

Role of Tissue Exposure and DNA Lesions for Organ-Specific Effects of Carcinogenic *trans*-4-Acetylaminostilbene in Rats

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trans-4-Acetylaminostilbene is acutely toxic to the glandular stomach and produces sebaceous gland tumors in rats quite specifically. Metabolism, tissue exposure to reactive metabolites, DNA binding and persistence of DNA lesions are implicated in tissue susceptibility, but nothing indicates that one of these parameters determines the biological effect. All tissues are exposed to reactive metabolites, liver as a nontarget tissue ranking highest. DNA binding in this tissue, however, is not irrelevant to tumor formation, but rather indicates the presence of initiating lesions. They can be amplified by partial hepatectomy and/or promoters, such as phenobarbital, DDT and diethylstilbestrol. Liver tumors are formed in high yields with these treatments, and mammary tumors also occur. *trans*-4-Acetylaminostilbene is therefore considered to be an incomplete carcinogen in these tissues and may initiate cells in other tissues as well. Apparently it lacks promoting properties which are supposed to be unrelated to reactive metabolites. It is concluded that DNA lesions do not reflect tissue risk, but rather secondary effects ultimately determine where the process of tumor formation starts and how fast it develops.

Introduction

An adequately performed animal experiment is still necessary to distinguish carcinogenic from non-carcinogenic chemicals. The recognition of carcinogens is straightforward if tumors arise in high yields and in tissues not forming tumors spontaneously in the species under test. A borderline activity is much more difficult to interpret, and species, strain and tissue-specific effects impede easy extrapolation to the human situation. A better understanding of the underlying mechanisms is therefore demanded.

Since the pioneering work of the Millers and their associates (1), it is widely accepted that aromatic amines have to be metabolically activated and that the reaction of metabolites with DNA produces a decisive lesion which initiates the process of tumor formation. Accordingly, attempts have been made to correlate the extent of DNA lesions with

the biological effect and to explain species, strain and tissue susceptibility by differences in exposure of the responsive tissue to reactive metabolites, due to specific balances between activating and inactivating metabolism. This concept had to be extended when the modulating properties of repair processes were detected. However, even if persistence of the DNA lesion is included in the considerations, it is difficult to find satisfactory correlations. From our studies with aminostilbene derivatives, which are carcinogenic and produce tumors quite selectively in sebaceous glands of rats, we conclude that substance-related effects not involving metabolic activation as well as tissue specific parameters contribute and may ultimately determine the biological effect (2, 3). Tissue-specific proliferation rates and their modulation and factors which influence processes collectively called promotion, including hormonal control, are such secondary parameters. These aspects have been widely neglected in cell-free and cell culture system investigations of the mechanism of metabolic activation. Recent results from studies of the fate of aminostilbene derivatives in rats will therefore be described to illustrate some of the problems involved in whole animal experiments and

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to support the proposed role of secondary factors in tumor formation.

Tissue Exposure and Primary Lesion

It is difficult to follow the concentration of individual metabolites in specific tissues with time in the intact animal. Usually metabolites are isolated and identified in urine, bile or sometimes blood, but reactive intermediates are not readily obtained from these sources. In tissues, the final reaction products with cellular macromolecules are therefore considered to indicate their presence. Covalent binding to proteins may be used to approximate tissue exposure. Although protein adducts may not reflect the exact pattern of metabolites reacting with nucleic acids, they may be formed by common metabolic precursors or even the same ultimate reactive metabolites. We have tentatively identified one of the protein adducts as a sulfinic acid amide which results from the reaction of *trans*-4-nitrosostilbene with the SH-group of cysteine (4). The adduct can be analyzed by mild acidic hydrolysis which releases *trans*-4-aminostilbene (Fig. 1). It constitutes 70% of the adducts in hemoglobin and it is also present in plasma and tissue proteins to a smaller extent (5). This indicates the presence not only of *trans*-4-nitrosostilbene but also of *trans*-4-*N*-hydroxylaminostilbene in blood and tissue. Both compounds are direct mutagens and therefore have genotoxic potential. Methylmercapto derivatives of *trans*-4-acetylaminostilbene (*trans*-AAS) have also been identified after acidic hydrolysis of tissue proteins (5), which indicates the reaction of methionine with a hydroxamic acid ester, which is also potentially genotoxic.

