

ON THE EFFICACY OF COMMONLY USED RIBONUCLEASE INHIBITORS

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SUMMARY

The ability of a number of commonly used inhibitors to inhibit pancreatic ribonuclease has been studied. At ribonuclease concentrations of 10 or 100 g/ml, heparin, polyvinylsulfate and proteinase K, at concentrations reported for their use in the literature, were ineffective in inhibiting RNase digestion of ^3H -uridine labelled RNA from Streptomyces antibioticus. In contrast, macaloid, diethylpyrocarbonate and sodium dodecyl sulfate were all effective inhibitors, with the degree of effectiveness decreasing in the order stated. Further, at inhibitor concentrations which allowed RNase conversion of only 50% of the labelled RNA to acid soluble products, a larger percentage of the acid insoluble digestion products sedimented in the "high molecular weight" range (4-16s) when macaloid was the inhibitor used than when diethylpyrocarbonate was the inhibitor.

In recent years, the increased interest in the properties of RNA's from prokaryotic and eukaryotic cells has accentuated the need for effective inhibitors of ribonuclease to use in RNA isolation. A number of presumed inhibitors have been used in various laboratories, including sodium dodecyl sulfate (SDS, ref. 1), diethylpyrocarbonate (DEPC, refs. 2, 3), polyvinylsulfate (PVS, refs. 4, 5), heparin (HEP, refs. 6, 7), macaloid (MAC, refs. 8, 9), and proteinase K (5, 10). These presumed inhibitors are frequently employed without any reference to their inhibitory ability and it is extremely difficult to determine the most effective inhibitor from the available literature. It was thus reasoned that the literature dealing with RNA isolation techniques would benefit from a direct comparison of these presumed inhibitors in terms of inhibitory activity. These studies are described below.

MATERIALS AND METHODS

Materials - SDS was obtained from BDH Chemicals Ltd., DEPC and HEP (135 Units/mg) from Sigma, PVS from General Biochemicals, Macaloid from NL Industries and proteinase K from Merck. ^3H -labelled RNA was prepared by incubating 12 hr old cultures of Streptomyces antibioticus with ^3H -uridine and extracting the RNA as previously described (11). Pancreatic RNase was from Calbiochem.

Methods - Assays for RNase activity were performed in 0.1 ml reaction mixtures containing: Tris-HCl, pH 7.6, 10 μmole ; ^3H -RNA, 1.93 A_{260} units (equivalent to 6000 acid precipitable counts per minute); RNase, 1 or 10 μg (10 or 100 $\mu\text{g}/\text{ml}$) and inhibitors as indicated in the legends to Figs. 1 and 2. In each experiment, the range of inhibitor concentrations was chosen so as to encompass the concentrations used in published RNA isolation experiments. Assay tubes were incubated for 5 min at 30° , when 10% trichloroacetic acid (TCA) was added to precipitate remaining RNA. RNA precipitates were collected on glass fibre filters which were dried and examined by liquid scintillation counting. Results in Fig. 1 are expressed as percentage of the original acid insoluble counts remaining in each assay tube. In the absence of inhibitor, each of the two RNase levels used was sufficient to completely solubilize the added RNA.

In some experiments, 0.3 ml incubations were performed as described above, containing 10 $\mu\text{g}/\text{ml}$ RNase, and either no inhibitor, DEPC (2.5%) or MAC (0.5 mg/ml). At the end of the incubation, the RNA remaining was extracted (11), combined with 10 A_{260} units of Escherichia coli tRNA and precipitated with ethanol. RNA thus obtained was analyzed via sucrose gradient centrifugation as described previously (12).

