PURIFICATION AND PROPERTIES OF A LEUKEMIC CELL PHOSPHODIESTERASE

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SUMMARY

A phosphodiesterase has been partially purified from extracts of mouse leukemic cells (L4946) carried as an ascitic tumor. Purified fractions resemble snake venom phosphodiesterase in forming nucleoside 5'-phosphates from RNA, from polymers synthesized with polynucleotide phosphorylase, and from oligonucleotides as small as a dinucleotide. As with venom, the cleavage appears to be stepwise by splitting off a single mononucleotide unit at a time from an end of the chain. However, in contrast to the venom enzyme, the leukemic cell phosphodiesterase is unable to hydrolyze simple benzyl or *p*-nitrophenyl esters of nucleoside 5'-phosphates. It thus belongs to a new class of phosphodiesterases with a specificity requiring that two nucleoside moieties must be in ester linkage to a phosphate group.

The rates of hydrolysis of different polynucleotides differ very widely.

INTRODUCTION

Of the phosphodiesterases which hydrolyze nucleic acids to nucleoside monophosphates, two have been very extensively studied; these are snake venom phosphodiesterase, which hydrolyzes polynucleotides to nucleoside 5'-phosphates, and spleen phosphodiesterase, which forms nucleoside 3'-phosphates. Both of these enzymes also hydrolyze simple esters of appropriate mononucleotides, such as benzyl adenosine 5'-phosphate in the case of the venom enzyme[1] or benzyl adenosine 3'-phosphate for spleen phosphodiesterase[2]. It appears, therefore, that substrates suitable for these enzymes need to contain only a single nucleoside moiety. The relevant literature has been recently reviewed[3].

We now wish to report on a phosphodiesterase partially purified from tumor cells which falls into a new classification. This enzyme hydrolyzes poly A*, poly AU, RNA

* The system of abbreviations used for oligonucleotides is described in "Use of Abbreviations" in current issues of the *Journal of Biological Chemistry* and in ref. 34. Poly A, poly U, poly C, poly G, poly AU, and poly AGUC are the polymers made with polynucleotide phosphorylase from the corresponding nucleoside 5'-diphosphates. Other abbreviations used are: RNA, ribonucleic acid; 5'-AMP, adenosine 5'-phosphate; 5'-UMP, uridine 5'-phosphate; 5'-IMP, inosine 5'-phosphate; ADP, adenosine diphosphate; UDP, uridine diphosphate; GDP, guanosine diphosphate; ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)-aminomethane-HCl buffer; EDTA, ethylenediaminetetraacetate; DEAE-cellulose, diethylaminoethylcellulose.

(very slowly) and oligonucleotides with a 5′-phosphomonoester end group, giving rise to nucleoside 5′-phosphates. Both ribonucleotides, such as pApA, pApApA and pUpU, and deoxyribonucleotides such as pTpT are hydrolyzed. In these respects, the specificity of the enzyme resembles that of venom phosphodiesterase. However, a difference is found in that simple esters such as benzyl adenosine 5′-phosphate and *p*-nitrophenyl thymidine 5′-phosphate are not split; a substrate must have at least two nucleoside residues.

EXPERIMENTAL

Materials

Poly A, poly U, poly AU, poly C and poly AGUC were synthesized by a published procedure[4] using polynucleotide phosphorylase from *Escherichia coli*[5] or *Azotobacter agilis*[4]. Samples of poly A made with the enzyme from *Micrococcus lysodeikticus*[6] were also used for many of the assays; these were kindly furnished by Dr. R. F. BEERS, Jr., Children's Hospital School, Baltimore, Maryland. Poly G was synthesized with the *A. agilis* enzyme, using pApApA as a primer[7]. Previously published methods were used for the isolation of pApA[8], pApApA[8], pApApApA[8], pApApApApA[8], pUpU[9], pUpUpU[9], ApUp[10], ApApUp[10], ApCp[11], ApA[8], and ApApA[8]. The tetranucleotides pApApApU and pApApApG were isolated after incubating UDP[12] or GDP[7] with pApApA and polynucleotide phosphorylase. The oligodeoxyribonucleotides, d-pTpT, d-pTpTpT, d-pTpTpTpT and d-pApA were chemically synthesized[13,14], and were kindly donated by Drs. G. TENER and H.G. KHORANA, British Columbia Research Council, Vancouver, B.C.

