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Initiation/Promotion Versus Complete Carcinogenicity in the Rodent Liver

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4-*N*-Pyrrolidinylazobenzene (4N) is a close structural analog of the rodent liver carcinogen 4-dimethylaminoazobenzene (DAB). This structural similarity led us to evaluate it for genotoxic activity *in vitro*. We observed activity for 4N and DAB in the BHK cell transformation assay and subsequently in the Salmonella mutation assay of Ames. By a curious chance, Scribner, Miller and Miller, probably prompted by the same structural similarity, had synthesized 4N in the 1960s and found it to be noncarcinogenic to the rodent liver using a bioassay test protocol that detected DAB as carcinogenic. These findings were only described following the publication of our observations made *in vitro*.

The conflict that apparently exists between these data can be interpreted in two separate ways. (a) Scribner et al. have suggested that 4N may be a carcinogenic initiator as opposed to a complete carcinogen like DAB. They also suggested that promotion of 4N-treated rodents with phenobarbitone might lead to the production of liver tumors. (b) We have evaluated the simpler concept that the activities observed for 4N *in vitro* define a carcinogenic potential that is not realized *in vivo* due to its rapid detoxification, at least in rodents.

The first of these explanations implies that pure carcinogenic initiators may form a separate class of genotoxic agents from complete carcinogens, and perhaps of greater interest, that 4N might provide a valuable model compound for the study of carcinogenic promotion in the rodent liver. The second explanation regards potential carcinogenicity as a single property that can be defined *in vitro* and which may or may not be expressed *in vivo* depending on the enzymic environments encountered by the test chemical. It is clearly important to evaluate these different propositions in order to aid progress in the study of carcinogenic promotion, especially in the rodent liver. The presentation will describe our recent studies *in vitro* and *in vivo* in this connection.

Introduction

Dramatic progress has been made during the past decade in both our understanding of the mode of action of organic chemical carcinogens and in the development of techniques for their detection. It is now generally accepted that an initiating event is induced in the nucleus of somatic cells by reaction of the carcinogen with DNA. Using this as a starting point, it is now possible to discern chemicals capable of such reactions using one or more bacterial or mammalian cell mutation assays. The fact that the majority of organic carcinogens give a positive response in such assays has led to various laws in several countries aimed at regulating the use of new chemicals found positive in these assays. This assumed equivalence of mutation *in vitro* and carcinogenicity *in vivo* has fostered a perhaps deceptively simple approach to the control of environmental carcinogens.

The legislative initiatives referred to above, together with the attendant battery of short-term tests that these laws require, take no account of the possible modulation of the carcinogenic expression of compound A by concomitant exposure of the host animal to chemicals B, C, etc. The significance of this omission is illustrated by the fact that several modulating agents are known that either enhance or attenuate the 'degree' of carcinogenicity of some carcinogens but which are themselves neither mutagenic nor carcinogenic. The several modulating effects produced by the noncarcinogenic nonmutagen disulfiram illustrate this concern (*1*, *2*).

The present symposium is concerned with carcinogenic promotion, i.e., the effect of chemical B on the carcinogenicity of chemical A. This "promotion" can take the form of either an increase in the ob-

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served tumor yield expected of a given dose of the carcinogen or a reduction in the latent period required to produce the original tumor yield. A major obstacle to a rational approach to the study of carcinogen promoting agents is that although, almost without exception, these agents are nonmutagenic *in vitro*, most have been shown to be weakly carcinogenic to rodents. Thus, not only are the promoting properties of such agents hard to anticipate in the absence of animal bioassays, but their "carcinogenicity" is not correlated with mutagenic activity *in vitro*.

It is suggested by several authors elsewhere herein that the carcinogenicity to the mouse liver of liver promoting agents such as phenobarbitone, DDT or dieldrin, may simply provide evidence of their ability to promote pre-existing and preneoplastic lesions in that organ. The species and strain specificity of the carcinogenicity, but not the promoting properties of these agents supports this suggestion. If these suggestions are confirmed, complete (i.e., initiating and self-promoting) carcinogens and pure promoting agents will form two discrete groups of xenobiotics. In anticipation of this possible resolution, consideration of a third class of agent, pure initiators, forms the basis of this paper. The relationship between pure initiation and pure promotion (both noncarcinogenic events) and complete carcinogenicity and initiation/promotion (both carcinogenic events) is shown (Fig. 1).

