

Reduction and Enhancement by Phenobarbital of Hepatocarcinogenesis Induced in the Rat by 2-Acetylaminofluorene¹

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SUMMARY

The effects of dietary phenobarbital on hepatocarcinogenesis in rats fed 2-acetylaminofluorene (AAF) were studied. The simultaneous feeding of AAF and phenobarbital reduced the hepatocarcinogenic effects of AAF. In contrast, the sequential feeding of AAF and phenobarbital resulted in a significant increase in the incidence of hepatomas. No ill effects were observed in rats fed phenobarbital alone.

AAF and phenobarbital each stimulated the proliferation of hepatocytes, but, whereas the effect of AAF was maintained for the duration of exposure, the effect of phenobarbital was short lived. Phenobarbital fed simultaneously with AAF decreased the effect of AAF on liver cell proliferation.

INTRODUCTION

The numerous effects of phenobarbital on the morphology and metabolism of the liver cell have been described extensively (6, 19, 21, 37, 48, 49). Phenobarbital causes proliferation of the smooth endoplasmic reticulum, coupled with an increased synthesis of RNA and drug-metabolizing enzymes. Evidence also exists that phenobarbital produces an increase in the mitotic activity of hepatocytes (1, 3, 7, 17) without, however, any indication that such activity represents regeneration as a consequence of toxicity. No carcinogenic potential has been demonstrated for phenobarbital to date.

In view of this ability of phenobarbital to affect liver ultrastructure and function without producing hepatomas, experiments were undertaken to determine whether phenobarbital treatment could alter the incidence of neoplasia induced by the hepatocarcinogen, AAF.² The results showed that phenobarbital, when fed simultaneously with AAF, retarded the appearance of tumors. When phenobarbital was fed after the rats had been exposed to AAF, however, tumor production was enhanced.

MATERIALS AND METHODS

Rats (male weanling albinos, Sprague-Dawley strain) were obtained from the animal-breeding facilities at Argonne

¹This work was supported by the United States Atomic Energy Commission.

²The abbreviations used are: AAF, 2-acetylaminofluorene; TdR-³H, tritiated thymidine.

Received March 11, 1971; accepted June 4, 1971.

National Laboratory, Argonne, Ill. (rat code designation SD/Anl[Anl 66]) or were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. [rat code designation CRL:CD (SD)]. The change in the source of rats was necessitated by the termination of the Argonne rat breeding colony during the course of these experiments. All rats were placed on experiment at 22 days of age and were housed, 4/cage, in a room with controlled temperature (25°) and lighting (12 hr light; 12 hr dark). The cages were clear plastic of the shoebox type with stainless steel covers. The bedding was ground, dried, sanitized corncobs (Sanicel, Paxton Processing Co., Paxton, Ill.)

Diets (General Biochemicals, Inc., Chagrin Falls, Ohio) contained 30% casein and were nutritionally adequate in other components. Phenobarbital and AAF were incorporated into the appropriate diets, during their preparation, at the percentages indicated below. All diets were pelleted. The following substances were obtained from the sources indicated: phenobarbital (reagent grade, free acid), Mallinckrodt Chemical Works, St. Louis, MO.; AAF, Eastman Organic Chemicals, Rochester, N. Y.; TdR-³H (0.36 Ci/mmmole), International Chemical and Nuclear Corp., Irvine, Calif.

Simultaneous Feeding of AAF and Phenobarbital

Three hundred rats (SD/Anl[Anl 66]) were divided into 6 groups. All groups were fed *ad libitum* the 30% casein diet supplemented as follows: Group 1, no additions (control diet in all experiments); Group 2, 0.05% phenobarbital; Group 3, 0.01% AAF; Group 4, 0.05% phenobarbital and 0.01% AAF; Group 5, 0.02% AAF; Group 6, 0.05% phenobarbital and 0.02% AAF. Three rats from each group were weighed and killed at regular intervals over a 6-month period (see "Results"), and their livers were weighed and examined grossly for tumors. Samples of liver then were taken and processed for light microscopy.

Three rats from each of the groups were given i.p. injections of TdR-³H (0.5 μ Ci/g, 0.36 Ci/mmmole) at 2, 4, and 8 weeks after commencement of the experiment and were killed 1 hr later. Autoradiograms were prepared with the use of sections stained by the periodic acid-Schiff method. The labeling indexes (percentage of labeled nuclei) of both hepatocytes and the lining cells of the sinusoids (littoral cells) were determined. Depending on the proliferative activity, the number of hepatocytes counted per animal varied from 1000 to over 3000.

Sequential Feeding of AAF and Phenobarbital

Mortality Study. Four hundred rats [CRL:CD (SD)] were fed the diet containing 0.02% AAF. At intervals designated by the data points in Fig. 2 (see "Results"), 20 of these rats were transferred to the control diet, and another 20 were placed on the diet containing 0.05% phenobarbital. The date of death of each rat was recorded, and each liver was examined grossly for the appearance of tumors. The duration of the experiment was 240 days.