Three days after administration of radiolabeled *trans*-AAS to rats, protein-bound metabolites are found in all tissues, and protein binding roughly parallels total radioactivity in a given tissue at that time (Table 1). This allows approximation of tissue exposure even in tissues from which macromolecules have not been isolated. According to these criteria, all tissues measured are exposed to reactive

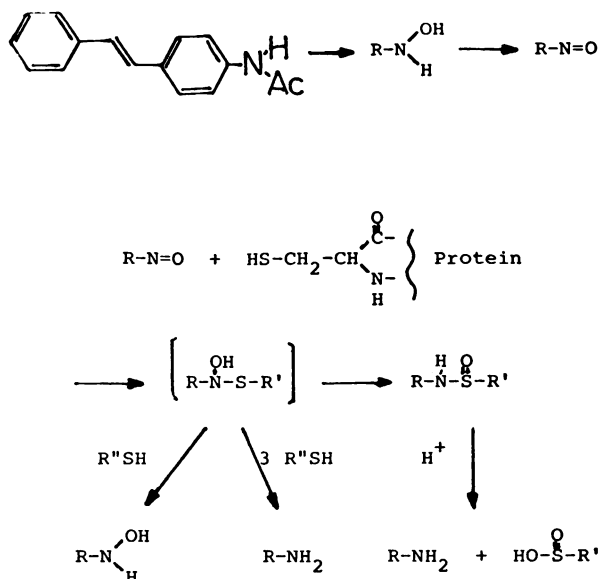


FIGURE 1. Protein adducts with aryl nitroso compounds. *trans*-4-Nitrosostilbene is a metabolite of *trans* AAS and reacts with HS-groups of cysteine (4). Part of the primary intermediate may be further reduced, another part rearranges and forms a stable sulfinic acid amide which may be hydrolyzed under mild acidic conditions.

metabolites, and exposure appears to vary only by a factor of 10, with liver and kidney ranking highest, and with lung, Zymbal's gland, glandular stomach and mammary tissue following in decreasing order (Table 1). The ratio between protein and DNA binding appears not to be constant in different tissues. This may be explained by different turnover and secretion of proteins and by different inactivation of reactive metabolites on their course from the cytosol to the cell nucleus. In the experiment described, the ratio varied from 18:1 in lung to 1:1 in forestomach (data not shown) 3 days after dosing. Tissue exposure and exposure of DNA are therefore not identical, and we prefer to describe the latter as the primary lesion. Even on this level however, all tissues are found to be affected, and the extent of the lesion does not permit discrimination between susceptible and nonsusceptible tissues.

Table 1. Exposure and DNA binding in different rat tissues.^a

	Liver	Kidney	Lung	Zymbal's gland	Glandular stomach	Mammary gland
Total radioactivity, pmole/mg wet weight	2.4	1.8	0.7	0.4	0.3	0.2
Protein binding, pmole/mg	6.6	2.0	1.4	N.D.	0.7	N.D.
DNA binding, pmole/mg	5.2	0.7	0.1	N.D.	0.6	N.D.

^a(³H)-*trans*-AAS (5 μmole/kg) was orally administered to female Wistar rats. After 3 days, total radioactivity, protein-bound and DNA-bound radioactivities were determined in various tissues (\bar{x} , $n = 3$; standard deviation <25%). N.D. = not determined.