Evidence Supporting the Existence of Noncarcinogenic Initiating Agents

The majority of carcinogen promoting studies employ a low dose-level of an established carcinogen as the initiating agent and subsequent repeated administration of a promoting agent. The test protocols are usually so adjusted that neither the initiating agent nor the promoting agent alone produces tumors within the time course of the study; the carcinogenic effects observed are therefore the clear product of initiation/promotion. Higher dose levels of the initiating agent are carcinogenic without auxiliary promotion, and most of these agents are active in short-term tests such as the Salmonella mutation assay of Ames (*3*, *4*). This coincidence of activities has led to the general assumption that a chemical found to be active in an established *in vitro* assay is potentially able both to initiate the carcinogenic sequence, and, at appropriate dose levels, to produce tumors.

Contrary to this assumption, several instances of putative noncarcinogens showing activity in *in vitro* mutagenicity assays, but not in short-term assays conducted *in vivo* have recently been encountered. Several such instances were observed in a recent publication (*4*) as illustrated by the contrasting test profiles of the carcinogen 2-acetylaminofluorene (2-

SCHEME I

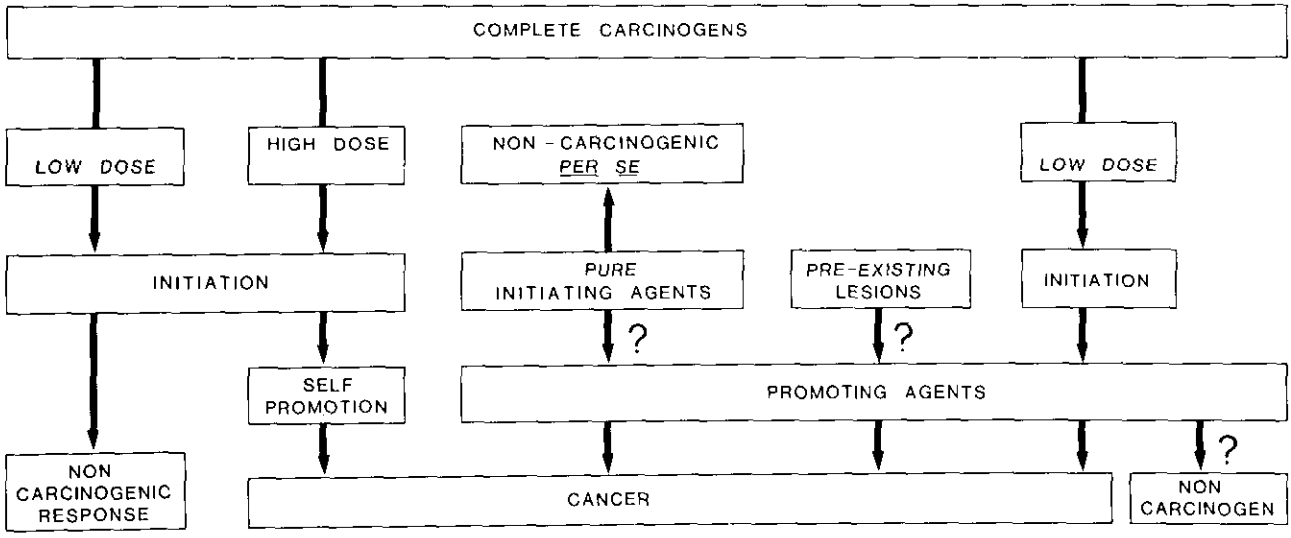
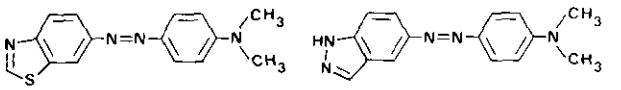


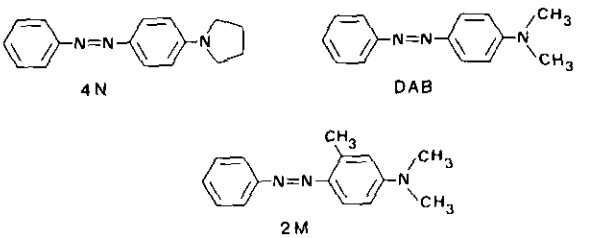
FIGURE 1. Relationships between carcinogenic initiation/promotion and complete carcinogenicity. The existence of pure initiating agents and noncarcinogenic (i.e., pure) promoting agents is speculative and forms the basis of the present discussion.

