Influence of Monofunctional Alkylation on the Reversibility of Heat Denaturation of DNA. Preliminary Report

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Several alkanesulfonic esters are strongly mutagenic in higher organisms.[1-4] They exhibit, however, variations in the pattern of biological effects, the analysis of which may give clues to their action mechanisms. In root meristems, thus, methyl methanesulfonate (MMS), thus, induces chromosomal aberrations,[5,6] whereas ethyl methanesulfonate (EMS) excerts this action only under restricted conditions such as the presence of certain metal ions,[7,8] or after a protracted post-treatment storage of the seeds.[9] In barley isopropyl methanesulfonate (iPMS) induces mutation proportionally to the concentration, thus differing from EMS and MMS for which a multihit type concentration dependence is observed.[3]

Many facts indicate that alkylation of DNA is a primary cause of the observed genetic effects, although additional mechanisms such as enzyme inactivation may be involved, especially in the case of a high selectivity,[10] e.g. measured as RS^-/RO^- alkylation ratio. For the above mentioned compounds, this ratio decreases in the order MMS > EMS > iPMS.[11,3]

It is indicated that DNA phosphates are primarily alkylated at least in the case of EMS,[12] although the alkyls appear later on purine bases, which are slowly dissociated off.[13] In order to evaluate the importance of these events or later consequences to the DNA such as strand scission or cross-linking to the genetical effects, adequate physico-chemical methods are needed to measure the last-mentioned effects. In a few experiments we have studied the usefulness for such purposes of the "Type I" reversibility of heat denaturation of DNA described by Geiduschek:[14] a de-

creased reversibility of partial denaturation will indicate strand scission, whereas an induced reversibility of full denaturation, i.e. after complete strand separation, may indicate cross-linking.

Experimental. Chemicals. Calf thymus DNA (Type I) from Sigma, and commercial qualities of MMS and EMS from Eastman Organic Chemicals, and iPMS from Light and Koch were used.

Procedure. DNA (2.0 mM with respect to nucleotide P) was alkylated in 0.05−0.9 M $NaHCO_3$ at 25°. Concns., reaction times, and degrees of alkylation (alkyls per P calcd. from rate constants[16]) were as follows:

    MMS 0.03 M,   0.5−5 h,
      0.006−0.05 alkyls/P
    EMS 0.07−0.7 M, 12 h,
      0.07−0.53 alkyls/P
    iPMS 0.07−0.29 M, 7 h,
      0.1−0.4 alkyls/P

The reaction was rapidly interrupted by filtration through a 50 nm Millipore filter (VM). After washing, the DNA was dissolved in 1.4×10^{-3} M saline-citrate buffer pH 7.2.

Denaturation and (Type I) renaturation studies were undertaken according to Geiduschek[14] on alkylated DNA, immediately after alkylation or after 40−90 h incubation at 37°, using DNA solns. diluted 1:10 with 8 M $NaClO_4 + 10^{-3}$ M EDTA (pH 7). The thermostated spectrophotometer used was constructed in the Institute and will be described elsewhere.[16] In our experiments the same cuvette was alternatingly heated to different temperatures for the determination of denaturation and, after attaining absorbance equilibrium (after 15 min), cooled to 25°C, where absorbance, was measured again for the determination of the reversibility of the denaturation.

The results are exemplified in Figs. 1−2, which show effects of EMS treatment giving ca. 0.03 ethyls/P and iPMS treatment giving ca. 0.4 isopropyls/P, respectively. In the case of ethylation T_m (50% denaturation temperature) decreased from the control value, 43, to 41.5 and by incubation for 40−90 h further to 39.5°. The latter decrease may be provoked by depurination of the type described by Lawley and Brookes.[13] iPMS treatment gave rise to a slight decrease of T_m, which was not changed further by incubation at 37° (Fig. 2).

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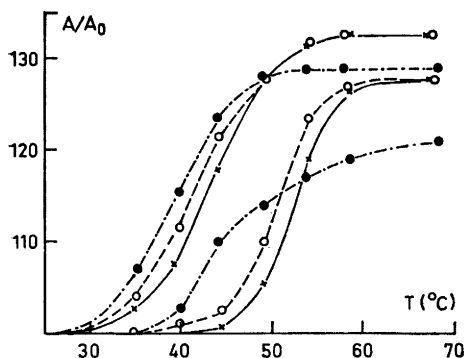
Fig. 1. Thermal denaturation (upper curves) and its reversibility (lower curves) of ethylated DNA (0.3 alkyls/P) immediately after alkylation (O) and after 90 h incubation at 37°C (●). The control DNA (×) was incubated for 12 h at 25°C. A/A_0 = absorbance at 254 nm relative to that of native DNA (100 %).