Tumor Incidence Study. This study consisted of 3 replicate experiments which were started at successive 1-week intervals. In each experiment, 288 rats were fed the 0.02% AAF diet for 11, 16, 21, or 26 days. At each of these intervals, 36 rats were transferred to the control diet, and another 36 were transferred to the 0.05% phenobarbital diet. Rats were caged by random selection, and the cages were then assigned, by random numbers, to the various experimental groups. The cages in each replicate experiment were kept as a unit in separate locations in the same room.

Four rats from each group were killed at 21-day intervals beginning 91 days after the beginning of the experiment. At autopsy, the rats were examined for tumors and other lesions. In the majority of cases, where hepatomas were obvious or where careful examination of all lobes revealed no abnormality, no liver samples were taken for histological examination. When appearance was abnormal and in a number of apparently normal or tumorous livers, samples were taken for preparation of sections. The incidence of hepatomas was compiled from both the macro- and microscopic examinations.

Effect of Phenobarbital on Proliferative Activity

For determination of the initial response of proliferative activity to phenobarbital, rats were put on the diet containing 0.05% phenobarbital, for 60 to 72 hr. Rats were then given injections of TdR-³H as described above, and were sacrificed 1 hr later. Autoradiograms were prepared, and the labeling indexes of both hepatocytes and littoral cells were determined. Two age groups were used to determine whether the response to phenobarbital varied over part of the age range that was of interest in these experiments.

RESULTS

Simultaneous Feeding of AAF and Phenobarbital. Chart 1 shows the change in the ratio of liver weight to body weight and in tumor incidence in rats fed phenobarbital and 0.02% AAF, either individually or in combination. Rats fed 0.05% phenobarbital in the diet had a consistently higher liver-to-body ratio than did the controls. The growth rate of the rats on phenobarbital was identical with that of the controls (not shown). Rats fed 0.02% AAF in the diet showed an increase in liver/body ratio after 6 weeks, accompanied by severe cirrhosis. Highly differentiated hepatomas (41) began to appear by Day 72. The size of the livers increased progressively, and the incidence of hepatomas also increased. All rats in this group examined after 120 days had multiple large hepatomas. By Day 175, all rats in the group receiving

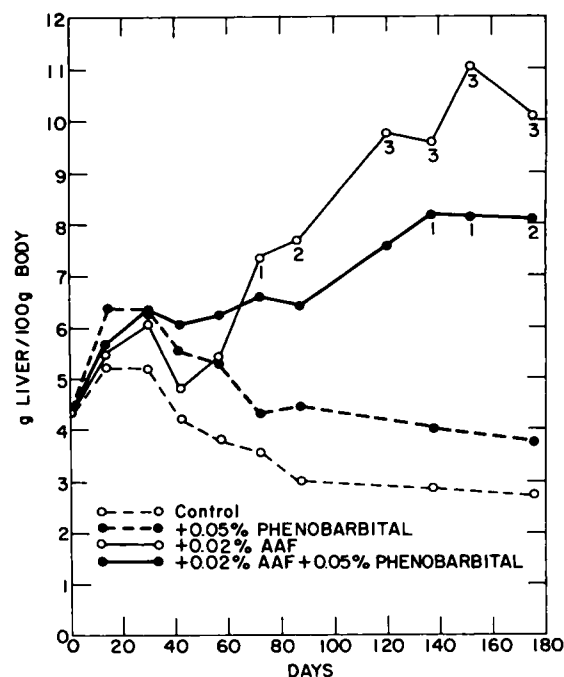


Chart 1. Effects of AAF and phenobarbital, fed alone or in combination, on the ratios of liver to body weights and on tumor incidence in rats over a period of 175 days. Numbers below points on the curves indicate how many rats had macroscopic hepatomas in each group of 3 rats killed at that time.

0.02% AAF alone also had mammary and other tumors. Fourteen of the remaining 17 rats in this group died within 2 weeks after Day 175. In rats receiving the 0.01% AAF diets, the tumor incidence was low and will not be considered here.

Before Day 57, rats fed both 0.02% AAF and 0.05% phenobarbital in the diet had a higher liver-to-body ratio than did rats fed only 0.02% AAF. Subsequently, however, this ratio was maintained at a lower level in rats receiving both phenobarbital and AAF than in rats receiving only AAF. Rats receiving both phenobarbital and AAF did not develop hepatomas until approximately 60 days after tumors appeared in rats receiving only AAF. In gross appearance, the livers of rats receiving both compounds were less cirrhotic than those of rats on AAF alone. No other tumors were found in rats receiving both phenobarbital and AAF. Only 3 of the remaining 20 rats in this group died during the 2 weeks after day 175.

Sequential Feeding of AAF and Phenobarbital. The results of the mortality experiment, involving the feeding of the 0.02% AAF diet for periods of 20 to 135 days, followed by the feeding of the control diet or one containing phenobarbital, are shown in Chart 2. The longer the rats were maintained on AAF, the higher was the mortality at 240 days. Rats returned to the phenobarbital diet after periods of 20 to 90 days on AAF had a higher mortality than did rats changed to the control diet. We concluded from this experiment that the lethal effects of the AAF diet, fed for periods of 20 to 90 days, were enhanced by subsequent feeding with the phenobarbital diet. Because all animals examined at autopsy had hepatomas, it appeared that the phenobarbital was influencing either the time of onset or the incidence of tumors, or perhaps both.

