Interaction of Diethylpyrocarbonate with Poliovirus Double-Stranded RNA

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SUMMARY

Diethylpyrocarbonate appears not to react with double-stranded nucleic acids at moderate temperatures, and has been used successfully in the preparation of biologically active double stranded RNA and DNA from bacterial and viral sources. Under conditions of elevated temperature, however, reaction with diethylpyrocarbonate renders poliovirus double-stranded RNA subsequently susceptible to digestion with ribonuclease. Exposure to diethylpyrocarbonate for 10 min at 90° followed by digestion with ribonuclease causes complete conversion to acid-soluble material; the same treatment in the absence of diethylpyrocarbonate causes no degradation of RNA.

INTRODUCTION

Diethyl pyrocarbonate (DEP)† is a reactive ester that forms carbethoxy derivatives of the amino group of proteins and nucleic acids. Reaction with DEP destroys the infectivity of poliovirus (1) and of T_4 bacteriophage (2). Since RNA obtained from inactivated poliovirus is still infectious, the loss of viral infectivity is presumably due to modification of the virus protein coat, preventing adsorption to host cell receptors (1). However, reaction with DEP also inactivates the infectivity of purified poliovirus single-stranded RNA (1) and purified TMV RNA (3, 4) and destroys the amino acid accepting activities of several purified tRNAs (5). Polyadenylic acid (poly A) binds one carbethoxy group per base, but the reactivity of poly G and poly C under non-denaturing conditions was significantly less; and poly U, without an amino group, did not react at all (6). The conformation of the RNA molecule is an important factor in the binding capacity; the double helix poly A-poly U is not carbe-

† Abbreviations: DEP, diethylpyrocarbonate
SSC, 0.15M NaCl, 0.015M Na citrate
NETS, 0.1M NaCl, 0.02M EDTA, 0.17 SDS 0.01M Tris HCl, pH 7.4

thoxylated, nor is native double-stranded adenovirus DNA (6).

The inability of double-stranded nucleic acids to react with DEP supports the observations that there was no affect of DEP treatment on the transforming activity of purified bacterial DNA (2), the infectivity of double-stranded poliovirus RNA (1), or the biological activity of protein-free transfectious DNA isolated from the Bacillus subtilis phage 3NT (7). Both the binding studies and the assays of functional activity suggest that DEP does not react with double-stranded DNA or RNA since the amino groups are hydrogen-bonded and unavailable for carbethoxylation.

In view of these properties of DEP and the reports that it is an effective inhibitor of nucleases (8) (and despite the reported inefficiency of DEP as a ribonuclease inhibitor in the presence of moderate amounts of RNA (9)), it is widely used in the preparation of biologically active double-stranded nucleic acids.

This study shows that under conditions of decreased stability of double-stranded RNA such as elevated temperatures well below the melting temperature, DEP reacts with and alters the properties of double-helical molecules, rendering them subsequently susceptible to ribonuclease digestion.

METHODS

Radioactive poliovirus double-stranded RNA was prepared as previously described (10) from infected HeLa cells grown in suspension culture in Eagle's minimum essential medium containing 10 μC/ml of [^{3}H] uridine. The procedure included phenol extraction and ETOH precipitation followed by extraction with 2M LiCl. The virus-specific double-stranded RNA in the LiCl-soluble fraction was purified from low molecular weight RNA by sucrose gradient velocity sedimentation.

RESULTS

Fig. 1 shows that viral double-stranded RNA becomes increasingly sensitive to digestion by pancreatic ribonuclease after treatment with 0.069M DEP at elevated temperatures. In

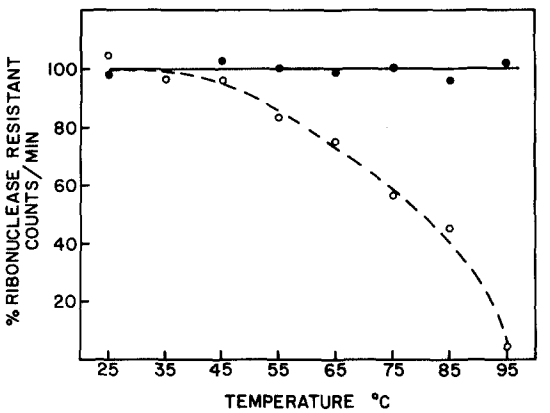

Fig. 1

Samples of poliovirus double-stranded RNA containing approximately 1200 counts/min were diluted to 2 ml in 0.1 x SSC (●——●) or in 0.1 x SSC containing 0.069 M DEP (generously supplied as Baycovin by Naftone, Inc. N.Y., N.Y. (o——o). The samples were heated to the indicated temperature for 10 min and plunged into a dry ice ETOH bath. All samples were thawed at 0°, the solutions were adjusted to 2XSSC and 20 μg of ribonuclease A (Worthington Biochemicals) was added. Samples were incubated for 15 min at 37° before precipitation with 5% CCl_3COOH in the presence of carrier bovine albumin. The acid-precipitable material was filtered on Whatman GF/C filters, washed with 5% TCA, dried and counted in a scintillation spectrometer in a triton-toluene scintillant.