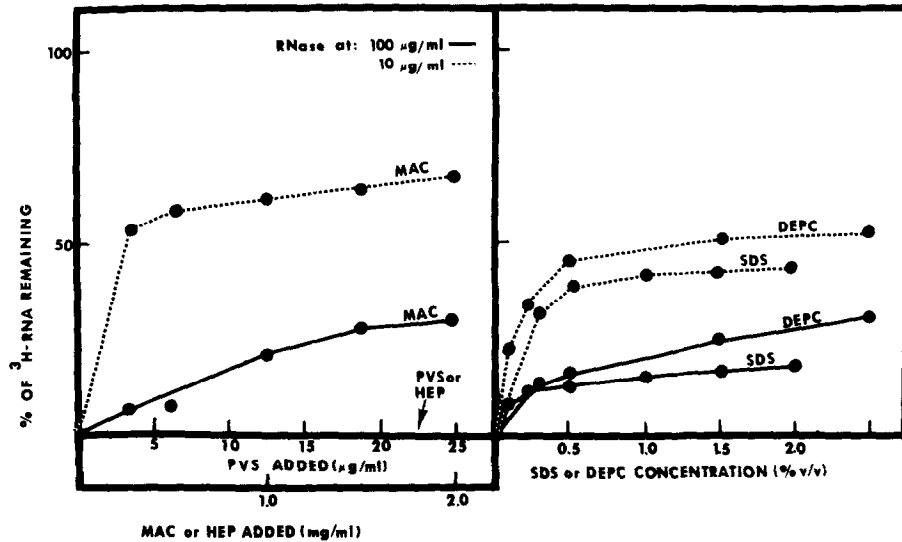


Fig. 1 - Abilities of various inhibitors to prevent solubilization of ³H-RNA by pancreatic RNase. Assay conditions were as described in Materials and Methods and inhibitors were present at the concentrations indicated. Results are expressed as the percentage of the acid insoluble RNA remaining after the incubation.

RESULTS

The data of Fig. 1 depict the ability of five of the substances tested to inhibit pancreatic RNase present at 10 or 100 µg/ml. It can be seen that PVS and HEP were completely ineffective at RNase inhibition at the concentrations used. In experiments not shown, it was also observed that proteinase K was an ineffective inhibitor under the assay conditions employed here. In contrast, SDS, DEPC and MAC were all effective inhibitors at some concentration. At 100 µg/ml RNase, the presence of DEPC and MAC prevented the solubilization of about 30% of the added RNA while SDS prevented the solubilization of about 20% of the added RNA. At 10 µg/ml RNase, SDS, DEPC and MAC prevented solubilization of 43, 49 and 67% of the added RNA, respectively.

The data presented above suggested that DEPC and MAC were the most effective of the RNase inhibitors tested. However, the assay used mea-

