The Effect of Diethyl Pyrocarbonate on the Biological Activity of Messenger RNA and Transfer RNA

M. DENIĆ,* L. EHRENBERG, I. FEDORCSÁK and F. SOLYMOSY

Institute of Genetics, Eötvös L. University, Budapest, Hungary, and Wallenberg Laboratory, Stockholm University, Lilla Frescati, S-104 05 Stockholm, Sweden

Diethyl pyrocarbonate (DEP), the diethyl ester of oxydiformic acid, is known to react with, *e.g.*, sulfhydryl groups, primary and secondary amino groups, and phenolic groups. It decomposes rather quickly in water (half life *ca.* 1.15 h at 20°C) according to:

$$C_2H_5-O-CO-O-CO-O-C_2H_5+H_2O \rightarrow 2C_2H_5OH+2CO_2$$

In reactions of DEP with proteins the primary reaction products contain carbethoxy groups bound to sulfur, nitrogen, or oxygen. The carbethoxylation of these groups may explain the later conformational changes of the proteins, and the rapid loss of their biological activity which has been encountered in several cases. Several enzymes including deoxyribonuclease and ribonuclease are inactivated by DEP,[1-4] and the compound inactivates DNA phages [5] and RNA viruses like polio virus [6] evidently by denaturing protein components of these particles. However, in the case of tobacco mosaic virus (TMV) where the role played by the coat protein is rather a protective one than an active function in virus invasion, the virus particles are insensitive to DEP.[7]

It has been suggested by Fedorcsák and Ehrenberg [1] in 1966 to utilize the nuclease inhibitory effect of DEP in procedures for the extraction of undegraded nucleic acids from various sources. The experiments carried out in the last years in different laboratories have proved this idea to be correct: using DEP as a nuclease inhibitor high molecular RNA was extracted from plant tissues,[8,9] *e.g.* with the purpose of studying template activity [10] or polyribosome distribution.[11] The application of DEP was further extended to extract RNA from *E. coli* [12] and to extract tRNA with high biological activity from human placenta.[13] It is evident that, under the conditions of extraction, DEP does not interfere with the biological activity of RNA, as shown for the infectivity of TMV RNA [7] and the template activity of RNA fractions from barley embryos.[10] Further, double-strandedness has been found to protect nucleic acids from inactivation by DEP, as demonstrated for double-stranded polio virus RNA [6] and transforming DNA.[5]

On the basis of its general reactivity one should expect, however, that DEP is able to react with, *e.g.*, free amino groups of nucleotides contained in nucleic acids, and render the latter biologically inactive. In this paper we present some preliminary evidence that, under certain conditions, DEP is able to react with nucleic acids with a loss of biological activity in consequence.

Fig. 1 shows the losses of the template activity of TMV RNA and of the phenylalanine acceptor activity of tRNA caused by treatment with different concentrations of DEP. The acceptor activities of the tRNA for lysine and alanine showed practically the same sensitivity to DEP as that for phenylalanine, and the template activity of mRNA from other sources, *e.g.* barley embryos,[10] is inactivated by DEP at the same rate as that of TMV RNA. The protective action of protein present during the incubation with DEP (see Fig. 1) explains at least partly why the inactivation of RNA does not occur during extraction from tissue. In this respect the RNA inactivation differs from the inactivation of enzymes where the presence of other proteins gives no protection.[1]

In a control experiment we used DEP as a nuclease inhibitor, according to the method described earlier,[8] in order to isolate undegraded nucleic acid from *E. coli* cells. The extracted nucleic acids were fractionated by sucrose density gradient centrifugation, the tRNA fraction was collected, reprecipitated with ethanol, and tested in a cell free protein synthetizing system from *E. coli*. Neither the amino acid acceptor nor the adaptor activity (for phenylalanine, using TMV RNA or poly U as mRNA) exhibited any decrease compared to the untreated standard tRNA preparation.

Compared to untreated samples no considerable spectral changes are observed in bacterial tRNA inactivated by 40 mM DEP,

* Present address: INEP, Zemun, Yugoslavia.

although purine nucleotides (AMP and GMP) treated with DEP under similar conditions show pronounced changes with an increased absorbance below 240 mμ and decreased absorbance at 260 mμ.[14]

The results presented indicate so far that the successful use of DEP as a nuclease inhibitor in preparative work depends on a masking or protection of, *e.g.*, free amino groups of the nucleic acids by proteins or other cell components or by double-strandedness. In extraction procedures amounts of DEP exceeding its water solubility (approx. 30 mmol l^{-1} at 20°C) have been applied.[8] The occurrence of threshold concentrations below which the respective RNA remains functionally intact (see Fig. 1) may indicate that the reactions leading to inactivation may be favored by a lipophilic phase of DEP droplets present at the beginning of the incubation. In this respect, too, the inactivation of RNA differs from that of enzymes where no threshold concentration has been found.[1] The non-occurrence of RNA inactivation when DEP is used in extraction of nucleic acids from tissue may then have an additional cause in a faster dispersion of the agent in the water phase and to lipophilic regions, *e.g.* in proteins and lipids. A requirement for several carbethoxylations per RNA molecule for the loss of the transfer or template activity of DEP-treated RNA is an alternative explanation of the thresholds.

In current experiments information is collected about the specificity of the reactions of DEP leading to RNA inactivation. These reactions may offer a useful tool in nucleic acid chemistry.

