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# ENHANCEMENT BY PHENOBARBITAL OF SOME EFFECTS OF CARBON TETRACHLORIDE ON MOUSE LIVER*

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**Abstract** Corn oil or a corn oil–$CCl_4$ mixture was administered orally twice a week for 7 or 8 weeks to male Swiss mice maintained without or with phenobarbital Na in the drinking water. There was a significant increase in liver weight in $CCl_4$-treated mice maintained without phenobarbital Na in the drinking water and killed 48 hr after the last treatment. Histologically, livers from these $CCl_4$-treated mice were composed of areas of necrosis within lobules of regenerating hepatocytes, but deposition of collagen was scant. Synthesis of DNA, protein and fatty acids *in vitro* was increased in liver tissue from $CCl_4$-treated mice. Combined treatment with phenobarbital and $CCl_4$ produced additional significant increases in liver weight but collagen deposition was meager. In liver tissue from mice treated with both phenobarbital and $CCl_4$, synthesis of DNA *in vitro* was significantly increased, protein synthesis was modestly increased and fatty acid synthesis was unchanged when compared with liver tissue from $CCl_4$-treated mice not additionally given the barbiturate. Thus, phenobarbital did not accelerate or promote cirrhosis in livers of $CCl_4$-treated mice but did enhance the effects of chronic $CCl_4$ treatment on liver weight, liver DNA synthesis and to a lesser extent on liver protein synthesis.

When $CCl_4$ is administered chronically to mice over periods of several weeks, the repeated necrotizing effects of $CCl_4$ stimulate continued regeneration of liver cells [1]. Histologically, the liver is composed of areas of necrotic tissue surrounding or intermingled with masses of regenerating hepatocytes [1, 2]. Long-term administration of $CCl_4$ to mice over periods of several months results in the deposition of collagen and in the formation of hepatomas [2, 3]. We have been employing this model to study the sequential changes in liver metabolism during the phase of hepatocyte regeneration, subsequently during the emergence and development of neoplastic tissue and what relationship may exist between regeneration and neoplasia. Our initial studies have been concerned with the regenerative phase of the mouse liver's response to long-term $CCl_4$ administration and with methods of manipulating this response.

Phenobarbital administration markedly enhances the acute hepatotoxicity of $CCl_4$ in rats [4] and in dogs [5]. The chronic administration of a combined regimen of phenobarbital and $CCl_4$ to rats produces within 8 weeks of treatment severe cirrhosis of the liver [6]. Preliminary experiments indicated to us that comparable combined treatment of mice did not produce liver cirrhosis but rather intensified selectively some of the metabolic effects of chronic $CCl_4$ administration in mouse liver. We have expanded this preliminary group of experiments and report here on the interaction of chronic $CCl_4$ and phenobarbital administration to mice.

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## METHODS

Male Swiss mice were maintained four to five per cage and given constant access to water and to food. The average body weight of the mice at the start of the experiment was 30 g and mice were randomly divided into each of the treatment and control groups. $CCl_4$ was added to corn oil to provide a 1:15 (v/v) mixture and the mixture was administered by gavage twice a week on a Thursday–Monday or Friday–Tuesday sequence. The dose of $CCl_4$ was 0·2 to 0·3 $\mu$l/g of body weight in a total volume of 0·10 to 0·15 ml of the mixture. Comparable volumes of corn oil were administered to control mice on the same schedule.

### *First series of experiments*

Mice were treated over periods of 46 or 54 days and were killed 48 hr after the last dose of either the mixture or the corn oil. The total duration of the experiment was 48–56 days.

### *Second series of experiments*

All mice were maintained on drinking water containing phenobarbital sodium (30 mg/100 ml) for 10 days prior to and during the first 3 weeks of the experimental period. The phenobarbital sodium concentration was then increased to 45 mg/100 ml for the remainder of the experiment. Our intent was to provide a daily intake of the barbiturate of 75–85 mg/g of body weight. Based on average water consumption, this would require a concentration of 45 mg phenobarbital Na/100 ml of drinking water. However, this higher concentration resulted in severe depression, ataxia and loss of weight when given to nontolerant mice. $CCl_4$ in doses as low as 0·05 $\mu$l/g of body weight resulted in 100 per cent mortality when given to mice maintained for 10 days on drinking water containing

phenobarbital Na, 45 mg/100 ml. The lower concentration of phenobarbital Na, 30 mg/100 ml of drinking water, was not accompanied by severe side effects, permitted the administration of $CCl_4$ without excessive mortality and apparently resulted in tolerance so that the higher concentration could be given as the experiment progressed.

