incubation with phenobarbital the level of the reductase is induced three-fold and may therefore be intimately linked to the increase in *O*-dealkylation activity.

To demonstrate the *de novo* biosynthesis of NADPH-cytochrome P-450 reductase during phenobarbital induction, we followed the incorporation of ^{14}C-leucine into mucosal cell homogenate (Table 2). Compared with the control, incubation with phenobarbital activated the incorporation of the label about two-fold. This effect was not seen in the presence of $1{\cdot}5 \times 10^{-5}$ M puromycin. Although this experiment by no means proves the synthesis of new reductase protein it suggests that phenobarbital incubation induces protein synthesis in mucosal cells.

TABLE 2. ^{14}C-LEUCINE INCORPORATION INTO MUCOSA CELL PROTEINS OF THE JEJUNUM

Mucosa cell homogenate of incubated jejunum	Counts/min per mg of mucosal protein
Control	1800 ± 200
Control + Puromycin ($1{\cdot}5 \times 10^{-5}$ M)	1200 ± 200
Phenobarbital-treated (5×10^{-4} M)	3900 ± 200
Phenobarbital-treated (5×10^{-4} M) + Puromycin ($1{\cdot}5 \times 10^{-5}$ M)	1400 ± 200

^{14}C-Leucine (5 μCi/0·1 μmol) was dissolved in 700 ml Tyrode solution. Phenobarbital (5×10^{-4} M) was added as indicated. After 120 min of incubation the proteins were precipitated and solubilized as described in Methods.

DISCUSSION

The results obtained in this study recommend the isolated small intestine as a model for investigations on the mechanism of phenobarbital induction of drug monooxygenase activity. In support of our previous results,[17] the *O*-dealkylation of 7-ethoxycoumarin could clearly be characterized as a cytochrome P-450–mediated reaction. The induction effect seems to be restricted to an increase in cytochrome P-450 reductase, thus stimulating the turnover of the cytochrome P-450 monooxygenase cycle. Final proof of this assumption, the direct demonstration of *de novo* synthesis of the flavoprotein will be presented in a future publication.[31] Thus the immediate response of the system to phenobarbital *in vitro* differs from the prolonged administration of phenobarbital (1–5 days) which results mainly in an increase in cytochrome P-450.

The inhibition experiments with puromycin strongly support the involvement of protein synthesis as the basis of the enhancement of *O*-dealkylation activity.[32,33] Interestingly, puromycin also inhibits the basal rate of dealkylation in controls to about 50 per cent. The simplest explanation of this effect would be a rapid turnover of one of the components, presumably the cytochrome P-450 reductase, during the incubation. Indeed, the occurrence of NADPH-cytochrome *c* reductase with a half life of about 2 hr has been demonstrated by Negeshi and Omura.[21] Further, the reductase from liver is immunologically identical to the reductase of small intestine.[34]

The fact that actinomycin D at a concentration which completely inhibits phenobarbital induction does not block the basal rate of *O*-dealkylation suggests that protein synthesis in controls is dependent on preformed, stable messenger RNA. Phenobarbital application probably causes an increase in the mRNA levels by *de novo* synthesis. This is supported by the inhibition caused by α-amanitin which is known to block RNA-polymerase specifically.[35,36]

That the mechanism of transcription and translation of the genetic information is even more complex is suggested by the paradoxical effect of actinomycin D which results in the phenomenon of "superinduction" at higher concentrations. Similar results have been obtained with the induction of tyrosine aminotransferase,[28] tryptophan pyrrolase[29] and other enzymes.[30] A possible explanation for superinduction is discussed by Tomkins *et al.*[28] These authors assume reprimation of a relatively stable mRNA by a protein repressor. This repressor should have a rapid turnover rate so that blocking of its synthesis by high actinomycin D concentrations preserves more mRNA for the translation process. An alternative explanation was proposed by Schimke *et al.*[37] based on the various half lives of mRNA species. A complete inhibition of mRNA synthesis would lead to a higher availability of ribosomes for the translation of stable mRNA. So far, one cannot prove or disprove either of these models and more data on the phenobarbital effect on the small intestine may be helpful in elucidating the mechanism of induction in higher organisms.

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