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NUCLEIC ACIDS—X.

ENZYMATIC HYDROLYSIS OF DINUCLEOSIDE PHOSPHATES CONTAINING *ARA*-CYTIDINE (CYTARABINE)

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Abstract—Soluble extracts of mouse liver, rabbit liver and rabbit kidney contain an alkaline phosphodiesterase which hydrolyzes the *p*-nitrophenyl ester of thymidine-5′-phosphate. The kidney and liver preparations have specific activities 30–150 times greater than similar enzyme preparations from KB human epidermoid carcinoma cells or L1210 mouse leukemia cells.

The mouse liver enzyme and rabbit kidney enzyme can hydrolyze 2′-5′ linked dinucleoside phosphates containing *ara*-cytidine. Dinucleoside phosphates linked 3′-5′ and 5′-5′ are also hydrolyzed by the kidney enzyme. The rate of hydrolysis is greater with 3′-5′ linked dinucleoside phosphates than with the 5′-5′ and 2′-5′ linked compounds. The rate of hydrolysis varies in parallel with the known antiviral activity of the compounds. The data on the enzymatic hydrolysis *in vitro* tend to support the hypothesis that the antiviral or cytotoxic action of the dinucleoside phosphates containing *ara*-cytidine is dependent on prior hydrolysis to *ara*-cytidine.

A SERIES of dinucleoside phosphates derived from the cytotoxic, antiviral and immunosuppressive nucleoside, *ara*-cytidine, have been synthesized in the hope of increasing the cellular selectivity of this antimetabolite (*ara*-C) either by alternative mechanisms of action or by alternative transport mechanisms.[1] Studies *in vivo* and *in vitro* on the antiviral and cytotoxic properties of these dinucleoside phosphates suggested that they owe their biological activity to hydrolysis and liberation of *ara*-C.[2,3] Direct evidence of hydrolysis by the target tissue used for the antiviral or cytotoxicity assays, which would prove that this was indeed their mode of action, has not been obtained to the present time. A single report of the hydrolysis of a 2′-5′ linked dinucleoside (rA 2′*p*5′rU)* by a mammalian enzyme has recently appeared which indicates that a rat liver phosphodiesterase will hydrolyze various oligonucleotides with a specificity much like that of snake venom phosphodiesterase.

The present report describes the hydrolysis of 2′-5′, 3′-5′ and 5′-5′ linked dinucleoside phosphates containing *ara*-C by extracts of rabbit kidney at pH 8·8. It has also

* The abbreviations used to describe the dinucleoside phosphates in this paper were first used by Richards *et al.*[4] to describe similar compounds. Nucleotides are abbreviated by two letters, a small letter denoting the sugar and a capital letter denoting the base. Thus aC is *ara*-C, rC is cytidine and dC is deoxyribocytidine. Three types of internucleotide linkages are abbreviated by placing appropriate numbers designating the carbons involved, before and after *p*, as: 2′*p*5′, 3′*p*5′ and 5′*p*5′. Other abbreviations used are MES for 2-(*N*-morpholino)-ethanesulfonic acid.

been demonstrated that KB cells contain an enzyme which hydrolyzes 3′-5′ linked dinucleoside phosphates at pH 6·0, but not at pH 8·8.

MATERIALS AND METHODS

Materials. Dinucleoside phosphates containing *ara*-C were prepared by Wechter.[1] Tritium labeled aC 3′*P*5′rA and aC 2′*P*5′rA were also prepared by Wechter.[5] Other dinucleoside phosphates were purchased from Zellstoffabrik Waldhof; *p*-nitrophenyl ester of thymidine-5′-phosphate was purchased from Calbiochem; and *p*-nitrophenyl ester of thymidine-3′-phosphate was obtained from W. J. Wechter.

Preparation of enzyme extracts. Extracts of mouse liver, rabbit liver and rabbit kidney were prepared as described by Futai and Mizuno.[6] Five g of fresh tissue was minced with scissors and suspended in 10 ml of 0·25 M sucrose. This suspension was homogenized by using a teflon pestle in a glass homogenizer. The homogenate was passed through one layer of silk gauze and centrifuged at 20,000 *g* for 20 min. The supernatant fluid was removed and the pellet suspended in 8 ml of 0·01 M Tris buffer pH 8·8. One-fifth volume of 1-butanol, previously cooled in dry ice–acetone, was added dropwise to the stirred pellet suspension. After addition of all the 1-butanol, the suspension was stirred for an additional hour. The suspension was centrifuged at 10,000 *g* for 20 min and the butanol layer, gel phase and pellet were discarded. The supernatant was dialyzed overnight against 650 ml of 0·01 M Tris buffer, pH 8·8, in the cold. The cloudy dialysate was centrifuged at 20,000 *g* for 20 min and the supernatant was used as the enzyme source. The solution can be stored frozen in 0·5 ml aliquots without loss of activity for at least 1 month.

