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PRELIMINARY COMMUNICATIONS

N, α-ACETOXYETHYL- *N*-ETHYL-NITROSAMINE: A PRECURSOR OF THE BIOLOGICALLY EFFECTIVE METABOLITE OF *N*, *N*-DIETHYLNITROSAMINE

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The toxicological activation of dialkylnitrosamines through metabolic oxidation, possibly on an alkyl α-carbon, has long been suspected largely on biochemical grounds[1,2]. Yet, there was no way for the experimental verification of this concept, because of the expected chemical instability of the *in vivo* oxidative products, particularly with the short chain dialkyl derivatives, such as dimethylnitrosamine (DMN) and probably also its diethyl analogue (DEN). Thus, α-carbon oxidation of a methyl group of DMN would result in the α-hydroxymethyl compound, or its aldehydic derivative, neither of which would be expected to be sufficiently stable for chemical isolation. Recently, however, chemical techniques have been developed for the synthesis of the α-acetoxy derivatives of dialkylnitrosamines[3-5], including that for the dimethyl compound (AcODMN) and its diethyl analogue: *N*, α-acetoxyethyl-*N*,ethyl-nitrosamine (AcODEN). These esters were shown to be hydrolysable in aqueous solutions[5] - especially in the presence of esterases[6] - with the liberation of the corresponding α-hydroxyalkyl derivatives. The same hydrolytic processes would be expected to occur more readily *in vivo*, because of the widespread distribution of esterases in animal tissues. The acetoxyalkyl nitrosamines could accordingly be regarded as *in vivo* precursors of the hydroxyalkylnitrosamines, thus permitting an experimental approach to the assessment of the relevance of such oxidative metabolites to the various toxicological manifestations of the parent nitrosamines.

The first indications for the considerable enhancement in the biological activity of dialkylnitrosamines through α-carbon acylation emerged from comparative genetic studies in *Drosophila* with DMN and AcODMN[7,8]. These studies revealed that AcODMN exerted identical mutagenic effects as DMN, but at a much lower molarity, which was - indeed - compatible with the chemical expectation that it was a precursor of the amine's biologically effective metabolite. This was further supported by the later demonstration that AcODMN was carcinogenic in the gastrointestinal tract of rats[9] and was markedly more effective in this

respect than DMN[6]. There seemed little doubt, therefore, that α-carbon hydroxylation was the first step in the metabolic activation of DMN, both in mutagenesis and carcinogenesis. The question then arose as to how far the α-carbon oxidation was also relevant in the activation of nitrosamines with longer alkyl chains. The present communication deals with the diethyl derivatives and was approached through a comparative study of the genetic properties of DEN and AcODEN.

DEN[$C_2H_5N(NO)C_2H_5$] was a commercial product (Schuchardt, Munich, West Germany) and AcODEN[$C_2H_5N(NO)CH(CH_3)OCOCH_3$] was synthesized according to the procedure previously described by Wiessler[3]. The biological study was undertaken in *Drosophila melanogaster* (Oregon-K strain) according to the techniques developed in our laboratory for the analysis of the molecular and genetic mechanisms of action of carcinogens[7-15]. These techniques have been described in detail elsewhere[10-12] and need only be mentioned here in brief outline. The test compounds were dissolved at the required concentration in dimethylformamide/Arachis oil (2%, v/v) and administered by micro-injection in the haemocoel of adult XY^{-bb} males, according to a standard technique which ensured homogenous dosimetry between males at each level of treatment. Biological activity was measured for all stages of spermatogenesis in relation to both cytotoxicity and mutagenicity. The genetic effects were assayed with respect to the overall response of the whole X-chromosome (recessive lethals and visibles) as well as at the specific RNA-forming genes: those for tRNA which yield the dominant *Minute (M)* deletions and the rDNA sites that mutate to the sex-linked recessive *bobbed (bb)*.

TABLE 1. The F_1 *Minute (M)* and *bobbed (bb)* mutations relative to the F_2 sex-linked recessive lethals and visibles [$X(1 + v)$], with DEN and AcODEN. Mosaic mutants are entered in brackets.

Genetic function	DEN : 20 mM			AcODEN : 2 mM		
	Gametes	Mutants	Per 10^3	Gametes	Mutants	Per 10^3
F_1-Mutations						
Phenotypic *M + bb*	8503	126(67)	14.8 ± 1.3	13163	270(153)	20.5 ± 1.2
Transmitted *M*	16238	22(10)	1.4 ± 0.3	24974	88(45)	3.5 ± 0.4
Transmitted *bb*	8503	33(15)	3.9 ± 0.7	13163	43(24)	3.3 ± 0.5
F_2-Mutations						
$X(1 + v)$	2052	161(54)	78.5 ± 5.9	2080	285(61)	137.0 ± 7.5
rDNA Selectivity Index*		4.48 ± 0.93			2.17 ± 0.38	

*Calculated as the induced frequency of $bb/[bb + X(1 + v)]$ 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 $X(1 + v)$.

