for the binding of DMBC to diol dehydratase, a direct effect on the binding of DMBC to β-lysine mutase seems less likely since potassium addition is most effective after the enzyme is activated rather than in the process during which DMBC is bound.

Unlike diol dehydratase (Toraya *et al.*, 1971), the relative effectiveness of the various monovalent cations in stimulating β-lysine mutase activity correlates poorly with their ionic radii. Although the relative activities of K^+, Rb^+, Na^+, and Cs^+ do correlate well there is appreciable deviation with Li^+ and NH_4^+. Li gives a higher value than expected. At low concentrations, ammonium ion also is more effective than would be predicted on the basis of its size. At high concentrations ammonium ion is inhibitory.

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Inhibition of Pancreatic Ribonuclease A by Arabinonucleotides†

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ABSTRACT: Changes in the carbohydrate, the base, or the phosphate component of nucleosidic or nucleotidic ligands to pancreatic ribonuclease A have been evaluated. The association constants with ribonuclease A of various compounds which can be obtained by combinations of uracil, cytosine, ribose, arabinose, and the phosphate group were determined. The hydrolysis of Cyd-2′:3′-P was studied spectrophotometrically at 25°, pH 7.0, at an ionic strength of 0.2. Kinetic rate constants were determined at three substrate concentrations and four inhibitor concentrations. In general, higher K values were obtained for the arabino derivatives than for the ribo derivatives, the highest being that of ara-Cyt-3′-P. These findings which can be interpreted primarily by geometric considerations add new emphasis to the importance of the 2′-hydroxyl group in the mechanism of RNase binding.

Knowledge of the tertiary crystalline structure of pancreatic ribonuclease (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967) has undoubtedly contributed to the continued interest in the mechanism of action of this enzyme. Inhibition studies have proven very useful to the understanding of how ligands bind to the active center. The possible inhibition of RNase action during the isolation of various RNAs also presents an important practical aspect of such studies. It was found that small inorganic anions, particularly phosphate and nitrate (Nelson *et al.*, 1962; Anderson *et al.*, 1968), exhibit a moderate binding to RNase by virtue of Coulombic interactions with the active site. More effective inhibitors are the polyanions such as polyphosphates (Anderson *et al.*, 1968) and polyglucose sulfate (Mora, 1962). Not unexpectedly, the best inhibitors of small molecular weight are the mononucleotidic end products of RNA hydrolysis. The association constants of these compounds with RNase have been determined by several authors (Anderson *et al.*, 1968; Barnard and Ramel, 1962; Harries *et al.*, 1962; Hummel and Dryer, 1962).

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The carbohydrate moiety is one factor which has some bearing on the energy of binding of nucleotides to RNase. Deoxyribonucleosides seem to bind slightly less than ribonucleosides, and xylonucleosides seem to be much more effective than ribonucleosides (Ukita *et al.*, 1961). A polynucleotide analog based on D-arabinose, poly(arabinouridylate), was found to be a potent inhibitor of RNA hydrolysis by Schramm and Ulmer-Schürnbrand (1967). Some oligoarabinouridylates obtained in our laboratory proved to be competitive inhibitors of the hydrolysis of Cyd-2′:3′-P[1] (Nagyvary *et al.*, 1968). The role of chain length was emphasized by both groups but no detailed investigation of the kinetics has yet been reported.

While oligo(arabinouridylates) do not seem to possess any biological activity, ara-Cyt and its mono- and dinucleotides (Wechter, 1967) have been shown to be active on DNA viruses (Renis *et al.*, 1967) and also effective in the treatment of human leukemia (Carey and Ellison, 1965). The cytotoxicity of ara-Cyt is believed to be a result of inhibition of ribonucleotide reductase by ara-Cyt-5′-PP (Chu and Fischer, 1962) or of DNA polymerase by ara-Cyt-5′-PPP (Cohen, 1966). However, it may be that even greater complexity is involved in the real biological action of ara-Cyt than has been recognized to date. It seemed to us worthwhile to investigate the inhibition of the RNase-catalyzed hydrolysis of Cyd-2′:3′-P by various arabinonucleotides.