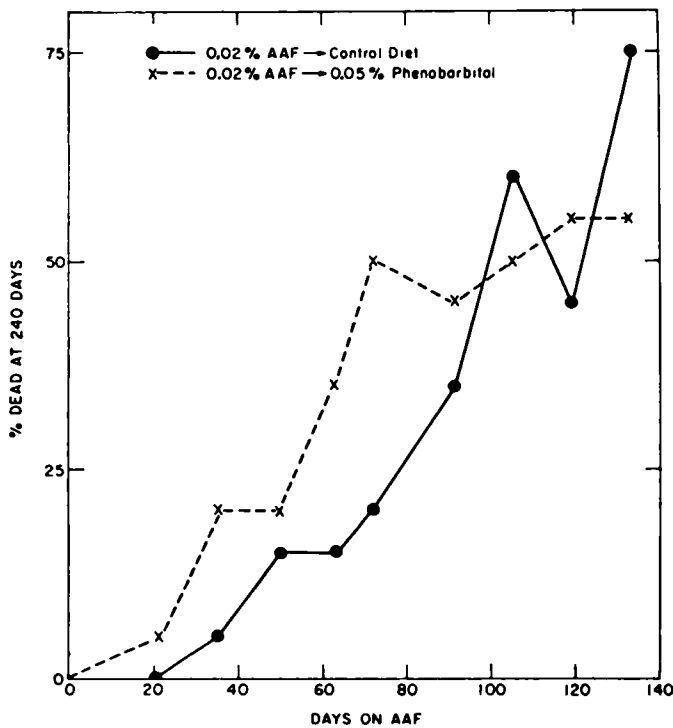


Chart 2. Mortality in rats fed the AAF diet for various periods, followed by the control or the phenobarbital diet. Each data point represents the percentage of rats dead at 240 days among a group of 20 rats.

As a consequence of the results of the mortality study, the study on tumor incidence in relation to sequential feeding of AAF and phenobarbital was carried out. The experiment was divided into 3 replicates, as described. Since the total tumor incidence for the replicate experiments did not differ significantly (at the 1% level), the results were pooled and are shown in Table 1 and Chart 3.

The lesions and progressive changes seen in the development of the tumors were similar to those described so well by Reuber (41). Areas of hyperplasia with cells containing lipid were found. In a number of rats that were given injections of TdR-³H, the areas of hyperplasia had fewer labeled cells than did the tumors. Proliferation of small bile ducts was seen, and a number of rats had cholangiomas, most of which occurred with hepatomas. Because the rats were fed the carcinogen for relatively short periods, the incidence of cirrhosis and hyperplastic nodules (41) was lower in this study than in the simultaneous feeding experiment.

Table 1 shows the total number of rats with tumors for the groups fed either the control or the phenobarbital diet after the initial treatment with the AAF diet for varying periods. For each period of AAF administration, the data for the 9 sacrifice intervals were pooled. The data show that the number of rats with hepatomas increased as the duration of AAF feeding was lengthened. For each of the 4 periods of AAF treatment, the number of rats with hepatomas was greater when phenobarbital was given subsequent to the AAF. A 2-way analysis of variance indicated that both the phenobarbital diet and the duration of feeding of AAF significantly affected the incidence of tumors. The enhancing

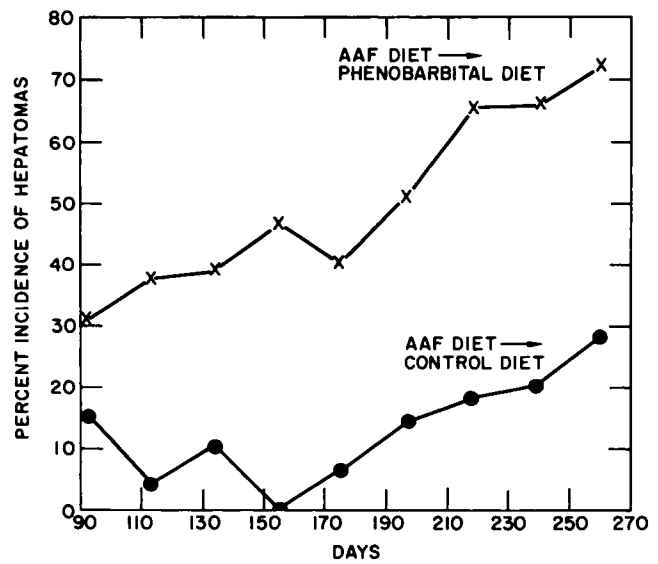


Chart 3. Tumor incidence at each sacrifice interval in rats fed the AAF diet for 11 to 26 days followed by the control or the phenobarbital diet. Each data point represents the percentage of rats with hepatomas among a group of 44 to 48 rats.

