

Comparative Enhancing Effects of Phenobarbital, Amobarbital, Diphenylhydantoin, and Dichlorodiphenyltrichloroethane on 2-Acetylaminofluorene-induced Hepatic Tumorigenesis in the Rat¹

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SUMMARY

Earlier studies showed that phenobarbital feeding enhanced hepatic tumorigenesis in rats previously fed 2-acetylaminofluorene for a brief period. As part of an investigation of the mechanism of this enhancement, the present study evaluated the relative enhancing abilities of amobarbital, diphenylhydantoin, and dichlorodiphenyltrichloroethane (DDT), agents that resemble phenobarbital to varying degrees in their effects on liver structure and metabolism. A comparison of hepatic tumor yields in rats fed 2-acetylaminofluorene, followed by the test substance (sequential treatment), showed that amobarbital and diphenylhydantoin had no enhancing activity, whereas the enhancing effect of DDT was similar to that of phenobarbital. These results show that the sequential treatment technique readily distinguishes among substances differing in enhancing ability and should prove useful in screening additional substances for this activity. The comparative biochemical effects of these substances in the liver can then be correlated with their relative enhancing abilities to provide information on the molecular events specifically associated with enhancement. Such correlations were initiated in this study by comparing the effects of the four test substances on liver weight and DNA synthesis. The results showed that the enhancers, phenobarbital and DDT, each stimulated liver DNA synthesis and increased liver weight, whereas the nonenhancers, amobarbital and diphenylhydantoin, had neither effect.

Phenobarbital and DDT both increased the early tumor incidence rate and maintained an increment in tumor incidence over that in the other treatment groups throughout the experiment, although it is not clear whether this increment would persist indefinitely. In addition, although the spectrum of tumor types observed ranged from highly differentiated to poorly differentiated in all treatment groups, DDT and phenobarbital selectively increased the incidence of highly differentiated tumors throughout most of the experiment.

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INTRODUCTION

In earlier studies (27, 29), the prolonged feeding of phenobarbital to rats that had previously been fed AAF³ for a brief period markedly increased the subsequent incidence of liver tumors. Using this sequential feeding regime, the present study evaluated the relative tumorigenic enhancing abilities of other compounds that, to varying degrees, resemble phenobarbital in their effects on liver structure and metabolism. The substances examined were amobarbital, diphenylhydantoin, and DDT. Table 1 compares relevant characteristics of these agents with those of phenobarbital, reviewed previously (27, 29). Amobarbital is closest to phenobarbital in structure and, like phenobarbital, alters the metabolism of other drugs by the liver. Diphenylhydantoin is less closely related to phenobarbital structurally but has analogous effects on DNA synthesis and drug metabolism. DDT and phenobarbital are structurally dissimilar but are closely analogous in their effects on the liver.

The major objective of this study was the identification of a series of compounds that differ in ability to enhance liver tumorigenesis. Correlations of these differing enhancing abilities with the effects of these compounds on metabolic processes in the liver can then be undertaken. Such correlations should facilitate the identification of those molecular events that are essential to enhancement.

MATERIALS AND METHODS

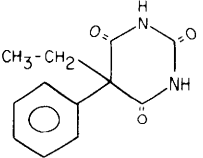
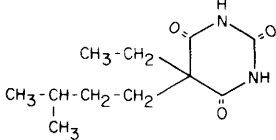
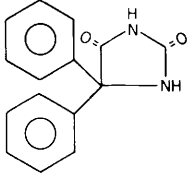
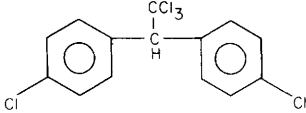
Tumor Incidence Study. Male Sprague-Dawley rats at 22 days of age were fed either a control diet or one containing 0.02% AAF for 18 days, after which they were all fed the control diet for 7 days. The control and AAF-fed rats were then separately divided into 5 groups. Of the rats not fed AAF, each group contained 48 animals; of those fed AAF each group contained 120 rats. Both sets of groups were given the following diets for the duration of the experiment: Group 1, control; Group 2, phenobarbital (Mallinckrodt Chemical Works, St. Louis, Mo.); Group 3, amobarbital (Sigma Chemical Co., St. Louis, Mo.); Group 4, diphenyl-

³ The abbreviations used are: AAF, 2-acetylaminofluorene; DDT, dichlorodiphenyltrichloroethane or 1,1,1-trichloro-2,2 bis(*p*-chlorophenyl)ethane.