Materials

Bovine pancreatic ribonuclease A (EC 2.7.7.6.), five-times recrystallized, salt free, grade A, was purchased from Calbiochem and used without further purification. Its concentration was measured spectrophotometrically at 278 nm using an extinction coefficient of 9800 cm^{-1} M^{-1} (Anderson *et al.*, 1968). Ultra pure Tris base was acquired from Schwarz-Mann. All other chemicals were reagent grade. All solutions were prepared in distilled, deionized water.

The nucleosides and nucleotides were checked for purity by spectral analysis, paper chromatography, and electrophoresis as described in Methods.

Cyd-2′:3′-P, Na salt, was either purchased from Sigma or prepared according to our modification of Michelson's method (Michelson, 1959). In this process, the cyclic phosphate is formed in pyridine–H_2O (9:1) following the addition of only 2 equiv of ethyl chloroformate. After repeated evaporations with dry pyridine, the pyridinium salt of Cyd-2′:3′-P was obtained (40% yield) in crystalline form, free of chlorides. The Na salt was prepared by adjusting the pH of an aqueous solution to 7.5, passing this solution through a Dowex 50 column (Na^+ form), and then freeze-drying. Purity was checked by electrophoresis using 10 OD_{268} units of material; the absence of detectable amounts of Cyd-2′(3′)-P in this test indicated higher than 98% purity. The following inhibitors were obtained from commercial sources: Cyd, Urd (Sigma); ara-Ura, L-ara-Ura, *cyclo*-ara-Cyt, ara-Cyt-5′-P, ara-Ura-5′-P (Terra-Marine), and Cyd-2′(3′)-P (Boehringer). The Cyd-2′-P and Cyd-3′-P were separated on a Dowex 1 column according to Hummel *et al.* (1961). Ara-Cyt, ara-Cyt-3′-P, *cyclo*-ara-Cyt-3′-P, aU-rU, and aU-aU-rU were synthesized by methods developed in this laboratory (Nagyvary, 1969; Nagyvary and Provenzale, 1969; Provenzale and Nagyvary, 1970). 2′:3′-*O*-Isopropylidene-O^2:5′-cyclouridine (Brown *et al.*, 1957) was prepared according to our improved method (Nagyvary, 1966).

Ara-Ura-3′-P was prepared as follows. Sodium nitrite (300 mg) and ara-Cyt-3′-P (100 mg) were dissolved in 1.0 ml of water, the pH was adjusted to 4.2 with glacial acetic acid, and the solution was incubated at 50° for 24 hr. The sample was freeze-dried, redissolved in water, and precipitated with barium acetate and ethanol. Pure ara-Ura-3′-P, sodium salt, was obtained by passing the barium salt through 10 ml of Dowex 50 (H^+) resin and neutralizing the solution with sodium hydroxide.

The concentrations of all nucleotides were determined spectrophotometrically in buffer pH 7.0 using the following extinction coefficient values: Cyd-2′:3′-P, ϵ_{268} 8400 (Anderson *et al.*, 1968); Cyd-2′- and -3′-P, ϵ_{260} 7600 (Anderson *et al.*, 1968); ara-Cyt and ara-Cyt-3′-P, ϵ_{272} 9200[2]; ara-Ura, ϵ_{262} 10,500 (Brown *et al.*, 1956); ara-Ura-3′-P, ϵ_{262} 10,000[2]; aU-rU, ϵ_{261} 18,600 (Nagyvary and Provenzale, 1969); aU-aU-rU, ϵ_{261} 28,000.[2]

Methods

Analytical. Descending paper chromatography was performed on Whatman No. 3MM paper using the following solvent systems: 1-butanol–water (86:14), 1-butanol–ethanol–0.5 M ammonium acetate, pH 7.0 (5:3:2), and 2-propanol–concentrated NH_4OH–water (7:1:2). Electrophoresis of nucleotides was carried out in a Savant flatplate apparatus in 0.05 M sodium phosphate buffers at pH 5.0 and 7.3, respectively. The ara- and ribonucleosides were separated in borate buffer according to Gordon *et al.* (1968).

A Gilford automatic recording photometer, Model 2000, equipped with thermosensor and offset control was used in all kinetic studies.