Mononucleotides with a 2′,3′-cyclic phosphoryl group, such as adenosine 2′,3′-phosphate were purchased from Schwarz Laboratories, Mt. Vernon, N. Y. Benzyl cytidine 3′-phosphate and benzyl adenosine 3′-phosphate were chemically synthesized[2,15]. Benzyl adenosine 5′-phosphate[1] was a gift from Dr. D. M. BROWN, The Chemical Laboratory, Cambridge, England. The *p*-nitrophenyl esters of thymidine 5′-phosphate[16], thymidine 3′-phosphate[17] and uridine 5′-phosphate[18] were gifts from Dr. H. G. KHORANA and Dr. W. E. RAZZELL. Highly polymerized RNA isolated from turnip yellow mosaic virus[19] was kindly supplied by Dr. R. MARKHAM, Plant Virus Unit, Agricultural Research Council, Cambridge, England. Other polymer substrates included yeast RNA[19], and bacterial RNA from *A. agilis* kindly furnished by Dr. R. M. S. SMELLIE, Department of Biochemistry, University of Glasgow, Glasgow, Scotland. Purified 5′-nucleotidase was prepared from bull seminal plasma[21]; it was free of phosphodiesterase activity. DEAE-cellulose was purchased from Eastman Kodak Company, Rochester, N.Y. Protamine sulfate was obtained from Eli Lilly and Co., Indianapolis, Indiana.

METHODS

Enzyme assays

Assay of the leukemic cell phosphodiesterase was based on the formation of perchloric acid soluble nucleotides from poly A. The reaction mixture (0.06 ml) contained poly A equivalent to 0.2 μmole of adenine, 0.15 μmole of $MgCl_2$, 0.5 μmole of glutathione, 0.005 μmole of EDTA, 4 μmoles of potassium phosphate buffer, pH 7.6,

and from 0.015 to 0.15 unit of enzyme*. The mixture was incubated at 37° for 30 min* and the reaction was terminated by the addition of 0.34 ml of 3 % perchloric acid. After being kept for 10 min at 0°, the mixture was centrifuged at 1500 × *g* for 5 min to remove coagulated protein and poly A. A 0.2-ml aliquot of the supernatant fluid was mixed with 0.8 ml of water and the O.D. was measured at 250 mμ in the Beckman model DU spectrophotometer, using silica cells with a 1-cm light path. This wave length was chosen because it is an isosbestic point for 5'-AMP and 5'-IMP. Both of these products were formed by the crude extracts that contain 5'-AMP deaminase. Control incubations lacking poly A or enzyme were run, and these readings were subtracted from the experimental readings. The blank due to poly A was usually negligible.

Activity toward the *p*-nitrophenyl esters was measured as follows: The reaction mixture (0.125 ml) contained 1 μmole of the ester, 12.5 μmoles of Tris, pH 8.8, 1 μmole of $MgCl_2$, and enzyme. The mixture was incubated at 37° for periods up to 1 h after which 0.475 ml of 0.1 *N* NaOH was added. Any turbidity, if present, was removed by centrifugation at 2,000 × *g* for 2 min, and the O.D. at 400 mμ was measured immediately.

In both assays, one unit of enzyme activity is defined as that amount of enzyme which forms 1 μmole of product/h. Specific activity is calculated as units/mg of protein. Protein was measured by the method of LOWRY *et al*[22].