Table 1

Comparison of known effects of phenobarbital, amobarbital, diphenylhydantoin, and DDT in the liver

The numbers in parentheses are references, including primary sources and review articles.

Agent	Structure	Effect in liver				
		DNA synthesis and hyperplasia	RNA synthesis	RNA degradation	Hypertrophy and smooth endoplasmic reticular proliferation	Microsomal enzyme activity
Phenobarbital		Increases (1, 27, 29)	Increases (27, 29)	Decreases (27, 29)	Increases (1, 27, 29)	Increases (13, 27, 29)
Amobarbital		Unknown	Unknown	Unknown	Unknown	Increases (21)
Diphenylhydantoin		Increases (36)	Unknown	Unknown	No effect (14)	Increases (7, 10, 12, 14, 35, 37, 38, 44)
DDT		Increases (16)	Increases (24)	Decreases (24)	Increases (15, 25, 26, 30)	Increases (34, 11)

hydantoin (Sigma); Group 5, DDT⁴ (Matheson, Coleman, and Bell, Cincinnati, Ohio). The dietary concentration of each agent was 0.05%. Based on measurements of food consumption, it was estimated that the average daily intake of these agents ranged from approximately 50 mg/kg of body weight during the 1st 4 to 6 months of the experiment down to approximately 20 mg/kg of body weight by the 15th month. No symptoms of sedation or toxicity were observed in any of the rats throughout the experiment.

Rats were killed at intervals (see "Results"; Chart 1) throughout the experiment and examined for tumors. Livers were sliced with a razor blade into sections that were 5 mm thick to reveal tumors beneath the surface. The largest diameter of each tumor was recorded, and samples of these and other grossly observable lesions were taken for light microscopy. Additional experimental details (rats, diets, husbandry, and histological preparations) were described previously (27, 29).

Acute Exposure Study. Weanling rats were fed the control diet for 25 days before being exposed to the test substances.

⁴ Technical grade, containing: *p,p'*-DDT (see Footnote 3), 70%; *o,p'*-DDT [1,1,1-trichloro-2,2-(*o*-chlorophenyl-*para*-chlorophenyl)ethane], 12%; *p,p'*-DDT [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane], 3% *p,p'*-DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene], 15%. Analysis, courtesy of the Department of Agricultural Chemistry, Oregon State University, Corvallis, Ore.

Beginning on Day 26, the rats were given single daily i.p. injections (between 7 a.m. and 9 a.m.) of phenobarbital sodium (83 mg/kg), amobarbital (75 mg/kg), diphenylhydantoin sodium (44 mg/kg), or DDT (112 mg/kg). The DDT was dissolved in cottonseed oil; the other agents were dissolved in distilled water. Control rats received equivalent volumes of water or cottonseed oil. The dosages of phenobarbital, amobarbital, and DDT were equimolar; the dosage of diphenylhydantoin was one-half that of the other agents, owing to the mortality observed in rats given higher doses. The rats given phenobarbital or amobarbital slept for 2 to 3 hr following treatment.

Twenty-four hr after receiving its last dose of test agent, each rat was given an i.p. injection of [³H]thymidine (500 μCi/kg; specific activity, 0.36 Ci/mole; Schwarz/Mann, Orangeburg, N. Y.) and was killed by cervical dislocation 1 hr later. Liver nuclei were isolated by the method of Blobel and Potter (3), and were counted in a hemocytometer.

For the determination of acid-insoluble radioactivity, 0.5-ml aliquots of the nuclear suspensions were placed on 9-cm filter papers folded to fit into Coplin jars. The samples were then dried under a heat lamp and given a series of 10-min washes, 3 times with 10% trichloroacetic acid, then twice with 95% ethanol. The samples were dried again, combusted in a Packard sample oxidizer, and counted in a liquid scintillation spectrometer.

