ACTA CHEMICA SCANDINAVICA 20 (1966) 107–112

Effects of Diethyl Pyrocarbonate and Methyl Methanesulfonate on Nucleic Acids and Nucleases

I. FEDORCSÁK and L. EHRENBERG

Institute of Biochemistry, University of Stockholm, Stockholm, Sweden

Biological and biochemical effects of diethyl pyrocarbonate (DEP) and methyl methanesulfonate (MMS) were compared. Whereas most biological effects of MMS may be referred to damage of the gene material, DEP acts as an antimetabolite not provoking mutation and chromosomal aberrations primarily.

On the biochemical level DEP inhibits enzymes, a.o. RNase and DNase, without affecting nucleic acids. Contrarily, MMS leaves the RNase and DNase activities intact but provokes a decomposition of DNA (formation of acid soluble products).

The enzyme inhibiting properties of DEP will be used for the preparation of mRNA from tissue.

Diethyl pyrocarbonate (DEP) is a bactericidal agent which rather quickly (half-life 1.15 h at 20°C) decomposes in water to CO_2 and ethanol:

$$C_2H_5{-}O{-}CO{-}O{-}CO{-}O{-}C_2H_5 + H_2O \longrightarrow 2\ C_2H_5OH + 2\ CO_2$$

Several authors suggest the use of DEP for food preservation.[1] The mechanism of its bactericidal action is still unclear, although it seems that reactions with proteins play a primary role. In earlier experiments we thus found that DEP, although it possesses a phage inhibiting activity, does not alter the transforming properties of DNA.[2] A strong trypsin inhibiting activity was also found.[3]

In order to get additional viewpoints on the biological action mechanisms of DEP, and in order to evaluate its indicated[2,3] usefulness as an enzyme inhibitor in the preparation of nucleic acids, we have investigated the effects of the substance under comparable conditions on nucleic acids (RNA and DNA) and nucleases (RNase and DNase). The effects of DEP were compared with those of a mutagenic[4,5] alkylating agent, the methyl methanesulfonate (MMS). The latter compound is known to react with nucleic acids,[6] and also at comparable rates with proteins,[5] although the former effect seems more significant for the biological effects provoked by MMS.

For comparison with known data for MMS, the mutagenicity and chromosome breaking ability of DEP were tested in a higher organism, barley.

MATERIALS AND METHODS

The compounds (source in parentheses) used were: RNase, DNase (Sigma Chemical Company); RNA (Hefenucleinsäure, Merck); DNA (highly polymerised *ex* salmon sperm, Calbiochem); DEP ("Baycovin", Bayer); and MMS (Eastman Organic Chemicals).

The action of the compounds on chromosomes was studied in the first cell division following treatment of barley seeds. Feulgen stained squash preparations of the roots fixed in 3:1 acetic acid-ethanol were analysed at anaphase. Part of the treated seeds were sown in the field, and the chlorophyll mutation rate [7] was determined in the following generation.

The enzyme assays used were: The method of Anfinsen *et al.*[8] for the determination of RNase activity and slightly modified versions of this method for the test of RNase at pH 7.2 as well as for the determination of DNase. For details of assays see legends of tables. The DEP and MMS were used from 10 % alcoholic solutions freshly prepared. All experiments were run in duplicate, and in critical cases the limits of variation are given in the tables. Further details of methods are presented in the table heads.

RESULTS

1. *Biological effects of DEP and MMS.* Frequencies of chromosomal aberrations observed at the anaphase of the first mitosis after the onset of germination and frequencies of progenies from X_1 spikes segregating for chlorophyll mutation [7] after 5 h treatment of seeds at 20°C were investigated (*cf.* Table 1). In neither respect were genetical changes observed, significantly

Table 1. Biological effects in barley following treatment of resting seeds with DEP solutions at 20°C.

Concentration, mole/l	0.037	0.019	0.009	0.0047	0.0023	Control
Survival at maturity [a]; % (200 seeds sown)	11.5	37.5	62.5	83.0	68.5	83.0
Fertility of spikes of X_1 plants, % (*ca.* 100 spikes investigated)	70.5	93.8	96.3	96.7	—[b]	96.5
Chlorophyll mutations, number/ number of investigated spikes progenies	0/4	0/157	0/174	0/193	—[b]	1/149
Chromosomal aberrations, number/number of investigated anaphases	5/173[c]	—[b]	0/200	1/102	0/100	0/110

[a] The figures for survival are nearly identical with those for germination in the field.
[b] Not analysed.
[c] Later experiment, see Natarajan *et al.*[9]

Table 2. Action of DEP on RNase and RNA at pH 5. First incubation: In a final volume of 1 ml, 0.25—0.40 units of RNase or 4 mg of RNA were incubated in NaAc buffer at pH 5.0 and in the presence of DEP for 20 h at 4°C or 15 min at 20°C. (The shorter incubation could be used after it had been shown that the enzymatic reaction rests unaffected by possible reactions of DEP with RNA). The enzymatic reaction (second incubation) was started by adding the given amounts of RNA or RNase, respectively, in 0.25 ml aq. The enzyme reaction (15 min, 25°C) was stopped by the addition of 0.25 ml 0.75 % uranyl acetate in 25 % $HClO_4$. After centrifugation 0.1 ml supernatant was diluted with water to 3.0 ml and the OD at 260 mμ was determined against a corresponding blank without enzyme.

