DIETHYL PYROCARBONATE DOES NOT DEGRADE RNA

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SUMMARY: Diethyl pyrocarbonate, a compound extensively used as a nuclease inhibitor, reacts with low molecular weight RNA and forms products which are less precipitable with certain precipitating reagents than untreated RNA. This phenomenon, erroneously interpreted by Wiegers and Hilz (Biochem. Biophys. Res. Commun. 44 (1971) 513) as degradation of RNA by diethyl pyrocarbonate, is now shown to be due to increased solubility of the treated low molecular weight RNA in precipitating reagents. This conclusion is based on (a) unaltered sedimentation pattern, (b) unchanged content in dialyzable fragments and (c) identical chain length of diethyl pyrocarbonate-treated RNA as compared to the untreated control.

INTRODUCTION: Diethyl pyrocarbonate ($(EtOCO)_2O$) a potent RNase inhibitor (1) has been introduced into nucleic acid biochemistry as an additive to media used for the extraction of undegraded nucleic acids (2). For almost a decade $(EtOCO)_2O$ has been found in a number of laboratories to be a useful reagent securing a nuclease-free milieu under a variety of experimental conditions and thus preventing nucleic acid breakdown (cf. 3). However, in the hands of Wiegers and Hilz (4,5) $(EtOCO)_2O$ acted as an "RNA degrading agent" which formed non-precipitable products ("acid-soluble nucleotides"!) in a time-dependent reaction with commercially available low molecular weight yeast RNA (4). In the present communication we show that $(EtOCO)_2O$-treated and untreated RNA molecules have an identical chain-length and that the formation of non-precipitable products is due to the increased solubility in various precipitating reagents of low molecular weight RNA after reaction with $(EtOCO)_2O$.

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MATERIALS AND METHODS: Low molecular weight RNA (Yeast nucleic acid, Cat. No. 4941) and high molecular weight RNA (rRNA from wheat germ, A grade, Cat. No. 557111) were purchased from Calbiochem, Los Angeles, Calif. $(EtOCO)_2O$ ("Baycovin") was a generous gift of Farbenfabriken, Bayer, Leverkusen. Inorganic chemicals were obtained from Merck AG, Darmstadt, all other chemicals and biochemicals from Sigma Chem. Co., Saint Louis, Mo. Methods are described in the legends to the Figures and Table.

RESULTS: The solubility of low molecular weight commercial RNA in an alcohol-containing precipitating reagent was found to increase with the time of exposure to a saturated solution of $(EtOCO)_2O$ (Fig. 1). A smaller increase in solubility was observed when an acidic precipitating reagent containing uranyl acetate was used (Fig. 1). The solubility of high molecular weight RNA treated with $(EtOCO)_2O$ in the same way as the low molecular weight RNA did not change in either of the precipitating reagents (Fig. 1). In order to work under the same conditions as those described by Wiegers and Hilz (4) the incubation medium used in the experiments presented in Fig. 1 contained both EDTA and $MgCl_2$. In control experiments the above components were included separately in the media and it was found that $MgCl_2$ did not influence, but EDTA increased, the solubility of low molecular weight RNA in the precipitating reagents.

The sedimentation pattern of low molecular weight RNA treated with $(EtOCO)_2O$ and showing an increased solubility in the precipitating reagents did not change as compared to that of the untreated control (Fig. 2).

Both $(EtOCO)_2O$-treated and untreated low molecular weight RNA samples were dialyzed against 0.1 M acetic acid (pH 3.0) to ensure denaturing conditions (6), for 40 hours. In order to estimate the amounts of degradation products of RNA the optical density of the acetic acid outside the dialysis bag was measured at 260 nm. As shown in Table 1 the RNA sample treated with $(EtOCO)_2O$ contained the same amount of dialyzable products as the untreated control.