AAF) and its putatively noncarcinogenic 4-substituted analog 4-AAF (Fig. 2).

The immediate question posed by these findings is whether the mutagenic noncarcinogen 4-AAF may be effective as an initiating agent but be incapable of promoting itself. This would coincide with the present definition of a pure initiating agent. This conclusion cannot be drawn at present due to the paucity of the available negative carcinogenicity data. A related example from our own researches (*6*) is illustrated by the similar mutagenicities *in vitro* of the potent liver carcinogen 6-dimethylaminophenylazobenzthiazole (6BT) and the apparent noncarcinogen 5-dimethylaminophenylazoindazole (5I) (Fig. 3).



A further possible example of a pure initiating agent has been suggested by Scribner et al. (*8*), namely, 4-*N*-pyrrolidinylazobenzene (4N). We have demonstrated (*9*) that this structural analog of the rodent liver carcinogen DAB is active in both the BHK cell transformation (*10*) and *Salmonella* mutation assays (Fig. 4). DAB gives a positive response in each of these assays (*7, 12*; data not shown). However, Scribner et al. (*8*) have shown that 4N is inactive in a limited rodent carcinogenicity bioassay that concurrently detected DAB as positive. Similarly, 2-methyl-4-dimethylaminoazobenzene (2M) is known to bind covalently to hepatic DNA, RNA and protein (*13*) but not to produce tumors in that organ (*14*). If however, the treated animals are subsequently promoted with phenobarbitone, tumors are produced (*14*).



The above examples provide evidence in favor of the concept that some bacterial mutagens may be capable of initiating preneoplastic events within the nucleus of somatic cells but be incapable themselves of promoting these lesions—they therefore appear to be noncarcinogenic and could consequently be referred to as pure initiating agents.

The immediacy of the need for studies designed to explore the possible existence of a discrete class of noncarcinogenic initiating agents is illustrated by the weight of evidence currently in favor of their existence, and by the influence that they would have, if adequately defined, on the study of carcinogen promotion (cf. the unresolved assumptions im-