Inhibition Kinetics. The kinetics of the RNase-catalyzed hydrolysis of Cyd-3′:3′-P were studied spectrophotometrically using the method of Crook *et al.* (1960). The change in absorption was followed at 292 nm using a scale expansion of 0.1 OD/full scale, and a chart speed of 1 in./min. The temperature was maintained at 25 ± 0.1° using a thermoplated cell compartment.

The solvent was a buffer, pH 7.0, consisting of 0.05 M Tris-HCl, 0.05 M CH_3COONa, and 0.1 M NaCl. A Radiometer pH meter was used in all experiments to adjust the pH to 7.00 ± 0.03 using acetic acid or sodium hydroxide. The RNase concentration was 2×10^{-7} M for all experiments.

Kinetic rate constants were determined at three substrate concentrations and four inhibitor concentrations with each experiment being performed at least in duplicate. The initial velocities were obtained in all experiments with an estimated uncertainty of 5%. To determine the type of inhibition, the data were evaluated according to Lineweaver and Burk (1934). The method of Dixon (1953) was used to determine the association constants. The data were analyzed graphically and by the method of least squares. Inhibition constants could be determined with an estimated uncertainty of ±15%.

Results and Discussion

To validate the experimental procedure of the kinetic experiments several preliminary experiments were conducted.

[1] The abbreviations used for nucleotides follow the recommendations of the IUPAC-IUB commission as reported in *Biochemistry* 9, 4025 (1970). Other abbreviations: L-ara-Ura, 1-β-L-arabinofuranosyluracil; *cyclo*-Urd, 2′:3′-*O*-isopropylidene-O^2:5′-cyclouridine; *cyclo*-ara-Cyt, O^2:2′-cycloarabinosylcytosine; aU-rU and aU-aU-rU, dinucleoside phosphate and trinucleoside diphosphate containing ara-Ura and Urd.

[2] As determined in our laboratory.

The pH was checked after the addition of RNase to Cyd-2′:3′-P for 30 min and was found to be constant at pH 7.0. Further, the change in absorbance was followed for at least 10 min and it was found to be linear in the presence of inhibitor, indicating that no detectable product inhibition due to substrate hydrolysis had occurred under the conditions of the experiments. Substrate concentrations of 4×10^{-4}, 7×10^{-4}, and 1×10^{-3} M were found suitable. The inhibitors were employed at 5×10^{-4}, 7.5×10^{-4}, 1×10^{-3}, and 2×10^{-3} M. The data were analyzed by the method of Lineweaver and Burk (1934) and the Michaelis constant was calculated. This value varied from 3.2×10^{-3} M^{-1} to 3.5×10^{-3} M^{-1} for all preparations, which is in good agreement with published results (Herries *et al.*, 1962).

The Lineweaver–Burk analysis gave plots characteristic of competitive inhibition for all compounds. The K_I values of the inhibitors were determined by the method of Dixon (1953) and are listed in Table I. Inaccuracy was determined by repeating the inhibition experiments three separate times and calculating a K_I for each experiment. K_I values are given as association constants.

Note that our association constant of Cyd-3′-P at pH 7.0 of 1.77×10^3 M^{-1} compares favorably with the value of 1.61×10^3 M^{-1} determined by Anderson *et al.* (1968). For comparative purposes we have included several association constants determined by Anderson *et al.* (1968) in Table I.

Because of the possible physiological implications of RNase inhibition, all these studies were conducted at pH 7.0. It should be realized that a comparison of nucleotide inhibition at this pH does not reflect differences at optimum binding conditions, but it is likely that the relative order of association constants would be the same. The negative charge on the phosphomonoesters shows little variation, and it can be assumed that the differences in the association constants are the result of more complex factors which involve the whole nucleotide structure.

A vast amount of information that is available on the binding of inhibitors to RNase has been discussed and interpreted by others (Ukita *et al.*, 1961; Nelson *et al.*, 1968; Meadows *et al.*, 1969). Meadows *et al.* (1969) also provide structural models for the binding of various cytidine nucleotides to RNase which account for the order of the observed association constants: 2′-CMP > 3′-CMP > 5′-CMP. The uracil ring binds less strongly, and the binding of the various uracil-containing nucleotides is weaker than that of the corresponding cytosine nucleotides. The role of the carbohydrate moiety in the binding process is less clear at this time. For the nucleosides the following order of association was reported (Ukita *et al.*, 1961): deoxyribo < ribo < xylo; on the other hand, dUrd-3′-P binds stronger than Urd-3′-P at all tested pH values (Walz, 1971). It is believed that histidine 12 interacts with the carbohydrate moiety in some fashion, but nonpolar interactions are also possible.