Preliminary experiments indicated that hexobarbital sleeping time in mice given phenobarbital sodium (30 mg/100 ml) in the drinking water for 10 days was reduced by 50–55 per cent when compared to controls. Using hexobarbital at a dose of 100 $\mu$g/g of body weight, sleeping times in control animals were 30 ± 1·7 min (mean ± 1 S.E.M.: N = 10) and in phenobarbital-treated mice were 14 ± 1·9 min (N = 10). Thus, when treatment with $CCl_4$ was begun, induction of liver drug-metabolizing enzyme system (DMES) activity presumably had occurred.

Mice were treated over a period of 46 or 54 days and were killed 48 hr after the last dose of $CCl_4$ or corn oil.

*Metabolic experiments*

Each mouse was killed by decapitation and exsanguination. The liver was quickly removed, placed into an iced beaker and slices of liver were then cut with a Stadie Riggs tissue slicer. Liver slices weighing 500 ± 25 mg or 200 ± 15 mg were placed in 50-ml Erlenmeyer flasks to which was added 6 ml Krebs–Henseleit solution [7]. The flasks were capped with a rubber sleeve, equilibrated for 5 min with 95% $O_2$–5% $CO_2$ and incubated for 2–3 hr depending upon the substrate used. The substrates incorporated in the incubation solution, and the incubation times are given below.

*Lipid metabolism.* The Krebs Henseleit solution contained sodium pyruvate (5 M) to which was added sodium [3-$^{14}$C]pyruvate to provide an initial specific radioactivity of 50,000 cpm/$\mu$mole of pyruvate. The incubation period was 3 hr and, at its conclusion, liver tissue was removed and placed into 1·5 ml of 30% KOH and an aliquot of the incubation solution was taken for recovery of bicarbonate as $CO_2$. Methods used for determining the incorporation of $^{14}$C from [3-$^{14}$C]pyruvate into bicarbonate, cholesterol and fatty acids were those described by Gans and Cater [8].

*Protein metabolism.* An amino acid mixture containing 20 amino acids having an average molecular weight of 144 was prepared as described by Gans and Cater [9]. This amino acid mixture was incorporated into the incubation solution to provide a concentration of 1 mM, and $^{14}$C was added as [U-$^{14}$C]-L-phenylalanine to provide an initial specific radioactivity of 150,000 cpm/ml of incubation solution. The incubation period was 2 hr and at its conclusion the liver tissue was placed into a 5% solution of trichloroacetic acid and an aliquot of the incubation solution was taken for the recovery of bicarbonate as $CO_2$.

Tissue protein was isolated according to the method of Manchester and Krahl [10] and the protein was dissolved in 0·05 N NaOH to provide a final solution of 5 ml. A 0·2-ml aliquot of the protein solution was added to a counting vial containing 0·5 ml Hyamine. Gentle swirling of the flask permitted complete solution of the protein in Hyamine, and 2 ml ethanol and 10 ml of a toluene-base fluor mixture (0·4% 2,5-diphenyloxazolyl + 0·01% *p*-bis[2-(4-methyl-5-phenyloxazolyl)] benzene) were then added. The incorporation of $^{14}$C into protein was determined by liquid scintillation spectrometry. Protein concentration was determined with the Biuret reagent [11].

*DNA metabolism.* Liver slices weighing 200 ± 15 mg were used for this series of experiments. Sodium pyruvate (5 mM) was incorporated in the Krebs–Henseleit solution to provide excess substrate and [methyl-$^3$H]thymidine was added to provide an initial specific radioactivity of 500,000 cpm/ml. The period of incubation was 2 hr and at its conclusion the liver tissue was homogenized in 7 ml of iced 10% trichloroacetic acid. DNA was isolated according to the method outlined by Munro and Fleck [12] including an alkaline digest with 0·3 N NaOH to remove RNA. The final precipitate containing DNA and protein was heated for 45 min at 90° in 5% trichloroacetic acid. The trichloroacetic acid solution was cooled to room temperature, its volume measured (usually a final solution of 5 ml) and 0·1 ml was added to a counting vial. Ethanol (2 ml) and 10 ml of the fluor mixture were added to the counting vial and the incorporation of $^3$H into DNA was determined by liquid scintillation spectrometry.