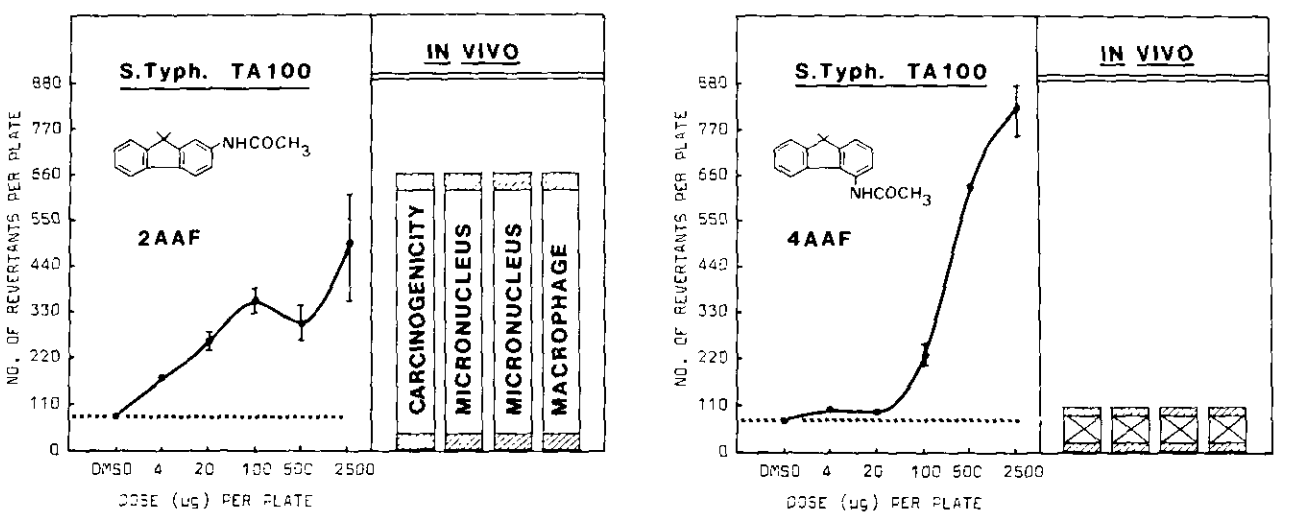


FIGURE 2. Comparison of the gross genotoxicity profiles of 2-acetylaminofluorene (2-AAF) and its 4-acetylamino isomer (4-AAF). The mutagenic dose-response curve observed for each chemical (+ S9) in Salmonella is derived from the data submitted by Trueman to the International Study (4). The mouse micronucleus *in vivo* assay data were similarly submitted to that study by Salamone et al. and Tschuchimoto et al. These two chemicals were recently evaluated independently by Nashed et al.(5) in their rat peritoneal macrophage assay, a positive and negative response being observed, respectively, as shown. The carcinogenicity classifications were as used in the International Study (4) and are discussed in more detail therein by Purchase et al. (Chapter 4).

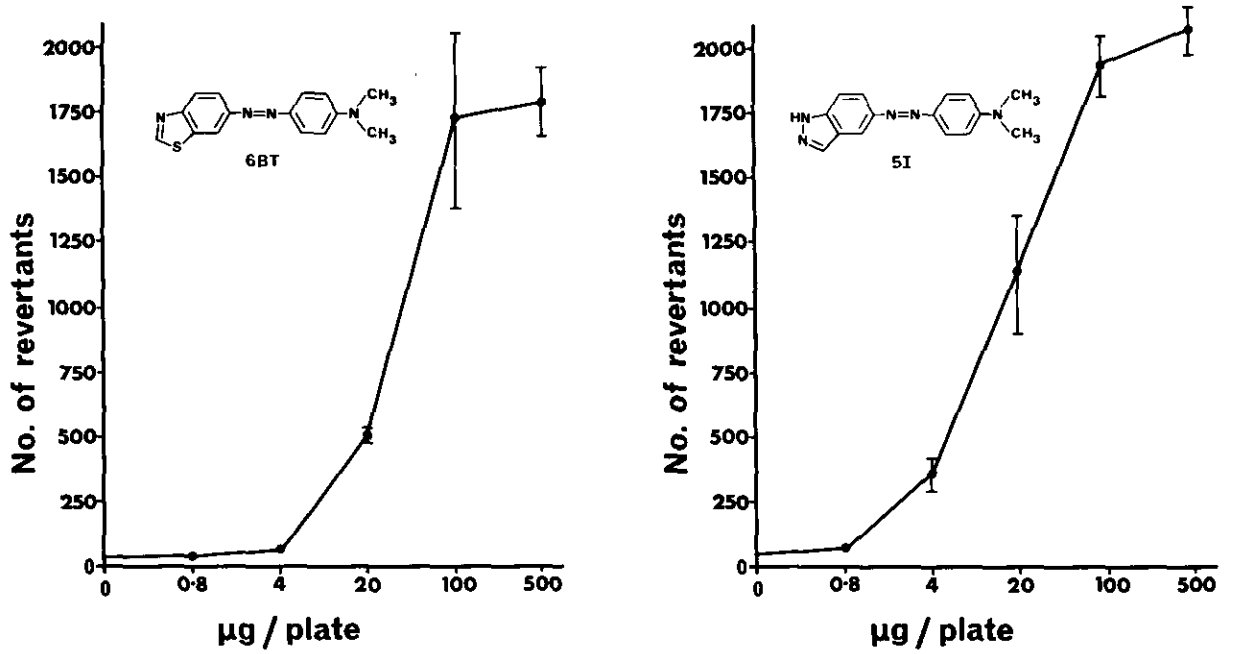


FIGURE 3. Mutagenic activity (*6*) of 6-dimethylaminophenylazobenzthiazole (6BT) and 5-dimethylaminophenylazoindazole (5I) when evaluated in *S. typhimurium* (strain TA98) using a pre-incubation assay (*7*) and Aroclor 1254-induced rat liver S9 mix.