Finally, to gain direct information about the size of low molecular weight RNA, the average chain-length was determined for both the $(EtOCO)_2O$-treated and the untreated samples. As shown in Fig. 3 the number of chain terminal phosphate groups was remarkably decreased in the $(EtOCO)_2O$-treated sample, indicating an "increased apparent chain-length" as a consequence of the reaction with $(EtOCO)_2O$. However, upon mild acid treatment (7) the number of chain terminal phosphate groups rapidly

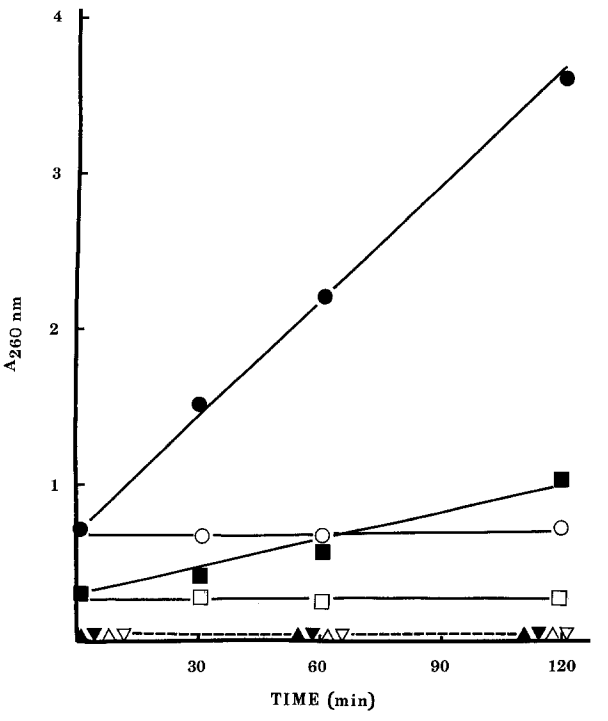

Fig. 1. Effect of $(EtOCO)_2O$ on the precipitability of RNA. Low molecular weight RNA and high molecular weight RNA were incubated without or with $(EtOCO)_2O$ (45 mg per ml) at 37°C at a concentration of 75 $A_{260\ nm}$ units (3mg) per ml in 10 mM tris (hydroxymethyl) aminomethane, pH 7.4, containing 7.5 mM ethylendiamine tetraacetate and 10 mM $MgCl_2$. At given times 0.3 ml samples were precipitated with 2.7 ml ice cold ethanolic precipitating reagent as described by Wiegers and Hilz (4), and after centrifugation the absorbance of the supernatant was measured at 260 nm. Simultaneously, at given times 0.5 ml samples were precipitated at 0°C with 0.1 ml ice cold acidic precipitatiny reagent (25 % $HClO_4$ containing 0.75 % uranyl acetate). After centrifugation 0.36 ml supernatant was diluted with 2.64 ml distilled water and the absorbance was measured at 260 nm. Low molecular weight RNA - ethanolic precipitating reagent (○——○), low molecular weight RNA + $(EtOCO)_2O$ - ethanolic precipitating reagent (●——●), low molecular weight RNA - acidic precipitating reagent (□——□), low molecular weight RNA + $(EtOCO)_2O$ - acidic precipitating reagent (■——■), high molecular weight RNA - ethanolic precipitating reagent (△——△), high molecular weight RNA + $(EtOCO)_2O$ - ethanolic precipitating reagent (▲——▲), high molecular weight RNA - acidic precipitating reagent (▽——▽), high molecular weight RNA + $(EtOCO)_2O$ - acidic precipitating reagent (▼——▼).

increased and within 40 minutes the ratio of total phosphate to chain terminal phosphate was identical in the $(EtOCO)_2O$-treated and non-treated samples.