RESULTS

Tumor Incidence Study. The observed hepatic lesions were classified into the following types. (a) Hyperplasia and hypertrophy. While these lesions appeared to represent a new cell population with an altered proliferation rate and glycogen metabolism, which are attributes of neoplasia, the main characteristics of normal liver structure were maintained, (b) Adenomas. The characteristics of these lesions were increased hepatocyte proliferation and highly differentiated cells and structure, but also abnormal cords and loss of lobular configuration. Some of these tumors grew to replace most of the normal hepatic tissue but did not show the characteristic invasive properties indicative of cancer, nor did they appear to metastasize. (c) Hepatocellular carcinomas. These tumors included types characterized as trabecular, because of the thickness of the cords, adenomatous, because the cell arrangement was acinar, and those showing both trabecular and adenomatous properties. The degree of pleomorphism, the degree of proliferation, the frequency of obviously abnormal mitoses, and the incidence of metastases varied considerably in these tumors. Only adenomas and carcinomas, as defined above, were counted as tumors.

Amobarbital and diphenylhydantoin were completely ineffective as enhancers under the conditions used, failing to increase the final tumor incidence (Table 2) or alter the kinetics of tumor appearance (Chart 1). DDT, on the other hand, showed an enhancing ability similar to that of phenobarbital (Table 2; Chart 1). In 14 of the rats included in Table 2, virtually the entire liver was replaced by tumor, and it was not possible to count individual tumors. These lesions were counted as single large tumors although there may have been multiple tumors that had coalesced. Of these rats, 1 was in the group given AAF alone, 1 was in the AAF-diphenylhydantoin group, 7 were in the AAF-phenobarbital group, and 5 were in the AAF-DDT group.

None of the rats given the test substances without prior exposure to AAF showed evidence of tumors throughout the experiment, but the number (48 rats) in each group was insufficient for adequately testing carcinogenic potential. These groups were used to study the comparative long-term effects of these compounds on the cytology of the hepato-

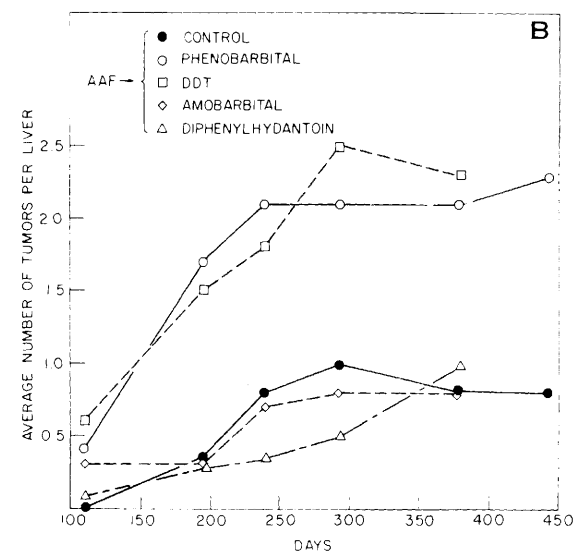
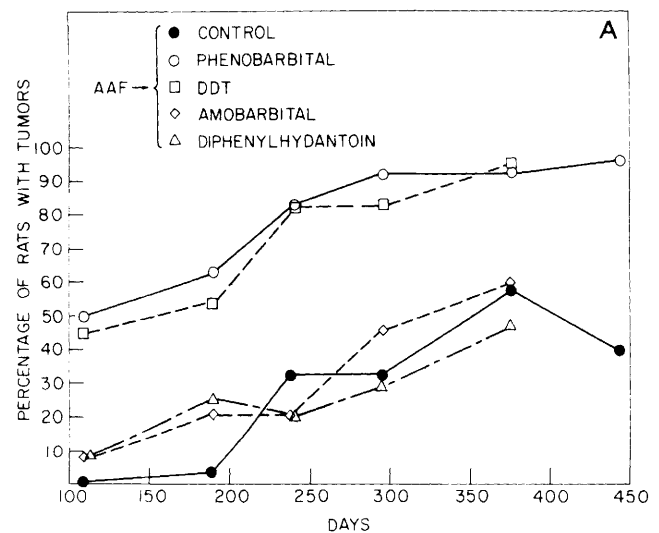


Chart 1. A, the percentage of rats with either adenomas or carcinomas; B, the average number of tumors per rat, as a function of time after cessation of treatment with AAF. The data for each interval have been obtained for 12 to 26 rats killed within 3 weeks of the times indicated.

cytes. Among those fed phenobarbital alone, an area of hyperplasia (32) was found in 1 rat. At all sacrifice intervals hypertrophy was observed, particularly of cells in the central part of the lobule, in both the phenobarbital and DDT groups; these rats also showed elevated liver weights (Table 3). Histological indications of hypertrophy were not observed in rats fed amobarbital or diphenylhydantoin alone, nor did the liver weights in these rats exceed those in untreated controls (Table 3).