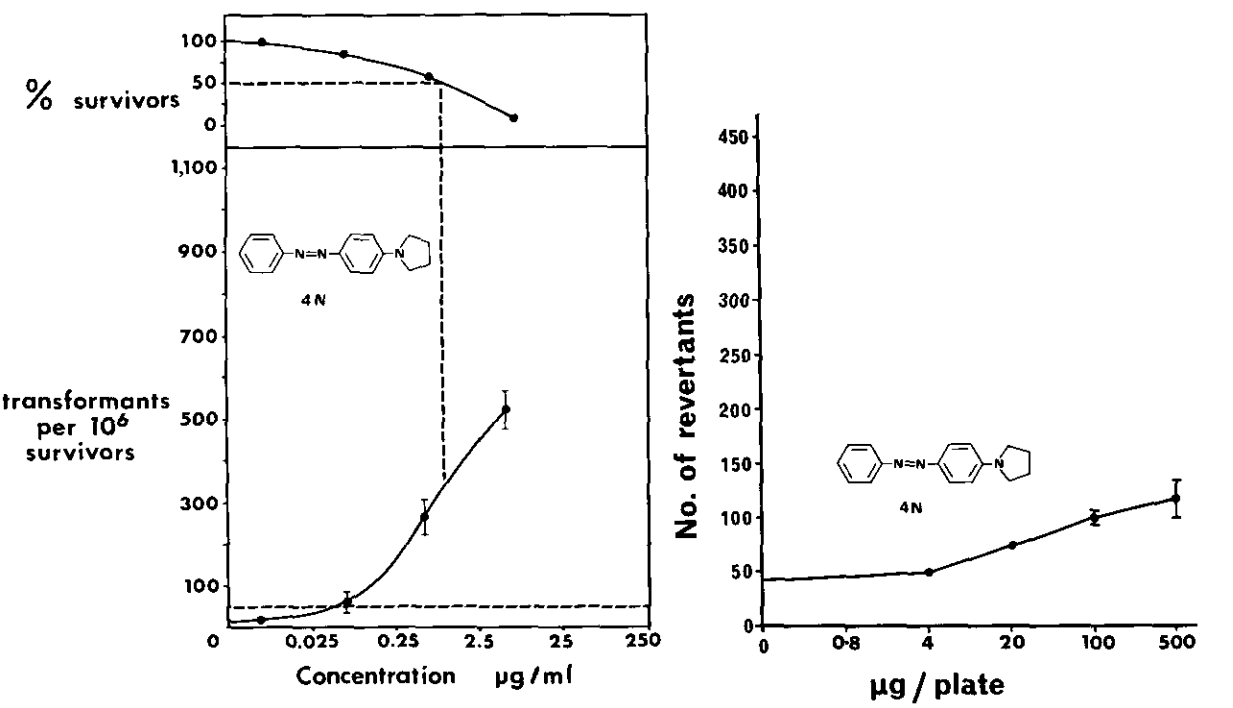


FIGURE 4. Positive response (*9*) observed for 4-*N*-pyrrolidinylazobenzene (4N) in the BHK cell transformation assay (*10*) and the Salmonella mutation assay (*11*) (strain TA 98, pre-incubation test[10]). Both experiments were conducted in the presence of an Aroclor 1254-induced S9 mix.

plicit in Figure 1). However, this evidence, especially that relating to the alleged noncarcinogenicity of such agents, must first be carefully assessed, as attempted below.

Assessment of the Evidence in Favor of the Existence of Pure Initiating Agents

The most important variable in initiation/promotion studies is the dose level at which the initiating carcinogen is administered. The level selected is usually the highest that will not, in the absence of promotion, produce a significant increase in cancer incidence within the time course of the study. But the fact that higher dose levels of the same agent are able to produce a carcinogenic response clearly defines the critical importance of the dosimetry in these studies. Within this context, the credibility that can be accorded to a candidate for pure initiating agent is inexorably linked to the dose levels at which it has been evaluated for carcinogenicity. This is a particularly important consideration because, until recently, chemicals were typically assayed for carcinogenicity via administration to between 10 and 50 rats followed by only a limited histopathological assessment of their major organs. Further, these studies were often terminated after only 1 year of exposure to the chemical. Such methods, while adequate for detection of "potent" carcinogens, are insufficient to define noncarcinogenicity, but their implicit use for this purpose in the past suggests that a proportion of the chemicals currently regarded as being "noncarcinogenic" may actually be weak carcinogens (*6*).

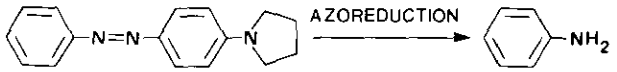
In the case of the three azo compounds mentioned earlier, i.e., 6BT (*17*), 5I (*18*) and 4N (*8*), the carcinogenicity bioassays were conducted at a single dose level, which in the case of the two noncarcinogens, are shown herein to be only a fraction of their maximum tolerated dose (MLD) level (Table 1).