Table 1. Effect of $CCl_4$ and of phenobarbital on mouse liver weight*

| Treatment | N† | Phenobarbital Na in the drinking water | Body wt (g)‡ | Liver wt‡ (g wet wt) | Liver wt‡ (% body wt) | Liver wt§ (% body wt) |
|---|---|---|---|---|---|---|
| Corn oil | 10 | – | 39·5 ± 1·3 | 2·2 ± 0·07 | 5·6 ± 0·2 | 5·2 ± 0·2 |
| $CCl_4$ | 8 | – | 42·0 ± 1·4 | 3·2 ± 0·2 | 7·5 ± 0·2 ‖ | 5·7 ± 0·3 |
| Corn oil | 12 | + | 40·0 ± 0·6 | 2·8 ± 0·2 | 6·8 ± 0·2 | 7·1 ± 0·6 |
| $CCl_4$ | 12 | + | 41·9 ± 0·7 | 4·5 ± 0·1 | 11·0 ± 0·3** ¶ | 9·6 ± 0·6†† |

* All data in this and subsequent tables are expressed as the mean ±1 S.E.M.

† Number of animals.

‡ Mice killed 48 hr after last dose of corn oil or of $CCl_4$.

§ Mice killed 7 days after last dose of corn oil or of $CCl_4$. Mice treated with the combined regimen of $CCl_4$ and phenobarbital and their appropriate controls were maintained on drinking water containing phenobarbital Na during this 7-day period following the last dose of corn oil or of $CCl_4$.

‖ Difference between $CCl_4$- and corn oil-treated groups: $P(t) < 0{\cdot}01$.

¶ Difference between the two $CCl_4$-treated groups: $P(t) < 0{\cdot}001$.

** Difference between $CCl_4$- and corn oil-treated groups: $P(t) < 0{\cdot}001$.

†† Difference between $CCl_4$- and corn oil-treated groups: $P(t) < 0{\cdot}02$.

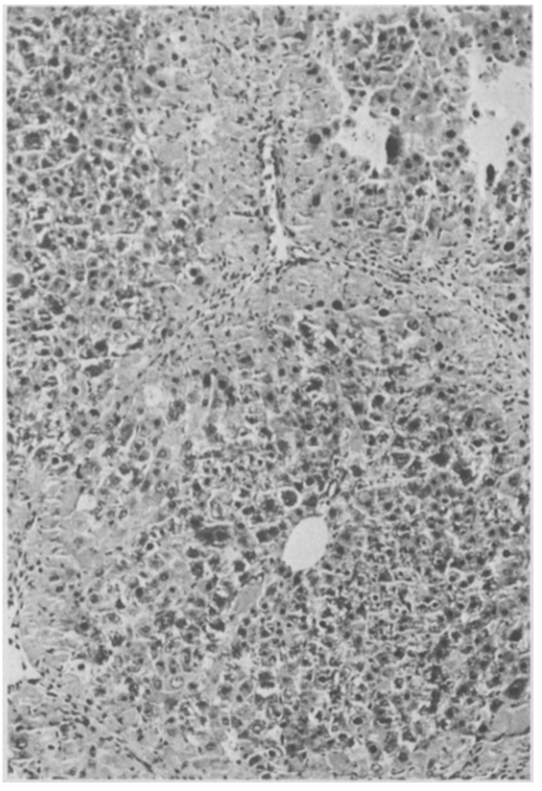

Fig. 1. Section from liver of mouse treated with the combination of phenobarbital and $CCl_4$ for 8 weeks. Extensive bands of necrosis extending from one portal area to the next enclose lobules of regenerating hepatocytes. The necrotic areas show some infiltration with mononuclear cells. Hematoxylin–eosin stain. Magnification, 100×.

*Histology*

Mice were maintained with or without phenobarbital Na in the drinking water as described and within each group mice were treated with either corn oil or $CCl_4$ for a period of 54 days. Mice were killed either 48 hr or 7 days after the last dose of the vehicle or $CCl_4$. In those mice maintained with phenobarbital Na in the drinking water, the barbiturate solution was used through the termination of the experiment.