Our kinetically determined association constants of nucleosides and nucleotides (Table I) are in agreement with the well-established pattern with regard to the kind of base and the position of phosphate. The most interesting result is the enhanced binding of arabinonucleotides compared to the corresponding ribonucleotides. The highest association constant was exhibited by ara-Cyt-3′-P, $K = 10{,}000$ M^{-1}, which is about five times greater than the value for Cyd-3′-P, as measured by Anderson *et al.* (1968) and by us. Thus, ara-Cyt-3′-P is a good mononucleotidic inhibitor of RNase, even better than Cyd-2′-P by a factor of 1.5. It is also remarkable that ara-Cyt approaches the inhibitory action of Cyd-3′-P although nucleosides are generally inferior to nucleotides. Similar results are found with uracil nucleotides where ara-Ura-3′-P is a better inhibitor than Urd-3′-P. As mentioned above, the 3′-phosphates are better inhibitors than the 5′-phosphates, an observation which is valid also for ara-Cyt-3′- and -5′-P.

TABLE I: Association Constants of Arabinonucleotides with RNase I.[a]

Compounds	K (M^{-1})
Urd	440
Urd-3′-P	2,270[b]
ara-Ura	130
L-ara-Ura	130
ara-Ura-3′-P	7,140
aU-rU	2,080
aU-aU-rU	2,900
Cyd	770
Cyd-3′-P	1,770 (1,610[b])
Cyd-2′-P	6,750[b]
ara-Cyt	1,420
ara-Cyt-3′-P	10,000
ara-Cyt-5′-P	1,540

[a] The hydrolysis of Cyd-2′:3′-P was studied spectrophotometrically according to Crook *et al.* (1960) at 25°, pH 7.0, at an ionic strength of 0.2. Kinetic rate constants were determined at three substrate concentrations and four inhibitor concentrations. The association constants were obtained by the method of Dixon (1953) with an estimated uncertainty of ±15%. [b] Anderson *et al.* (1968).

The extremely weak binding of the uracil moiety may explain that even the short oligonucleotides aU-rU and aU-aU-rU possess rather low K values, that of the latter being higher (Nagyvary *et al.*, 1968). *cyclo*-ara-Cyt, *cyclo*-ara-Cyt-3′-P, and *cyclo*-Urd did not inhibit the hydrolysis of Cyd-2′:3′-P up to a concentration of 2×10^{-2} M. These three compounds have one structural feature in common, *i.e.*, the cyclic isourea ether group in place of the C_2=O. In addition, the planes of the bases are fixed at a torsion angle which is perpendicular or opposite to the natural anti conformation. In this regard, it should be noted that O^2:5′-cyclouridine 2:3′-cyclic phosphate was found to be resistant to RNase (Nagyvary and Provenzale, 1971).

The recently determined X-ray diffraction structure of ara-Cyt shows a high steric barrier to rotation around the glycosidic linkage (Sherfinsky and Marsh, 1971). The optical rotatory dispersion (ORD) data of arabinonucleosides have previously been interpreted in terms of fixation in the anti conformation (Emerson *et al.*, 1967). We believe that this particular aspect of the geometry of arabinonucleotides is the main reason that accounts for the high association constants with RNase I. It can be visualized that a stiff base plane which has the proper conformation would lend itself better to both horizontal and vertical interactions than an oscillating base plane. It is apparent that the decrease of rotational freedom in the nucleosides, ribo > xylo > arabino, parallels the increase of their binding power to RNase (Ukita *et al.*, 1961). The geometrical peculiarity of ara-Cyt may also underlie the physiologically significant binding of its 5′-polyphosphates to other enzymes. However, data presented in Table I do not

substantiate our assumption that the mononucleotides could possess physiological significance by inhibiting RNase.

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