Table 1

Total incidence of hepatomas, at the end of 260 days, in rats fed a diet containing 0.02% AAF for various periods, followed by the feeding of either a control diet or one containing 0.05% phenobarbital

Duration of AAF treatment (days)	Total no. of rats with hepatomas/total no. of rats in group given	
	AAF diet followed by control diet	AAF diet followed by phenobarbital diet
11	2/105	17/106
16	7/101	42/104
21	18/103	64/102
26	27/103	86/108
Totals	54/412	209/420
Deaths	20/432	12/432

Analysis of variance on arcsine transforms

Source of variation	d.f.	Sum of squares	Variance ratio	p
Time	3	1.35	15.0	<0.05
Treatment	1	1.52	51	<0.01
Interaction	3	0.091		

effect of phenobarbital on the incidence of tumors was decreased as the duration of AAF feeding increased, but the overall incidence in the rats receiving phenobarbital was about 4 times greater than in those given the control diet.

Chart 3 shows the percentage of rats with hepatomas at each of the 9 sacrifice intervals for rats fed AAF, followed by the control or the phenobarbital diet. In this case, the data for different periods of exposure to AAF were pooled. At every sacrifice interval, the number of rats with tumors was higher in the phenobarbital group. The hepatomas occurring at the early sacrifice intervals were found only in the groups which had

been fed AAF for the 2 longer periods. Unfortunately, a definitive comparison of the shapes of the 2 curves cannot be made because of the scatter of the data points, and it is not possible to determine whether the latent periods for tumor incidence are different in the 2 groups.

Effects of AAF and Phenobarbital on Proliferative Activity. The results of the autoradiographic studies are shown in Tables 2 and 3. The expected decrease with increasing age in the number of cells in DNA synthesis is clearly shown by the decrease in the labeling index. The index decreased 75% in the 2 weeks between 36 and 50 days in both the control and 0.05% phenobarbital groups.

The effect of the AAF on proliferative activity became more clear-cut with time, because the absolute number of cells in DNA synthesis decreased with time on the diet containing the AAF, but the decrease was less than that in the controls (Table 2). For example, in the group receiving 0.02% AAF, the labeling index of the hepatocytes dropped from 3.5 to 1.7%

between 2 and 8 weeks. The 2-week value (3.5 ± 0.6) was not significantly different from that found in the control animals at this time (2.8 ± 0.6), whereas the 8-week value (1.7 ± 0.3) was significantly different from the corresponding control value of 0.4 ± 0.08 . At 8 weeks, the number of cells in DNA synthesis was greater in rats receiving 0.02% AAF than in those receiving 0.01% AAF.

The influence of simultaneous feeding of phenobarbital on reducing the effect of AAF on proliferative activity also became more clear-cut at 8 weeks, at which time the labeling index in neither the 0.01% AAF group nor the 0.02% AAF group with phenobarbital added was significantly different from that of the controls. As a result of the choice of test intervals used (Table 2), it appeared that continuous treatment with 0.05% phenobarbital alone over the age range tested did not cause an increase in proliferative activity. Previously, we also found no increase in labeling or mitotic index after 137 and 213 days on the 0.05% phenobarbital diet. However, it

Table 2
The labeling index of liver cells in rats fed various diets for 2, 4, and 8 weeks

Treatment	Time on diet (wk)	Age (days)	Labeling index ^a			Littoral cells
			Hepatocytes	n	p ^b	
Control diet	2	36	2.8 ± 0.6^c	3		5.8 ± 0.8
	4	50	0.7 ± 0.2	3		1.7 ± 0.8
	8	78	0.4 ± 0.01	3		1.4 ± 0.8
Phenobarbital, 0.05%	2	36	3.2 ± 1.7	3		6.9 ± 5.0
	4	50	0.8 ± 0.1	2		2.5 ± 0.9
	8	78	0.2 ± 0.07	3		1.2 ± 0.6
AAF, 0.01%	2	36	2.1 ± 1.9	3		5.0 ± 2.9
	4	50	1.9 ± 1.2	3	< 0.01	2.9 ± 1.1
	8	78	0.9 ± 0.2	3		2.0 ± 1.2
AAF, 0.02%	2	36	3.5 ± 0.6	3		6.6 ± 0.2
	4	50	2.0 ± 0.5	3	< 0.02	4.4 ± 1.0
	8	78	1.7 ± 0.3	3	< 0.005	4.0 ± 1.2
AAF, 0.01% + phenobarbital, 0.05%	2	36	4.3 ± 0.4	3	< 0.025	8.0 ± 0.8
	4	50	2.2 ± 1.2	3		3.9 ± 0.3
	8	78	0.7 ± 0.2	3		1.5 ± 0.2
AAF, 0.02% + phenobarbital, 0.05%	2	36	2.6 ± 1.0	2		6.8 ± 0.1
	4	49	2.2 ± 0.4	2	< 0.005	3.7 ± 0.2
	8	78	0.6 ± 0.1	3		1.9 ± 0.8

^a Percentage of nuclei labeled 1 hr after i.p. injection of TdR-³H.

^b Values have been determined by Student's *t* test on arcsine transforms of the data for the groups given AAF and/or phenobarbital and for the group receiving the control diet for the corresponding time period.

^c Mean \pm S.D.