A comparison of the published carcinogenicity data on 6BT, 5I and 4N with their relative toxicities as observed in our laboratory is shown in Figure 5. The substantial differences in the relative toxicity of both 4N and 5I to the carcinogen 6BT reflects adversely upon the reliability of their classification as noncarcinogens. In particular, the probable fact that the "noncarcinogenicity" of these compounds was established at only ~4% of their maximum tolerated dose (MTD) should be viewed within the context that many established carcinogens prove inactive when tested at such reduced dose-levels and for so short a period of time (cf. the basis for conducting initiation/promotion studies).

The true carcinogenic status of 4N and 5I can only be determined via further bioassays conducted at higher dose levels and for longer periods of time, however, an analysis of the short-term (1-6 week) effects they produce in rodents may indicate the likely outcome of such studies (see below and Table 2).

Biological Profile of 4N

This material failed to affect significantly the rate of growth of the test animals up to dose levels of 500 mg/kg over a period of 6 weeks (Fig. 6). In addition, the livers of the treated rats were normal in terms of pathology and nuclear ploidy (cf. Table 2 for 6BT and 5I). Further, evaluation of cellular protein for covalent addition of 4N gave negative results at the 2-week and 6-week points. In contrast to these negative findings, the spleens of the treated animals were enlarged and their blood levels of methaemoglobin elevated (>8% compared to control levels of <1%). These findings are consistent with the rapid cleavage of 4N *in vivo* (either in the gut or in the liver) to yield aniline a known methemoglobinemic agent (*23*) and rodent spleen carcinogen (*24*).



4N Aniline

These data therefore suggest that 4N is unlikely to be a rodent liver carcinogen at any dose level. However, the effects seen in the spleen suggest

Table 1. Comparison of published data on 6BT, 5I and 4N.[a]

Chemical	Activity *in vitro*	Carcinogenicity (SD rats)	Dose level administered, % in diet	MTD in AP rats via gavage, mg/kg	Estimated factor of MTD at which bioassay conducted	Predicted carcinogenicity at MTD
6BT	+	+	0.03	~7.5	~100%	+
5I	+	–	0.03	~200	~ 4%	+
4N	+	–	0.06	>500	< 3%	–

[a] The carcinogenicity bioassay data for 6BT and 5I in Sprague Dawley (SD) rats are from Brown et al. (*17, 18*) while those for 4N are from Scribner et al. (*8*). The studies in Alderley Park (AP) rats will be described by us in detail elsewhere (*11*). MTD = maximum tolerated dose by daily stomach gavage of a single bolus in corn oil.