Segments of liver were taken from each mouse and placed into 10% buffered formalin solution. Sections prepared for histologic examination were stained with hematoxylin–eosin, with the Masson Trichrome stain or with Wilder's Reticulin stain.

## RESULTS

Body weights of the $CCl_4$-treated mice were the same as those of the appropriate control mice (Table 1). Liver weights of the $CCl_4$-treated mice were significantly increased over those of the appropriate control mice (Table 1). Additionally, the livers from mice treated with both $CCl_4$ and phenobarbital were significantly heavier than the livers from $CCl_4$-treated mice maintained without phenobarbital Na in the drinking water (Table 1). Seven days after the last dose of the corn oil or the mixture, liver weights of mice which had been treated with $CCl_4$ were the same as those of the control mice (Table 1). However, livers of mice which were treated with both $CCl_4$ and phenobarbital were still significantly heavier than the livers of the appropriate phenobarbital-treated controls 7 days after the last dose of either the $CCl_4$ mixture or of corn oil (Table 1). No gross evidence of liver tumors or of metastases was observed in any of the control or treated mice.

Histologic examination of livers from $CCl_4$-treated mice killed 48 hr after the last treatment indicated patterns of change which were similar to those described by other investigators [1 3]. There were characteristic zonal and band-like areas of necrosis which separated sheets of regenerating hepatocytes. Necrotic areas were not uniformly centrolobular, but rather distributed randomly throughout the sections. In liver sections taken from mice treated with the combined regimen of phenobarbital and $CCl_4$ and killed 48 hr after the last treatment, the necrotic areas were predominantly in the periphery of the lobule, extending from the portal triads and producing a bridging effect (Fig. 1). The lobules were composed of masses of regenerating hepatocytes (Fig. 1). The interlobular "bridges" of necrotic tissue contained variable numbers of mononuclear cells and a few polymorphonuclear cells but were almost completely devoid of collagen (Fig. 2). Liver sections taken 7 days after the last treatment showed considerable but not complete restoration toward the appropriate "normal" condition.

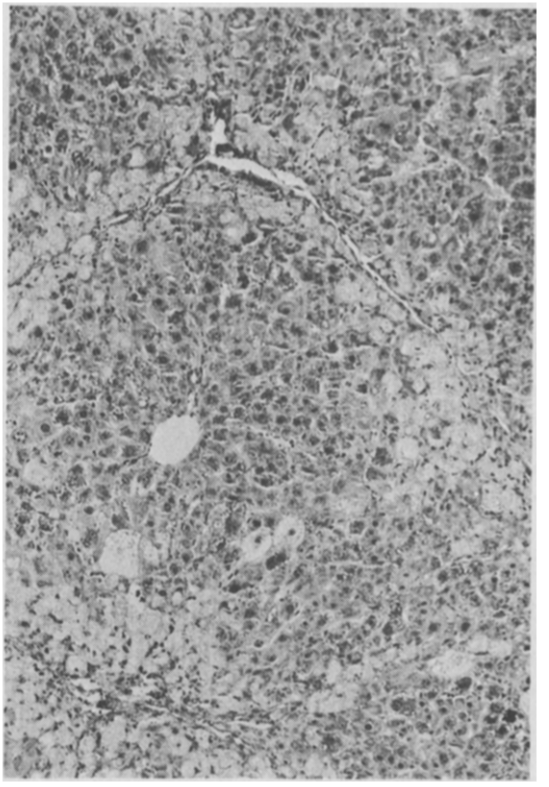

Fig. 2. Section contiguous to that of liver tissue shown in Fig. 1, but stained with Wilder's Reticulin stain. There is no evidence of fibrosis, a marked contrast to the effects of the combined regimen of phenobarbital and $CCl_4$ in rats [6]. Sections stained with the Masson Trichrome stain also indicated virtually no deposition of collagen. Magnification, 100×.

