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Use of 2-Acetamidophenanthrene and 2-Acetamidofluorene in Investigations of Mechanisms of Hepatocarcinogenesis

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Comparison studies have been undertaken on the hepatocarcinogen 2-acetamidofluorene (AAF) and its nonhepatocarcinogenic analog 2-acetamidophenanthrene (AAP). Previous studies have shown that amount of compound acutely and persistently bound to rat liver DNA is comparable for the two compounds following single injections into adult Fischer rats, but that AAP fails to initiate tumors in weanling Sprague-Dawley rats. In this work we show that the amount of bound adduct from AAF and AAP is also comparable after three weeks of feeding compound to weanling Sprague-Dawley male rats. Three of the adducts found in RNA of AAP-treated rats cochromatographed on Sephadex LH-20 with two adenosine adducts and one guanosine adduct prepared by reaction of the nucleosides with *N*-acetoxy-*N*-trifluoroacetyl-2-aminophenanthrene at neutrality.

Because of the lack of initiating ability of AAP in liver, we have also investigated early biochemical alterations in liver after various regimens. Feeding of either AAF or AAP to male weanling rats, followed by three weeks of DDT feeding produced no alteration in either histochemically detected γ-glutamyltranspeptidase or in ganglioside complement in total liver homogenate. Partial hepatectomy after feeding of either AAF or AAP resulted in the appearance of new fucoganglioside and alteration in the distribution of the major gangliosides. DDT feeding after partial hepatectomy resulted in foci of elevated gamma-glutamyltranspeptidase in AAF-fed rats but not in AAP-fed rats. These results support a previous proposal that AAP may initiate tumorigenesis in rat liver, but that the promoting regimens now in use lack the ability to cause further progression of the initiated cells. The data also suggests that ganglioside synthesis may be a more sensitive marker for early stages in carcinogenesis than are the various histochemical stains now in use.

As is evident from much of the material presented in this workshop, 2-acetamidofluorene (AAF) is a multipotent carcinogen which has stimulated extensive research over the last 40 years. Yet, as it is sometimes difficult to recognize normality except in its absence, it is difficult to recognize the roles in carcinogenesis of the many effects produced by AAF unless we devise some way to weigh each of these roles separately and to reconstitute the carcinogenic process. Comparative studies on structural analogs combined with initiation-promotion regimens may offer us the means to do this. 2-Acetamidophenanthrene (AAP) appears to be a valuable compound for such studies. About 25 years ago, the Millers showed that AAP fed to male rats at the same level as a hepatocarcinogenic dose of AAF failed to induce any liver tumors, although it was leukemogenic and induced ear duct tumors and others (*1*). In later work they showed that only a small fraction of the dose of AAP was excreted in the urine as *N*-hydroxy-AAP (*2*), in contrast with the much larger fraction of the hepatocarcinogen 7-fluoro-AAF which was excreted as its *N*-hydroxy derivative (*2*). The relative carcinogenicities of these compounds and 4-acetamidobiphenyl in extrahepatic tissues has, of course, never been explained in terms of urinary *N*-hydroxyamide and cannot be addressed here. We have shown, however, that AAF and AAP yield the same amount of DNA-bound metabolites (*3*). This result was obtained in adult male Fischer rats given a single injection of either compound. In addition, after rapid loss of some adducts, a substantial portion of the bound

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aminophenanthrene persisted for at least 4 weeks (*3*). This result suggested that if DNA damage constituted a pre-initiating event, initiation might be completed by feeding AAP immediately upon weaning, during a period of rapid liver growth. Promotion might then be accomplished with DDT, according to Peraino's regimen (*4*). We have recently reported the outcome of this experiment, which was negative with respect to the liver, although significant acceleration of mammary gland tumorigenesis was induced by DDT (*5*). In other work, now in press, we found that feeding of AAP to male rats had relatively little effect on activation for DNA binding and that AAF feeding may actually reduce the efficiency of the activation system (*6*). In this work, we present new data regarding adduct structure, binding during the initiation procedure, and early biochemical changes during initiation with both AAF and AAP, followed by promotion with DDT. The results from these studies confirm in our minds that AAP is indeed an initiator in rat liver, but that further events must be caused before the initiated population is subject to DDT promotion.

