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Murine DNA Polymerases
I. Distinguishing Characteristics of Two Activities Separated by Phosphocellulose Chromatography (38277)

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(Introduced by M. Laskowski, Sr.)

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Three DNA polymerizing enzymes C, A, and N have been separated from mammalian lymphoid cells by chromatography of crude cell homogenates on phosphocellulose (1).

We have shown that murine polymerases are further distinguished by their preferential usage of the templates: "activated" DNA, poly rA·dT $_{12-18}$, and poly rA·poly dT, respectively. Since polymerases C, A, and N were eluted with a KCl gradient, it was conceivable that the observed template preferences were due to the ionic conditions of the assay. Therefore, a study of the optimal ionic strength and pH for each of the three polymerases was undertaken. In addition, a possible effect of contaminating nucleases on template preferences was considered and excluded. The results show that although the activity of polymerases C and N are sensitive to salt concentration and pH, their template preferences are retained over a wide range of KCl concentrations. The data also provide salt and pH optima for polymerases C and N confirming their identification with polymerases described from other sources (2–5).

Materials and Methods. Preparation of crude extract. Spleen or liver was obtained from 12–20-week-old female C57BL/$_{6J}$ mice and immediately placed in cold Eagle's basal medium with Earle's salts (BME, Grand Island Biological Co.). All subsequent procedures were conducted at 0–4°. Lymphoid cells were obtained by perfusion of the spleen with BME while gently squeezing the splenic capsule with forceps. Nucleated cell counts averaged 1×10^8 per spleen. Cell suspensions were washed twice in BME by centrifugation at 1000*g* for 15 min and resuspended at 0.5 ml/spleen in TEM buffer (50 m*M* Tris–HCl, pH 7.8, 1 m*M* EDTA, 1 m*M* 2-mercaptoethanol). Removal of red blood cells by a brief exposure to distilled water, prior to resuspension in TEM, did not alter the recovery or the separation properties of DNA polymerases. Liver was finely minced with scissors, and the fragments were washed twice in BME and collected by centrifugation at 1000*g* for 15 min. Tissue fragments were resuspended at 20% v/v in TEM buffer.

Spleen cells or liver tissue fragments were disrupted by 10 strokes of a tight-fitting pestle in a Dounce homogenizer and subjected to three cycles of freezing and thawing. Triton X-100 was then added to a final concentration of 0.5%. After thorough mixing, the homogenate was centrifuged at 240,000*g* for 1 hr in a Spinco SW-50.1 rotor. The supernatant solution, adjusted with glycerol to a final concentration of 20% v/v, was used as crude extract.

Phosphocellulose chromatography. A column (0.9 × 2 cm) of phosphocellulose (Whatman P11, prewashed by the method of Burgess, Ref. 6) was equilibrated with TEM containing 20% glycerol (TEMG). A minimum of 20 mg protein, as determined by the method of Lowry (7), was applied to each column. Following a 5-ml wash of TEMG, the column was eluted with a linear KCl gradient (100 ml, 0–0.8 *M*) in TEMG. Fractions of 2.0 ml were collected at a flow rate of 8 ml/hr, and the salt concentrations of individual fractions were determined from standard curves using a conductivity bridge