The incubation at 37° led in all cases to an increased irreversibility of partial heat denaturation, indicating decomposition by (one-strand) scission. The method cannot be used, however, for a simple assessment of this effect since the incubated samples, except in the case of iPMS, also revealed a reversion of fully denatured DNA. This
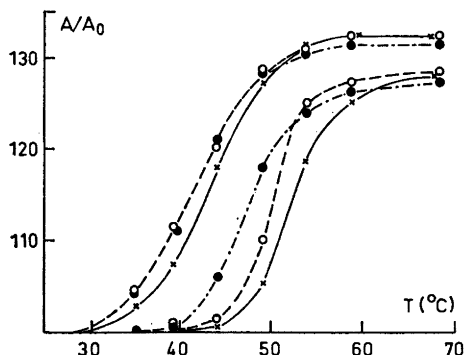

Fig. 2. Thermal denaturation (upper curves) and its reversibility (lower curves) of iso-propylated DNA (0.4 alkyls/P) immediately after alkylation (O) and after 90 h incubation at 37°C (●). The control (×) was incubated for 12 h at 25°C. A/A_0 = absorbance at 254 nm relative to that of native DNA (100 %).

reversibility may indicate a cross-linking of the strands, the effect being probably related to the cross-linking claimed to be caused by low pH depurination.[17] In contrast to the complete reversibility obtained with nitrogen mustard (3×10^{-4} M for 45 min at 25°, according to Geiduschek [14]), EMS and MMS do not seem to give more than partial reversibility, possibly due to simultaneous degradation of DNA. Other explanations for this type of reversibility, such as an unspecific aggregation of denatured DNA are at present not excluded.[12]

The mentioned effects are amplified by increased degree of alkylation and by post-treatment incubation. MMS is most effective in inducing all the effects, 3×10^{-2} methyls/P being equivalent to 7×10^{-2} ethyls/P or *ca.* 10×10^{-2} isopropyls/P.

The mutagenic effectiveness of iPMS is in reasonable agreement with the expectation from the alkylation rates.[3] Judging from the results of the present studies it seems therefore that the consequences of the DNA alkylation *per se* (by spectrophotometric data indicated to occur on the phosphates) and/or single strand scission, are sufficient causes of gene mutation in higher organisms.

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On the Ferromagnetism of CoS₂

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A neutron diffraction investigation of the ferromagnetic state of CoS_2 has been carried out in the course of a general study of the properties of transition metal chalcogenides and pnictides. CoS_2 crystallizes with the pyrite type structure [1-2] and its magnetic properties have previously been examined [3-7] by magnetic susceptibility and magnetization measurements.

The CoS_2 sample was prepared from a stoichiometric mixture of the components (99.999 % pure Co rod, Johnson, Matthey & Co., Ltd. and 99.999+ % pure S, American Smelting and Refining Co.) by heat treatment at 500°C for 7 days. After crushing, the sample was reannealed at 800, 700, and 600°C for 7 days, and at 400°C for 14 days and finally cooled slowly to room temperature over a period of 10 days. X-Ray diffraction data were obtained in a Guinier camera (80 mm diameter, monochromatized $CuK\alpha_1$-radiation ($\lambda = 1.54050$ Å), and KCl as internal standard) at room temperature. A General Electric powder diffractometer with cryostat attachment ($CuK\alpha$-radiation and diamond powder as internal standard) was used at liquid nitrogen temperature. Neutron diffraction data (neutron source JEEP I, $\lambda = 1.148$ Å) were collected at

liquid helium, liquid nitrogen, and room temperature using a vanadium sample holder.

The lattice constant $a = 5.528 \pm 0.005$ Å obtained at room temperature is in agreement with the previous lattice constant values.[1,2,7,8] Since room temperature is higher than the Curie temperature of $110-116°K$ [5-7] reported for CoS_2, the structural parameter x of the S atoms could be checked by neutron diffraction data in the paramagnetic state of the compound. (The pyrite type crystal structure with space group $Pa3$ places 4Co in (a) and 8S in (c).) Least-squares refinement gave $x = 0.389$ with $R = \sum |jF_o{}^2 - jF_c{}^2|/\sum jF_o{}^2 = 0.055$ (*cf.* Table 1). This x-value is identical with the value ($x = 0.389 \pm 0.001$) found in the X-ray diffraction study of Elliott.[2] The nuclear scattering lengths $b_{Co} = 0.28 \times 10^{-12}$ cm and $b_S = 0.31 \times 10^{-12}$ cm listed in *International Tables* [9] were used in these calculations. Roth [10] (see also van Laar [11]) has suggested the considerably smaller value $b_{Co} = 0.232 \times 10^{-12}$ cm. Least-squares refinement based on this b_{Co}-value was also tried, and gave the result $x = 0.392$ and $R = 0.081$. However, thermal vibrations of unreasonable magnitudes were found in the last case and this result together with the higher R-value indicate that the former set of parameters is more correct than the latter. $b_{Co} = 0.28 \times 10^{-12}$ cm was accordingly used throughout the remainder of this study.

The form factor for Co^{2+} given by Scatturin *et al.*[12] was used to calculate $F_{magn}{}^2$-values. The comparison of $jF_o{}^2$ and $jF_c{}^2$ at 4.2°K (Table 1) gave good agreement ($R = 0.040$) for a ferromagnetic arrangement of the moments with spin quantum number $S_{Co} = 0.5 \pm 0.1$. This value agrees within the accuracy stated with $\mu_p = 1.85$ B.M. observed for the paramagnetic state of CoS_2 by Néel and Benoit [5] and Benoit [6] whereas it differs somewhat from $\mu_p = 2.17$ B.M. recently reported by Heidelberg *et al.*[7]

The powder neutron diffraction technique leaves the direction of the moments undetermined within the cubic unit cell, *cf.* Shirane.[13] It should be mentioned, however, that a slight broadening of the peak profiles for some of the reflections has been observed at 4.2°K indicating a deformation of the cubic symmetry. A crystallographic distortion below T_c is quite probable, but attempts to verify a deformation at 80°K by X-ray diffraction experiments were unsuccessful. Further experiments will be carried out in order to resolve this ambiguity, and