DDT and phenobarbital increased both the percentage of rats bearing tumors (Chart 1A) and the average number of tumors per liver (Chart 1B) during the 1st 250 days after the cessation of AAF treatment. After this time, the tumor incidence in both these groups remained essentially unchanged. By 370 days, over 90% of both the phenobarbital- and DDT-treated rats bore tumors (Chart 1A) with a multiplicity of 2.0 to 2.5 tumors per liver (Chart 1B). At the same time, in rats given AAF, followed by either the

Table 2

Hepatic tumor incidence in rats continuously fed phenobarbital, amobarbital, diphenylhydantoin, or DDT for 389 days, starting 1 week after an 18-day period of AAF feeding

Group	Treatment	Rats/group	Rats with tumors	Tumors/group
1	AAF then control diet	108	31	69
2	AAF then phenobarbital	108	85	199
3	AAF then amobarbital	104	34	56
4	AAF then diphenylhydantoin	102	28	43
5	AAF then DDT	103	77	188

control, amobarbital, or diphenylhydantoin diet, the incidence of tumors was 40 to 50% (Chart 1A), with a multiplicity of 0.5 to 1.0 tumors per liver (Chart 1B).

Table 3

Relative liver weights in rats fed the control diet or diets containing 0.05% phenobarbital, amobarbital, diphenylhydantoin or DDT for 270 days, without prior exposure to dietary AAF

Treatment	g liver/100 g body wt ^a
Control	3.16 ± 0.08
Phenobarbital	3.89 ± 0.16 ^b
Amobarbital	3.01 ± 0.07
Diphenylhydantoin	3.05 ± 0.12
DDT	3.89 ± 0.13 ^b

^a Each value is the mean ± S.E. for a group of 12 rats.

^b Significantly different from control ($p < 0.001$)

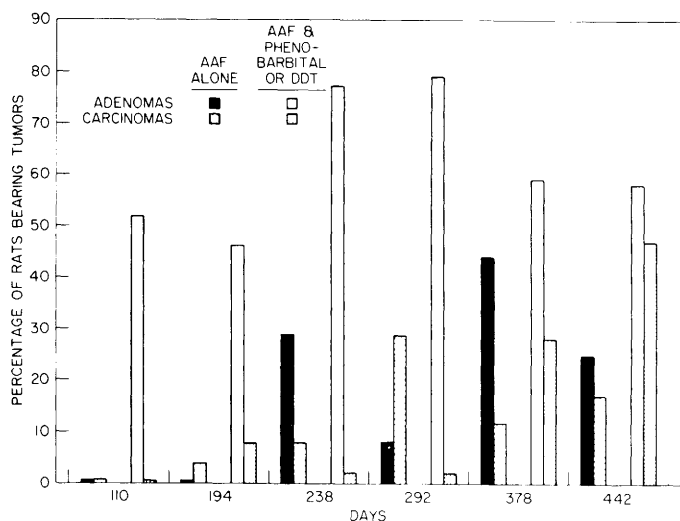


Chart 2. The percentage of rats bearing tumors at intervals after the cessation of treatment with AAF. The data for the groups of rats given AAF followed by DDT, and AAF followed by phenobarbital have been pooled. Thus, these data are based on 23, 48, 48, 48, 44, and 25 rats, respectively, for the times indicated.

Chart 2 compares the relative incidences of adenomas and carcinomas occurring throughout the experiment in rats given AAF followed by the control diet or by the phenobarbital or DDT diet. The results in the latter 2 groups were not different and were therefore combined. In rats given AAF alone, no consistent relationship was noted in the incidence of adenomas versus carcinomas. The feeding of phenobarbital or DDT after AAF markedly elevated the proportion of rats with adenomas at all time intervals but did not increase the overall incidence of carcinomas above that seen in rats given AAF alone, except at the last time interval.