Table 3
Effect of 0.05% phenobarbital in the diet on proliferative activity in hepatocytes and littoral cells of rats in 2 age groups

Treatment	Age (days)	Weight (g), mean \pm S.D.	No. of rats	Hepatocytes		Littoral cells	
				Mean \pm S.D.	p ^a	Mean \pm S.D.	
Control diet ^b	43	162.0 ± 20.0	4	0.8 ± 0.1		3.6 ± 0.6	
Phenobarbital ^c 0.05%	43	161.0 ± 9.0	4	2.0 ± 0.4	< 0.005	6.5 ± 1.2	< 0.005
Control diet	56	223.0 ± 9.0	4	0.6 ± 0.2		1.9 ± 0.4	
Phenobarbital, 0.05%	56	234.0 ± 15.0	4	2.0 ± 0.4	< 0.005	3.0 ± 0.7	< 0.005

^a *p* values are for the control versus phenobarbital diet in each age group.

^b Fed from age of 21 days.

^c Fed for 60 to 72 hr before injection of TdR-³H.

can be seen from Table 3 that, if the labeling index was determined within about 3 days of administering phenobarbital, a marked increase in the index was found for both hepatocyte and littoral cells. The results in Tables 2 and 3 suggest that the stimulating effect of phenobarbital is relatively rapid and short lived. The results in Table 3 also show that the proliferative effect of phenobarbital was not influenced by age over the range covered in this experiment. These results are in agreement with earlier observations that phenobarbital produced a transient increase in the mitotic index in the liver (1, 7, 17). The proliferative response, coupled with the histologically observable hypertrophy resulting from phenobarbital treatment, explains the character of the elevation in the liver-to-body ratio produced by phenobarbital (Chart 1). Thus, the initial increase in this ratio reflected a period of hyperplasia and hypertrophy. The liver then returned to its normal, relatively nonproliferative state, but a difference was maintained, with respect to liver cellularity and hypertrophy, between the control rats and those receiving phenobarbital. Indications of hyperplasia were not observed by Gumbmann and Williams (12), who measured total liver DNA in rats treated with phenobarbital for 5 to 7 days.

DISCUSSION

The protective effect of phenobarbital (when given simultaneously with AAF) against the hepatocarcinogenic action of AAF (Chart 1) is in accord with previous observations of the effects of phenobarbital on carcinogenesis induced by 4-dimethylaminoazobenzene (14) or diethylnitrosamine (22). Suppression of AAF hepatocarcinogenesis has also been obtained by the administration of methylcholanthrene (28, 31, 32), acetanilide (59), and chloramphenicol (40). The effects of phenobarbital and methylcholanthrene apparently result from the stimulation of liver enzymes that detoxify the carcinogens (11, 18, 20, 26, 27). It has also been shown that phenobarbital administration increases the urinary excretion of *N*-hydroxy-AAF in rats fed AAF, although methylcholanthrene does not have this effect (58). Several additional examples of the attenuating effects of various agents on chemical hepatocarcinogenesis have been reported (15, 16, 46, 47).

In contrast to the protective action of phenobarbital fed simultaneously with the carcinogen, an enhancing effect of phenobarbital on tumor incidence was observed when it was fed after the rats had been exposed to carcinogen (Table 1). Although our evidence on the lack of tumorigenesis with phenobarbital is based on only about 50 rats and cannot be considered conclusive, to our knowledge there is no reported experimental or clinical evidence that phenobarbital has a carcinogenic effect. If the lack of carcinogenic activity is confirmed, phenobarbital can be considered a singularly clear-cut promoter, at least under the conditions of the present experiments. Among the possible explanations for the enhancing action of phenobarbital are the following.

1. Phenobarbital increased the probability that an initiating event would be produced by metabolites of AAF previously bound to macromolecules. These bound metabolites remain in liver cells for weeks (8), and therefore

the effect on initiation could occur even with sequential feeding.

2. Phenobarbital enhanced the expression of a carcinogenic molecular event previously produced by AAF. This possibility is suggested by the numerous known effects of phenobarbital on nucleic acid and protein metabolism (6, 19, 21, 33, 38, 43, 48), including the observation that phenobarbital binds strongly to adenine (23).

3. (a) By increasing the number of cells entering DNA synthesis, phenobarbital increased the probability of the fixation of the neoplastic transformation (53). (b) The effect of phenobarbital on proliferative activity stimulated the growth of tumor cells previously induced by AAF, thereby accelerating the formation of gross tumors. The promoting effect of compounds and treatments that increase proliferation has been studied and reviewed by Cole and Nowell (5). The importance of the temporal relationship of the presence of AAF and phenobarbital to the induction of tumors, as well as the induction of synthetic and proliferative activity, is now being investigated.

4. Phenobarbital acted as an immunosuppressive agent and thus enhanced the growth of tumor cells induced by AAF. The immunosuppressive effect of physical (52), chemical (29, 39, 50, 51), and viral (45) carcinogens has been documented. Furthermore, it was shown that croton oil, which is considered a promoter, depressed the plaque-forming cell response in spleens of mice immunized to sheep erythrocytes (50) and the antibody γ -globulin antibody production in guinea pigs (2). However, the possibility of a connection between immunosuppression and carcinogenesis is not supported by the finding that the immunosuppressant, azathioprine, did not increase hepatocarcinogenesis induced by *N*-hydroxy-AAF (9). Similarly, a reduction in graft rejection ability, produced by treatment with antilymphocytic serum, did not increase the incidence of sarcomas induced in mice by methylcholanthrene (57).

