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PRELIMINARY COMMUNICATIONS

α-ACETOXY-DIMETHYLNITROSAMINE: A PROXIMATE METABOLITE OF THE CARCINOGENIC AMINE

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The discovery that dimethylnitrosamine (DMN) possessed carcinogenic potential[1], initiated extensive chemical and biological studies on a host of related nitroso compounds, and the results have sporadically been reviewed and evaluated[2-4]. The preponderance of evidence appeared to be compatible with the possibility that the biological effects of such compounds might well be an outcome of their indirect alkylation of cellular macromolecules, especially the nucleic acid bases. The alkylating moieties were thought to be derived from the breakdown of the initial molecules either during the oxidative dealkylation of the dialkylnitrosamines[5], or in the course of intracellular chemical reactions with the nitrosamides and nitrosamidines, because of their intrinsic reactivities towards thiol and free amino groups[6].

The biological activities of nitroso compounds could not be fully reconciled with the alkylation hypothesis, neither with respect to carcinogenesis nor mutagenesis. The major difficulty arose in connection with the differential oncogenic and genetic effects obtained with compounds expected to effect the same DNA alkylations, as was observed with methylating or ethylating agents from different nitroso subseries: amines, amides or amidines (details in refs. 7 & 8). This raised the suspicion that reactive species, other than alkyl carbonium ions, might be responsible for some biological effects and led to the search for reactivation mechanisms that do not involve the complete breakdown of the initial nitroso compound. Foremost in this connection is Schoental's recent hypothesis[7] that the proximate carcinogenic forms of dialkylnitrosamines might be enzymatic oxidation products which retained the alkylnitrosamino moiety, but had acquired a carbonyl function through the coversion of an alkyl group to the corresponding aldehydic side chain. The resulting metabolite would thus become bifunctional and could then effect DNA/protein cross-linkage at specific genic sites, which were thought might result in the mutagenic events most relevant to cancer initiation.

The generation of an aldehydic side chain during the metabolism of DMN would not be detectable chemically, since the oxidation of either of its alkyl α-carbons would lead to highly reactive and unstable intermediates; namely, the hydroxymethyl and the corresponding aldehydic derivatives. Any evidence for

the formation of such metabolites must accordingly be inferential and was thought might well be revealed by the genetic techniques developed in *Drosophila*, based on the mutagenic selectivity of carcinogens for rDNA[9-17]. A research project has accordingly been designed in our laboratory for the analysis of the mutagenic properties of a series of structurally related *N*-methyl and *N*-ethyl-*N*-nitrosamino derivatives, with various intrinsic or potential reactive centres and with different carcinogenic potencies. The full results of these investigations are beyond this communication and are being serialized elsewhere[8,18]. This note is intended to draw attention to the fact that α-acetoxy-dimethylnitrosamine (AcODMN) exerted identical genetic effects as the unsubstituted parent, but at a much lower molarity, which would indicate that it could well be a precursor of the amine's biologically effective metabolite.

DMN [$CH_3N(NO)CH_3$] was a commercial product (Schuchardt, Munich, West Germany) and AcODMN [$CH_3N(NO)CH_2OCOCH_3$] was synthesized and kindly supplied by Dr. M. Wiessler of the Cancer Institute, Heidelberg, West Germany. Mutagenicity assays were undertaken in *Drosophila melanogaster* (Oregon-K strain) according to techniques previously utilized with other carcinogenic chemical series[9-17], including some nitroso compounds[8,18]. The compounds were dissolved at the required molar concentration in an oily vehicle (2% v/v, dimethylformamide in Arachis oil) and administered by micro-injection in the haemocoel of adult XY^{bb} males of roughly the same average age (25 ± 5 hours) and weight (0.85 ± 0.05 mg) so that each received the same volume of solution (0.25 ± 0.05μl). Genetic activity was examined with respect to the non-specific effects on the whole X-chromosome (recessive lethals and visibles) relative to the specific effects on the RNA-forming genes: those for tRNA, throughout the genome, which yield the dominant *Minute (M)* deletions and the rDNA sites that mutate to the sex-linked recessive *bobbed (bb)*. The various mutations were simultaneously assayed in the same population of treated gametes according to the techniques devised for this purpose (cf.10, 12,15). The mutagenicity of the two test compounds on the successive stages of spermatogenesis was assessed by the customary progeny fractionation - or 'brood' - technique [8-18], under strictly identical breeding conditions, so as to ensure maximal homogeneity in the speed of sampling of the various testicular sectors.

The biological activity of AcODMN was invariably higher than that of the parent amine. This was first noticed with respect to cytotoxic activity, as indicated by the size of the progeny per injected male relative to the controls; the substituted compound was completely sterilizing at 5.0 mM, whereas this dose of the parent only effected some reduction in fertility (10-20%). The mutagenicity assays with the ester were accordingly conducted within a much lower dose range (0.1 - 2.5 mM) than that previously investigated with the amine[18] (1 - 10 mM), so as to enable the comparison of their genetic activities at equitoxic doses. It was found that the two compounds exerted the same mean

TABLE 1. The F_1 *Minute (M)* and *bobbed (bb)* mutations relative to the F_2 sex-linked recessive lethals and visibles [X(*l* + *v*)], showing the X10 dose equivalence for DMN as compared to AcODMN. Mosaic mutants are entered in brackets.

