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Effects of β-Hydroxyethylation and β-Methoxyethylation on DNA *in vitro*

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Yeast ribonucleic acid is known to be hydrolysed in alkaline solution to mononucleotides.[1] An alkali catalysed interaction between the vicinal 2-OH and the phosphoryl group will give a cyclic ester, which is hydrolysed by P—O fission.[2–4] Alkylated RNA has been found to be hydrolysed even in neutral solution. Kriek and Emmelot [5] observed a degradation of RNA after treatment with diazomethane (*cf.* also Brimacombe *et al.*[6] and Holy and Scheit [7]). The transformation of RNA phosphate diester groups to triester would make the molecule more disposed to hydrolysis as phosphate triesters are generally more labile than diesters.[8] As a parallel, it might be assumed that the labilization of DNA produced by alkylation of phosphate to triester would be considerably enhanced, if the alkyl was equipped with an OH-group in β-position. This would render DNA susceptible to alkaline hydrolysis through a mechanism similar to that of RNA hydrolysis. In order to test this hypothesis, DNA was alkylated *in vitro* with β-hydroxyethyl methanesulfonate [9] (HOEMS) and, for comparison, β-methoxyethyl methanesulfonate [10] (MOEMS). Observed changes of the secondary structure of the alkylated DNA are described in this paper.

Calf thymus DNA (Type I) (from Sigma Chemical Co.; 0.75 mg/ml) was alkylated (0.4 M alkylating agent) in phosphate buffer (0.4 M) at pH 7.0 for 16 h at 25°C. The DNA was separated from small molecules by chromatography on a Sephadex G-25 column. The DNA fraction was collected and concentrated in vacuum at room temperature. pH was adjusted to 7.0 and 8.5, respectively, with sodium carbonate. Samples of the concentrated DNA were incubated for different times at 37°C.

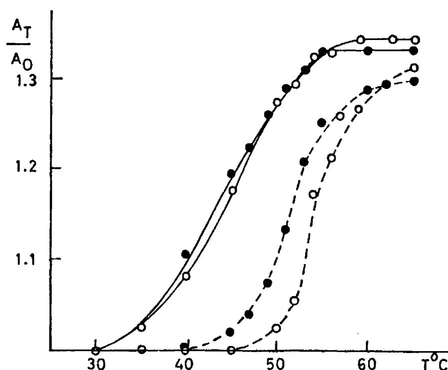

Fig. 1. Thermal denaturation and its irreversibility of HOEMS-treated calf thymus DNA. Absorbance at 254 mμ as a function of temperature immediately after treatment (○) and after 16 h of incubation at 37°C and pH 8.5 (●). The dashed curves illustrate the corresponding absorbance values measured after cooling from the different denaturation temperatures to 25°C.

Table 1. Irreversibility of DNA treated with MOEMS and HOEMS after incubation for different times at 37°C.

pH	Time (h) of incubation at 37°C	Irreversibility (%)		
		Control	MOEMS	HOEMS
	0	14	12	18
7.0	1	—	17	19
	4	15	15	20
	16	12	14	26
	0	14	14	18
8.5	1	15	20	22
	4	18	17	29
	16	13	12	30

Denaturation and reversibility (Type I) were studied according to Geiduschek.[11] The measurements were performed in a solution of 7.2 M $NaClO_4$ at pH 7.0. The denaturation and irreversibility curves were illustrated by plotting the ratio A_T/A_0 (A is absorbance at 254 mμ) as a function of T in °C (Fig. 1). At each temperature the transition fractions[12]

$$f_{t,d}\left(=\frac{A_{T,d}-A_0}{A_{max}}\right) \text{ and } f_{t,i}\left(=\frac{A_{T,25}-A_0}{A_{max}}\right)$$

were calculated. In these expressions A_0 is the absorbance at 25° of double stranded DNA; A_{max} the absorbance of completely denatured DNA (measured at temps. above 60°, *cf.* Fig. 1); $A_{T,d}$ the equilibrium absorbance of partially denatured DNA heated to temperature T; $A_{T,i}$ the absorbance after cooling to 25° of a sample heated to T. At complete irreversibility $f_{t,i}$ is equal to $f_{t,d}$ at all temperatures (the diagonal in the diagram, Fig. 2). The area limited by the curve $f_{t,i}=F$ ($f_{t,d}$) and the lines $f_{t,i}=0$ and $f_{t,d}=1$ was determined and used as a measure of the degree of irreversibility. Values for these areas, expressed as per cent of the triangle at complete irreversibility, are given in Table 1.