DISCUSSION: In agreement with the finding of Wiegers and Hilz (4) our results

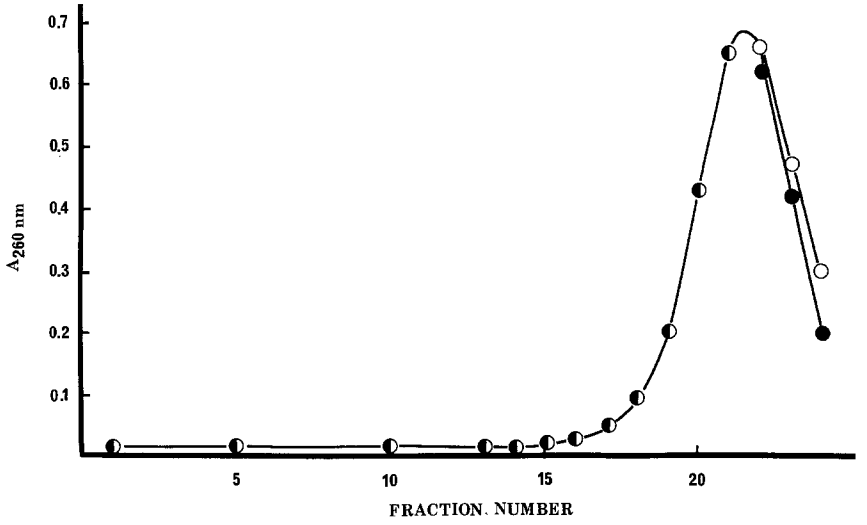

Fig. 2. Sedimentation patterns of low molecular weight RNA incubated with or without $(EtOCO)_2O$ for 2 hours as described in the legend to Fig. 1. At the end of the incubation 0.12 ml samples were layered on top of 5 to 20 % linear, nuclease free (2) sucrose gradients containing 0.05 M tris (hydroxymethyl) aminomethane, pH 7.4, plus 0.1 M NaCl and spun in the Spinco SW 39 rotor at 38000 rev./min for 7 hours. The tubes were then pierced and 0.2 ml fractions were collected. Each fraction was diluted with 1.3 ml distilled water and the absorbance was measured at 260 nm with a light path of 0.5 cm. Low molecular weight RNA incubated without $(EtOCO)_2O$ (o ——— o), low molecular weight RNA incubated with $(EtOCO)_2O$ (● ——— ●).

demonstrate that commercially available low molecular weight yeast RNA becomes less precipitable after treatment with $(EtOCO)_2O$ (Fig. 1). A decreased precipitability, however, does not necessarily mean degradation even if hydrolysis of nucleic acids, e.g., with nucleases or acid is often assayed by the appearence of non-precipitable products. Since no degradation products were detected by either ultracentrifugation or dialysis (Fig. 2 and Table 1) we concluded that the decreased precipitability was due to a reaction of $(EtOCO)_2O$ with low molecular weight RNA rather than to degradation. Carbethoxylation, however, is an expected reaction to occur (cf. 3) and carbethoxylated low molecular weight RNA may have an increased solubility in various precipitating reagents. This conclusion was further supported by data concerning the average chain-length of both the $(EtOCO)_2O$-treated and untreated low molecular weight RNAs. Degradation of RNA should show up as a decreased ratio of total to