Extracts of KB cells and L1210 cells were prepared in a variety of ways in order to obtain preparations active at pH 8·8. The most active enzyme preparations of KB cells were obtained by repeated freeze-thawing of a 50 per cent suspension (v/v) of KB cells in 0·001 M 2-mercaptoethanol. After four freeze-thaw cycles, the suspension was centrifuged at 12,000 *g* for 10 min and the supernatant liquid was frozen in 2-ml aliquots. A pH 5·0 extract was prepared from the above freeze-thaw supernatant by adding 0·1 vol. of 0·5 M sodium acetate, pH 5·0, and allowing the mixture to stand for 20 min. The precipitate was removed by centrifugation at 35,000 *g* for 15 min and the clear supernatant was used as pH 5·0 enzyme.

Extracts of L1210 cells were obtained by freeze-thaw as described above or alternatively by sonication followed by centrifugation at 100,000 *g* for 60 min. The supernatant was treated with a $\frac{1}{3}$ vol. of 0·5 per cent protamine sulfate and centrifuged at 30,000 *g* for 15 min to remove the precipitate. The supernatant was used as the enzyme source.

Protein was determined by the method of Lowry *et al.*[7] Descending paper chromatography was performed employing Whatman paper No. 44 or No. 2. Thin-layer chromatography was carried out on plates or sheets coated with microcrystalline cellulose (Brinkman or Eastman) by the ascending technique. The solvent systems used routinely were: isopropanol–concentrated NH_4OH–H_2O (7:1:2, v/v) (solvent A); or isobutyric acid–1 M NH_4OH–0·1 M Na_2EDTA (100:60:1·6) (solvent B). Chromatograms were scanned for radioactivity by using a Packard radioscanner and for 260 mμ absorbing materials by a Cary model 14 spectrophotometer with attachment for paper strips or visually by using a 254 mμ light source. The area under each peak on the scans was determined with a Technicon planimeter/calculator.

A variety of assays were used and each is detailed in the appropriate table or figure.

RESULTS

Since Smith *et al.*[3] showed that the dinucleoside phosphates containing *ara*-C were cytotoxic to KB human epidermal carcinoma cells, we chose this as our initial enzyme source. When freeze-thaw extracts of KB cells were incubated at pH 7·0 with rA3′*p*5′rA or aC3′*p*5′rA, considerable hydrolysis was observed. Hydrolysis was not detected at pH 8·0 or 8·5. The extent of hydrolysis of aC3′*p*5′rA (^{3}H) at various pH values is shown in Table 1. At all pH values run, aC2′*p*5′rA (^{3}H) was stable. Extracts of L1210 mouse leukemia cells, prepared like the KB extracts, also hydrolyzed aC3′*p*5′rA but not aC2′*p*5′rA at pH 6·0.

TABLE 1. EXTENT OF HYDROLYSIS OF aC3′*p*5′rA (^{3}H)*

pH of reaction mixture	% Hydrolyzed
9·0 (Tris)	0
8·5 (Tris)	3
7·0 (Tris)	50
6·5 (MES)†	73
6.0 (MES)	81
5·5 (MES)	70
5·0 (acetate)	80
4·5 (acetate)	47

* Each reaction mixture contained 2 μmoles $MgSO_4$, 5 μmoles buffer, 1·5 mg KB cell extract protein, 46 mμc aC3′p5′rA (^{3}H) in a volume of 120 μl. After 2·5 hr at 37°, 15 μl aliquots were chromatographed in solvent A on cellulose TLC plates. The per cent hydrolysis was determined from the area under each peak after scanning for radioactivity.

† 2-(*N*-morpholino)ethanesulfonic acid.

With rA3′*p*5′rA as substrate, the earliest products seen by paper chromatography in solvent A were inosine and adenosine monophosphate. The adenosine monophosphate slowly disappeared until only inosine was left after 5 hr of incubation. The presence of adenosine deaminase activity in the KB extracts was confirmed. The adenosine is rapidly deaminated while 3′-AMP and 5′-AMP were very slowly deaminated.