Modulation of Tissue Exposure by Metabolism

Tissue-specific effects are often thought to be caused by tissue-specific metabolic activation. Total binding to proteins and nucleic acids does not account for the possible presence of particularly critical metabolites. Therefore, the metabolic pathways, distribution and sites of formation for reactive metabolites must be determined. Studying the metabolism of acute toxic doses of *trans*-AAS, we have now obtained additional data which support our conclusion that liver metabolism largely determines exposure of extrahepatic tissues (6). Acute toxic doses of *trans*-AAS quite specifically damage the proliferating isthmus cells in the antrum of the glandular stomach and the blood cell-forming bone marrow (7). Since these effects can be suppressed by pretreatment with methylcholanthrene (MC), but not with phenobarbital (PB), this indicates a role of metabolic activation (Table 2). The consequences of MC pretreatment were therefore studied on the level of metabolism, tissue exposure and DNA binding. Phase I and phase II metabolism are altered considerably (Tables 3 and 4). The conjugation pattern in urine and bile indicates that the conjugation capacity is increased by the protecting pretreatment. Binding to blood proteins, particularly hemoglobin, is lowered, which indicates that less *N*-oxidized, i.e., activated, metabolites are distributed by the blood

Table 2. Acute toxicity of *trans*-AAS.^a

Treatment	Dose, mg/kg	Lethal effects	Latency, days	Weight loss, %
<i>trans</i> -AAS (propanediol)	50	5/6 ^b	12	40
<i>trans</i> -AAS (tricaprylin/30% DMSO)	75	6/6	13	15
MC + <i>trans</i> -AAS	75	0/6 ^c	—	10

^a*trans*-AAS was administered by gavage to female Wistar rats.

^bOne animal developed a mammary tumor after 30 weeks.

^cFive animals developed tumors within 1 year.

stream (Table 5). As a consequence, all extrahepatic tissues are less exposed (Table 6). In the toxic situation, the glandular stomach as a target tissue is exposed to the same extent as other extrahepatic tissues, and the protective pretreatment lowers exposure and DNA damage in all tissues. It is therefore unlikely that metabolic activation specific to this tissue causes toxicity and that tissue-specific inactivation provides protection. This is in accordance with previous experiments with nontoxic doses, in which a similar pattern of DNA adducts revealed that all four of the above studied tissues are exposed to the same reactive metabolites (10). However, this may not hold true if the carcinogen is administered chronically, because drug-metabolizing enzymes in liver or extrahepatic tissues may be induced. However, this confounding effect can be excluded be-

Table 3. Excretion of *trans*-AAS metabolites in urine^a.

Pretreatment	Metabolites, nmole					
	AAS	4'-OH-AAS	4'-OH-AS	3-OH-AAS	erythrodiol	threodiol
Without MC	50	200	32	183	770	320
With MC	29	46	9	104	185	39

^a(³H)-*trans*-AAS (210 μmole/kg) was administered orally to female Wistar rats and urine was collected for 24 hr. Conjugates were separated by chromatography on alumina (cf. Table 4), unconjugated metabolites, sulfates and glucuronides were analyzed by HPLC separately after hydrolysis (Pfeifer and Neumann, unpublished), and the metabolites contained in these three fractions added. AS = 4-aminostilbene, diol = 4-acetylaminobenzyl- α,β -diol. Animals pretreated with MC excreted larger amounts of unidentified metabolites than untreated animals. (\bar{x} , $n = 2$).

Table 4. Metabolite pattern in urine and bile after toxic *trans*-AAS doses.^a

	Metabolites, %			
	Urine		Bile	
	No pretreatment	MC pretreatment	No pretreatment	MC pretreatment
Unconjugated metabolites	39.4	19.3	3.2	2.7
Sulfates	23.1	42.2	25.1	2.9
Glucuronides	12.0	18.8	21.1	25.6
Polar metabolites (Mercapturic acids)	24.5	16.7	46.5	65.6

^a(³H)-*trans*-AAS (210 μmole/kg) was administered orally to female Wistar rats with and without MC pretreatment (3 × 20 mg/kg). Metabolites in urine (24 hr) and bile (12 hr) were analyzed by chromatography on alumina (8); (\bar{x} , $n = 2$, deviation <25%).

cause when 12 doses were administered within 6 weeks, tissue exposure remained constant for each dose, and DNA damage accumulated (Table 7).