Previous genetic studies with DEN indicated its lower mutagenicity compared to related ethylating nitrosamides[16,17]. It was accordingly decided to adjust its dose level relative to AcODEN, so as to enable a comparison of the genetic effects of the two compounds at equivalent cytotoxic levels; this was achieved at a molarity ratio of 10 for DEN/AcODEN. The comparative genetic results over the investigated dose ranges of the two compounds will

be published elsewhere; Table 1, illustrates the situation at the maximal tested molarity for DEN (20 mM) as compared to the equitoxic level of AcODEN (2 mM). In spite of the 10-fold higher molarity of DEN, AcODEN induced a significantly higher mutation frequency with respect to the collective effects on the RNA genes (M + bb), largely due to the higher activity on the tRNA loci, which was expressed in the doubling of the frequency of their mutations (transmitted M). The corresponding yield of rDNA mutations (bb) was virtually the same, but that for the overall X-chromosome recessives [X(l + v)] was 2-fold higher in favour of AcODEN, thus resulting in the rDNA selectivity index for DEN being double that for AcODEN (bottom row, Table 1). It might be inferred, therefore, that at equimolar injected doses of the two compounds, mutagenic potency would be higher for AcODEN by a factor of about 20-fold for the non-specific point-mutations, but only 10-fold for the specific rDNA deletions expressed as bb. This shows that the two mutational classes could not be attributed to the same reactive species (ethyl carbonium ions), which again points to the possibility that the specific deletions were induced by a difunctional metabolite, as suggested by Schoental[18] and indicated by genetical results with other nitroso compounds[7,8,15].

TABLE 2. The sex-linked mutations in successive sectors of the male germ line for DEN and AcODEN. Mosaic mutants are entered in brackets.

Broods*	DEN : 20 mM			AcODEN : 2 mM		
	Chromosomes	Mutants	Per 10^3	Chromosomes	Mutants	Per 10^3
I	527	34(17)	64.5 ±10.7	435	54(14)	124.1 ± 15.8
II	527	52(12)	98.7 ±13.0	486	69(14)	142.0 ± 15.8
III	475	46(20)	96.8 ±13.6	581	84(19)	144.6 ± 14.6
IV	523	29(5)	55.4 ±10.0	578	78(14)	134.9 ± 14.2
Total	2052	161(54)	78.5 ±5.9	2080	285(61)	137.0 ± 7.5

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

The relative mutagenic activities of DEN and AcODEN on the various stages of spermatogenesis could be gathered from the yield of the sex-linked recessive mutations in the successive progeny fractions (or broods) from the injected males (Table 2). The pattern of yield of these mutations between broods was qualitatively similar for the two compounds, both exerting a slightly higher activity in the metabolizing spermatids and spermatocytes (broods II and III) than on the metabolically inert sperm (brood I). The relative yield from the spermatogonia (brood IV) appeared to vary between experiments with each agent, probably due to complications of germinal selection and DNA repair. The quantitative aspects of mutagenic cell stage response with the two compounds was further analysed statistically on the basis of their brood mutability data detailed in Table 2. The ratio of the

mean mutation frequencies for the whole testicular tissue (broods I-IV) at the compared doses of AcODEN/DEN was 1.7 ± 0.2, and proved to be statistically equal to the values representing the response of the separate germ line sectors, which ranged from a minimum of 1.4 ± 0.2 in brood II, to a maximum of 2.4 ± 0.5 in brood IV. It would be noticed, however, that the proportion of mosaics among the DEN X-recessives in broods I and III was considerably higher than its level in all other broods with both DEN and AcODEN. This deviation in the extent of mosaicism between compounds was not observed among any of the F_1 mutational classes (Table 1) and would, therefore, appear to be an artifact of mutant recovery in the DEN F_2 brood experiment.

The considerably higher genetic activity of AcODEN as compared to equimolar doses of DEN justifies the conclusion that it is the amine's proximate mutagen. This supports the chemical expectation that α-carbon hydroxylation could, indeed, be the crucial first step in the metabolic activation of DEN, as well as DMN[7,8], and might accordingly be of general applicability to all dialkylnitrosamines. Significantly also, the higher mutagenicity of the acetoxy derivatives was noticeable on the RNA genes, the response of which was shown to be a sensitive indicator of carcinogenic potential among a diversity of chemical series, including the nitroso compounds[7-15]. It was on the basis of such genetic information that the higher carcinogenicity of AcODMN over that of DMN was predicted[7] before it was experimentally observed[6]. In the light of the present genetic results, a comparable situation could reasonably be anticipated for the carcinogenicity of AcODEN as compared to DEN. However, the difference in carcinogenic potency for these compounds could well be less pronounced than with the methyl analogues, in view of the higher rDNA selectivity index for DEN as compared to AcODEN, which might compensate-partially at least - for its comparatively lower absolute mutagenicity on a dose basis.

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