Additional evidence for the presence of an acid phosphodiesterase in these extracts was obtained by using the pH 5·0 KB enzyme. A variety of dinucleoside phosphates were incubated with the pH 5·0 KB enzyme and aliquots were examined for hydrolysis by thin layer chromatography in solvent A or by paper chromatography in solvent B. The dinucleoside phosphates, aC*p*rA, rA*p*rA, rA*p*rC, rG*p*rA, rA*p*rG and rG*p*rC linked 3′-5′ were all hydrolyzed, while aCr*p*A, rA*p*rC and aC*p*dA linked 2′-5′ or aC5′p5′rA were stable.

The pH 5·0 enzyme was incubated with DNA or with yeast RNA at pH 6·0 and hydrolysis followed by hyperchromicity at 260 mμ with a Gilford spectrophotometer. There was no change in the $O.D._{260\ m\mu}$ with either polymeric substrate. If the assay was changed so that release of acid-soluble nucleotides was measured, as is used for exonuclease, then hydrolysis of both DNA and RNA is detected which is proportional to enzyme concentration. The results with each substrate are shown in Fig. 1.

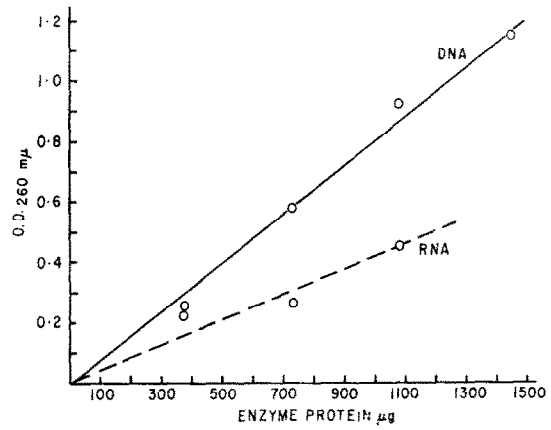

FIG. 1. Exonuclease activity of pH 5·0 KB enzyme. Each reaction mixture contained: 325 μmoles MES buffer, pH 6·0; salmon sperm DNA 0·14 mg, or yeast RNA, 0·15 mg; and the indicated amount of enzyme protein in a volume of 1·5 ml. After 10 min of incubation at 37°, 1 mg bovine serum albumin in 0·2 ml water and 0·5 ml of 2·5 N perchloric acid were added. The $O.D._{200}$ was determined on the supernatant liquid after centrifugation.

The hydrolytic activity of the pH 5·0 extract, with the synthetic *p*-nitrophenyl esters of thymidine 3′- or 5′-phosphate as substrates at pH 6·0 is shown in Table 2. The results indicate that only the 3′-ester is hydrolyzed by the pH 5·0 KB enzyme, thus indicating that the enzymatic activity measured was a 3′-exonuclease similar to spleen phosphodiesterase II.

TABLE 2. HYDROLYSIS OF *p*-NITROPHENYL ESTERS OF THYMIDINE PHOSPHATE*

Time (hr)	O.D. 400 mμ	
	Thymidine-3′-ester	Thymidine-5′-ester
0	0	0
1·0	0·087	0·019
1·5	0·169	0·005
12	0·931	0·020

* Incubation mixtures contained per 0·3 ml: 1·2 μmoles *p*-nitrophenyl ester; 50 μmoles MES buffer, pH 6·0; 300 μg pH 5·0 KB enzyme protein. 50-μl aliquots were removed and diluted in 1·0 ml of 0·1 M NaOH. Read O.D. $_{400m\mu}$.

At pH 9·0, there was no hydrolysis of the 3′-ester even in the freeze-thaw preparation containing 1·85 mg of KB cell protein. At pH 6·0, approximately 0·1 μmole/hr of the 3′-ester was hydrolyzed. Hydrolysis of the 5′-nitrophenyl ester is much slower but does occur at pH 9·0 with these extracts. The KB cell extracts, which did not hydrolyze aC3′*p*5′rA at pH 8·8, were able to hydrolyze polyadenylic acid. Anderson and Heppel[8] reported that mouse leukemia L4946 cells as well as L1210 and HeLa cells contain a phosphodiesterase active at a pH above 7, which hydrolyzes polyadenylic acid, but not *p*-nitrophenyl esters of nucleoside 5′-phosphates. They mention

in their paper that crude extracts of the L1210 cells do hydrolyze the *p*-nitrophenyl ester of thymidine-5′-phosphate.