Chromatography

Descending chromatography on Whatman No. 3MM paper was carried out with the following solvent systems: Solvent 1, *n*-propanol–concentrated NH_4OH–water (60:30:10, v/v/v)[23]; Solvent 2, saturated ammonium sulfate–isopropanol–1 *M* sodium acetate (80:2:18, v/v/v)[24]; Solvent 3, isobutyric acid–1 *M* NH_4OH–0.2 *M* EDTA (100:60:0.8, v/v/v)[25]; Solvent 4, isopropanol–water (70:30, v/v) with NH_3 in the vapor phase[26]. Paper electrophoresis of nucleotides was performed according to MARKHAM AND SMITH[26] on strips (56 cm × 9 cm) of Whatman No. 3MM paper saturated with 0.05 *M* ammonium formate–formic acid buffer, pH 3.5. Purine- and pyrimidine-containing compounds were located on paper with a Mineralight lamp. For quantitative estimation, the compounds were eluted from paper with 0.1 *N* HCl and estimated spectrophotometrically, using appropriate paper blanks. For enzymic identification the materials were eluted with water.

DEAE-cellulose for column chromatography[27] was washed extensively with water and then with a solution of 0.03 *M* Tris, pH 7.2, that also contained 0.001 *M* EDTA and 0.005 *M* β-mercaptoethanol[28].

RESULTS

Purification of the leukemic cell phosphodiesterase

Mouse lymphocytic leukemia, L4946, in ascitic form, was originally obtained from Dr. LLOYD LAW of the National Cancer Institute and Dr. R. JORDAN of the City of Hope Medical Centre; both of these lines gave similar results. The tumor was

* For most fractions, the extent of reaction was proportional to amount of enzyme between these limits which correspond to 3–30 % hydrolysis. With heated crude extracts, however, the range of proportionality was more narrow. The reaction was also linear with time up to 30 % hydrolysis.

carried, and harvests grown, in $(C_3H \times AKR)F_1$ hybrid mice[29]. Cell growth from an inoculum of $2 \cdot 10^6$ cells was harvested after 6 days. The ascites cells were washed with 0.9% NaCl and disrupted in 0.1 *M* Tris, pH 7.3, by means of high frequency vibration, using stainless steel beads and a Syntron paper jogger (Type PI3, Style 1783, from Syntron Co., Homer City, Penn.). All steps in the fractionation were done at 3° except as stated. The mixture was centrifuged at 15,000 × *g* for 10 min and the precipitate discarded. The crude extract was diluted with 0.1 *M* Tris, pH 7.3, to a final volume equal to 3 times the volume of the packed ascites cells, and β-mercaptoethanol was added to a concentration of 0.005 *M* (crude extract, 100 ml, Table I). The pH was adjusted to pH 6.9 with 2 ml of 0.1 *M* sodium acetate–acetic acid buffer, pH 3.5 and the extract was heated for 10 min at 53°. The mixture was immediately cooled to 3°, and a precipitate was removed by centrifugation at 15,000 × *g* for 3 min. This step resulted in some loss of activity, but gave a 2.5-fold purification and resulted in complete destruction of contaminating adenosine 5′-phosphate deaminase.

TABLE I

PURIFICATION OF THE PHOSPHODIESTERASE FROM CELLS OF MOUSE LEUKEMIA L4946

Fraction	*Volume of fraction (ml)*	*Total activity (units)*	*Overall yield (%)*	*Specific activity units/mg protein*
Crude extract	100	700	100	1.5*
Heated extract	93	575	82	3.6
Supernatant from Protamine I	93	585	84	3.9
Eluate from Protamine II	37	520	74**	15.5

* Crude extracts vary in specific activity from this level up to 4 units/mg protein; from more active extracts final protamine eluates with specific activities up to 27 were prepared.
** Final yields were frequently below this figure and vary from 30% to 75%.

To the supernatant solution (heated extract, 93 ml, Table I) was slowly added 4.2 ml of 1% protamine sulfate. The mixture was kept at 3° for 15 min, with occasional stirring. An inactive precipitate was removed by centrifuging for about 1 min at 15,000 × *g**. The supernatant solution (protamine I, 93 ml, Table I) was diluted 6-fold with cold distilled water. Enough additional β-mercaptoethanol was added to make the final concentration 0.0025 *M*, and the pH was adjusted to pH 7.4 with 0.2 *N* NH_4OH.