Repair

Although accumulation of DNA damage in the previous experiment already precluded tissue-specific repair of the initial lesions as a gross modulating factor, we measured the elimination rate of DNA-bound metabolites at different times during the course of administration and found it to remain constant up to the 12th dose (Table 7). Repair processes are therefore neither induced nor inhibited with this protocol.

Table 5. Covalent binding to blood proteins^a.

Pretreatment	Protein binding, pmole/mg	
	Plasma proteins	Hemoglobin
Without MC	151 ± 10	674 ± 136
With MC (3 × 20 mg/kg)	57 ± 8	29 ± 9

³H)-*trans*-AAS (210 μmole/kg) was administered orally to female Wistar rats with and without MC pretreatment. Blood was obtained after 24 hr, and proteins were processed (9); (\bar{x} , $n = 2$).

Initiation-Promotion

At this point, the question had to be answered whether or not the persistent and accumulating DNA damage could be regarded at all as indicating an initial lesion in terms of tumor initiation. The possibility had to be ruled out that the total binding measured with DNA obtained from whole tissue homogenates is entirely irrelevant with respect to tumor formation. Actually one should expect only that some, as yet undefined, critical lesion, which constitutes only a small fraction of total binding, would correlate with biological effects. However, pharmacokinetic considerations make it quite likely that a certain correlation also exists between a critical lesion and the total DNA lesion. As long as the critical event cannot be defined, this correlation cannot be determined. It should be possible, however, to demonstrate whether or not there is a critical lesion at all among the persistent DNA-lesions, if secondary effects can be found which amplify the primary effect. For liver, partial hepatectomy is known to increase cell proliferation and therefore to improve fixation of an initial lesion (11, 12). In addition, promoters like PB and DDT may enhance the growth of initiated cells (13-15). We have therefore

Table 6. Exposure and DNA binding in different rat tissues^a.

Tissue	Protein/binding, pmole/mg		DNA/binding, pmole/mg	
	No pretreatment	MC pretreatment	No pretreatment	MC pretreatment
Liver	219	329	80	50
Kidney	52	36	20	5
Lung	72	9	3	2
Glandular stomach	60	9	15	5

³H)-*trans*-AAS (210 μmole/kg) was administered orally to female Wistar rats with and without MC pretreatment (3 × 20 mg/kg). After 24 hr, proteins and DNA were isolated from different tissues (10); (\bar{x} , $n = 2$, deviation <17%).

Table 7. Accumulation and elimination of DNA-bound metabolites^a.

Tissue	Accumulation, pmole/mg, 3 days after 12 doses		Elimination of DNA-bound metabolites, $t_{1/2}$ between day 3 and 17 after dosing, days				
	Total tissue	DNA binding	1 dose	4 doses	8 doses	12 doses	Average
Liver	7.2	24.4	23	38	17	11	22
Kidney	7.0	17.1	persistent				
Lung	3.0	1.9	15	26	16	15	18
Zymbal's gland	1.4	N.D.	N.D.				
Glandular stomach	1.3	3.9	10	7	10	10	9
Mammary tissue	0.8	N.D.	N.D.				

^aThe results of two different experiments are summarized: (1) to determine accumulation, 12 (³H)-*trans*-AAS doses (5 μmole/kg, twice weekly) were orally administered and total radioactivity as well as DNA binding were measured 3 days after the last dose. (\bar{x} , $n = 3$, standard deviation <25%); (2) to determine the elimination rate during the administration period, only one labeled dose was administered after various unlabeled *trans*-AAS doses, and DNA binding was measured 3 and 17 days after the last dose (\bar{x} , $n = 2$).

performed an initiation-promotion experiment and included diethylstilbestrol (DES) to test the promoting properties of estrogenic hormones in liver (16, 17). The protocol is shown in Figure 2 and the results are shown in Table 8.

The *trans*-AAS dose turned out to be moderately carcinogenic, producing sebaceous gland tumors in half the animals within one year. Up to this time, neither enzyme-deficient foci nor hyperplastic nodules could be detected in the liver. If *trans*-AAS administration was followed by partial hepatectomy, preneoplastic lesions as well as hepatomas were observed. Feeding DDT and DES alone to pretreated animals produced similar effects. Tumor formation was particularly increased and the latent period shortened if partial hepatectomy and promoter feeding were combined.

