# THE USE OF DIETHYLPYROCARBONATE FOR SEQUENCING ADENINES AND GUANINES IN DNA

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## 1. Introduction

Gel sequencing methods have greatly facilitated studies of nucleic acids sequences. One subgroup of these methods is based on a specific chemical degradation approach [1,2]. Several chemical reactions of base modification, in addition to those initially suggested, have already been adapted for the purposes of sequencing [3–7], but the somewhat laborious procedure of chemical degradation justifies a search for new reactions that would simplify the technique without influencing its reliability. One candidate of this kind is the reaction of nucleic acids bases with diethylpyrocarbonate (diethoxyformic anhydride, DEPC). In aqueous solutions DEPC reacts with both purines to give carbethoxy-derivatives, that have their imidazole rings opened [8]. In [2] this reaction was used to sequence adenines in RNA. Here, I report chemical conditions in which this reaction can be used for sequencing adenines and/or guanines in DNA.

## 2. Experimental

### 2.1. *Materials*

Diethylpyrocarbonate was from E. Merck (FRG). Reagents for gel electrophoresis were from Reanal (Hungary). T4-polynucleotide kinase was isolated as in [9]. [$\gamma$-$^{32}$P]ATP, 2000 Ci/mmol was from the Radiochemical Centre Amersham (UK). All other reagents and buffer salts were of reagent grade unless specially indicated.

### 2.2. *Conventional procedures for DNA sequencing*

DNA sequencing was performed essentially according to a revised version of the Maxam-Gilbert method [10], with several exceptions, that are described below.

Unlabelled DNA fragments were eluted from 3–5% polyacrylamide gels by the procedure of [11], which employs DNA precipitation with cetyltrimethylammonium bromide to remove depolymerized acrylamide. After 5′-end labelling the unreacted ATP was removed by centrifugation of the reaction mixture through a 1 ml column of Sephadex G-50, as in [12]. The centrifuged-through solution was mixed with dyes and directly applied to polyacrylamide gel for fractionation or, otherwise, mixed with the restriction enzyme buffer (for secondary cleavage) or with an equal volume of dimethylsulfoxide (for strand separation). DNA fragments were visualized by autoradiography and eluted by the procedure of [11], with 15–20 $\mu$g carrier RNA.

Chemical reactions were those in [6,10], except that all volumes were reduced 2-fold and carrier DNA omitted. Cleavage products were fractionated on thin polyacrylamide-urea gels [13] and gels were exposed to preflashed medical X-ray films with intensifying screens at −70°C [14].

### 2.3. *Reactions of DNA with DEPC*

#### 2.3.1. A+G-specific cleavage

A portion of $^{32}$P-labelled DNA fragment was mixed on ice with 150 $\mu$l 50 mM sodium acetate buffer pH 5 (measured at 22°C), containing 1 mM EDTA; 2 $\mu$l of the 10% DEPC solution in ethanol (freshly prepared) were added, the tube sealed, vortexed for a few seconds and placed in a water bath at 90°C for 5 min. The reaction mixture was then briefly cooled, mixed with 15 $\mu$l 3 M sodium acetate buffer (pH 6) and 500 $\mu$l ethanol. The DNA was then precipitated, rinsed with ethanol, dried and treated with 1 M aqueous piperidine as in [10].