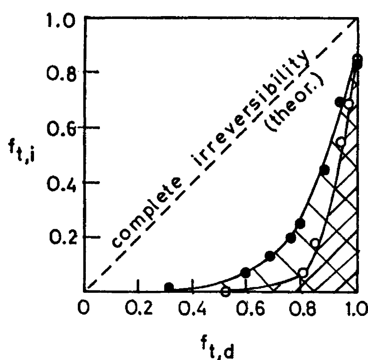

Fig. 2. "Irreversibility plot" of calf thymus DNA denaturation. The experiments are the same as in Fig. 1. The degree of irreversibility was measured by the areas (shadowed) under the experimental curves.

A significant increase of the irreversibility was observed after incubation of the β-hydroxyethylated DNA. This increase is more rapid at pH 8.5 than at pH 7.0. Treatment with β-methoxyethyl methanesulfonate does not give any remaining change of the irreversibility. These results support the hypothesis, that introduction of a β-hydroxyethyl group into the DNA molecule would lead to degradation of the molecule by single strand backbone breaks (*cf.* also Gichner *et al.*[13]). There was no indication of base alkylation according to UV spectra even at severe treatment (0.4 M HOEMS, 2 M phosphate buffer at pH 7.0 at 37°C for 50 h).

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Chelating Agents and Regulation of Lactate Dehydrogenase Synthesis

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Changes in lactate dehydrogenase (LDH) activity induced by exposure to chelating agents have been demonstrated in established cell strains, cultured *in vitro*.[1,2] In the cell strain used in the present study (Chang liver), addition of 2,2′-bipyridine or 1,10-phenanthroline to the culture medium, brought about an increased synthesis of LDH. Furthermore, the inducing action of the two compounds was enhanced by the addition of equimolar amounts of ferrous ions.[2]

In order to clarify the mechanism of the action of 2,2′-bipyridine and 1,10-phenanthroline on the regulation of LDH synthesis, it was considered necessary to establish the extent to which their chelating properties are essential for their ability to induce synthesis of LDH. Consequently, a number of structurally related compounds, some of them with no ability to chelate metal ions, were tested to ascertain their effect on LDH synthesis in Chang cells. Tests were also made, where the compounds were added together with equimolar amounts of ferrous ions, in order to investigate if the potentiating action of these ions could be correlated with the formation of a ferrous complex of the compound.

Experimental. Chang cells were grown in suspension cultures, continuously exposed to an atmosphere of 95% air and 5% carbon dioxide. The cultivation and the experiments were carried out in a manner similar to that used for 2,2′-bipyridine and 1,10-phenanthroline in a previous report [2] and the results should thus be directly comparable. 2,2′-Bipyridine, 4,4′-bipyridine, 1,10-phenanthroline, and ferrous ions were added to the cultures in a small volume of saline, the other compounds were dissolved in a small amount of ethanol. The control cultures received a corresponding amount of pure solvent. 2,2′-Bipyridine and 1,10-phenanthroline were obtained from Merck, Darmstadt, Germany, and the other compounds listed in Table 1 from Th. Schuchardt, Munich, Germany.

LDH activity was determined by the method of Stambaugh and Post,[3] and the nitrogen content of the homogenates by a micro-Kjeldahl method.

Results. Of the compounds with chelating abilities listed in Table 1, *i.e.* 2,2′-bipyridine, 1,10-phenanthroline, and its substituted derivatives, 5-nitro-1,10-phenanthroline was the most potent inducer of LDH synthesis, with a high activity in a concentration of 10^{-5} M. This compound was too toxic to be used in the same concentrations as the other compounds ($5\times10^{-5}-10^{-4}$ M). The action on LDH synthesis was similar to and in the same range as that obtained when the cells were exposed to 2,2′-bipyridine or 1,10-phenanthroline.[2] The effect of 5-nitro-1,10-phenanthroline was enhanced by the addition of ferrous ions. Cells exposed to 4,7-dimethyl-1,10-phenanthroline did not show any significant increase in LDH activity when the compound was used alone. However, upon addition of ferrous ions, this compound was able to stimulate the synthesis of LDH. In contrast to the other chelating compounds used, 2,9-dimethyl-1,10-phenanthroline (Neocuproine) failed to bring about an increased LDH synthesis, but caused a