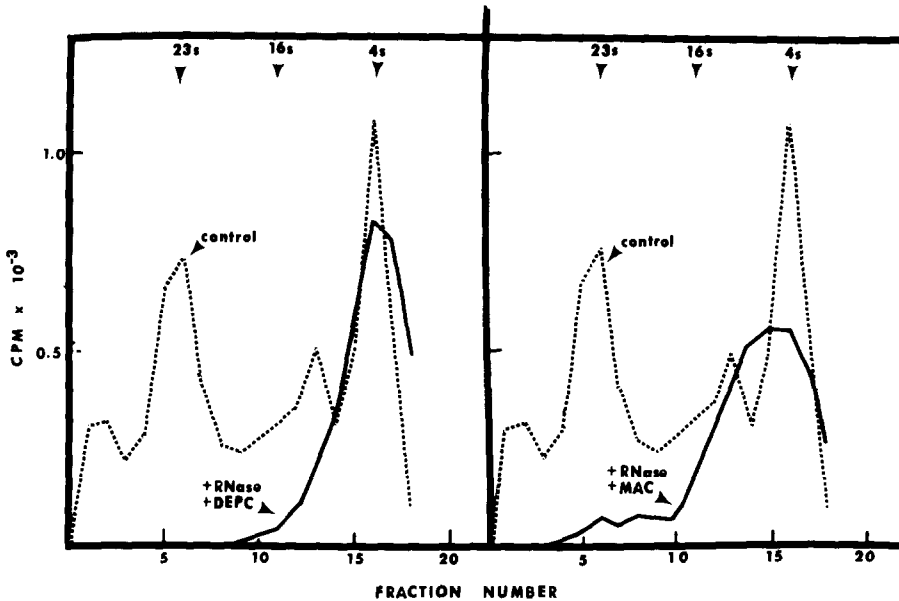


Fig. 2. - Sucrose gradient analysis of the RNA remaining after incubation of ^3H -RNA with 10 g/ml RNase in the presence or absence of DEPC or MAC. See text for details of the incubation and RNA extraction. Gradients (5-20%) were centrifuged for 13.5 hr at 36,000 rpm and were collected and assayed as described previously (12). Radioactivity values have been corrected for background and the direction of sedimentation is from right to left. The positions of 23s, 16s and 4s RNA's were determined using marker RNA prepared from *E. coli* cells and run on a parallel gradient.

sured only the ability of each inhibitor to prevent conversion of the added RNA to an acid soluble form. It seemed possible that an inhibitor might prevent complete solubilization of the added RNA, but still allow significant conversion of higher molecular weight RNA to lower molecular weight RNA which was still insoluble in 10% TCA. To examine this possibility, three large scale (0.3 ml) RNase assay mixtures were prepared as described in Materials and Methods containing no inhibitor, 2.5% DEPC or 0.5 mg/ml MAC, respectively, and 10 $\mu\text{g}/\text{ml}$ RNase. The RNA was extracted after the incubation and analyzed by sucrose gradient centrifugation as described in Fig. 2. In the incubations described, the concentrations of DEPC and MAC were chosen so as to produce about equal levels of protection against RNase, and in each case, about 50%

of the RNA was recovered as compared with the control. In addition, the recovery of TCA precipitable RNA from the sucrose gradients was quite similar for the incubations containing DEPC and MAC (3500 cpm and 3700 cpm, respectively). It is evident from Fig. 2 that, although both DEPC and MAC prevented complete solubilization of about 50% of the added RNA, much more of the high molecular weight RNA was converted to lower molecular weight RNA in the presence of DEPC than in the presence of MAC. For example, if one calculates the percentage of the total RNA recovered in gradient fractions 1-13, one observes that only 5% of the RNA from the incubation containing DEPC sediments in this region. In contrast, 37% of the RNA from the incubation containing MAC is recovered in tubes 1-13. Thus, DEPC is somewhat less effective than MAC in preventing conversion of higher to lower molecular weight RNA by RNase.

DISCUSSION

In summary, the data presented indicate that SDS, DEPC and MAC are all capable of inhibiting RNase at appropriate concentrations. It should be noted, however, that under the assay conditions employed, the concentrations of each inhibitor required to produce maximal inhibition were higher than the concentrations commonly employed in RNA isolation experiments (1, 4, 9). This observation may lead to difficulties when SDS and DEPC are employed for RNA isolation, since high concentrations of SDS might necessitate repeated ethanol precipitation of the RNA for its removal, and since DEPC has been reported to react with RNA (13). Macaloid, on the other hand, is a relatively inert acidic clay, whose presence in isolation mixtures apparently does not interfere with the preparation of RNA. In addition, it seems possible that MAC could be included as an RNase inhibitor in solutions of purified RNA, and could be removed by centrifugation prior to the use of the RNA in biological assays. Although they are soluble in aqueous

solutions, the properties of SDS and DEPC would require their being absent from RNA solutions to be used in biological assays. These considerations, coupled with the observation that MAC is more effective than DEPC in preventing conversion of higher to lower molecular weight RNA (Fig. 2) would seem to make macaloid the inhibitor of choice in RNA isolation procedures.

As regards the other presumed inhibitors tested, PVS, HEP and proteinase K, it may be argued that these substances would have been effective at lower RNase concentrations. Indeed, it seems reasonable to conclude that the RNase concentrations used in these experiments were higher than one would expect to observe in cellular extracts. However, it is clear that even at this high RNase concentration, three of the six inhibitors tested are quite effective. It seems reasonable to conclude that the same relative activity would be manifested at lower RNase concentrations.

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