Numerous studies have been made of the enhancement, by various treatments, of the carcinogenic effects of chemical hepatocarcinogens (4, 10, 11, 13, 16, 24, 25, 28, 30, 34–36, 42, 44, 54–56). There are several possible ways in which such enhancing agents may act. Elucidation of the mechanism is complicated because of the toxic or carcinogenic effects that most if not all of these agents possess. It appears that phenobarbital may be an exception, in that it does not cause cell death or tumors. Therefore, phenobarbital may make testing of the alternative hypotheses for the mechanism of tumor enhancement more feasible.

ACKNOWLEDGMENTS.

We gratefully acknowledge the advice of Mr. Sylvanus A. Tyler and the assistance of Mr. W. Hart, C. Colegrove, Mr. W. McDade, and Mr. N. Odrowski.

REFERENCES

1. Argyris, T. S., and Magnus, D. R. The Stimulation of Liver Growth and Demethylase Activity following Phenobarbital Treatment. *Develop. Biol.*, 17: 187–201, 1968.
2. Bluestein, H. G., and Green, I. Croton Oil Induced Suppression of

- the Immune Response of Guinea-Pigs. *Nature*, *228*: 871–872, 1970.
3. Burger, P. C., and Herdson, P. B. Phenobarbital Induced Fine Structural Changes in Rat Liver. *Am. J. Pathol.*, *48*: 793–809, 1966.
 4. Chernozemski, I., and Warwick, G. P., Liver Regeneration and Induction of Hepatomas in B6AF₁ Mice by Urethan. *Cancer Res.*, *30*: 2685–2690, 1970.
 5. Cole, L. J., and Nowell, P. C. Radiation Carcinogenesis: The Sequence of Events. *Science*, *50*: 1787–1788, 1965.
 6. Conney, A. H. Pharmacological Implications of Microsomal Enzyme Induction. *Pharmacol. Rev.*, *19*: 317–366, 1967.
 7. Conney, A. H., and Gilman, A. G. Puromycin Inhibition of Enzyme Induction by 3-Methylcholanthrene and Phenobarbital. *J. Biol. Chem.*, *238*: 3682–3685, 1963.
 8. Epstein, S. M., McNary, J., Bartus, B., and Farber, E. Chemical Carcinogenesis: Persistence of Bound Forms at 2-Fluorenylacetylamide. *Science*, *162*: 907–908, 1968.
 9. Frankel, H. H., Yamamoto, R. S., Weisburger, E. K., and Weisburger, J. H. Chronic Toxicity of Azathioprine and the Effects of This Immunosuppressant on Liver Tumor Induction by the Carcinogen *N*-Hydroxy-*N*-2-fluorenylacetylamide. *Toxicol. Appl. Pharmacol.*, *17*: 462–480, 1970.
 10. Glinos, A. D., Bucher, N. L. R., and Aub, J. C. The Effect of Liver Regeneration on Tumor Formation in Rats Fed 4-Dimethylaminoazobenzene. *J. Exptl. Med.*, *93*: 313–324, 1951.
 11. Gram, T. E., Rogers, L. A., and Fouts, J. R. Effect of Pretreatment of Rabbits with Phenobarbital or 3-Methylcholanthrene on the Distribution of Drug-metabolizing Enzyme Activity in Subfractions of Hepatic Microsomes. *J. Pharmacol. Exptl. Therap.*, *157*: 435–445, 1967.
 12. Gumbmann, M. F., and Williams, S. N. Effect of Phenobarbital Pretreatment on the Ability of Aflatoxin B₁ to Inhibit Ribonucleic Acid Synthesis in Rat Liver. *Biochem. Pharmacol.*, *19*: 2861–2866, 1970.
 13. Hollander, C. F., and Bentvelzen, P. Enhancement of Urethan Induction of Hepatomas in Mice by Partial Hepatectomy. *J. Natl. Cancer Inst.*, *41*: 1303–1306, 1968.
 14. Ishidate, M., Watanabe, M., and Odashima, S. Effect of Barbital on Carcinogenic Action and Metabolism of 4-Dimethylaminoazobenzene. *Gann*, *58*: 267–281, 1967.
 15. Ito, N., and Farber, E. Effects of Trypan Blue on Hepatocarcinogenesis in Rats Given Ethionine or *N*-2-Fluorenyl Acetylamide. *J. Natl. Cancer Inst.*, *37*: 775–785, 1966.
 16. Ito, N., Hiasa, Y., Konishi, Y., and Maugami, M. The Development of Carcinoma in Liver of Rats Treated with *m*-Toluenediamine and the Synergistic and Antagonistic Effects with Other Chemicals. *Cancer Res.*, *29*: 1137–1145, 1969.
 17. Japundžić, M., Knežević, B., Djordjević-Čamba, V., and Japundžić, I. The Influence of Phenobarbital-Na on the Mitotic Activity of Parenchymal Liver Cells during Rat Liver Regeneration. *Exptl. Cell Res.*, *48*: 163–167, 1967.
 18. Jellinck, P. H., Smith, G., and Fletcher, R. Nature of the Water-soluble Metabolites of 7,12-Dimethylbenz(a)anthracene Formed by Liver Microsomes of Normal and 3-Methylcholanthrene-treated Rats. *Cancer Res.*, *30*: 1715–1721, 1970.