Table 2. Incorporation of $^{14}C$ from [3-$^{14}C$]pyruvate into bicarbonate, cholesterol and fatty acids by mouse liver slices *in vitro*: Effects of $CCl_4$ and of phenobarbital

| Treatment | N | Phenobarbital Na in the drinking water | % incorp. added $^{14}C$ 500 mg tissue 3 hr into: Bicarbonate | Cholesterol | Fatty acids |
|---|---|---|---|---|---|
| Corn oil | 10 | – | 10·2 ± 0·5 | 0·35 ± 0·12 | 2·1 ± 0·2* |
| $CCl_4$ | 10 | – | 9·9 ± 0·5 | 0·24 ± 0·06 | 5·6 ± 0·5† |
| Corn oil | 7 | + | 8·6 ± 0·6 | 0·16 ± 0·02 | 0·7 ± 0·4* |
| $CCl_4$ | 7 | + | 8·8 ± 0·8 | 0·25 ± 0·08 | 3·9 ± 0·6† |

* Difference between the two corn oil-treated groups: P(t) < 0·05.
† Difference between each $CCl_4$-treated group and appropirate corn oil-treated group: P(t) < 0·01.

*Liver protein concentrations.* The concentrations of liver protein, both prior to and following incubation, expressed as mg/g wet weight, were the same in all groups. These negative results have not been tabulated.

*Pyruvate metabolism.* The results of these experiments are summarized in Table 2. Incorporation of $^{14}C$ into bicarbonate and into cholesterol by liver slices from $CCl_4$-treated mice was the same as that observed in liver slices from the corn oil-treated groups (Table 2). Incorporation of $^{14}C$ into fatty acids was significantly increased in liver slices from $CCl_4$-treated mice when compared to the values obtained from liver tissue of corn oil-treated mice.

The pattern of effects of $CCl_4$ on hepatic pyruvate metabolism *in vitro* in liver slices from mice maintained on drinking water containing phenobarbital Na was similar to that in liver slices from mice maintained without phenobarbital. There was, however, a decrease in $^{14}C$-fatty acid formation in liver slices from mice treated with phenobarbital when compared to that in liver slices from mice maintained without phenobarbital Na in the drinking water.

*Phenylalanine metabolism.* These results are summarized in Table 3. Incorporation of $^{14}C$ from [U-$^{14}$C]-L-phenylalanine into protein *in vitro* was significantly greater in liver tissue from $CCl_4$-treated mice when compared with that in liver tissue from corn oil-treated mice (Table 3). Incorporation of $^{14}C$ into protein was significantly increased in liver tissue from $CCl_4$-treated mice concurrently maintained on drinking water containing phenobarbital Na, when compared with that of liver tissue from the appropriate corn oil-treated group as well as from mice treated with $CCl_4$ and maintained without phenobarbital in the drinking water (Table 3).

*[$^3$H]thymidine incorporation into DNA.* These data are summarized in Table 4. There was a marked increase in the formation of [$^3$H]DNA by liver slices from $CCl_4$-treated mice when compared with mice treated with corn oil (Table 4). Liver slices from mice treated with both $CCl_4$ and phenobarbital showed a further highly significant increment in [$^3$H]DNA formation (Table 4).

## DISCUSSION

The results of these experiments demonstrate a marked species difference between the hepatic responses by mice and rats to combined treatment with phenobarbital and $CCl_4$. This regimen, which produces heavy scarring in rat liver within an 8-week treatment period [6], fails to induce the deposition of collagen in mouse liver. This failure to scar the liver may underlie the very significant increase in liver weight produced by combined treatment of mice with phenobarbital and $CCl_4$. The chronic administration of $CCl_4$ to both species of rodents produces repetitive cell death followed by regeneration. The liver DMES inducer phenobarbital [13], when given concurrently with $CCl_4$, apparently enhances this continuing sequence of necrosis and regeneration. In mouse liver, the failure to deposit collagen leads to an increase in both necrotic tissue and hepatic parenchymal substance.

A large increase in DNA synthesis and in protein synthesis by liver slices from $CCl_4$-treated mice would be anticipated, since peak regenerative activity in mouse liver occurs at approximately 48 hr after administering $CCl_4$ [14]. The further very considerable increment, particularly in DNA synthesis, produced by combined treatment with phenobarbital and $CCl_4$ seems paradoxical, since treatment with phenobarbital Na alone, in this investigation, did not increase [$^{14}$C]protein or [$^3$H]DNA formation by mouse liver tissue. An increase, particularly in protein synthesis, would have been anticipated from the results of many experiments in other species of laboratory animals [15]. This does not reflect a species difference because livers from mice treated for short periods of time with