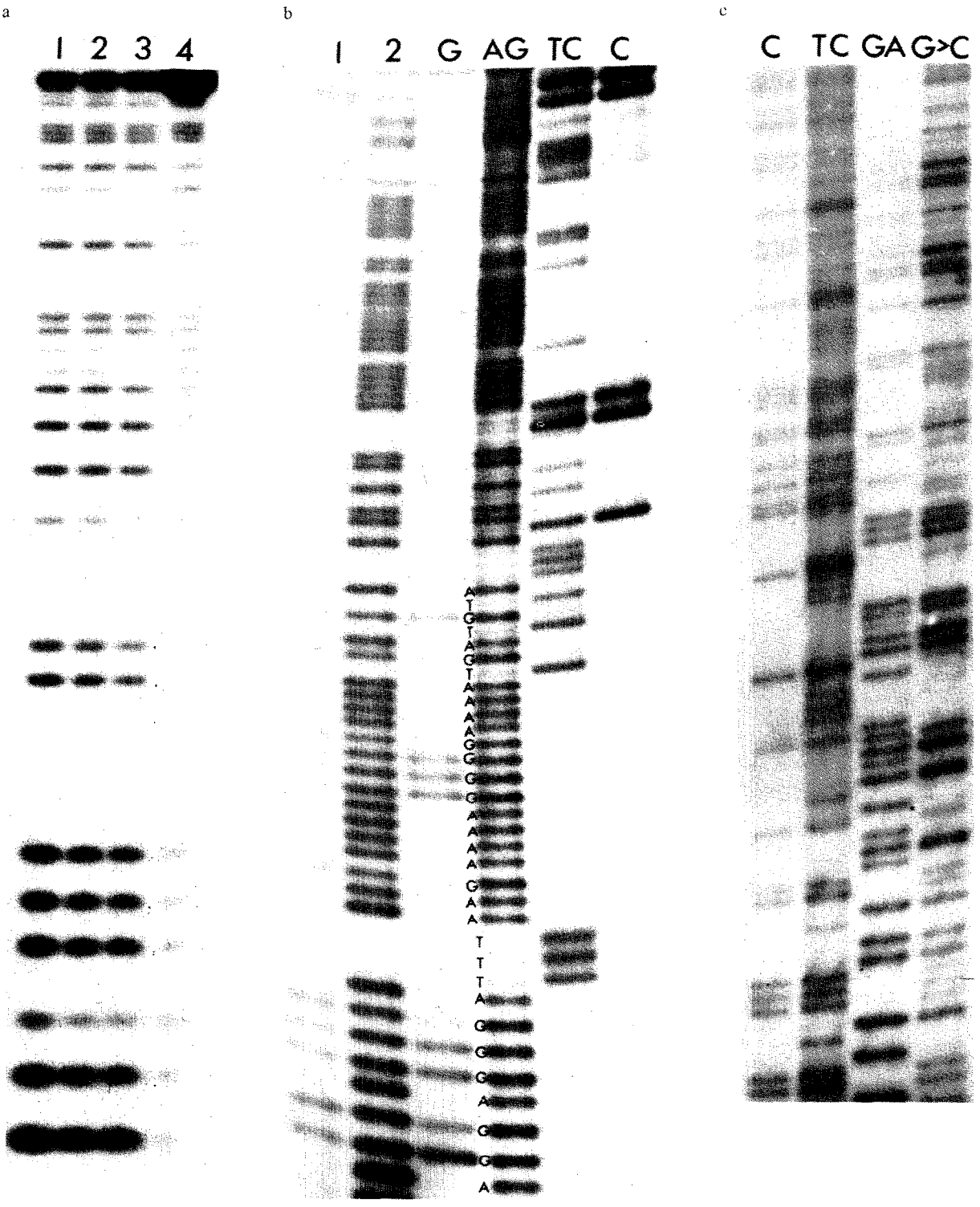


Fig. 1. Sequencing gels, displaying products of the base-specific cleavage of DNA after treatment with DEPC, fractionated alongside with the cleavage products, obtained with conventional chemical reactions: (a) lanes 1–3, DEPC-treated DNA, at pH 5 for 5, 10 and 30 min, respectively. Lane 4 is acid depurination according to [6]; (b) Lanes G, A+G, T+C and C, products of chemical cleavages according to [10]. Lanes 1 and 2, DEPC-treated DNA at pH8 and pH 5, respectively; (c) An example of a sequence ladder, obtained with two DEPC cleavages (designated G+A and G > C) and with hydrazine according to [10].

2.3.2. G > C-specific cleavage

All procedures were as in section 2.3.1., except that the buffer for treatment with DEPC was the DMS-buffer of Maxam and Gilbert [1], and 5 μl 10% ethanolic DEPC were added.

## 3. Results and discussion

Fig.1a shows the comparison of chemical cleavage products, obtained from the same labelled DNA fragment by acid depurination and by treatment with DEPC at pH 5. Obviously, the products are essentially the same in both cases, only some variation in the intensity of particular bands occurs in the DEPC case. As also evident from the picture, the extent of DNA cleavage with DEPC is virtually the same at incubation times >5 min. This result is easily explained by the well-known fact that DEPC readily decomposes at high temperatures. Therefore, the extent of cleavage can be regulated by changing initial DEPC concentration rather then the time of incubation. The conditions, specified in section 2 are appropriate for obtaining cleavage products with the lengths of 1–300 nucleotides.

Fig.1b displays the comparison of a complete conventional set of chemical cleavage products with the products of DEPC-cleaved fragment, at pH 5 and at pH 8. As evident from the picture, in the pH 8 reaction only cleavage at guanine is observed, which is accompanied with a weak cleavage at cytosines (faint bands, usually seen only on overexposure). The picture also shows an advantage that the pH 8 DEPC cleavage offers over the dimethylsulphate one – it gives bands of uniform intensity in runs of several guanine residues, while the dimethylsulphate reaction in the same case results in a series of bands, which intensity decreases in the $5' \rightarrow 3'$ direction, sometimes making identification of the last G's ambiguous.

Vincze et al. [8] have shown that the modification of nucleic acids bases with DEPC is almost independent of pH within values of 4–9. The difference in cleavage products, observed in reaction conditions, specified here, should then be attributed to the features of the base removal and/or strand scission step, rather than the features of the modification reaction. The difference, in fact, could be explained by the assumption, that the base removal step takes place during initial heating, after most of the DEPC has decomposed. The subsequent treatment with piperidine reveals this difference in the base removal rate, which is pH-dependent for a given purine. Otherwise, some rearrangements take place during the heating step, that only at acidic pH make the carbethoxy-derivative of adenine vulnerable to piperidine cleavage. Though proof for this assumption has not been made, it is substantiated by the observation, that at pH between 5 and 7 cleavage of the G > A type is obtained, the A-bands intensity gradually decreasing while the pH value increases (not shown).

The temperature of the reaction was chosen to be 90°C for several reasons:

(i) The reaction rate at room temperature is too low to obtain cleavage times convenient for a sequencing procedure, and the initial DEPC concentration cannot be deliberately varied due to its limited solubility in aqueous solutions.

(ii) Preliminary experiments, performed at room temperature, resulted in cleavage patterns, that were inappropriate for the purpose of sequencing due to considerable variation in band intensity (not shown).

(iii) Fast DEPC decomposition at high temperature could be turned to advantage, for it required no special means for stopping the modification reaction.

The limited set of reaction conditions tested thus far gave results that make this reaction useful for sequencing adenines and guanines in DNA, by using the pH 5 and pH 8 cleavages in parallel. The sequencing gel in fig.1c shows, that the 2 cleavages can efficiently substitute the 2 conventional purine-specific cleavages. A fairly long electrophoretic run proves that DEPC chemistry apparently works well far from the labelled end.

Whether the reaction described in this paper would give rise to occasional mistakes when used for sequencing is not clear at present. Certainly only considerable experience will clarify this point. Nevertheless, its technical simplicity makes it a viable alternative to the conventional base-specific cleavages commonly used in the Maxam-Gilbert procedure.

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