To this solution was slowly added 3.16 ml of 1% protamine sulfate. After 10 min at 3° with occasional stirring, the resulting precipitate was collected by centrifugation as above, and the supernatant solution was discarded. The tubes were allowed to drain briefly (1–2 min), and the precipitate was disrupted with a glass rod and mixed with 0.2 *M* phosphate buffer, pH 7.2, containing 0.005 *M* β-mercaptoethanol. The volume of eluting buffer was 0.4 that of the protamine I fraction. The mixture was centrifuged as before and the supernatant solution collected (Protamine II, 37 ml,

* This step accomplished little purification but did appreciably reduce the blank absorption at 250 mμ, thereby markedly increasing the sensitivity of the assay.

Table I)*. Except where otherwise specified, this fraction was used for the studies reported in this paper.

As shown in Table I, this procedure gave a 10-fold purification. Further, it resulted in complete removal of phosphomonoesterase and adenosine 5'-phosphate deaminase activities. All efforts to obtain further purification by the older methods of fractionation were unsuccessful. However, a preparation with a specific activity about 3-fold greater than that of the protamine eluate was obtained by DEAE-cellulose chromatography.

Heated extract, prepared as described above, was diluted 2-fold with cold distilled water and applied to a DEAE-cellulose column. Elution was carried out with a gradient of 0 to 0.5 *M* NaCl in 0.03 *M* Tris–0.001 *M* EDTA–0.005 *M* β-mercaptoethanol. Certain experimental details and the results are presented in Table II. Active protein with approximately the same specific activity as the protamine eluate was readily eluted in a single peak. However, by pooling fractions from the centre of the peak, a preparation of greater purity but smaller yield could be obtained (Table II).

TABLE II

PURIFICATION OF LEUKEMIC CELL PHOSPHODIESTERASE BY CHROMATOGRAPHY ON DEAE-CELLULOSE

Experiment No.	*Fraction*	*Total activity units*	*Overall yield %*	*Specific activity units/mg protein*
1	Heated extract (3.75 ml)*	48.6	100	5.4
	DEAE eluate**			
	Tubes 12–18, 18 ml	24.5	50.5	—
	Tubes 14–15, 5.5 ml	14.3	29.4	63
2	Heated extract (9.5 ml)*	125	100	4.5
	DEAE eluate***			
	Tubes 15–21, 50 ml	75.1	60	15.5
	Tube 20, 5.2 ml	18.7	15	51

* These heated extracts are comparable to those used for further purification with protamine (Table I).

** For this experiment, the mixing chamber contained 52.5 ml of 0.03 *M* Tris, pH 7.2, 0.001 *M* with respect to EDTA and 0.005 *M* with mercaptoethanol. The reservoir solution (52.5 ml) was similar in composition except that it also contained 0.5 *M* NaCl. The dimensions of the DEAE column were 0.9 × 9 cm and the flow rate was 1 ml/min. Tube 12 was collected after 17.2 ml had passed through the column and tube 14 after 23.2 ml.

*** Conditions were similar except that the reservoir and mixing chamber each contained 127 ml, the column measured 2.2 × 11 cm, and flow rate was 2 ml/min. Tube 15 was collected after 104 ml and tube 20 after 144 ml.

Properties of the enzyme

pH optimum and the effect of sulfhydryl reagents and of various ions: The enzyme has a rather broad pH optimum between pH 7 and pH 8 (Fig. 1). The reaction is stimulated to a variable extent by sulfhydryl reagents; β-mercaptoethanol or glutathione was, therefore, routinely used to stabilize the enzyme during purification and storage. The hydrolysis of poly A requires Mg^{++}ions; the optimal concentration is $3 \cdot 10^{-3}$ *M*

* This step usually gave satisfactory results, but did, upon occasion, produce a fraction which for unknown reasons, was distinctly lower in specific activity than the usual product.

and concentrations 3 times as high or higher are inhibitory. This inhibition is not observed in the hydrolysis of oligonucleotides, such as pApA and pApApA and with such compounds good activity is observed with concentrations as high as $8 \cdot 10^{-3}$ *M* Mg^{++}.