Acute Exposure Study. Both liver weight and DNA synthesis were increased after acute exposure to phenobarbital (Chart 3A). The stimulatory effect of phenobarbital on DNA synthesis was transient, reaching a peak on Day 3 and returning to control levels by Day 5, despite continued phenobarbital administration. The weight of the liver continued to increase throughout the experiment, however. DDT produced similar although less pronounced effects under these conditions, and the rate of DNA synthesis had not declined to control values by the end of the experiment (Chart 3A).

Treatment with amobarbital did not affect liver weight but did reduce liver DNA synthesis (Chart 3B). DNA synthesis was also lower in rats given diphenylhydantoin, and the liver weight decreased during the 1st day of treatment but returned to control levels after the 2nd day (Chart 3B).

DISCUSSION

The sequential exposure technique (AAF followed by test substance) used in this study clearly distinguished between the equivalent abilities of phenobarbital and DDT to enhance tumorigenesis on the one hand and the apparent absence of such enhancing ability in amobarbital and diphenylhydantoin on the other. The similarity in the effects of phenobarbital and DDT indicates that enhancers,

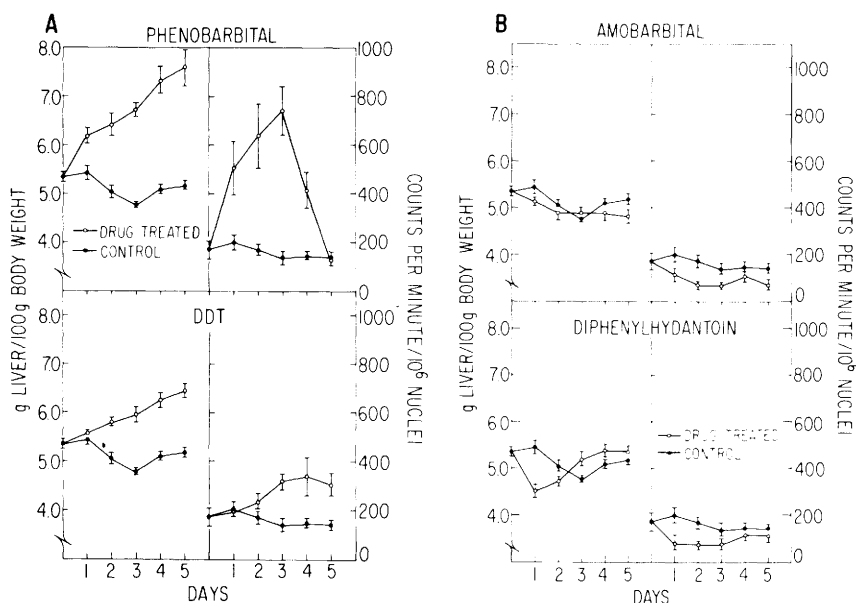


Chart 3. Relative liver weight and liver DNA synthesis in rats treated acutely with (A) phenobarbital or DDT, or (B) amobarbital or diphenylhydantoin. Each data point represents the mean ± S.E. for a group of 9 to 29 rats. Left, relative liver weights; right, nuclear labeling.

like carcinogens, can differ substantially in molecular structure although, again like carcinogens, they evidently possess common molecular characteristics that enable them to affect similar tumorigenically important processes within the liver cell. However, the failure of amobarbital and diphenylhydantoin to enhance tumorigenesis, when fed at doses similar to that of phenobarbital, suggests that relatively minor modifications in the structure of an enhancer can markedly alter its effectiveness.

It is not known whether increases in the dietary concentrations of amobarbital or diphenylhydantoin would produce enhancement, but the complete ineffectiveness of these agents at the dosages used suggests that much higher dietary concentrations would be required to test this possibility. When these agents were administered acutely at dosages comparable to the amounts ingested daily in the diet, however, amobarbital produced sedation and diphenylhydantoin produced significant mortality. In addition, both agents suppressed liver DNA synthesis (Chart 3B), in contrast to the stimulation of DNA synthesis produced by phenobarbital and DDT under the same conditions (Chart 3A). The results of the acute experiments suggest that substantial increases in the dietary levels of amobarbital or diphenylhydantoin over the amounts presently used for the long-term feeding studies might produce toxic side effects that would complicate the interpretation of effects on liver tumor incidence.