 19. Jones, A. L., and Fawcett, D. W. Hypertrophy of the Agranular Endoplasmic Reticulum in Hamster Liver Induced by Phenobarbital (with a Review on the Functions of this Organelle in Liver). *J. Histochem. Cytochem.*, *14*: 215–232, 1966.
 20. Kato, R., Shoji, H., and Takanaka, A. Metabolism of Carcinogenic Compounds. I. Effect of Phenobarbital and Methylcholanthrene on the Activities of *N*-Demethylation of Carcinogenic Compounds by Liver Microsomes of Male and Female Rats. *Gann*, *58*: 467–469, 1967.
 21. Kuntzman, R. Drugs and Enzyme Induction. *Ann. Rev. Pharmacol.*, *9*: 21–36, 1969.
 22. Kunz, W., Schaude, G., and Thomas, C. The Effect of Phenobarbital and Halogenated Hydrocarbons on Nitrosamine Carcinogenesis. *Z. Krebsforsch.*, *72*: 291–304, 1969.
 23. Kyogoku, Y., Lord, R. C., and Rich, A. Specific Hydrogen Bonding of Barbiturates to Adenine Derivatives. *Nature*, *218*: 69–72, 1968.
 24. Lane, M., Liebelt, A., Calvert, J., and Liebelt, R. A. Effect of Partial Hepatectomy on Tumor Incidence in BALB/c Mice Treated with Urethan. *Cancer Res.*, *30*: 1812–1816, 1970.
 25. Laws, J. O. Tissue Regeneration and Tumor Development. *Brit. J. Cancer*, *13*: 669–674, 1959.
 26. Lotlikar, P. D. Effects of 3-Methylcholanthrene Pretreatment on Microsomal Hydroxylation of 2-Acetylaminofluorene by Various Rat Hepatomas. *Biochem. J.*, *118*: 513–518, 1970.
 27. Lotlikar, P. D., Enomoto, M., Miller, J. A., and Miller, E. C. Species Variations in the *N*- and Ring-Hydroxylation of 2-Acetylaminofluorene and Effects of 3-Methylcholanthrene Pretreatment. *Proc. Soc. Exptl. Biol. Med.*, *125*: 341–346, 1967.
 28. MacDonald, J. C., Miller, E. C., Miller, J. A., and Rusch, H. P. The Synergistic Action of Mixtures of Certain Hepatic Carcinogens. *Cancer Res.*, *12*: 50–54, 1952.
 29. Malmgren, R. A., Bennisson, B. E., and McKinley, T. W., Jr. Reduced Antibody Titers in Mice Treated with Carcinogenic and Cancer Chemotherapeutic Agents. *Proc. Soc. Exptl. Biol. Med.*, *79*: 484–488, 1952.
 30. Maltoni, C., Peretti S., and Ghetti, G. Synergistic Oncogenic Effect of *N*-2-Fluorenylacetylamide and Carbon Tetrachloride on Rat Liver. *Cancro*, *21*: 63–72, 1968.
 31. Maragami, M., Ito, N., Konishi, Y., Miasa, Y., and Farber, E. Influence of 3-Methylcholanthrene on Liver Carcinogenesis in Rats Ingesting DL-Ethionine, 3'-Methyl-4-dimethylaminoazobenzene, and *N*-2-Fluorenylacetylamide. *Cancer Res.*, *27*: 2011–2019, 1967.
 32. Miller, E. C., Miller, J. A., Brown, R. R., and MacDonald, J. C. On the Protective Action of Certain Polycyclic Aromatic Hydrocarbons against Carcinogenesis by Aminoazo Dyes and 2-Acetylaminofluorene. *Cancer Res.*, *18*: 469–477, 1958.
 33. Mycek, M. J. Microsomal Nucleic Acid Breakdown in Livers of Phenobarbital-treated Rats. *Biochem. Pharmacol.*, *20*: 325–337, 1971.
 34. Newberne, P. M., Harrington, D. M., and Wogan, G. N. Effects of Cirrhosis and Other Liver Insults on Induction of Liver Tumors by Aflatoxin in Rats. *Lab. Invest.*, *15*: 962–999, 1966.
 35. Odashima, S. Development of Liver Cancers in the Rat by 20-Methylcholanthrene Painting Following Initial 4-Dimethylaminoazobenzene Feeding. *Gann*, *50*: 321–345, 1959.
 36. Pai, S. R., Yamamoto, R. S., and Weisburger, J. M. Rapid Liver Tumor Induction by Concerted Action of *N*-Hydroxy-2-acetylaminofluorene and Hormonal Stimulation. *Nature*, *199*: 1299–1300, 1963.
 37. Peraino, C., Lamar, C., Jr., and Pitot, H. C. Studies on the Induction and Repression of Enzymes in Rat Liver. IV. Effects of Cortisone and Phenobarbital. *J. Biol. Chem.*, *241*: 2944–2948, 1966.
 38. Piper, W. N., and Bousquet, W. F. Phenobarbital and Methylcholanthrene Stimulation of Rat Liver Chromatin Template Activity. *Biochem. Biophys. Res. Commun.*, *33*: 602–605, 1968.
 39. Prehn, R. T. Function of Depressed Immunologic Reactivity during Carcinogenesis. *J. Natl. Cancer Inst.*, *31*: 791–805, 1963.
 40. Puro, R., and Firminger, H. I. Protection against Induced Cirrhosis and Hepatocellular Carcinoma in Rats by Chloramphenicol. *J. Natl. Cancer Inst.*, *35*: 29–37, 1965.
 41. Reuber, M. D. Development of Preneoplastic and Neoplastic Lesions of the Liver in Male Rats Given 0.025% *N*-2-Fluorenyl-diacetylamide. *J. Natl. Cancer Inst.*, *34*: 697–723, 1965.