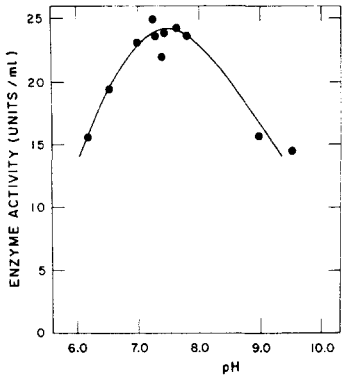

Fig. 1. Effect of pH on the rate of hydrolysis of poly A by purified leukemic cell phosphodiesterase. The assay was carried out as described under METHODS, but using phosphate buffers at a final concentration of 0.09 *M* for pH values up to 7.8. For the value at pH 9 the reaction mixture contained 0.1 *M* Tris, and for pH 9.5, 0.1 *M* glycine. The pH values are those of the final reaction mixture. The enzyme fraction was the protamine eluate (Table I).

At $3 \cdot 10^{-3}$ *M*, Mn^{++} stimulates the hydrolysis of poly A two-thirds as much as Mg^{++}, and Ca^{++} is even less effective. The effect of several other ions on the hydrolysis of poly A was tested under routine assay conditions, except that the concentration of $MgCl_2$ was 0.008 *M*. A 40 % inhibition is found with 0.01 *M* inorganic pyrophosphate and complete inhibition with 0.04 *M* fluoride. A 4-fold stimulation results from the addition of 0.04 *M* phosphate; this is not found with oligonucleotide substrates, and probably represents an interaction involving poly A and both phosphate and Mg^{++}.

Stability: Crude extracts of the ascites cells can be stored for at least 6 months at $-15°$ without significant loss in activity. Heated extracts occasionally show some loss after several days. The protamine eluate (Table I) usually shows no loss after 2 months at $-15°$, but in several instances up to 50 % loss of activity has occurred. Fractions from DEAE-cellulose are very unstable as eluted from the columns, but they can be stored for at least 3 weeks if they are concentrated by ammonium sulfate precipitation, and the precipitate redissolved in one-fifteenth of the original volume of a solution containing 0.04 *M* Tris, pH 7.2, 0.004 *M* β-mercaptoethanol, 0.001 *M* EDTA and 25 % glycerol.

Heating to 50–55° for 10 min at pH 6.9 results in loss of one-third of the activity (Table I). Heating at a lower pH (5.5 to 6.2) or at a higher temperature (57°–59°) produces significantly more inactivation.

Characteristics of the hydrolysis of poly A

A substrate saturation curve for poly A shows K_m to be approx. $2.0 \cdot 10^{-3}$ *M*, expressed as adenine equivalents, and substrate saturation is achieved at a concentration of $5.0 \cdot 10^{-3}$ *M*. These values are not affected by varying the concentration of Mg^{++} from $2 \cdot 10^{-3}$ *M* to $8 \cdot 10^{-3}$ *M*. The hydrolysis of poly A is proportional to amount of enzyme only up to the point at which $1 \cdot 10^{-3}$ *M* 5′-AMP is formed, corresponding

to 30 % hydrolysis in the standard assay. With more enzyme, very little additional reaction occurs. The hydrolysis of poly A is linear with time, but only as long as the concentration of 5'-AMP formed in the reaction is less than 1 to 1.5 μmoles/ml. Thereafter the time curve is almost flat. No satisfactory explanation for this behavior is available, for 5'-AMP at this concentration does not inhibit the enzyme (Table III). The hydrolysis of poly A is inhibited by oligonucleotides such as pApA (Table III), but these could not be detected as intermediates. It is possible that comparatively large polynucleotide fragments are formed from poly A and that such intermediates have a high affinity for the enzyme but undergo very slow further hydrolysis.

TABLE III

INHIBITION OF THE HYDROLYSIS OF POLY A

Routine assay conditions were used, as described under METHODS. The enzyme fraction was a protamine supernatant (Table I) for the experiments with 5'-AMP and adenosine, and a protamine eluate for the experiments with pApA.