Experimental

Binding of Radioactive Compounds to Rat Liver Nucleic Acids

Weanling male Sprague-Dawley rats were obtained from Inglemoor Breeding Laboratories, Kirkland, WA. The animals were immediately placed on powdered stock diet (Wayne Lab-Blox) containing 1.68 mmole/kg of either AAF or AAP, and containing 4 mCi/kg of [ring-^{3}H]-arylamide. After 3 weeks, the rats were sacrificed and the nucleic acids isolated as described previously (*3*).

Adducts of 2-Phenanthrylnitrenium Ion with Nucleosides

N-Acetoxy-*N*-trifluoroacetyl-2-aminophenanthrene was prepared by a modification of the method of Weeks et al. for obtaining *N*-hydroxytrifluoroamide (*7*). 2-Nitrophenanthrene (*8*) was reduced to hydroxylamine according to Westra (*9*), then acylated directly without workup with trifluoroacetic anhydride in tetrahydrofuran. The product was isolated according to Weeks et al. (*7*), then acetylated (0.2 g) with 0.5 mL acetic anhydride and 0.2 mL 2, 6-lutidine in 10 mL of benzene at room temperature overnight. The product was separated by chromatography on silica gel in 1:1 benzene/petroleum ether. After several preparations carried out in this fashion, the preparation of hydroxamic acid failed, and we have not been able to discover the reason.

Solutions of adenosine or guanosine in water were treated with acetone solutions (equal volume) of *N*-acetoxy-*N*-trifluoro-2-aminophenanthrene. After 2 hr at room temperature, the acetone was removed under reduced pressure, the aminophenanthrene solvolysis products were extracted with ether, and the products were extracted with ethyl acetate. After removal of the solvent, the residue was taken up in a small amount of ethanol/water and chromatographed on Sephadex LH-20.

Perturbation Calculations for the Reaction of 2-Phenanthrylnitrenium Ion with Nucleosides

Hückel approximation molecular orbital calculations were carried out for 2-phenanthrylnitrenium ion and nucleic acid bases as described previously (*10*). Perturbation calculations with correction for steric hindrance were carried out as before (*11*).

Assays for γ-Glutamyltranspeptidase and Altered Ganglioside Biosynthesis

Rats to be analyzed for altered ganglioside synthesis received injections of 0.5 mCi [C^3H_3]-fucose (New England Nuclear, 10-15 mCi/μmole) 4 hr before sacrifice. Upon sacrifice, liver slices for histochemistry were immediately placed in ice-cold acetone, and the remainder was frozen for storage until ganglioside isolation. The acetone-fixed slices were processed as described by Ogawa et al. (*12*). Low-melting paraffin (49°C) is no longer available, and we substituted a preparation of 52°C paraffin (Surgi-Path) which was melted and mixed with 11% (v/v) heptane, then cooled. Sections 5 μm thick, were cut and stained either for γ-glutamyltranspeptidase (GGT) (*13*) or with hematoxylin and eosin. Fresh kidney slices were used as positive control for each complete operation of mounting and staining for GGT. Total gangliosides were isolated and separated by thin-layer chromatography as described previously (*14*).

Treatment of Rats

Weanling rats (21-day-old male Sprague-Dawley rats from Inglemoor Breeding Laboratories) were placed on AAF, AAP or control diet immediately upon arrival. The animals were maintained in plastic cages with food and water available *ad libitum* on wood chips in enclosed racks with filtered air flow over the cages and exhausted directly to the outside. At the end of 3 weeks, the rats were sacrificed, placed on control diet, or placed on

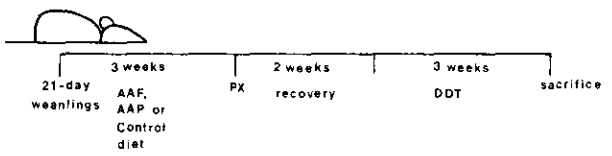


FIGURE 1. Regimen for induction of fucoganglioside synthesis and GGT induction. Weanling Sprague-Dawley male rats were subject to the regimen shown, or were sacrificed after the first 3 weeks or 5 weeks of treatment. Some animals were carried through the entire regimen, but without partial hepatectomy.