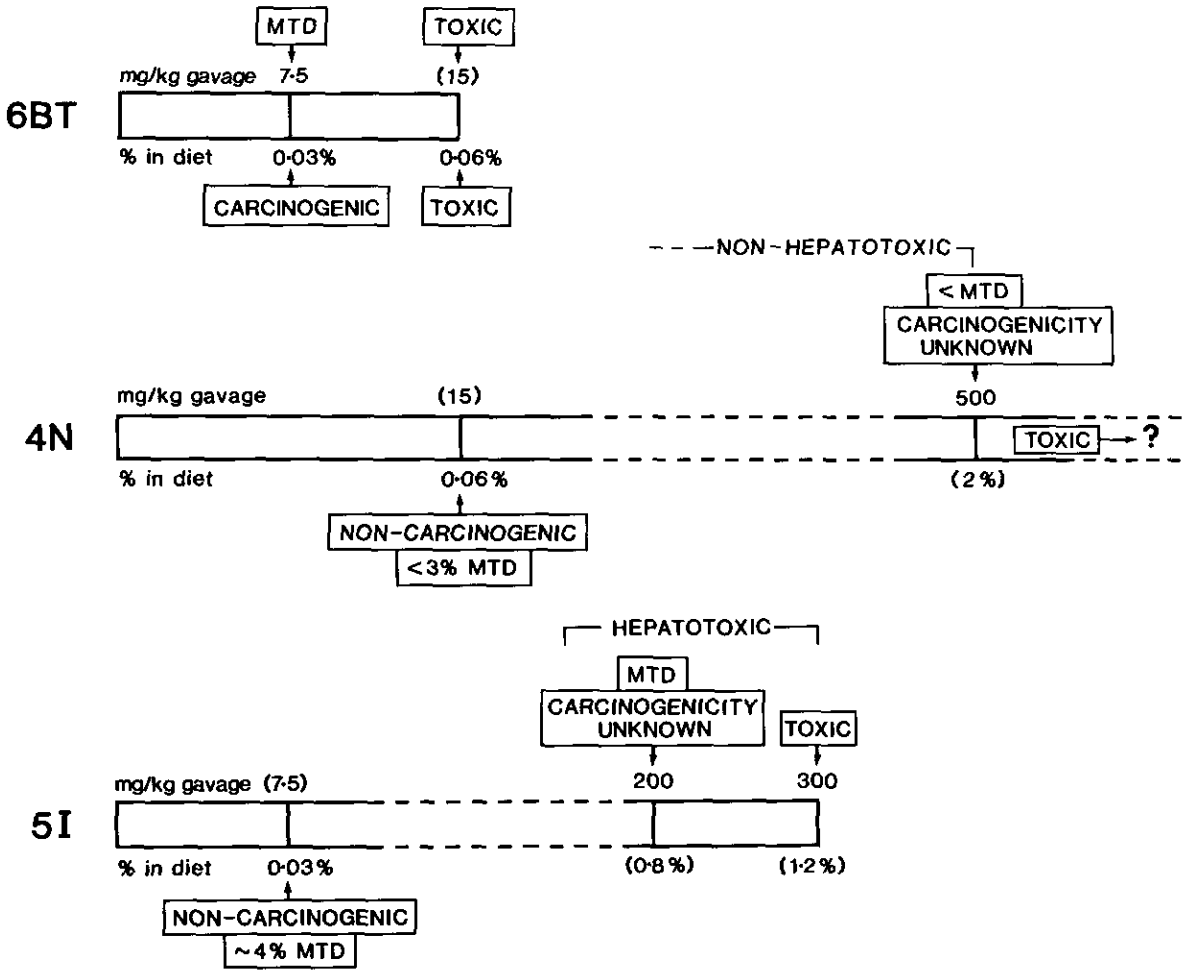


FIGURE 5. Composite data derived in rats for 6BT, 4N and 5I. The lower axis of each histogram represents previous observations made by Brown et al. (*17, 18*) for 6BT and 5I and Scribner et al. (*8*) for 4N, each derived from bioassays in which the test chemicals were administered *via* the diet. The top axis of each histogram represents the data obtained in our laboratory (*11*), doses being expressed as mg/kg via gavage. The correspondence between the MTD of 6BT (~7.5mg/kg) in the present study and 0.03% in diet in the study of Brown et al. (*17*) has been used to calibrate the remainder of the histograms. Doses shown in parentheses are estimated based on observations made in the complementary study (cf. complementary axis). Use of the word "toxic" implies observation of moribund animals or recorded deaths. "Subtoxic" doses do not, therefore, preclude cellular changes induced in the liver. The potent hepatotoxicity and hepatocarcinogenicity of 6BT has been confirmed in this laboratory by using both the gavage and dietary routes and both SD and AP rats (*11*).

Table 2. Comparative activities of 6BT, 5I and 4N, administered via gavage, in the livers of Alderly Park rats.[a]

Chemical	MTD in AP rats (mg/kg, gavage)	Effects on body weight	Evidence of covalent addition to protein	Reduction in relative proportion of tetraploid nuclei	Evidence of mitotic activity in liver	Evidence of pre-neoplastic changes in the liver (oval cells, etc.)
6BT	~7.5	+	–[b]	+	+	+
5I	~200	+	+	+	+	+
4N	>500	–	–	–	–	–

[a] All determinations were made within the first 4 weeks of dosing. The nuclear ploidy determinations (based themselves on the findings of Neal and Butler (*19*) were made using a flow cytometer and will be published elsewhere (*20*). The liver histopathology (mitotic activity and H & E section analysis) will also be discussed elsewhere (*11*). The dye-binding experiments were determined optically by using the method of Miller and Miller (*11, 21*).

[b] The failure to detect protein-bound dye may be associated with the low dose levels employed; further studies are in progress.

