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Effects of Dietary Phenobarbital on the Binding of 2-Acetylaminofluorene to Rat Liver Nuclear DNA[1] (37911)

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(Introduced by J. F. Thomson)
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Although phenobarbital itself has no apparent tumorigenic potential (1, 2), phenobarbital feeding enhances hepatic tumorigenesis in rats previously fed the carcinogen 2-acetylaminofluorene (AAF) (2, 3). In contrast, phenobarbital fed simultaneously with AAF decreases tumor incidence (2). Because *in vivo* binding of AAF metabolites to rat-liver DNA may be important in hepatic tumorigenesis (4, 5), the present experiment was conducted to determine how dietary phenobarbital affects AAF binding to DNA under conditions whereby phenobarbital would be expected, on the basis of the earlier studies (2, 3), either to reduce or to enhance AAF-induced hepatic tumorigenesis.

Methods. Fifteen male rats [rat code designation CRL:CD(SD)] at 22 days of age were obtained from Charles River Laboratories and were housed as previously described (2). The rats were evenly divided at random into three groups. Groups 1 and 2 received a nutritionally adequate diet containing 30% casein and Group 3 received the same diet supplemented with 0.05% phenobarbital. These diets are the same as those used to study the effects of phenobarbital on tumorigenesis (2, 3).

On Day 78 of this regimen each rat was given an ip injection of AAF-9-^{14}C (New England Nuclear, sp act 10.9 Ci/mole) at a dose of 372 μCi(7.1 mg)/kg body wt. The AAF was dissolved in a minimum volume of dimethylsulfoxide and 9 vol of cottonseed oil were added. A stable emulsion was then produced by brief sonication. Each rat received approximately 0.5 ml of this emulsion. Three days after the injections (to allow the binding of AAF metabolites to DNA to occur), Group 2 was changed to the 0.05% phenobarbital diet.

The three groups of the present study bear the following analogy to treatments used in the earlier tumorigenesis study (2):

Group 1: AAF fed alone—control level of tumorigenesis.
Group 2: AAF feeding followed by phenobarbital feeding—enhancement of tumorigenesis.
Group 3: AAF and phenobarbital fed simultaneously—reduction of tumorigenesis.

At 5-day intervals, beginning 42 days after the AAF injections, one rat from each group was killed by cervical dislocation. The liver was quickly excised and nuclei were prepared by the method of Blobel and Potter (6). (None of the livers showed tumors or other gross evidence of damage from the injected AAF.)

The DNA isolation procedure was designed for extracting pure DNA from nuclei from a liver weighing between 15 and 25 g and was a combination and modification of the methods of Irving and Veazey (7) and Thomas *et al.* (8). All operations

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were performed at room temperature unless specified otherwise.

The nuclei were suspended in 50 ml of lysing medium (pH 8.0) which was 0.2% Sarkosyl (Ciba-Geigy), 0.1 *M* NaCl, and 0.01 *M* EDTA. Following the addition of 15 mg of pronase (Calbiochem) (previously dissolved in distilled water and incubated for 2 hr at a concentration of 5 mg/ml to inactivate contaminating DNase), the lysate was swirled gently at 37° for 3 hr. After incubation, the mixture was extracted with 1 vol of liquified phenol (Mallinckrodt) (previously adjusted to pH 8.0 with 1 *M* Tris buffer, and containing 1 g/liter of 8-hydroxyquinoline) for 15 min at room temperature and then placed in an ice bath for 2 min to facilitate the separation between the aqueous and phenol phases. The suspension was centrifuged at 5° for 10 min at 5000 rpm in a Sorvall SS-34 rotor. The phenol layer was carefully removed by suction and discarded. The aqueous phase was extracted again with phenol and then twice with 1 vol of 24:1 (v/v) chloroform–isoamyl alcohol (9) by first swirling for 10 min and then centrifuging in a Sorvall SS-34 rotor for 5 min at 1000 rpm. The chloroform layer was discarded. The aqueous phase was transferred to a small beaker, and 2 vol of ice-cold ethanol were added. The DNA was removed by spooling onto a glass rod, suspended in 50 ml of 0.01 *M* Tris–0.001 *M* EDTA, and gently swirled overnight at 4°.

The opalescent solution was made 0.01 *M* in NaCl and centrifuged in a Beckman Model L-2 ultracentrifuge at 40,000 rpm in a 50 Ti rotor for 1 hr at 5°. The clear supernatant containing the DNA was decanted, and the glycogen pellet was discarded. The DNA solution was warmed to room temperature, and 3 mg of RNase were added. The RNase (Worthington) was added as a solution (1 mg/ml) in 0.1 *M* NaCl and 0.01 *M* Na $C_2H_3O_2$ (pH 5) which had been boiled previously for 10 min to inactivate contaminating DNase. The mixture was incubated at 37° for 30 min, then extracted three times with phenol, and twice with chloroform–isoamyl alcohol. DNA was precipitated from the aqueous phase with 2 vol of ice-cold ethanol and spooled out on a glass rod. The DNA was washed for about 30 sec in 75% ethanol–1% NaCl, then in 75% ethanol, and finally in 95% ethanol.