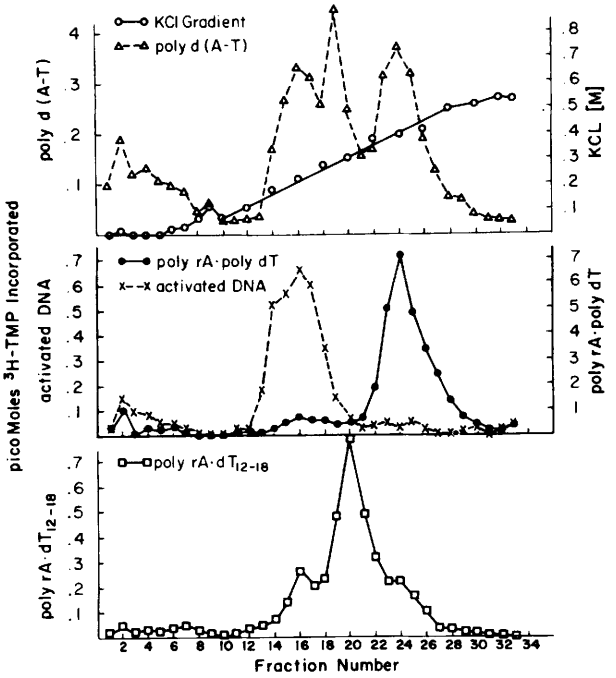

FIG. 1. Phosphocellulose chromatography of murine spleen extract prepared in TEMG as described in Materials and Methods. Twenty-four milligrams of crude extract protein were applied to the column.

(Model 31, Yellow Springs Instrument Co.).

Peak fractions from phosphocellulose columns of spleen cell extracts were pooled (for example, see Fig. 1) and will be referred to as peak I (fractions 15–17), peak II (fractions 19–21), and peak III (fractions 23–25). Pooled peak fractions were dialyzed against two changes of TEMG, 100 × volume. All experiments using phosphocellulose-purified peaks of enzyme activity were performed two or more times with pooled material obtained from different extracts chromatographed separately on phosphocellulose columns. Virtually all of the protein (99%) applied to the column was recovered in the column wash. The protein concentration in column fractions containing DNA polymerase activity was below the level of accurate detection either by absorption at 280 nm or by the method of Lowry (7).

DNA polymerase assays. DNA polymerase activity was routinely assayed using four templates: "activated" calf thymus DNA, poly rA·poly dT, poly rA·dT_{12-18}, and poly d(A-T). All polymers were stored at −20° in distilled water at a concentration of 2.5 A_{260} U/ml. Molar concentrations of polynucleotide templates were calculated from absorbance at 260 nm using the following coefficients (E_{260}): poly rA·poly dT, 6.7×10^3; poly rA, 9.81×10^3; poly dA, 9.79×10^3; poly dT, 8.09×10^3; oligo dT_{12-18}, 8.4×10^3; oligo dG_{12-18}, 9×10^3.

All reactions were conducted in plastic microtiter trays (Cooke Engineering) with 0.125-ml "V"-shape wells for 1 hr at 37°. Reactions were terminated by absorbing the entire reaction mixture onto a Whatman 2.3-mm filter paper circle. Filters were immediately submerged in cold 10% trichloroacetic acid (TCA) containing 1% sodium pyrophosphate for 15 min in an ice bath. Filters were washed six times in 5% TCA, 15 min each; twice in 90% ethanol, 10 min each; twice in acetone for 5 min, and air dried (8). Acid-insoluble radioactivity was determined in a toluene-base scintillation fluid containing 0.5% PPO, 0.03% POPOP (Beckman Instruments) in a Packard model 3003 liquid scintillation spectrometer. Background radioactivity was determined by incubation of complete reaction mixtures to which no enzyme containing fraction was added. Counting efficiency was 20% as determined by double-label with ^{3}H-dTTP and α ^{32}P-dTTP, assuming 100%

counting efficiency for ^{32}P. Data are reported in picomoles of dTMP incorporated into acid-insoluble material.

"Activated" DNA. Calf thymus DNA was activated by the method of Loeb (9). Assays for enzyme activity stimulated by "activated" DNA were conducted in 30 m*M* $NaPO_4$, pH 7.2, 9 m*M* $MgCl_2$, 1.2 m*M* 2-mercaptoethanol, 60 μM each dATP, dCTP, dGTP, and ^{3}H-dTTP (specific activity 433 cpm/pmole), 5 μg "activated" DNA, and 10 μl of enzyme in a total volume of 60 μl.