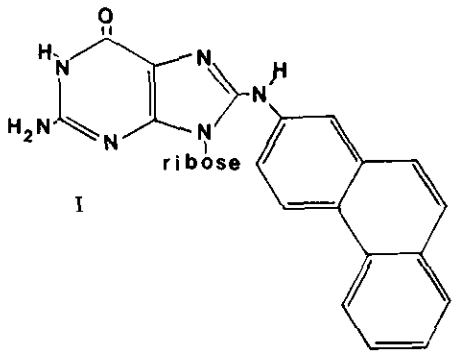
control diet after being subjected to two-thirds partial hepatectomy (Fig. 1). Animals kept alive at this point were kept on control diet for 2 weeks to allow regrowth of liver, then were sacrificed or placed on DDT diet (0.05%) for an additional 3 weeks, after which they were sacrificed. All animals were sacrificed by exsanguination under ether anesthesia.

Results

Feeding of labeled AAF and AAP to weanling Sprague-Dawley rats resulted in comparable binding of label from the two compounds (Table 1). Adduct distribution will be determined at a later date.

In our initial attempts to prepare *N*-acetoxy-*N*-trifluoroacetyl-2-aminophenanthrene by the method described, we were able to obtain a 25% yield based on 2-nitrophenanthrene. However, for unknown reasons this preparation failed at the point of isolation of hydroxamic acid, and our repeated success in our initial attempts appears to have been exceptional, based on discussions with others who have attempted this synthesis.

Standard reaction conditions for the treatment of adenosine or guanosine with *N*-acetoxy-*N*-trifluoroamide have not yet been established, because of difficulty in preparing starting material. The amount of acetone in the mixture governs the distribution of adducts apparently, but a relatively high yield (about 10-20%) of mixed adducts is obtained, which may be separated by Sephadex LH-20 chromatography. Two guanosine adducts which are eluted in 35% ethanol decomposed under conditions of preparation for mass spectrometry, while a third adduct (the first peak in 70% ethanol) was found to be 8-(*N*-2-fluorenylamino)guanosine (I). Two adenosine adducts, which are eluted in 70% ethanol, have not yet been analyzed. Figure 2 shows a comparison between radioactive adducts obtained from RNA of a rat given labeled AAP and nonradioactive products obtained from the reactions of nucleosides with *N*-acetoxytrifluoroamide. Apparently overlap is seen between an early (35% ethanol) guanosine adduct and one radioactive compound and between the two adenosine adducts and two more radioactive compounds. Further characterization of these adducts is planned.



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The structures of these adducts can be anticipated from perturbation calculations, which have been remarkably useful in predicting the products of similar reactions (*11, 15*). Table 2 shows that an anticipated secondary product of reaction of phenanthrylnitrenium ion with guanosine would be the ArN-O^6 type of adduct found by Kadlubar from treatment of DNA with 1-naphthylhydroxylamine (*16*), while the major adduct with adenosine might actually be a hydrazine.

So far, we have not observed foci of increased levels of GGT in weanling rats fed only AAF or AAP for three weeks, with no other treatment. Following partial hepatectomy and a 2-week recovery period, microscopic foci (visible only at 400× magnification) were seen in AAF-fed rats, but not in AAP-fed rats. After DDT promotion, these were enlarged to macroscopic foci of 0.5-1.0 mm in diameter. From eight slices, an average of about 10 foci/cm^2 were seen. After the complete regimen (Fig. 1), however, foci were still undetectable in AAP-treated rats. In contrast, synthesis of new fucoganglioside was detected in both AAP- and AAF-treated livers (Fig. 3), although the level of labeled ganglioside in AAF-treated rats was roughly twice that in AAP-treated

Table 1. Binding of labeled AAF and AAP to livers of weanling Sprague-Dawley rats. Determined after 3 weeks of feeding labeled compound in powdered diet. (4 mCi/kg).