Renis *et al.*[2] reported that the antiviral activity of the *ara*-C containing dinucleotide phosphates is dependent on the linkage. In general, the 3′-5′ linked compounds were the best viral inhibitors in the rabbit kidney monolayers. Kidney and liver have high levels of phosphodiesterase I.[9,10] Futai and Mizuno[6] reported that a phosphodiesterase from rat liver hydrolyzes *p*-nitrophenyl esters of nucleoside-5′- monophosphates and oligonucleotides, including a 2′-5′-dinucleoside phosphate. By using their method of enzyme purification, a phosphodiesterase from mouse liver, rabbit liver, and rabbit kidney was isolated. The mouse liver and rabbit kidney enzyme hydrolyzed aC2′*p*5′rA. In Table 3 are the specific activities of the various enzyme

TABLE 3. PHOSPHODIESTERASE I ACTIVITY IN VARIOUS CELL FRACTIONS*

Cell fraction	Specific activity (μmoleHydrolyzed/hr/mg protein)
L1210 sonicate 100,000 *g* pellet	0·12
L1210 protamine supernatant	1·57
KB cell supernatant 100,000 *g*	0·25
KB cell pellet	0·22
Mouse liver pellet	0·75
Mouse liver supernatant	0·13
Mouse liver soluble pellet enzyme	6·9
Rabbit liver pellet	0·70
Rabbit liver supernatant	0·54
Rabbit liver soluble pellet enzyme	4·1
Rabbit kidney pellet	2·56
Rabbit kidney supernatant	0·87
Rabbit kidney soluble pellet enzyme	22·0

* The assay is the same as that described in Table 3, with *p*-nitrophenyl ester of thymidine-5′-phosphate and Tris buffer pH 9·0.

preparations from kidney and liver as well as from KB and L1210 cells with *p*-nitrophenyl ester of thymidine-5′-phosphate as substrate. The specific activity of the phosphodiesterase is 30–150 times higher in the liver and kidney preparations than in the KB or L1210 preparations.

A homogenate of cells obtained from primary rabbit kidney monolayer cultures[2] after centrifugation was assayed for phosphodiesterase I activity using *p*-nitrophenyl ester of thymidine-5′-phosphate. Enzymatic activity was detected in both the 20,000 *g* supernatant and the resuspended pellet, with the bulk of the activity in the latter. The activity is about 1 μmole substrate hydrolyzed/hr/mg protein.

The hydrolysis of various *ara*-C containing dinucleoside phosphates was studied by using purified kidney enzyme. The initial rates for aC3′*p*5′rA, aC2′*p*5′rA and aC5′*p*5′rA are shown in Fig. 2. Thus aC3′*p*5′rA is hydrolyzed 2·5 to 3 times as fast as is the 2′-5′ linked compound. The K_m values for the hydrolysis of aC*p*rA are $2{\cdot}9 \times 10^{-4}$, 6×10^{-4} and 2×10^{-4}, respectively, for the 3′-5′, 2′-5′ and 5′-5′ linked dinucleoside while the K_m value for the methyl ester of *ara*-C 5′-phosphate was $2{\cdot}5 \times 10^{-3}$.

The phenyl ester of *ara*-C 5′-phosphate hydrolyzes too rapidly for determination of a K_m value. The poorest substrate among the 3′-5′ linked dinucleoside phosphates

was rA3′*p*5′aC, which hydrolyzed about as fast as did aC2′*p*5′rA. The poorest substrate for the kidney enzyme was aC2′*p*5′dU, which showed no hydrolysis in 20 min. These data compare favorably with the data of Richards *et al.*[4] for the same compounds using a highly purified exonuclease from venom. The results are also consistent with the antiviral activity in rabbit kidney monolayers,[2] where the aC3′*p*5′rA and aC5′*p*5′rA

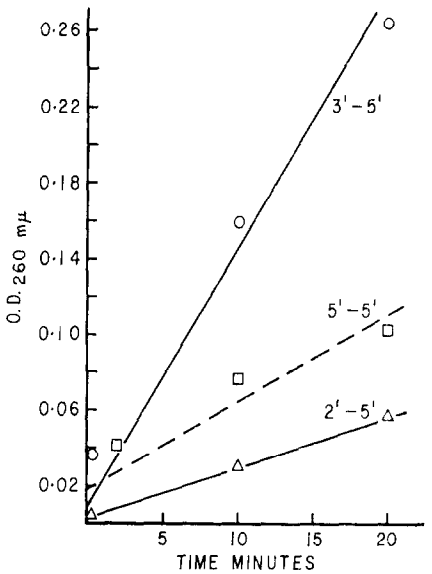

FIG. 2. Hydrolysis of *ara*-cytidine containing dinucleoside phosphates by rabbit kidney phosphodiesterase. Each incubation mixture contained: 5 μmole Tris buffer, pH 8·8; 1μmole $MgCl_2$; 0·3 μmole $CaCl_2$0 225 μg kidney phosphodiesterase protein; 0·28 μmole dinucleoside phosphate in final volume of 200 μl. Aliquots (50 μl) were removed and chromatographed in solvent A on Whatman No. 2 paper. O.D.$_{260}$ was determined by using a Cary spectrophotometer with attachment to scan paper strips.