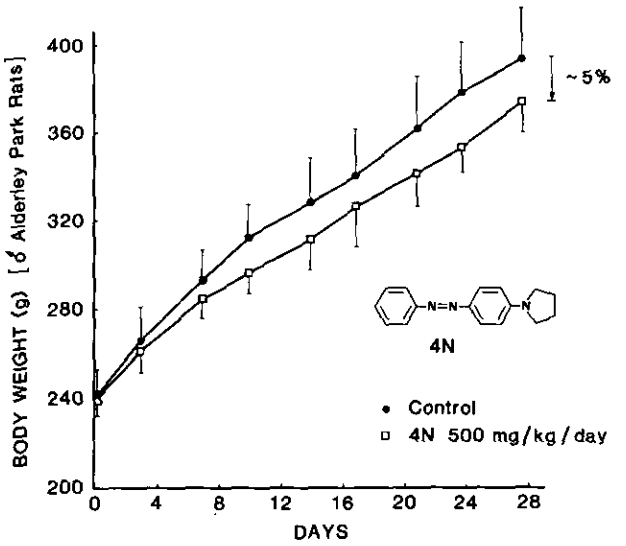


FIGURE 6. Growth curves for control(– ● –) and 4N-treated (– □ –) male Alderly Park rats over the first 4 weeks of treatment. Each data point represents the average of five animals (± SD). No deaths occurred during this experiment. The ~5% depression in body weight between the treated and control groups at 28 days was derived by comparison of means.

that the induction of cancer by 4N in this organ cannot be excluded. Such an effect, if established, would probably be associated with the secondary toxicity and not with the primary potential (liver) carcinogenicity discerned for this chemical by structural considerations and the activities observed for it *in vitro*. Further, we suggest that it is unlikely that 4N will even prove to be an initiating agent in the rodent liver. In particular, no evidence of any change in the liver was discerned at the elevated dose level of 500 mg/kg.

Biological Profile of 5I

The hepatic effects observed for this chemical at ~200 mg/kg (Table 2) suggest that it may prove carcinogenic to the liver at these dose levels. These changes included histopathological disturbances similar to those seen in the preneoplastic phase of the azo dye-induced carcinogenicity (*16*), a reduction in the tetraploid component of the hepatic nuclei and covalent addition of the test chemical to cellular protein. These findings suggest that the reported noncarcinogenicity of this material may be an illusion created by the low dose levels employed (~4% of the MTD level) and the short duration (8 months) of the only available carcinogenicity bioassay, and consequently, that it is unlikely to be defined ultimately as a pure (i.e., noncarcinogenic) initiating agent.

Biological Profile of 2M

This chemical has apparently good credentials as a pure initiating agent: it binds to hepatic nuclear DNA and RNA after oral administration to rats but fails to produce liver tumors. These findings are nonetheless subject to at least two restraints. First, the dose level employed in the carcinogenicity bioassay of 2M by Kitagawa et al. (*14*) (the standard 0.06% in diet) was not related to its MTD. Second, Roe et al.(*15*) have provided limited evidence of the carcinogenicity of this chemical to the mouse liver. A further and more detailed carcinogenicity bioassay of 2M is therefore required before it can be classed as a pure initiating agent. This material has not been studied in our laboratory, thus it does not figure in Tables 1 and 2; however, appropriate experiments with it are planned.

Conclusions

The search for a noncarcinogenic initiating agent is made attractive by the order it would bring to carcinogen promotion studies. However, the candidate examples currently available are insecurely based due to the inadequacy of their carcinogenicity bioassay data. This conclusion is not based solely on the examples discussed herein; pyrene, methyl orange, α-naphthylamine, dinitrosopentamethylenetetramine and 3-methyl-4-nitroquinoline *N*-oxide have been similarly classified (*4*). The issue of pure initiators is, however, of sufficient philosophical importance for it to be pursued even if it is finally established that as a class they do not exist. The evidence currently available suggests that the bacterial mutagens that have so far proven to be noncarcinogenic to rodents are either rapidly detoxified *in vivo*, and are therefore incapable of reaching the target organ of interest in high enough dose levels to initiate preneoplastic lesions (e.g., 4N?), or that they only appear to be non-carcinogenic because they have not been adequately tested for carcinogenicity (e.g., 5I?). Appropriate studies on 4N and 5I are currently in hand in these laboratories to answer these questions for these particular compounds. In the meantime, initiation/promotion studies will have to employ low dose levels of a complete carcinogen as the initiating species (*16*).

NOTE ADDED IN PROOF: The bacterial mutagenicity of 4N has subsequently been published (*25*).

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