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the absence of DEP, it can be seen that poliovirus double-stranded RNA remains totally resistant to ribonuclease digestion even after heating for 10 min to temperatures up to 95°C followed by immediate quenching in dry ice. This stability of poliovirus double-stranded RNA has been noted previously (11). In the same experiment, poliovirus single-stranded RNA was more than 95% sensitive to ribonuclease at room temperature in the presence or absence of DEP.

Fig. 2 shows a sucrose gradient analysis of the DEP-treated viral double-stranded RNA before and after ribonuclease digestion. At temperatures up to approximately 37°,

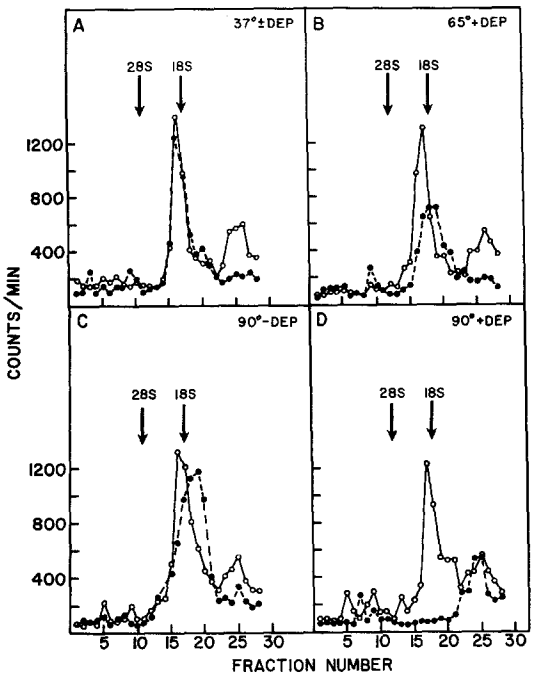

Fig. 2

Samples of poliovirus double-stranted RNA were diluted with 0.1 x SSC in the presence or absence of 0.069M DEP as described in the legend to Fig. 1. These were incubated for 10 min at 37°, 65° or 90°, as indicated, and immediately frozen in dry ice - ETOH. Samples were thawed at 0°, adjusted to 2 x SSC and half of each sample was treated with 10 μg/ml of ribonuclease for 15 min at 37°. Each sample was mixed with uninfected HeLa cell cytoplasmic RNA to serve as absorbance markers, and was layered on a linear 15-30% sucrose gradient prepared in NETS buffer. Centrifugation was for 17 hr at 23,000 rev/min, 23°, in the Spinco SW 27 rotor. The gradients were collected by pumping through a Gilford recording spectrophotometer to measure absorption at 260 nm to a fraction collector collecting 1-ml samples. Carrier albumin was added to each fraction, and they were precipitated with CCl_3COOH and counted as described.

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both treated and untreated molecules show no change in sedimentation properties after enzyme digestion (Fig. 2A). However, at 65°, although there is no apparent change in sedimentation rate resulting from treatment of the double-stranded RNA with DEP, it is

clear that the molecules are now rendered more susceptible to attack by ribonuclease (Fig. 2B). Treatment with DEP at 90° results in total nuclease sensitivity (Fig. 2D), in confirmation of the complete loss of CCl_3COOH precipitable radioactivity shown in Fig. 1. In contrast, as predicted by the resistance of the acid-precipitable radioactivity to ribonuclease after heating in the absence of DEP, Fig. 2C shows that double-stranded RNA remains fairly intact after nuclease digestion of samples heated to 90°, although there is a slight reduction in sedimentation rate and a skewing of the peak to the light side, probably indicating nicked or broken molecules.

We have also observed that in some experiments, treatment of RNA with DEP at elevated temperatures causes degradation of the molecules even in the absence of added ribonuclease, although contamination of the double-stranded RNA preparation with nuclease activity was not excluded.

DISCUSSION

Although the temperatures at which DEP causes poliovirus double-stranded RNA to become susceptible to ribonuclease attack are well below the melting temperature for this molecule, it is likely that local melting of regions of the double-strand makes the amino groups of nucleotides available for reaction with DEP. It thus appears from the data in Fig. 1 as if DEP may facilitate the denaturation of double-stranded RNA and cause an apparent lowering of its melting temperature. Probably this appearance is not real: double-stranded RNA has a strong tendency to renature spontaneously even when rapidly cooled after heating, and the reaction of DEP with denatured regions of the RNA would simply prevent this spontaneous renaturation.

It is not clear whether carbethoxylation resulting in adenosine ring opening (4) occurs in the reaction of DEP with RNA at elevated temperatures, or whether these modifications would be likely to render the RNA molecules more susceptible to thermal scission.

Extraction of nucleic acids from biological materials is frequently done at elevated temperatures to improve yields and facilitate deproteinization. Recognition of the demonstrable reaction of DEP with double-stranded nucleic acids is important when the reagent is used for nuclease inhibition in the preparation of biologically active RNA.

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