Since the present study suggests that the sequential exposure technique may be useful as a method of screening for liver tumorigenic enhancers, it now becomes important to test for enhancing activity in other agents that are known to affect liver structure and metabolism. Examples of such agents are: the commonly used food antioxidant, butylated hydroxytoluene (8); the insecticide, dieldrin (34, 45); and the polychlorinated biphenyl species of industrial pollutants (6, 23). Once relative enhancing abilities are established for a series of agents, the effects of these agents on biochemical processes in the liver, such as those cited in Table 1, can be compared. [It has been recently found that phenobarbital modifies chromosomal proteins (2, 31) and the transport of RNA from the nucleus (20). These processes also may be relevant to enhancement.] Correlations of the relative enhancing abilities of these agents and their relative biochemical effects will then facilitate the identification of those molecular events that are associated specifically with enhancement.

In order to obtain data for initial correlations, the effects of the test agents on liver growth were investigated, using DNA synthesis and relative liver weight as indices of liver growth (Chart 3; Table 3). These data, when compared with the data for tumor incidence (Chart 1; Table 2), suggest that the enhancement of liver tumorigenesis may be associated with the stimulation of liver growth. It is likely, however, that the critical growth response related to tumorigenic enhancement involves more than the early onset of increased DNA synthesis (Ref. 27; Chart 3A), since the period of exposure to phenobarbital required for enhancement (29) was longer than that required to produce the transient stimulation of DNA synthesis observed whether the pheno-

barbital was fed (27) or injected (Ref. 1; Chart 3A). This supports the possibility that additional molecular effects (Table 1) responsible for the maintenance of increased liver size with continued enhancer administration (Ref. 27; Table 3) are involved in the enhancement process. The dependence of tumorigenic enhancement on molecular events additional to DNA synthesis has also been observed in studies of skin tumorigenesis (4, 5).

The data in Chart 1 show that rats given phenobarbital or DDT following AAF treatment maintained a higher tumor incidence for the duration of the experiment than did rats given AAF alone, or AAF followed by nonenhancers. It is not yet clear, however, whether this difference is due only to an increased rate of tumor appearance, or, in addition, reflects a true increment in net tumor production. This question could be resolved if definite plateau levels of tumor incidence were demonstrated in both enhancer-treated and control (AAF alone) rats. Higher plateau levels in enhancer-treated rats would suggest that the enhancer completed the neoplastic transformation in cells that, through prior exposure to AAF, had been partially altered, but not enough to exhibit loss of growth control. On the other hand, a higher initial incidence of liver tumors in enhancer-treated rats, with the subsequent attainment of a similar plateau level in rats given carcinogen alone, would suggest that the enhancers simply accelerated tumor appearance in cells that were destined to produce tumors in any case. The data in Chart 1 do not yet distinguish unequivocally between these 2 possibilities because the plateau phase of tumor incidence was not sufficiently well defined in rats that did not receive phenobarbital or DDT (Chart 1A). Therefore, it will be necessary to extend the duration of future experiments in order to establish the plateau phase and permit definitive comparisons of tumor incidences during this period.

Further experiments of longer duration are also necessary to determine whether the increase in carcinomas seen at the last sacrifice interval in the enhancer-treated rats (Chart 2) continues. A critical examination, over an extended period, of the relative incidences of liver carcinomas and adenomas in these animals may provide information about tumor progression.

Experiments have shown that long-term DDT feeding can increase the incidence of liver tumors in certain strains of mice and rats (16, 17, 22, 39, 41-43) and, on this basis, DDT is currently considered a liver carcinogen (33). The strains used in these studies, however, are characterized by a high (1 to 30%) spontaneous incidence of hepatic tumors (18, 39, 43). Liver tumorigenesis in such animals can also be increased markedly by dietary phenobarbital in the absence of carcinogen feeding (28, 40), although as yet there is no experimental or clinical (9) evidence that phenobarbital is intrinsically tumorigenic. Therefore, the observations to date on the effects of DDT on liver tumorigenesis do not constitute unequivocal evidence that DDT is a carcinogen (19, 40). The correspondence in the effects of DDT and phenobarbital on tumorigenesis seen in the present study does suggest, however, that DDT might be appropriately classified with phenobarbital as an enhancer.

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