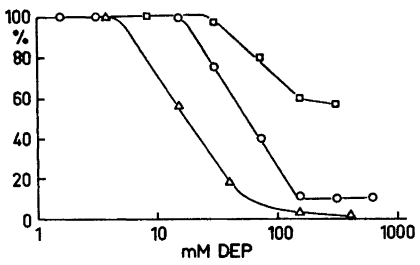

Fig. 1. The template activity of DEP treated TMV RNA (△), the phenylalanine acceptor activity of DEP treated tRNA (○), and the phenylalanine acceptor activity of tRNA treated with DEP in the presence of protein (□). 5 mg tRNA (tRNA *E. coli* B from Schwartz Bioresearch) or 3 mg TMV RNA (extracted from TMV, Strain *flavum*, according to Fraenkel-Conrat [15]) was dissolved in 1 ml of 0.05 M Tris-Cl buffer, pH=7.6, and was incubated with DEP ("Baycovin", from Bayer Ltd.) at 37°C for 1 h. Then the RNA was precipitated and washed with ethanol at 0°C and finally redissolved in 0.5 ml distilled water. In the case when tRNA was treated with DEP in the presence of protein, 50 mg RNA-free S-100 protein from *E. coli* B was added, and after the incubation with DEP the RNA was reextracted with water saturated phenol and then precipitated and washed with ethanol as described above. The assay for template activity was carried out as described earlier.[10] The amino acid acceptor activity was determined as described by Ehrenstein. [16] 100 % activity corresponds to the template activity or to the phenylalanine acceptor activity of the RNA sample treated in the same way except that DEP was omitted.

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Separation of Estriol Conjugates on Sephadex

M. J. TIKKANEN and H. ADLERCREUTZ

Department of Clinical Chemistry, University of Helsinki, Helsinki, Finland

Beling [1] used gel filtration on Sephadex G-25 to separate estrogen conjugates in late pregnancy urine into two major fractions, referred to as peaks I and II. This method eliminates most of the urinary constituents that would otherwise interfere with enzymatic hydrolysis,[1,2] and separation of estrogen 3-glucuronides from estrogen 16-glucuronides is achieved. However, sulfates and sulfoglucuronides cannot be separated from glucuronides by this procedure. Methods to subfractionate peaks I and II by reapplication on various hydrophilic Sephadex columns have been described.[3,4] A gel chromatographic system in which the Sephadex G-25 column was eluted with an organic solvent has been used in the separation of various peak I and II conjugates.[1,5-7]

The advantages of a lipophilic gel, Sephadex LH-20, for the purification and separation of neutral steroid conjugates [8-11] have been demonstrated but its possible merits in the separation of conjugated estrogens have seemingly been overlooked. Accordingly, we set out to explore the potentialities of Sephadex LH-20 for the separation of peak I and II conjugates. This communication describes preliminary studies on a method to separate four urinary estriol conjugates by sequential gel filtration on Sephadex G-25 and chromatography on Sephadex LH-20. A similar scheme can probably be used for the fractionation of a number of other urinary estrogen conjugates as well.

Methods. Sephadex G-25 and Sephadex LH-20 were purchased from Pharmacia AB, Uppsala, Sweden. Gel filtration of urine specimens on Sephadex G-25 columns was performed according to Beling.[1] When reference compounds were gel-filtered in the absence of urine, the samples were dissolved in 10 ml of 0.1 M phosphate buffer, pH 6.5, containing 8 mg of uric acid, which led to elution of peak I and II conjugates in the correct position.[3] Flow rate was maintained at 0.2 ml/min with a peristaltic pump, and fractions were collected automatically. The gel chromatographic procedures were modified from a method used in the separation of urinary neutral steroid conjugates.[11] Columns of 4 g of Sephadex LH-20 (300 × 10 mm) were prepared in chloroform/methanol 1:1 containing 0.01 mol/l sodium chloride giving a bed height of approximately 25 cm. The samples were applied in 2–3 ml of the eluting solvent, which consisted of chloroform/methanol mixtures containing 0.01 mol/l sodium chloride, or, when higher percentages of chloroform were used, the eluent was saturated with sodium chloride (Ref. 9). Solvent flow rate was about 0.5 ml/min and fractions were collected automatically. Radioactivity was measured in a Wallac NTL 314 liquid scintillation spectrometer.

Labeled reference compounds. Four labeled estriol conjugates, estriol-3-glucuronide (E_3–3Gl), estriol-3-sulfate, 16-glucuronide (E_3–3S, 16Gl), estriol-16-glucuronide (E_3–16Gl) and estriol-3-sulfate (E_3–3S), were prepared biosynthetically from estriol-6,7-^{3}H and estriol-4-^{14}C (New England Nuclear Corporation, Boston, Mass., U.S.A.). Gel filtration on Sephadex G-25 in the presence of uric acid (see above) proved to be a convenient method for the initial separation of estriol conjugates after the biosynthetic procedure. Labeled E_3–16Gl, synthesized according to Slaunwhite *et al.*,[12] was converted to E_3–3S,16Gl by incubating in a 100 000 *g* supernatant of homogenized guinea-pig liver in the presence of ATP and $MgSO_4$ (Levitz *et al.*[13]). Gel filtration on Sephadex G-25 in urate-phosphate buffer gave the synthesized E_3–3S,16Gl (50–90 % of the radioactivity) in the frac-