These results clearly demonstrate that *trans*-AAS induces critical lesions in liver. In addition, tumors grew in mammary tissue, a tissue which had been shown to be exposed the least (Table 1). DES

was particularly effective in promoting these tumors. This observation indicates a *trans*-AAS-related effect, since mammary tumors were not observed in any one of the control groups up to that time.

Several questions may be asked at this point: (1) does partial hepatectomy induce only the fixation of DNA damage? If this were the case, fixation would be sufficient to start the whole process; alternatively, does this procedure exert an additional promoting activity? (2) Are promoters able to fix an initial lesion like partial hepatectomy (for instance by stimulating cell proliferation), or is fixation in the sense of a mutational mechanism unnecessary to amplify the initial lesion?

Biochemical Effects of Partial Hepatectomy and Promoters

The stimulating effect of partial hepatectomy on cell proliferation can be directly demonstrated by

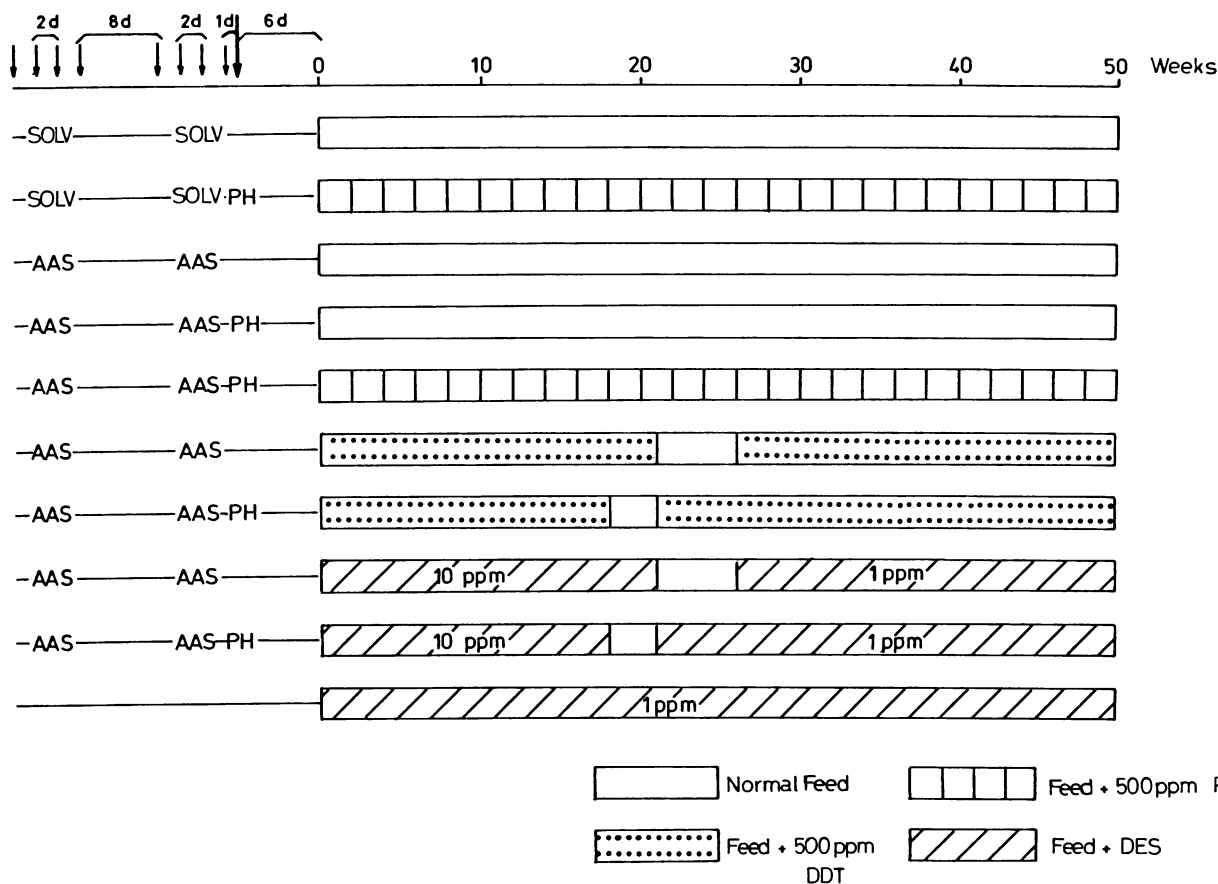


FIGURE 2. Protocol of the initiation-promotion experiment. After 24 and 46 weeks, two animals of each group were sacrificed, and livers were histologically examined for enzyme-deficient foci (alkaline phosphatase), increased glycogen storage and hyperplastic nodules. Solv = solvent; PH = partial hepatectomy.

Table 8. Effects of partial hepatectomy and promoters on tumor incidence after *trans*-AAS^a.

Treatment	n	Incidence	Tumor localization					
			Sebaceous glands		Liver			Mammary tissue
			Zymbal's gland	Lip	Hyperplastic nodule	Hepatoma	Cholangioma	
Solvent	10	0/10	—	—	—	—	—	—
Solvent + PH + PB	8	0/8	—	—	—	—	—	—
AAS	9	4/9	3	2	—	—	—	—
AAS + PH	10	8/9	1	—	6	2	—	2
AAS + PH + PB	12	11/11	3	2	4	7	1	—
AAS + DDT	10	6/6	1	—	2	4	2	1
AAS + PH + DDT	14	11/13	2	—	3	7	—	1
DES	10	0/10	—	—	—	—	—	—
AAS + DES	10	9/10	1	2	4	5	1	5
AAS + PH + DES	14	12/12	1	—	6	6	2	1

^aThe experiment was terminated after 54 weeks, except for the control groups. Tumors were histologically verified. Hyperplastic nodules are contained in the incidence rate. It was not attempted to specify the malignancy of liver tumors.