Addition	*Concentration μmoles/ml*	*Inhibition %*
5'-AMP	1.4	0
5'-AMP	2.8	30
Adenosine	1.9	70
pApA	0.4	6
pApA	1.6	62

Hydrolysis of other polymers and of oligonucleotides

Poly A is hydrolyzed more rapidly than any of the other polymers in the series. Poly U, poly G and poly AU are split about one-half as fast as poly A. The products of hydrolysis of these polymers were identified as 5'-mononucleotides by their absorption spectra, their chromatographic behavior in Solvents 1 and 3 and their cleavage by purified bull semen 5'-nucleotidase[21]. Other polymers are hydrolyzed to 5'-mononucleotides at rates that are less than 10 % of that observed with poly A. These include poly C, poly AGUC, yeast RNA and RNAs derived from turnip yellow mosaic virus and *A. agilis*. The reason for these low rates is not known, and may, perhaps, be related to degree of polymerization or secondary structure of the polymers.

The hydrolysis of the oligonucleotides, pApA, pApApA, pApApApA and pApApApApA was followed by chromatography in Solvents 1 and 2. The ultimate product was determined to be 5'-AMP by its spectrum, R_F in Solvents 1 and 2, electrophoretic mobility and by the fact that it was hydrolyzed by 5'-nucleotidase[21]. Smaller oligonucleotides accumulate as transient intermediates. Thus, during the hydrolysis of pApApApA to 5'-AMP one can observe the appearance of pApApA and pApA*. Table IV shows the rate of formation of 5'-AMP from these compounds, compared with that from poly A, all measured at saturating concentrations of substrate. Substrate saturation is achieved with $3.5 \cdot 10^{-4} M$ pApApApA, $4.5 \cdot 10^{-4} M$ pApApA,

* $R_{3'\text{-AMP}}$, which is the ratio of the R_F of a given compound to that of 3'-AMP has the following values in Solvent 1: pApA, 0.53; pApApA, 0.31; pApApApA, 0.18; pApApApApA, 0.09. In Solvent 4 the values are: pApA, 0.40; pApApA, 0.17; pApApApA, 0.06; pApApApApA, 0.03.

TABLE IV

RATE OF FORMATION OF 5′-AMP FROM POLY A AND FROM CERTAIN OLIGONUCLEOTIDES

The reaction mixture (0.06 ml) contained 0.15 μmole of $MgCl_2$, 2.5 μmoles of Tris, pH 8.2, 0.5 μmole of glutathione, 0.005 μmole of EDTA, substrate and 10 μg of protamine eluate fraction (Table I; specific activity, 15). The mixtures were incubated at 37° and chromatographed in Solvent 1. Regions of the paper containing the 5′-AMP were located in u.v. light and the nucleotide was quantitatively eluted with 0.01 *N* HCl. The O.D. was measured at 257 mμ. The time of incubation was 0.5 h for poly A and 5.5 h for the oligonucleotides.

Substrate	*Concentration (M)*	*5′-AMP formed μmoles/mg enzyme/h*
Poly A	$6.7 \cdot 10^{-3}$*	15.0
pApA	$2.4 \cdot 10^{-3}$	0.8
pApApA	$1.5 \cdot 10^{-3}$	0.9
pApApApA	$1.4 \cdot 10^{-3}$	1.0

* Expressed as adenine equivalents. All other concentrations are expressed as molar concentration of the given compound. All concentrations were well above the level required to saturate the enzyme.

and $7 \cdot 10^{-4}$ *M* pApA. It is evident from Table IV that 5′-AMP is formed at least 15 times as fast from poly A as from the oligonucleotides*. The pyrimidine derivatives pUpU and pUpUpU are split nearly twice as rapidly as the corresponding adenine oligonucleotides. Mononucleotides with a 2′,3′-cyclic phosphoryl group such as adenosine 2′,3′-phosphate, are not attacked.