Conditions for reaction with DEP (first incubation)	% RNase activity after treatment with DEP conc. (mole/l): 0	0.0015	0.003	0.015	0.03
RNase treated for 20 h at 4°C	100		0		0
RNA treated for 20 h at 4°C	100				101 ± 3
RNase treated for 15 min at 20°C	100	60	44	0	0

Table 3. Effects of DEP and MMS on RNase, RNA, DNase, and DNA at pH 7.2. Conditions in general as in Table 2. The duration of the first incubation with DEP was 15 min at 20°C for the treatment of enzymes and 20 h at 20°C for the treatment of nucleic acids. MMS treatments lasted for 20 h at 20°C. 0.2 M phosphate (= P) or 0.2 M tris (= T) buffers were used giving a (final) pH = 7.2. Due to higher activity of RNase at pH 7.2 the 2nd incubation for the assay of this enzyme was restricted to 7 min. In the studies of DNase and DNA 2 μg enzyme or 1.8 mg DNA were first incubated with DEP or MMS in 1 ml tris buffer containing 0.01 M $MgSO_4$. The enzyme reaction was initiated by adding the given amounts of DNA or DNase, respectively, in 0.5 ml aq. After 5 min incubation at 25°C the reaction was stopped by the addition of 0.3 ml 25 % $HClO_4$. After centrifugation the OD of 0.1 ml supernatant diluted to 3.0 ml was then determined at 260 mμ.

	Treatment of RNase or DNase				Treatment of RNA or DNA		
Conc. of DEP (mole/l)	0	0.003	0.03	0	0	0.03	0
Conc. of MMS (mole/l)	0	0	0	0.045	0	0	0.045
RNase activity (% of control)	P:100 T:100	P:13 T:49	P:0 T:0	P:99 ± 2[a] —	P:100 T:100	P:102 T:99 ± 5	P:90 ± 2[a] —
DNase activity (% of control)	T:100	T:46	T:0	T:98	T:100	T:102	T:103

[a] Maximum variation in two different experiments.

higher than those in the untreated controls. A certain X_1 spike sterility remains unexplained; it may possibly be related to a production of haploid cells, which will be described in detail in a broader presentation of the biological action of DEP.[9]

Whereas the action of DEP on seeds is typical for an antimetabolite, possibly enzyme inhibitor, MMS gives, under the same conditions of treatment with 1—2 mM solutions, 25—100 % anaphases with chromosomal aberrations [10] and 10—20 % chlorophyll mutations.[4]

2. *Action of DEP and MMS on nucleases and nucleic acids.* The effects on RNase and RNA were first studied at pH 5 (Table 2), *i. e.* the pH originally used in Anfinsen's [8] method. Since biological media are more often neutral a second series of experiments was run at pH 7.2, using tris or phosphate buffers. This series comprised the action of the two compounds on DNase and DNA as well. The results of this series are presented, with some concentrations omitted, in Table 3.

From the two tables it may be concluded that DEP excerts a strong inhibiting action on both enzymes, whereas a possible action on the nucleic acids could not be detected as a reduced rate of enzymatic decomposition.

The inactivation of the enzyme is fairly rapid; see the time course of the inactivation of RNase at pH 5, 20°C (Fig. 1). It ought to be mentioned, too, that, within a broad range of concentrations, the relative inactivation of RNase provoked by DEP increases (or remains constant) with increasing RNase concentration. The presence of other proteins does not disturb the inactivation; thus, 0.03 molar DEP completely abolished the activity of RNase (0.4 units/ml) in the presence of 5 % blood albumin.

MMS, which was used at a definitely supratoxic concentration, was found to leave the enzyme activities completely intact. After treatment of the nucleic acids a significant reduction of the specific enzyme substrate function was found; this effect is too slight, however, to be used as a criterion on biological change of the RNA or DNA.

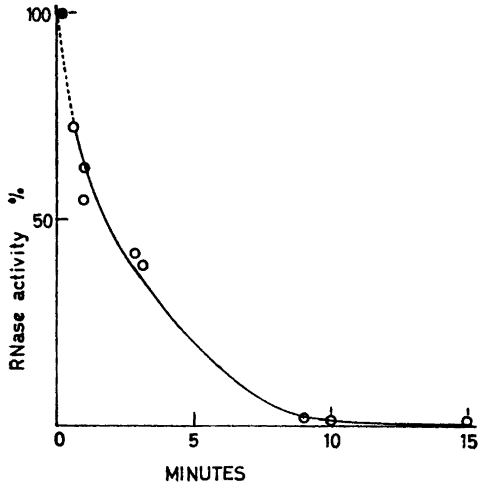

Fig. 1. The time-course of RNase inactivation by DEP at pH 5.0. Conditions are identical with those given in Table 2 except that the duration of the first incubation was varied at 20°C in the presence of 0.015 M DEP. [100 % RNase activity ● = the activity of RNase without DEP].