Table 9. Effects of partial hepatectomy (PH) on DNA binding.^a

Treatment	DNA binding, pmole/mg							
	Liver		Kidney		Lung		Glandular stomach	
	1 day	17 days	1 day	17 days	1 day	17 days	1 day	17 days
AAS	7.8	2.9	0.7	0.7	0.25	0.10	0.5	0.18
PH 24 hr before AAS	5.8	0.6***	1.6***	1.4**	0.64**	0.40***	1.1**	0.45***
PH 24 hr after AAS	3.5	0.7***	0.9	0.8	0.30	0.16*	0.5	0.34***

^a(³H)-*trans*-AAS (5 μmole/kg) was administered orally to female Wistar rats. Either 24 hr before or 24 hr after dosing a two-thirds hepatectomy was performed and DNA binding was determined either 1 or 17 days after dosing (\bar{x} , $n = 3$). The significance of differences to the control values is indicated.

* $p < 0.1$.

** $p < 0.05$.

*** $p < 0.001$.

the more rapid reduction of DNA-bound metabolites compared to untreated controls. DNA synthesis dilutes specific binding, particularly during the first days after partial hepatectomy. Binding is reduced to 20% of that of the controls after 17 days (Table 9). The initial DNA binding is similar, regardless of whether or not partial hepatectomy is performed before or after dosing. This means that rapidly proliferating liver cells metabolize the carcinogen at the same rate as normal cells; in terms of DNA binding, this means that liver cells are not preferentially affected unless the two effects cancel each other out.

Promoters do not stimulate liver cell proliferation nearly as much, if at all, as partial hepatectomy. Correspondingly, specific DNA binding of *trans*-AAS-metabolites does not decrease in liver. With DDT it even increases somewhat (Table 10). It is therefore unlikely that promoters contribute to fixation of DNA damage to an extent comparable to that of partial hepatectomy. These findings would

support the notion that a specific lesion is amplified by partial hepatectomy directly, i.e., through DNA synthesis, and by promoters indirectly through stimulating the expression of altered cell properties. An irreversible change would be a prerequisite for both mechanisms.