The DNA was suspended in a small volume of distilled water and sonicated until dissolved. The yield of DNA was approximately 15 mg/g liver, as determined by the diphenylamine reaction (10). Spectral analyses and thermal denaturation measurements yielded values in agreement with those reported by Irving and Veasey (7). Tests for RNA (11) and protein (12) contamination were negative and a single band was obtained when the DNA was subjected to cesium chloride density gradient centrifugation (13).

For the determination of radioactivity, aliquots of DNA solution were dried on small filter paper discs and combusted in a Packard oxidizer. The samples were counted in a Beckman liquid scintillation counter. Each sample was then corrected for quenching by the addition of a ^{14}C-toluene internal standard.

Results and Discussion. DNA isolated from the livers of rats given a single injection of AAF-9-^{14}C contained bound label for at least 9 weeks after the injection. No significant decrease in DNA labeling was observed during the 3-week period in which the rats were killed, and consequently the data were averaged as shown in Table I. This apparent stability of binding is in agreement with earlier reports of the persistent binding of labeled AAF metabolites to DNA (14–16).

When phenobarbital feeding was begun *after* labeled AAF metabolites had become bound to DNA, no significant reduction in binding occurred despite a 42–62-day exposure to the phenobarbital diet (Group 2 vs Group 1, Table I). However, when phenobarbital was also fed for 77 days *prior* to the administration of the labeled AAF, the binding was reduced by approximately 80% (Group 3 vs Group 1). The latter results are in agreement with those of Matsushima *et al.* (17) who observed a 60% decrease in the short-term (51-hr) binding of AAF metabolites to DNA after

TABLE I. Persistent Binding of AAF Metabolites to Rat-Liver Nuclear DNA.

Group	Treatments[a]	Disintegrations/ min/mg DNA[b]
1	Control diet, 77 days, then AAF injection, then control diet, 42–63 days	28.04 ± 7.04
2	Control diet, 77 days, then AAF injection, then phenobarbital diet, 42–63 days	22.71 ± 5.69[c]
3	Phenobarbital diet, 77 days, then AAF injection, then phenobarbital diet, 42–63 days	5.90 ± 1.41[d]

[a] Further details in *Methods.*

[b] Corrected for quenching (see text). Each value is the mean ± SE for a group of 5 rats.

[c] Not significantly different from Group 1 ($P > 0.500$).

[d] Significantly different from Group 1 ($P < 0.020$) and from Group 2 ($P < 0.025$).

a relatively brief exposure (7 days) of rats to injected phenobarbital. This decrease in binding is probably a consequence of the induction by phenobarbital of microsomal enzymes (17) which degrade AAF metabolites before they can interact with DNA. After AAF metabolites become bound to DNA, however, they are apparently inaccessible to attack by such degradative enzymes (Group 2, Table I).

The results of the binding studies are consistent with earlier observations on the effects of phenobarbital on AAF-induced hepatic tumorigenesis (2). Thus, the protective effect of dietary phenobarbital against AAF-induced hepatic tumorigenesis (2) probably results from a reduction in the concentration of AAF metabolites able to bind to key target molecules such as DNA (17). On the other hand, once such binding occurs, it may be virtually irreversible (present study), and the resultant carcinogen–DNA complex may then become subject to phenobarbital-induced metabolic processes that enhance its tumorigenic potential (2, 3).

The results of the present study coupled with those reported earlier (2, 3) underline the necessity for the thorough analysis of the metabolic effects of "chemoprophylactic" (18) agents which might be developed as a means of reducing the hazards from environmental carcinogens (18, 19). Thus, while such agents may (like phenobarbital) increase the degradation of a carcinogen and substantially reduce the amount able to reach its site of action, they may also (again like phenobarbital) cause metabolic changes that increase the risk of tumorigenesis from any remaining carcinogen molecules that escape degradation and bind irreversibly to tumorigenically important target molecules.

Summary. Previous studies have shown that dietary phenobarbital reduces 2-acetylaminofluorene (AAF)-induced hepatic tumorigenesis when fed simultaneously with the carcinogen, but enhances tumorigenesis when fed after the cessation of AAF feeding. Since DNA may be a primary target of chemical carcinogens, the effects of phenobarbital on the binding *in vivo* of AAF metabolites to hepatic nuclear DNA were investigated. The results show that the prolonged feeding of phenobarbital prior to the injection of AAF-9-^{14}C reduced the binding of label to DNA, but the binding of label was not reduced if phenobarbital feeding was begun after the injection of AAF.

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