Table 4. Acid solubilization of DNA after treatment with DEP or MMS. 0.022 % DNA solution in 0.2 M phosphate, pH = 7.2, was incubated at 20°C for 20 h in the presence of DEP or MMS. Then the incubatum was dialyzed against 0.01 M NaCl for three days. One ml of the dialyzed sample was precipitated with 0.2 ml of 25 % $HClO_4$ and after centrifugation and suitable dilution the OD of the supernatant was measured at 260 mμ. Another 1.0 ml of dialyzed sample was heated at 100°C for 10 min, then cooled in ice and after the addition of 0.2 ml of 25 % $HClO_4$ the OD of the supernatant was measured in the same way as earlier. The DNA content of the supernatant was given as % of total amount of DNA present in supernatant plus precipitate.

Concentration of DEP (mole/l)	0	0.03	0
Concentration of MMS (mole/l)	0	0	0.045
% DNA present in supernatant before heating	5	5	5
% DNA present in supernatant after heating	5	5	28

For this reason a few additional experiments were run in order to measure the formation of acid soluble decomposition products from the nucleic acids. One experiment with DNA is presented in Table 4. It is demonstrated that MMS but not DEP is able to provoke changes in the DNA leading to the formation of acid soluble products when the DNA is heated. Similar results were obtained in experiments, where the dialysis was omitted (*cf.* table head) *i. e.* where $HClO_4$ precipitation was performed directly at the end of the treatment.

DISCUSSION

DEP and MMS have completely different spectra of biological effects. When tested on plant seeds DEP is lethal with only few or no chromosome aberrations and no mutation at sublethal concentrations, whereas MMS induces high frequencies of such genetical changes. It should be mentioned, however, that the criterion is not absolute; antimetabolites, *e. g.* affecting thymidylate synthesis, may very well give rise to chromosomal aberrations, but then always above a dose threshold.[11]

This difference seems to have a counterpart on the biochemical level, although the details of the reactions are still unknown. Whereas DEP is a strong enzyme inhibitor, as judged from its action on RNase and DNase, MMS was found completely inactive in this respect even at concentrations which are relatively high compared to the genetically active ones. On the other hand, of the two compounds only MMS was able to cause a solubilization in acids of DNA, indicating a decomposition of the nucleic acid.* The function on RNA

* In later experiments MMS and its higher homologues were found to affect the heat denaturation of DNA, whereas DEP was inactive in this respect (cooperation with S. Walles; data to be published).

and DNA as substrates to nucleases was not affected or (in the case of RNA, see Table 3) only slightly affected by any of the compounds, and could therefore not be used as a good criterion on chemical change of the nucleic acids. It is thus plausible that the lethal action of DEP is due to reactions with proteins (enzymes), with no genetical factors involved. As typical for an antimetabolite, the lethal effect of DEP may be described as a complete inhibition of germination, with practically normal growth of most surviving plants.[9] MMS is more "radiomimetic" in its action, with genetical causes of death, *i. e.* the seeds die some time after germination at intermediate concentrations. As earlier discussed for ethylene oxide and diepoxybutane, higher concentrations of MMS may be lethal through non-genetical changes.[12]

It is further strongly indicated that DEP may be used to destroy RNase and DNase, respectively, in the preparation of nucleic acids, especially messenger RNA, from biological materials.

Acknowledgement. The present investigation was supported financially by the *Swedish Agricultural Research Council.* Thanks are due to Drs. J. and M. Moutschen-Dahmen, Liège, for the determination of chromosomal aberrations and to Mrs. U. Lundqvist, Lund, for the analysis of mutation rates.

REFERENCES

1. Farbenfabriken Bayer AG. *Baycovin zur Kaltentkeimung alkoholfreier Getränke*, Ausgabe vom 1. Juli (1964).
2. Fedorcsák, I. and Turtóczky, I. *Nature.* **207** (1966) 830.
3. Hullan, L., Szontagh, T., Turtóczky, I. and Fedorcsák, I. *Acta Chem. Scand.* **19** (1965) 2440.
4. Ehrenberg, L. *Abh. Deut. Akad. Wiss. Berlin Kl. Med.* (1960) No. 1, 124.
5. Ehrenberg, L. *et al. Data to be published in Hereditas.*
6. Lawley, P. O. and Brookes, P. *Biochem. J.* **89** (1963) 127.
7. Gustafsson, Å. *Lunds Univ. Årsskr. N. F. Avd. 2* **36** (1940) No. 11.
8. Anfinsen, C. G., Redfield, R. R., Choate, W. L., Page, J. and Carrol, W. R. *J. Biol. Chem.* **207** (1954) 201.
9. Natarajan, A. T., Fedorcsák, I. and Ehrenberg, L. *Exptl. Cell. Res. In press.*
10. Moutschen, J. *Thesis* Univ. Liège (1964).
11. Taylor, J. H. *J. Cellular Comp. Physiol.* **62** *Suppl.* (1963) 73.
12. Ehrenberg, L. and Gustafsson, Å. *Hereditas* **43** (1957) 595.

Received September 28, 1965.