Purified protamine eluate fractions as well as fractions from DEAE-cellulose chromatography were tested with the oligodeoxyribonucleotides, d-pTpT, d-pTpTpT and d-pApA. All were studied at a concentration of 10^{-3} *M*; because of insufficient material, substrate saturation curves were not obtained. The rates of formation of mononucleotide from d-pTpTpT and d-pTpT are one-third and one-sixth, respectively, of that found with pApApA, while d-pApA is hydrolyzed about half as fast as d-pTpT.

Specificity requirements of the enzyme

Leukemic cell phosphodiesterase resembles snake venom phosphodiesterase in forming nucleoside 5′-phosphates from polymers and from oligonucleotides bearing a 5′-phosphomonoester end group. This resemblance is borne out in other results. Thus, the compounds ApCp, ApUp and ApApUp, which possess a 3′-phosphomonoester end group are not hydrolyzed. Also, the rates of hydrolysis of ApA and ApApA, two compounds without end groups are very slow. Similar findings have been reported for the venom enzyme[30,33]. An important difference between the two enzymes was discovered, however, when it was noted that various simple esters of nucleotides, such as benzyl adenosine 5′-phosphate and *p*-nitrophenyl uridine 5′-phosphate are not attacked by the tumor enzyme. Hydrolytic activity against such esters can be detected in crude extracts, but Table V shows that this is almost completely removed as a result of purification. The fractionation produces at least a 75-fold change in the ratio of enzymic activity against poly A to that against *p*-nitrophenyl uridine 5′-phosphate,

* It requires more poly A than oligonucleotides to give substrate saturation, based on adenine equivalents. The data in Table III are consistent with competitive inhibition, by pApA, of the splitting of poly A.

and the purified fractions hydrolyze several esters at rates that are less than 1/240 of that observed for poly A (Table V).

From the data presented here, it appears that a substrate for leukemic cell phosphodiesterase must contain at least two nucleoside residues.

TABLE V

REMOVAL OF HYDROLYTIC ACTIVITY AGAINST CERTAIN *p*-NITROPHENYL ESTERS IN THE COURSE OF PURIFICATION OF LEUKEMIC CELL PHOSPHODIESTERASE

Assays for rates of hydrolysis of poly A and of the *p*-nitrophenyl esters are given under METHODS. Units of activity are expressed as μmoles of product formed per hour at 37°. Comparable results were obtained in another experiment with other, similarly purified, enzyme fractions.

Fraction	*Specific activity (U/mg) against*				*Ratio of specific activities*	
	Poly A	*p-nitrophenyl uridine 5'-phosphate*	*p-nitrophenyl thymidine 5'-phosphate*	*p-nitrophenyl thymidine 3'-phosphate*	*Poly A / uridine 5'-ester*	*Poly A / thymidine 5'-ester*
Crude extract	2.42	0.74	1.0	0.2	3.27	2.4
Protamine eluate	16.1	<0.06*	<0.06*	<0.06	>244	>244
DEAE eluate	15.5	<0.07			>222	

* For these measurements the amount of enzyme used was 10 times that required to demonstrate hydrolysis of poly A, and the incubation time was twice as long. Under these conditions there was no detectable reaction with these esters.

Mechanism of the reaction

The cleavage reaction is hydrolytic. There is no absolute requirement for inorganic phosphate. Furthermore, no ADP or ATP can be detected during the splitting of poly A or of adenine-containing oligonucleotides. Control experiments indicate that even small amounts of these compounds are stable upon incubation with the enzyme.

The cleavage of poly A is apparently stepwise, with successive removal of mononucleotide units from an end of the chain, as has been observed for venom phosphodiesterase[31,32]. This conclusion derives from the fact that pApApApApA and smaller oligonucleotides were never detected at any stage of the hydrolysis of poly A. Random splitting throughout the polynucleotide chain would have given rise to such compounds. Further, if formed, they should have accumulated in the reaction mixture because they liberate 5'-AMP at only one-fifteenth of the rate observed with poly A (Table IV).