are the most active and the aC2′*p*5′rA and rA3′*p*5′a C are much less active. The methyl ester of *ara*-C 5′-phosphate was inactive in this assay.*

DISCUSSION

In this paper evidence has been obtained for hydrolysis of *ara*-C dinucleoside phosphates by extracts of the target tissue used for the antiviral or cytotoxicity assay. Renis *et al.*[2] using inhibition of a DNA virus yield in rabbit kidney monolayers, indicated that the 3′-5′ linked *ara*-C dinucleoside phosphates were about 3 times as active as the corresponding 2′-5′ linked compounds. A phosphodiesterase active at pH 8·8 was purified from rabbit kidney, which hydrolyzes the *ara*-C containing dinucleoside phosphates. The rates of hydrolysis vary in parallel with the antiviral activity of the compounds seen in the kidney monolayers. The 3′-5′ compounds are most readily hydrolyzed, followed by the 5′-5′, and the 2′-5′ are the slowest. An exception is the rA3′*p*5′aC which hydrolyzes only as fast as the aC2′*p*5′rA. The

* H. A. Renis, personal communication.

phosphodiesterase activity was also present in the cells from kidney monolayer cultures. Razzell[9, 10] in a survey of mammalian tissues reported that two distinct phosphodiesterases can be found: a phosphodiesterase I, hydrolyzing *p*-nitrophenyl thymidine-5′-phosphate, active at pH 9·0; and a phosphodiesterase II, hydrolyzing the 3′-nitrophenyl ester, active at pH 6·0. The mouse liver enzyme and the kidney enzyme both hydrolyze 2′-5′ *ara*-C containing dinucleoside phosphates. Whether the enzyme in kidney is identical to that described by Futai and Mizuno[6] in liver or is a distinct enzyme has not been determined, but both tissues have active phosphodiesterase I activities.

Using a 3-day cytotoxicity assay, Smith *et al.*[3] have shown that aC3′*p*5′rA is 5 times as potent as is the corresponding aC2′*p*5′rA. G. L. Neil (unpublished data) has calculated presumed hydrolysis half-lives in KB cells by two different approaches, one from data on inhibition of DNA synthesis and the other from data on uptake of radioactivity from tritium-labeled dinucleoside phosphates. The calculations indicate that aC3′*p*5′rA appears to be hydrolyzed at a rate about 5 times faster than is aC2′*p*5′rA. The extracts of KB cells and L1210 cells contain a phosphodiesterase I activity as measured by hydrolysis of the *p*-nitrophenyl ester of thymidine-5′-phosphate. The enzyme is 30–150 times less active in comparison to the activity found in liver or kidney. This very low specific activity is the probable explanation for our inability to show hydrolysis of the 2′-5′ or 5′-5′ linked dinucleoside phosphates by KB cell or L1210 cell extracts. Hydrolysis of aC3′*p*5′rA was readily demonstrated with KB cell extracts at pH values below 7. Data obtained by using various substrates indicate that the enzyme activity at pH 6·0 is different from that activity at pH 8·0 and resembles the phosphodiesterase II from spleen, which hydrolyzes oligonucleotides to the nucleotide-3′-monophosphate level.

These results support the hypothesis that selective and differential rates of cleavage of the *ara*-C containing dinucleoside phosphates account for the differences in biological activity measured by cytotoxicity or antiviral effects. Wechter[1] indicated that the *ara*-C containing dinucleoside phosphates were synthesized in the hopes of increasing cellular selectivity of such compounds, either by alternative mechanisms of action or by alternative transport mechanisms. The work presented in this paper and the previous biological work would tend to rule out a mechanism of action of the dinucleoside phosphates different from that of *ara*-C alone. However, the dinucleoside phosphates have alternative hydrolysis (transport) mechanisms. From this work, it would appear that dinucleoside phosphates of *ara*-C or other cytotoxic nucleosides would be more selective in the cell type that they would affect depending on the level and type of phosphodiesterase (I or II) present, which could hydrolyze the compound and liberate the cytotoxic *ara*-C or *ara*-C phosphate. Thus, further study of the phosphodiesterases and other phosphatases of animal cells combined with selective synthesis of oligonucleotides of *ara*-C or other cytotoxic nucleosides may yield the desired cellular and biological specificity to their action.

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