Table 3. Incorporation of $^{14}C$ from [U-$^{14}$C]-L-phenylalanine into bicarbonate and into protein by mouse liver slices *in vitro*: Effect of $CCl_4$ and of phenobarbital

| Treatment | N | Phenobarbital Na in the drinking water | % Incorp. added $^{14}C$ 500 mg tissue 2 hr into: Bicarbonate | Protein |
|---|---|---|---|---|
| Corn oil | 6 | – | 6·0 ± 0·1 | 2·8 ± 0·7 |
| $CCl_4$ | 8 | – | 4·4 ± 0·3 | 7·8 ± 0·7*† |
| Corn oil | 10 | + | 6·1 ± 0·5 | 3·7 ± 0·3 |
| $CCl_4$ | 10 | + | 3·8 ± 0·4 | 10·5 ± 0·7*† |

* Difference between each $CCl_4$-treated group and appropriate corn oil-treated group: P(t) < 0·001.
† Difference between two $CCl_4$-treated groups: P(t) < 0·02.

Table 4. Incorporation of [methyl $^3$H]thymidine into DNA by mouse liver slices *in vitro*: Effect of $CCl_4$ and of phenobarbital

| Treatment | N | Phenobarbital Na in the drinking water | % Incorp. added [$^3$H]/200 mg tissue/2 hr into DNA |
|---|---|---|---|
| Corn oil | 6 | − | 0·25 ± 0·06 |
| $CCl_4$ | 8 | − | 2·8 ± 0·4*† |
| Corn oil | 7 | + | 0·33 ± 0·05 |
| $CCl_4$ | 12 | + | 5·8 ± 0·4*† |

* Difference between each $CCl_4$-treated group and appropriate corn oil-treated group: $P(t) < 0·001$.

† Difference between the two $CCl_4$-treated groups: $P(t) < 0·001$.

phenobarbital incorporate increased amounts of radioactively labeled amino acid into liver protein [16]. Rather, degenerative changes in liver cell endoplasmic reticulum following long-term exposure to liver DMES inducers [17] probably diminished the capacity of the liver cells to maintain an increased rate of protein synthesis.

An entirely different situation may be present in mice given both phenobarbital and $CCl_4$. The necrotizing effects of each dose of $CCl_4$ with subsequent regeneration produce a new population of hepatocytes. Since regenerating parenchymal cells, following partial hepatectomy, respond positively to liver DMES inducers [18], the regenerating hepatocytes in livers from mice treated with $CCl_4$ should also be capable of a comparable positive reaction to phenobarbital. Moreover, the new hepatocytes would have been exposed to phenobarbital for only a short period and should have increased protein synthesis accordingly. Thus, the increase in protein synthetic activity, and possibly DNA synthesis as well, in livers from mice treated with phenobarbital and $CCl_4$ probably represent additive effects of each compound. Therefore, the mice maintained for 7–8 weeks with phenobarbital Na in the drinking water and treated with corn oil may have been an inappropriate "control" group for the mice treated with both phenobarbital and $CCl_4$.

An increase in liver fatty acid synthesis appears to be a characteristic metabolic response to chronic $CCl_4$ administration to mice [19]. Liver tissue from mice treated chronically with $CCl_4$ (tissue taken 48 hr after the last treatment) has a lower concentration of total lipid than does tissue from control mice [19]. If this decrease in liver total lipid concentrations also reflects a decrease in liver cell fatty acyl-CoA concentrations, increased fatty acid synthesis would result from diminished inhibition of acetyl-CoA carboxylase [20, 21]. It is clear, however, that the concurrent treatment with phenobarbital and $CCl_4$ did not increase liver fatty acid synthesis *in vitro* beyond the levels associated with $CCl_4$ treatment alone.

In summary, phenobarbital produces a selective enhancement of the effects of chronic $CCl_4$ administration to mice. The barbiturate results in a striking enhancement of $CCl_4$-induced DNA synthesis, more modest effects on protein synthesis and liver weight, and no effect on $CCl_4$-induced increases in liver fatty acid synthesis. The increments in DNA synthesis and protein synthesis in mice treated with both phenobarbital and $CCl_4$ may result from a summation of the effects of each chemical because of $CCl_4$-induced changes in the hepatocyte population, even though phenobarbital itself produces very few if any changes in these aspects of liver weight and metabolism.

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