The last two experiments demonstrate, in addition, that the applied procedures do not only affect the liver. A preceding partial hepatectomy increases DNA binding in all extrahepatic tissues (Table 9, in this situation extrahepatic metabolism contributes significantly), and decreases the elimination between day 3 and 17 (data not shown) of DNA-bound metabolites in glandular stomach and lung. The latter effect is also observed with subsequent partial hepatectomy and is more pronounced in lung than in glandular stomach and kidney. After 2 weeks of promoter feeding, specific DNA binding is greater in experimental than in control animals in all tissues studied. In kidney, even an absolute in-

Table 10. Effects of promoters on DNA binding.^a

Treatment	DNA binding, pmole/mg							
	Liver		Kidney		Lung		Glandular stomach	
	3 days	17 days	3 days	17 days	3 days	17 days	3 days	17 days
AAS	4.5	2.9	0.9	0.7	0.28	0.10	0.38	0.18
AAS + PB	5.5*	2.7	1.0	1.5**	0.22	0.15*	0.35	0.25**
AAS + DDT	5.9**	3.8*	1.1	1.9***	0.20	0.22**	0.38	0.30**
AAS + DES	4.5	2.3	1.3	2.7****	0.35	0.15*	0.58	0.28**

^a(³H)-*trans*-AAS (5 μmole/kg) was administered orally to female Wistar rats. Promoters were added to the feed from the next day at the following concentrations: PB, 500 ppm; DDT, 500 ppm, DES, 10 ppm. DNA binding was determined in different tissues 3 and 17 days after dosing ($x, n = 3$). Values were significantly different from the controls as indicated.

* $p < 0.1$.

** $p < 0.05$.

*** $p < 0.001$.

crease is observed; this is largest with DES (Table 10).

Conclusions

According to the definition outlined in the introduction, *trans*-AAS is a strong and complete carcinogen. This statement may now be modified: *trans*-AAS is a complete carcinogen for sebaceous glands, but an incomplete carcinogen for liver and mammary tissue, and it may be an incomplete carcinogen for other tissues as well. Since the extent of accumulating and persistent DNA damage, which apparently indicates the presence of initiating DNA lesions, does not correlate with the biological effects, it has to be postulated that secondary effects modulate the primary lesion and may ultimately determine where the process of tumor formation starts and how fast it develops. The proliferation rate and endogenous hormonal stimulation may be decisive in Zymbal's gland. In mammary tissue, an exogenous hormonal stimulus (or inhibition of prolactin inhibiting factor) was most effective. This raises the question, what does *trans*-AAS lack to make it a complete carcinogen for liver? Obviously, the promoting properties, rather than the initiating, are absent. 2-Fluorenylacetylamide has been shown to possess both properties. It induces drug metabolizing enzymes (18-20) and thus shares a trait common to other typical promoters, and it is also toxic to liver tissue. *Trans*-AAS, in contrast, neither induces enzymes (21) nor is toxic to liver, even in lethal doses (7).

Other examples of incomplete liver carcinogens have been described recently. 2-Methyl-4-dimethylaminoazobenzene (22, 23) was classified noncarcinogenic but induced tumors after additional promotion. Benzo(a)pyrene (24) is a complete carcinogen for many tissues after local administration, but produces liver tumors only with partial hepatectomy and promotion. *N*-Acetylamino-biphenyl (25), a

known carcinogen for mammary tissue in rats, has also been shown to initiate liver cells. It will therefore be necessary to include the multistage characteristics of carcinogenesis (26) in risk evaluations and to consider both genotoxic and epigenetic properties to assess the carcinogenic potential more precisely. The results described indicate that epigenetic effects may be unrelated to reactive metabolites, since *trans*-AAS metabolites react with DNA and proteins to the same extent as hepatotoxic and hepatocarcinogenic aromatic amines.

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