	AAF	AAP
Wt. diet consumed (two rats), g	450	430
DNA, cpm/mg	330[a]	315[a]
RNA, cpm/mg	168[a]	180[a]
Corrected for diet consumption		
DNA, cpm/mg/mCi	183	183
RNA, cpm/mg/mCi	93	105

[a]Average of two rats determined separately.

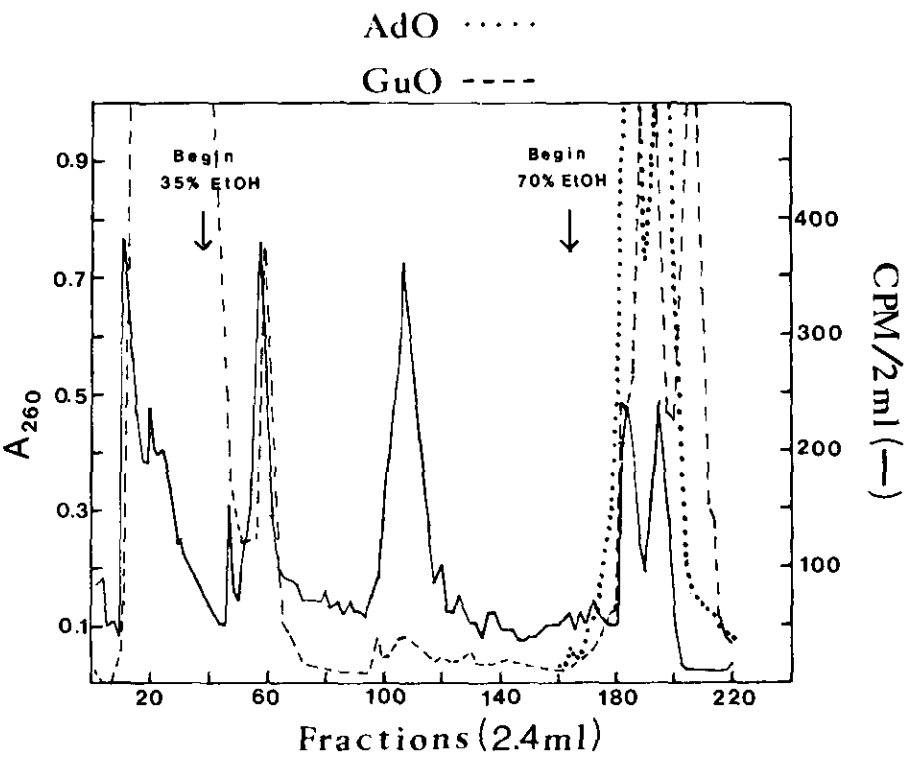

FIGURE 2. Sephadex LH-20 chromatogram of enzymatic digest (*3*) of RNA from Sprague-Dawley male rats given a single injection of [*ring*-^{3}H]-AAP 18 hr before sacrifice. The column contained 14.0 g of Sephadex (1.5 × 30 cm) and was eluted with 60 mL of water, 300 mL of 35% ethanol, and 150 mL of 70% ethanol. Absorbance indicates the location of product mixtures from reactions of guanosine (GuO) or adenosine (AdO) with *N*-acetoxy-*N*-trifluoroacetyl-2-aminophenanthrene.

Table 2. Reaction of 2-phenanthrylnitrenium ion with nucleosides.[a]

Position on nitrenium ion	Position on guanine	Guanosine	Position on adenine	Adenosine	Position on cytosine	Cytidine	Position on thymine	Thymidine
C-1	1	0.10	1	0.08	3	0.10	3	0.09
	3	0.04	3	0.02	O^2	0.04	O^2	0.03
	7	0.09	7	0.09	N^4	0.22	O^4	0.10
	8	0.22	8	0.18				
	N^2	0.23	N^6	0.23				
	O^6	0.11						
N	1	0.14	1	0.13	3	0.16	3	0.14
	3	0.10	3	0.07	O^2	0.34	O^2	0.27
	7	0.14	7	0.14	N^4	0.26	O^4	0.31
	8	0.43[b]	8	0.22				
	N^2	0.28	N^6	0.29				
	O^6	0.35						

[a]*Figures shown are solutions to the simple polyelectronic perturbation calculation corrected for steric hindrance by ribose (11).* The sign convention was chosen so that a higher number indicates a greater probability of reaction.
[b]Known adduct.

rats. Following DDT treatment, total isolated ganglioside was greatly reduced, and only traces of fucoganglioside were detectable (Fig. 3).

