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Determination of the Rate Constants for Alkylation of DNA *in vitro* with Methanesulfonic Esters

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The mutagenic alkylating agents, methyl, ethyl, and isopropyl methanesulfonate (MMS, EMS, and iPMS, respectively) give quantitatively and qualitatively different effects both with biological materials [1,2] and with DNA *in vitro*.[3,4] The site of alkylation of DNA for these substances is supposed to be at different groups, *i.e.* unesterified phosphate oxygens and purine base nitrogens.[5,6]

As a basis for a quantitative comparison of the effects of treatment with different alkylating agents, the reaction rate constants with DNA *in vitro* were determined. The site of alkylation will be discussed.

Experimental. Calf thymus DNA was purchased from Sigma Chemical Co. Tritium labelled MMS, EMS and iPMS were synthesized according to Wachtmeister *et al.*[7] Unlabelled MMS and EMS from Eastman Organic Chemicals and iPMS from Koch-Light Laboratories were used.

The labelled alkylating agents were diluted with inactive substance to the following specific activities: 8 mCi/mmole for MMS, 50 mCi/mmole for EMS, and 70 mCi/mmole for iPMS. The concentration of MMS and EMS were 3 mM and for iPMS 0.5, 0.75, and 1.5 mM. DNA was alkylated at a concentration of 1 mg/ml in a 0.02 M phosphate buffer of pH 7.0 at 25°C. Samples of 1 ml were withdrawn at different times. iPMS, which has a half life of 1 h at 25°,[1] was incubated for 8 h with DNA, *i.e.* to practically complete consumption of the ester. DNA was precipitated by 2 volumes of 95 % ethanol and washed four times with 70 % ethanol. It was then redissolved and hydrolyzed in 1 N HCl for 20 min at 100°C in vacuum. The samples were mixed with a scintillation solution consisting of dioxane-naphthalene-PPO-POPOP [8] to which was added 0.5 ml 1 N HCl and 0.5 ml of hydroxide of hyamine 10X (Packard Co.). The liquid scintillation spectrometer used was a Nuclear Chicago Unilux model 6851 (Packard Co.).

Results. During incubation with DNA in a buffer solution an alkylating agent reacting according to S_N2 (in the present case MMS and EMS [1,5]) is used up mainly in additive reactions with the nucleophiles present. Besides water and DNA, the phosphate ions are alkylated. Thus, the alkylating agent disappears with the rate constant k'

$$k' = [H_2O] \cdot k_{H_2O} + [HPO_4^{2-}] \cdot k_{HPO_4^{2-}} + [H_2PO_4^-] \cdot k_{H_2PO_4^-} + [\text{DNA-P}] \cdot k_{\text{DNA}}$$

where [DNA-P] is the concentration of DNA in mole/l nucleotide phosphorus. Under the reaction conditions used the factors $[H_2PO_4^-] \cdot k_{H_2PO_4^-}$ and $[\text{DNA-P}] \cdot k_{\text{DNA}}$ are negligible. $k_{H_2PO_4^-}$ is *ca.* 17 times less than $k_{HPO_4^{2-}}$ and [DNA-P] is about ten times lower than $[HPO_4^{2-}]$.[9] The constants for secondary phosphate and water have been determined by Osterman-Golkar *et al.*[10]

iPMS reacts predominantly according to an S_N1 mechanism, especially with weak nucleophiles,[5,8,10] and its total decay rate is therefore uninfluenced by nucleophiles present, although it may be affected by salts.[9] In the absence of competing reactions and at low concentrations of a dissolved nucleophile, *i.e.* under conditions which apply to DNA in the present case, it is, however, possible to assign formally

a second order rate constant also to an S_N1 reactant.[10]

The bimolecular rate constant for alkylation of DNA-P may be written

$$k = \frac{d[\text{DNA-PA}]}{dt[\text{AA}]\,[\text{DNA-P}]} \quad (1)$$

where [AA] is the concentration of the alkylating agent and [DNA-PA] the concentration of alkylated DNA nucleotide. This is valid, when AA is equal to MMS and EMS. For the reactions of MMS and EMS k was calculated from the concentration of DNA-PA at the time t presented as

$$[\text{DNA-PA}] = \frac{k[\text{DNA-P}]\,[\text{AA}]_0\,(1-e^{-k't})}{k'}$$

In the case of iPMS the expression used for DNA-PA at infinite time was as follows:

$$[\text{DNA-PA}] = \frac{k[\text{DNA-P}]\,[\text{AA}]_0}{k_{H_2O}[H_2O]}$$

$[AA]_0$ is the initial concentration of AA; [DNA-P] is practically unchanged. The rate constants (l mole^{-1}h^{-1}) at 25°C for reactions between DNA-P and a few alkyl methanesulfonates are: MMS 0.10 ± 0.01; EMS 0.005 ± 0.0005; iPMS 0.09 ± 0.006.