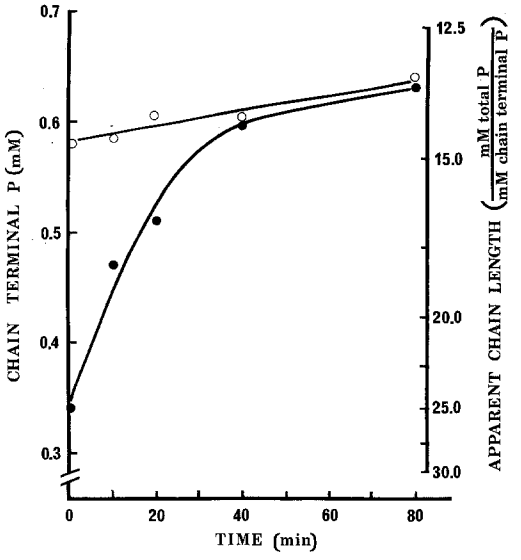

Fig. 3. Effect of mild acid treatment on the apparent chain length of $(EtOCO)_2O$-treated and untreated low molecular weight RNA. Low molecular weight RNA was incubated with or without $(EtOCO)_2O$ under the conditions described in the legend to Fig. 1. After incubation for 2 hours the samples were diluted 4 times with water, their pH adjusted to 1.8 with 0.1 N HCl (7) and incubated at 25°C. At the times indicated aliquots were withdrawn for total and chain terminal phosphorus determinations according to Seaman (8). The results are expressed as mM chain terminal phosphorus in the original untreated (o ——— o) or $(EtOCO)_2O$-treated (● ——— ●) sample containing 8.5 mM total phosphorus ($A_{260nm} = 75$).

chain terminal phosphate. Using this criterium, no degradation of RNA upon treatment with $(EtOCO)_2O$ was found. In fact, instead of a decrease we found an increase in the ratio of total to chain terminal phosphate indicating a reaction which rendered primary phosphate groups inaccessible for hydrolysis by phosphatase. The product of this reaction was found to be very labile in acidic solution: after 40 minutes incubation at 25°C and pH 1.8 (7) the number of chain terminal phosphate groups was found to be the same in the $(EtOCO)_2O$-treated and untreated RNA (Fig. 3) indicating an identical chain-length in both samples. It will be shown in a coming publication that the decrease in the detectable amounts of chain terminal phosphate is due to the formation of 2′ : 3′ cyclic phosphates in the $(EtOCO)_2O$-treated sample (9).

The above data demonstrate that the appearence of non-precipitable products

Table 1. Amounts of dialyzable fragments in untreated and $(EtOCO)_2O$-treated low molecular weight RNA.(x)

Sample	$A_{260\ nm}$	Volume ml	$A_{260\ nm} \times$ ml
Control, before dialysis	25.48	1.8	45.86
$(EtOCO)_2O$-treated, before dialysis	24.56	1.8	44.21
Control, after dialysis			
inside the dial. bag	19.48	2.0	38.96
outside the dial. bag	0.17	33.0	5.61
$(EtOCO)_2O$-treated, after dialysis			
inside the dial. bag	19.20	2.0	38.40
outside the dial. bag	0.17	33.0	5.61

(x) Low molecular weight RNA was incubated with or without $(EtOCO)_2O$ under the conditions described in the legend to Fig. 1. After incubation for 2 hours, 0.6 ml aliquots were taken, diluted with 1.2 ml 0.1 M acetic acid and dialyzed overnight against 33.2 ml 0.1 M acetic acid at 4°C. The absorbancy at 260 nm (1 cm light path) of the samples inside the bag and that of the acetic acid outside the bag was then measured.

in $(EtOCO)_2O$-treated low molecular weight RNA - the phenomenon which was interpreted by Wiegers and Hilz as degradation of RNA by $(EtOCO)_2O$ (4) - is related to the solubilization in the precipitating reagent rather than to the degradation of low molecular weight RNA reacted with $(EtOCO)_2O$. This agrees well with the results of Öberg (10,11) who demonstrated by polyacrylamide gel electrophoresis that the size of single-stranded poliovirus RNA remained the same after treatment with $(EtOCO)_2O$. Similar results were obtained by Oxelfelt and Årstrand (12) by comparing the sedimentation patterns of $(EtOCO)_2O$-treated and untreated tobacco mosaic virus RNA. The considerable loss of rapidly labelled RNA from $(EtOCO)_2O$-treated polysomes as described by Wiegers and Hilz (4,5) can be explained by the protein denaturing effect of $(EtOCO)_2O$ (13) rather than by assuming degradation of rapidly labelled RNA by $(EtOCO)_2O$. It is known that nucleic acids embedded in proteins denatured by $(EtOCO)_2O$ are less easily extractable (14).

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