Discussion

The role and influence of carcinogen adducts to DNA remain an enigma, dispite the conceptual role they are expected to play in carcinogenesis. While it is not difficult to devise a mechanism in which mutagenesis, and the lesions leading to mutagenesis, can result in transformation, it is now quite clear that chemical damage to DNA in and of itself is not a carcinogenic event. It may better fit the role of an initiating event, an irreversible change which pre-

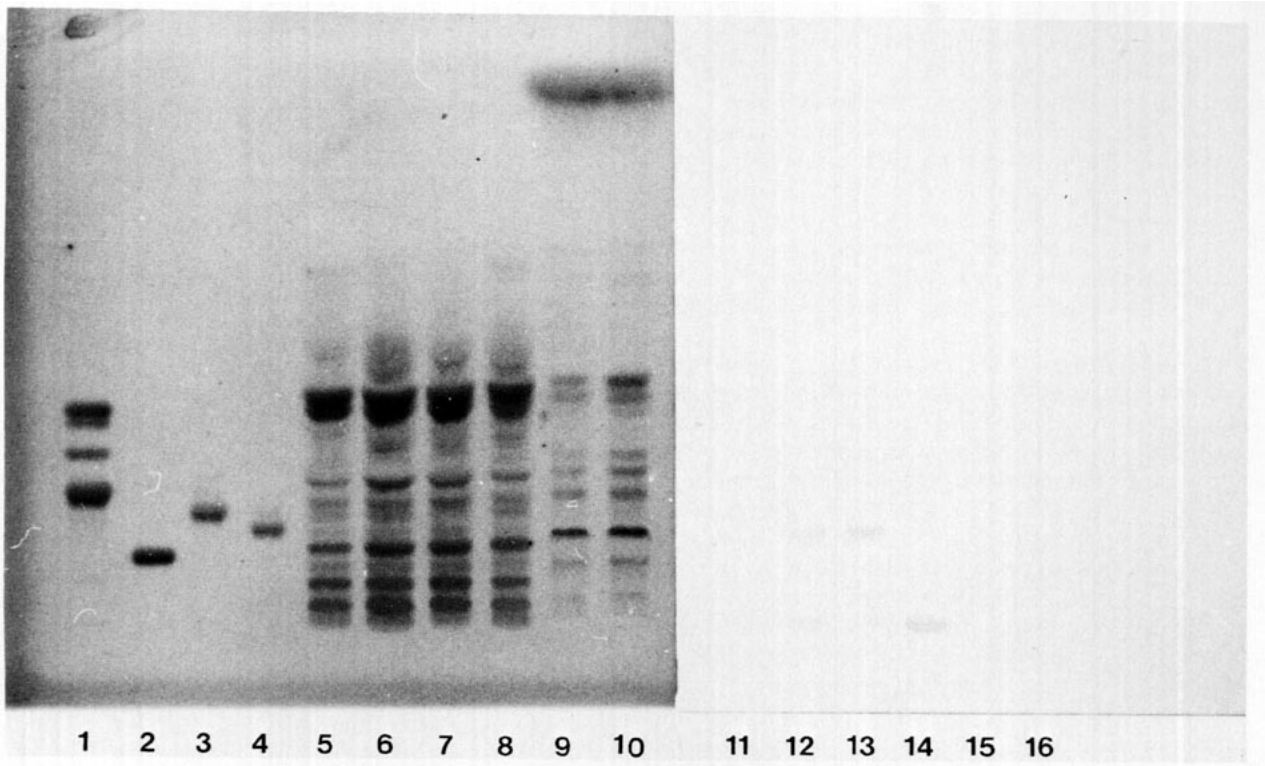

FIGURE 3. Thin-layer chromatography analysis of upper phase gangliosides from normal and treated rat livers. (1) GM_1, GM_2, GM_3 (highest mobility); (2) GD_{1a}; (3) fucosyl GM_1; (4) galactosyl-fucosyl GM, (14); (5) total gangliosides from normal liver; (6) total gangliosides from AAP-treated liver followed by partial hepatectomy and recovery; (7) total gangliosides from AAF-treated liver followed by partial hepatectomy and recovery; (8) total gangliosides from liver following partial hepatectomy and DDT treatment; (9) total gangliosides from liver following complete regimen (AAP, Fig. 1); (10) total gangliosides from liver following complete regimen (AAF); (11-16) autoradiograms of lanes (5)-(10). Silica gel, $CHCl_3$:CH_3OH:H_2O (60:40:9) containing 0.02% $CaCl_2$:H_2O. Gangliosides were visualized with resorcinol spray. Autoradiogaphy was done after spraying the plate with 0.4% diphenyloxazole, 10% toluene in 2-methylnaphthalene.

pares some unknown fraction of the tissue for further processes which can be carried out by additional DNA damage or by nongenotoxic alterations of the cell which can lead to tumors. We have demonstrated clearly that equal adducts to DNA during a period of rapid liver growth do not lead to equal responsiveness to DDT, whether in an early foci experiment or during a full initiation/promotion tumor experiment. However, liver growth is markedly less in rats fed AAP than in those fed AAF. In the labeling experiment, the livers of AAF-fed animals were 8 and 9 g at the time of sacrifice, while those of the AAP-fed rats were 7 and 4 g. The partial liver weights of animals subjected to partial hepatectomy were comparable, 21 g for four AAF-fed rats, and 16 g for four AAP-fed rats. Examination of slices stained with hematoxylin and ecosin (H&E) from AAF- and AAP-fed rats (not shown) revealed that the extensive vacuolization and cell-swelling found in AAF-treated liver was not apparent in AAP-treated liver.