Discussion. Using the mode of presentation of Hudson and Harper [11] the relative rate constants of MMS and iPMS for reaction with different nucleophiles are given with the corresponding rate constants of EMS as a standard [10] (Fig. 1). When the rate constants found for reaction with DNA are inserted into the diagram, it is observed that the reaction rate of EMS with DNA is close to its rate of reaction with primary phosphate, the nucleophility, n, of which is *ca.* 2.5 [10] in the Swain-Scott scale.[12] The reaction rate of iPMS, the substrate constant, S,[5,12] of which (*i.e.* the slope of the curve relative to the slope of the standard EMS curve) is very low, is also in acceptable agreement with this value. (The point may in fact have a lower n value considering the possibility of a rate enhancing influence of the strongly polar surroundings of the NaDNA. The point in parenthesis (×) in the figure reduces the reaction rate found by the enhancing influence of 1 M Na_2SO_4.[9]) The reaction rate with MMS is decidedly higher than expected from attack by a nucleophile with $n = 2.5$. Although it is still possible that the lower values for EMS and iPMS are due to steric hindrance on the side of the DNA, the values obtained

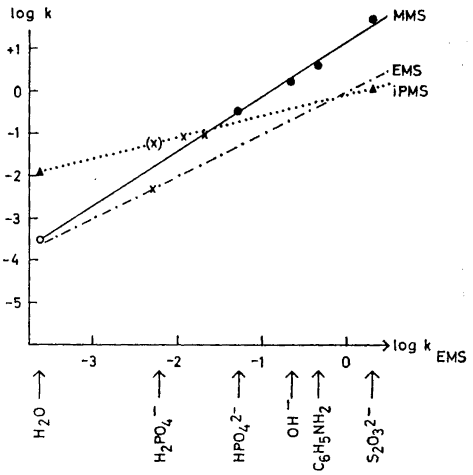

Fig. 1. log k_{MMS} and log k_{iPMS} for reaction with different nucleophiles as a function of the corresponding log k_{EMS} (data from Osterman-Golkar *et al.*[10]). The rates of reaction with DNA are denoted by ×. In the iPMS point (×) a roughly estimated rate-enhancing influence of Na poly-deoxyribonucleate was subtracted.

support the idea that MMS alkylates directly base nitrogens, especially guanine-N-7,[6,3] in contrast to EMS and, evidently, iPMS which primarily alkylates the phosphates of DNA. The n of guanine-N-7 is not known, but certainly higher than that of primary phosphate, in view of the realkylation found to occur when ethylated DNA is hydrolysed.

The relative mutagenic effectiveness in barley [10] and *E. coli* [13] of the three compounds studied agrees better with relative reaction rates at $n = 2.5$. It will therefore be investigated whether the *in vivo* rates of alkylation of DNA are different from those found *in vitro*.

A possible source of error in the rate constants between small molecules and DNA emanates from the adsorbent properties of the latter which may *either* lead to an increased concentration of the AA and changed solvent properties in the vicinity of its nucleophilic centres *or* to a contamination of the DNA with AA or its reaction products, in the present case especially alkyl phosphate. The latter possibility was excluded in the present case since addition of cold alkyl

phosphate before precipitation of the alkylated DNA did not influence the rate constant determined, and since, further, the rate constants for EMS and MMS were confirmed by an independent method (determination of alkylguanine formed in hydrolysis). In a preliminary investigation the rate constants were determined by separation of alkylated DNA from low-molecular labelled compounds by means of Sephadex G-25. This technique gave higher values, which in the case of iPMS were reduced to about the present values by addition of cold $iPrOPO_2^{2-}$ before separation.

The preliminary higher values obtained were used for a calculation of degrees of alkylation in a previous communication.[4] More correct degrees of alkylation used in that case should therefore be: Me-DNA 0.2–1.4; Et-DNA 0.4–2.9; and iPr-DNA 0.9–3.7 alkyls per 100 DNA phosphates.

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The Crystal and Molecular Structure of 4-Phenyl-1,2-dithiolium Bromide

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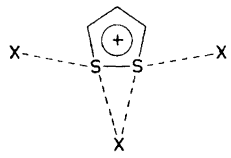
In crystals of 4-phenyl-1,2-dithiolium iodide [1] there are sulphur-iodine close contacts similar to those present in crystals of thiuret hydrohalides.[2–4] They occur in roughly linear halogen-sulphur-sulphur-halogen arrangements and triangular sulphur-halogen-sulphur arrangements, *cf.* Scheme 1.



Scheme 1. Arrangements of halide ions (X) relative to disulphide group.

The X···S distances in the linear arrangements in thiuret hydroiodide [2] and hydrobromide [3] are 0.4 Å shorter than the corresponding van der Waals distances. In thiuret hydrochloride hemihydrate [4] there is a close contact, 0.1 Å shorter than the van der Waals distance, on one side of the disulphide group only. The fact that the sulphur-sulphur bonds in the hydroiodide, $S-S=2.088\pm0.012$ Å, and in the hydrobromide, $S-S=2.081\pm0.008$ Å, are found to be longer than that in the hydrochloride hemihydrate, $S-S=2.063\pm0.004$ Å, indicates that the halogen-sulphur close contacts have caused a small lengthening of the sulphur-sulphur bonds. Such a lengthening might be anticipated if charge is partially transferred from the halide ions into the antibonding sulphur-sulphur σ-orbital.

Crystals of 4-phenyl-1,2-dithiolium bromide,[5] chloride monohydrate,[6] and