Genetic function	DMN : 10.0 mM			AcODMN : 1.0 mM		
	Gametes	Mutants	Per 10^3	Gametes	Mutants	Per 10^3
F_1-Mutations						
Phenotypic *M* + *bb*	8355	180(108)	21.5 ± 1.6	7332	164(106)	22.4 ± 1.7
Transmitted *bb*	8355	37(17)	4.4 ± 0.7	7332	33(18)	4.5 ± 0.8
Transmitted *M*	15915	31(15)	1.9 ± 0.3	13801	61(29)	4.4 ± 0.6
F_2-Mutations						
X(*l* + *v*)	2028	291(60)	143.5 ± 7.8	1984	294(69)	148.2 ± 8.0
rDNA Selectivity Index*		2.81 ± 0.50			2.79 ± 0.55	

*Calculated as the induced frequency of *bb*/[*bb* + X(*l* + *v*)] X100. The induced frequencies were obtained from the experimental values by the subtraction of the mean control contributions per 10^3 gametes: 0.3 ± 0.04 for the *bb*'s and 1.8 ± 0.5 for the X(*l* + *v*).

biological activities on the stages of the testis at a molarity ratio of 1:10, in favour of AcODMN. The comparative results for all the investigated doses will be published elsewhere; Table 1, being a representative example. The two compounds, at the compared doses (1.0 & 10.0 mM), induced virtually identical mutation frequencies both with respect to the specific effects on the RNA genes (*M* & *bb*) and the non-specific sex-linked recessives [X(*l* + *v*)]. However, there was a significantly higher yield of transmitted *M* with AcODMN, but this was not observed with other dose comparisons (*e.g.* 0.5 & 5.0 mM), where these mutations were recovered at the same frequencies.

The relative mutagenic activities of DMN and AcODMN, as described by the X10 dosage relationship, was clearest for the yield of the X-recessives during spermatogenesis, as shown in Table 2. These mutations were recovered at virtually equal frequencies from the same testis sectors (*i.e.*, within corresponding broods), thus indicating an identical pattern of cell stage response during spermatogenesis. Both compounds exerted much lower activity on the metabolically inert mature sperm than on the actively metabolizing spermatocytes and spermatogonia, and the increases in their activities between these testicular sectors were statistically comparable; the ratios of the mutation frequencies for broods III + IV/I were: 3.2 ± 0.7 and 2.0 ± 0.3 for the amine and the ester, respectively (Table 2). This showed that even the ester required further metabolic activation and should, therefore, be considered as the amine's proximate - rather than the ultimate - genetically effective metabolite. The metabolic pathway for the activation of the ester could well proceed through deacylation, with the production of the hydroxymethyl compound, which might then be oxidized to the corresponding aldehydic derivative[7].

The common mechanism of mutagenesis for DMN and AcODMN was most clearly manifested in the identical nature of their induced mutations. The two compounds gave statistically comparable frequencies of mosaic mutants among corresponding mutational classes and germ cell stages (Tables 1 and 2). This showed that the extent of DNA damage across the chromo-

TABLE 2. The sex-linked mutations in successive sectors of the male germ line at the doses of mutagenic equivalence for DMN and AcODMN. Mosaic mutants are entered in brackets.

Broods*	DMN : 10.0 mM			AcODMN : 1.0 mM		
	Chromosomes	Mutants	Per 10^3	Chromosomes	Mutants	Per 10^3
I	503	33(9)	65.6 ± 11.0	517	47(10)	90.9 ± 12.6
II	499	73(18)	146.3 ± 15.8	513	74(23)	144.2 ± 15.5
III	514	108(20)	210.1 ± 18.0	515	102(21)	198.1 ± 17.6
IV	512	77(13)	150.4 ± 15.8	439	71(15)	161.7 ± 17.6
Total	2028	291(60)	143.5 ± 7.8	1984	294(69)	148.2 ± 8.0

*Germ cell sectors sampled in the successive broods are: I, sperm; II, spermatids; III, spermatocytes; IV, spermatogonia.

some (single as opposed to double-strand effects) with the two compounds were the same, which would imply that the molecular mechanisms involved in their induction were also identical. Further evidence for the analogous modes of action of the amine and its ester - at the molecular level - was indicated by the fact that both compounds gave the same rDNA selectivity index (bottom row, Table 1). This feature has an added significance in relation to carcinogenesis, in view of the impressive correlation so far established between mutagenic selectivity for rDNA and oncogenic efficiency[8-18]. It would seem highly plausible, therefore, that AcODMN could well be a proximate metabolite of DMN in carcinogenesis, as it was in mutagenesis.

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