RAZZELL AND KHORANA[31] have found that oligodeoxyribonucleotides are degraded by snake venom phosphodiesterase in a stepwise manner, starting from the end of the oligonucleotide chain bearing a 3'-hydroxyl group. HILMOE[32] has confirmed this observation, using polyribonucleotides. In view of this work, an effort was made to discover from which end of the molecule the attack by leukemia phosphodiesterase begins. For this purpose the hydrolysis of pApApApU and pApApApG was investigated, and the reaction products were examined at various time intervals. Unfortunately, pApApA formed as an intermediate was hydrolyzed very much faster than the original tetranucleotides. Apparently (see Table IV) the presence of a terminal uridylic or guanylic acid residue results in a slower rate of attack by the enzyme. As a consequence no conclusions concerning the initial site of hydrolysis could be drawn from serial chromatograms of the reaction mixtures.

DISCUSSION

Leukemic cell phosphodiesterase resembles snake venom phosphodiesterase in its specificity except that various simple esters of nucleoside 5′-phosphates are not hydrolyzed. By contrast, the venom enzyme splits *p*-nitrophenyl thymidine 5′-phosphate more rapidly than any other substrate tested[33]. The tumor phosphodiesterase appears to be the first example of an enzyme hydrolyzing polynucleotides to the mononucleotide stage that requires a nucleoside residue to be present on each side of the susceptible phosphodiester bond. Simple esters in which phosphate is doubly esterified to a single nucleoside residue and to an alkyl or aryl group cannot be cleaved by this new kind of hydrolase. It may be mentioned in this connection that various nucleases such as pancreatic ribonuclease[15], and ryegrass ribonuclease[31] hydrolyze certain simple esters of nucleosides as well as polynucleotides. The same is true for spleen phosphodiesterase[2].

RAZZELL AND KHORANA (personal communication) have investigated the cleavage of the *p*-nitrophenyl esters of thymidine 5′-phosphate and thymidine 3′-phosphate. Both types of activity are widely distributed in animal tissues and also occur in bacteria. Enzymes comparable to leukemic cell phosphodiesterase may also be widely distributed, but they would be difficult to find for lack of an easy, specific assay. In most tissues the activity is largely obscured by high concentrations of other, interfering phosphodiesterases and nucleases. Another difficulty is caused by the presence of phosphomonoesterases; most tumor tissues, however, have comparatively low phosphatase activity[35]. Dr. M. F. SINGER (personal communications) has found that fractionation of lysates of mouse Ehrlich ascites cells resulted in a 10-fold increase in the ratio of activity against poly A to that against *p*-nitrophenyl uridine 5′-phosphate. This indicates the likely presence in another source of an enzyme that hydrolyzes polynucleotides but not simple esters.

Finally, it may be mentioned that we have demonstrated the formation of nucleoside 5′-phosphates from poly A, poly U, poly AU, pApA, pUpU, etc., in extracts of various other tumor tissues including: (a) an ascitic lymphocytic leukemia L1210[36], (b) an ascitic plasma cell neoplasm, 70429[37], (c) an ascitic mast cell tumor, P815[38], (d) a solid plasma cell tumor, X-5563[39]. These were obtained from Drs. LLOYD LAW and M. POTTER of the National Cancer Institute. Similar activity was found in extracts of cultured HeLa cells obtained from Drs. N. SALZMAN and H. EAGLE of the National Institute of Allergy and Infectious Diseases. The reactions observed are probably hydrolytic, but fractionation would be required to rule out a phosphorolytic mechanism.

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IN VITRO LIPID SYNTHESIS IN FOWL BLOOD

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SUMMARY

1. Fowl whole blood has been shown to incorporate labelled acetate into both cellular and plasma lipids.

2. Long chain saturated and unsaturated acids are shown to be synthesized and then incorporated into triglycerides, phospholipids and cholesterol esters.

3. "Sterol"-like unsaponifiable substances are also synthesized from acetate.

4. Comparison of the synthetic ability of leucocytes, young erythrocytes and old erythrocytes show that all cells contribute to the synthesis, the most active being the leucocyte and young erythrocyte preparations.