Thus, these results reflect the findings of Sarma, Farber and their associates, from experiments with diethylnitrosamine, that toxicity and regenerative hyperplasia are required during exposure to carcinogen for initiation to occur in liver (*17, 18*). Since partial hepatectomy takes place while there is still a significant level of carcinogen bound to DNA, one might ask whether the liver should not be considered to be exposed at this point to this powerful regenerative stimulus. If further chemical damage during regrowth is required, this is not likely, since intake was at a relatively low rate before hepatectomy and was not resumed afterward. The same, however, was also true for previously published single-dose initiation experiments (*17*). Such experiments may differ from ours only in the length of the promoting treatment. We, however, did observe foci, of different sizes, both before and after promotion of AAF-treated rats, with the conclusion being that DDT treatment simply expanded the size of the altered foci. Foci were never observed in AAP-

treated rats, however, even when slices were searched at high magnification after DDT treatment. Thus, our results with AAP differ significantly from earlier data in this respect. They offer us the opportunity to subdivide the initiation process more than has been possible previously and to ask biochemical questions about each stage in the process. The induction of synthesis of new fucogangliosides may represent a new marker for a very early stage in initiation, but this will require further study with other compounds and strains to be verified.

All of these results, however, fail to answer the question posed at the beginning of the discussion, namely, what is the role of the nucleic acid adducts in the carcinogenic process. Presumably, they are not toxic in themselves, since AAP, with the same level of binding to DNA and RNA as AAF, does not produce the same toxicity. Presumably the necessary toxicity need not arise from amide adducts, since female Sprague-Dawley rats, in which no amide adducts were detected (*6*), nonetheless develop liver tumors in high yield from 7-fluoro-AAF (*1*). The observation that activation for amide adduct formation disappears early in AAF feeding (within a week?) (*6, 19*) suggests that even the toxicity seen after prolonged feeding of AAF to male Sprague-Dawley rats is not due to amide adducts. It must be remembered that each of these qualifiers should be considered not as a denial of a particular hypothesis, but as a reminder that greater effort is needed to identify the operational and mechanistic stages in carcinogenesis in liver and other tissues.

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