42. Reuber, M. D. Effect of 3-Methylcholanthrene on Hyperplastic and Early Neoplastic Hepatic Lesions Induced in Rats by Carbon Tetrachloride. *J. Natl. Cancer Inst.*, *45*: 1237–1242, 1970.
43. Ruddon, R. W., and Rainey, C. H. Stimulation of Nuclear Protein Synthesis in Rat Liver after Phenobarbital Administration. *Biochem. Biophys. Res. Commun.*, *40*: 152–160, 1970.
44. Rzucidlo, Z., Szacki, J., Lawinski, M., and Grzebieluch, M. The Influence of Partial Resection and X-Irradiation of the Liver on the Latent Period of Chrysoidin Hepatoma in Mice. *Arch. Immunol. Terapii Doswiadczalnej*, *15*: 877–880, 1967.
45. Salaman, M. H., and Wedderburn, N. The Immunodepressive Effect of Friend Virus. *Immunology*, *10*: 445–458, 1966.
46. Sidransky, H., Ito, N., and Verney, E. Influence of α -Naphthyl-isothiocyanate on Liver Tumorigenesis in Rats Ingesting Ethionine and *N*-2-Fluorenylacetamide. *J. Natl. Cancer Inst.*, *37*: 677–686, 1966.
47. Sidransky, H., and Verney, E. Influence of Orotic Acid on Liver Tumorigenesis in Rats Ingesting Ethionine, *N*-2-Fluorenylacetamide, and 3'-Methyl-Dimethylaminoazobenzene. *J. Natl. Cancer Inst.*, *44*: 1201–1215, 1970.
48. Smuckler, E. A., and Arcasoy, M. Structural and Functional Changes of the Endoplasmic Reticulum of Hepatic Parenchymal Cells. *Intern. Rev. Exptl. Pathol.*, *7*: 305–418, 1969.
49. Staubli, W., Hess, R., and Weibel, E. R. Correlated Morphometric and Biochemical Studies on the Liver Cell. II. Effects of Phenobarbital on Rat Hepatocytes. *J. Cell Biol.*, *42*: 92–112, 1969.
50. Stjernsward, J. Further Immunological Studies of Chemical Carcinogenesis. *J. Natl. Cancer Inst.*, *38*: 515–526, 1967.
51. Stutman, O. Carcinogen-induced Immune Depression: Absence in Mice Resistant to Chemical Oncogenesis. *Science*, *166*: 620–621, 1969.
52. Taliaferro, W. H., Taliaferro, L. G., and Jaroslow, B. N. *Radiation and Immune Mechanisms*. New York: Academic Press, Inc., 1964.
53. Todaro, G. J., and Green, H. Cell Growth and the Initiation of Transformation by SV 40. *Proc. Natl. Acad. Sci. U. S.*, *55*: 302–308, 1966.
54. Ueda, Y. The Relation between Experimental Liver Carcinoma and Liver Cirrhosis Induced by Simultaneous Administration of *p*-Dimethylaminoazobenzene and Carbon Tetrachloride. *Tohoku J. Exptl. Med.*, *92*: 83–107, 1967.
55. Ueda, Y. The Relation between Experimental Liver Carcinoma and Liver Cirrhosis Induced by Successive Administration of *p*-Dimethylaminoazobenzene and Carbon Tetrachloride. *Tohoku J. Exptl. Med.*, *92*: 109–140, 1967.
56. Ueda, Y. Experimental Liver Carcinoma and Liver Cirrhosis Induced by *p*-Dimethylaminoazobenzene after Preliminary Carbon Tetrachloride Injections. *Tohoku J. Exptl. Med.*, *92*: 175–206, 1967.
57. Wagner, J. L., and Haughton, G. Immunosuppression by Antilymphocytic Serum and Its Effect on Tumors Induced by 3-Methylcholanthrene in Mice. *J. Natl. Cancer Inst.*, *46*: 1–10, 1971.
58. Whyatt, P. L., and Cramer, J. W. Urinary Excretion of *N*-Hydroxy-2-acetylaminofluorene by Rats Given Phenobarbital. *Proc. Am. Assoc. Cancer Res.*, *11*: 83, 1970.
59. Yamamoto, R. S., Glass, R. M., Frankel, H. H., Weisburger, E. K., and Weisburger, J. M. Inhibition of the Toxicity and Carcinogenicity of *N*-2-Fluorenylacetamide by Acetanilide. *Toxicol. Appl. Pharmacol.*, *13*: 108–117, 1968.