Poly rA·poly dT. Assays stimulated by poly rA·poly dT were performed in 20 m*M* Tris–HCl, pH 7.8, 2 m*M* dithiothreitol (DTT), 0.6 m*M* manganese acetate, 0.25 m*M* ^{3}H-dTTP (specific activity 87 cpm/pmole), 0.012 A_{260} U poly rA·poly dT (35.6 μM) with 10μl of enzyme in a total volume of 60 μl.

Poly d(A-T). Reactions stimulated by poly d(A-T) were performed in a total volume of 50 μl of 50 m*M* Tris–HCl, pH 8.3, 6 m*M* DTT, 6 m*M* magnesium acetate, 40 μM ^{3}H-dTTP (specific activity 1770 cpm/pmole), 0.46 m*M* dATP, 0.009 A_{260} U poly d(A-T) (35 μM), and 25 μl of enzyme.

Poly rA·dT_{12-18}. Reactions using poly rA·dT_{12-18} were performed in 50 m*M* Tris–HCl, pH 8.3, 6 m*M* 2-mercaptoethanol, 0.015 A_{260} U poly rA (30.6 μM), 0.007 A_{260} U dT_{12-18} (34 μM), 0.8 m*M* manganese acetate, 50 μM ^{3}H-dTTP (sp act 820 cpm/pmole), and 25 μl of enzyme, in a total volume of 50 μl.

Nucleolytic activity. Hydrolysis of templates by phosphocellulose-purified enzyme fractions was assayed using dialyzed, pooled fractions from phosphocellulose columns representing peaks I, II, and III. ^{3}H-poly rA (sp act 0.85 μCi/μmole at a final concentration of 0.5 A_{260} U) (102 μM) was mixed with either 0.025 A_{260} U oligo dT_{12-18} (122 μM) or 0.05 A_{260} U poly dT (120 μM) per reaction. ^{14}C-L cell DNA (sp act 6×10^3 cpm/μg) was used at a final concentration of 0.5 μg/assay. Hydrolysis by peak I was assayed in the reaction mixture routinely used for "activated" DNA. Hydrolysis by peak II was assayed in the reaction mixture described for poly rA·dT_{12-18}. Peak III was assayed for hydrolysis in the reaction mixture described for poly rA·poly dT. In all reactions, labeled polynucleotides replaced unlabeled templates and ^{3}H-dTTP. Control reactions contained labeled polynucleotide in the preferred reaction mixtures for that polynucleotide template but no added enzyme fraction. Data were recorded as acid-insoluble radioactivity remaining after 1 hr at 37°.

Synthetic template preferences. Dialyzed peak fractions from phosphocellulose columns were assayed with synthetic homopolymer template and primer combinations of poly rA, poly dA, poly dT, and oligo dT_{12-18}, or poly rC and oligo dG_{12-18}. All reaction mixtures contained a total of 0.012 A_{260} U of polynucleotide and 0.25 m*M* ^{3}H-dTTP or ^{3}H-dGTP (sp act 87 cpm/pmole) in a total volume of 50 μl. Complementary polynucleotides were present at approximately 1:1 molar ratios. Peak I was assayed in 30 m*M* $NaPO_4$, pH 7.2, 1.2 m*M* 2-mercaptoethanol. Peak III was assayed in 50 m*M* Tris–HCl, pH 8.3, 0.1 *M* KCl, 2 m*M* DTT. Manganese was present at 0.6 m*M*, and magnesium was present at 9 m*M*.

Reagents. Unlabeled deoxyribonucleoside triphosphates, dATP, dCTP, dGTP, and dTTP were purchased from General Biochemicals. ^{3}H-dTTP and ^{3}H-dGTP were purchased from Schwartz/Mann in a solution of ethanol–water, 1:1, and used without further treatment at a concentration of 5 μl per assay. α^{32}P-dTTP was purchased from New England Nuclear Corporation.

Calf thymus DNA, poly rA·poly dT, poly rA, poly dA, poly dT, poly d(A-T), and ^{3}H-poly rA were purchased from Miles Laboratories. Poly rC and the oligo nucleotides dT_{12-18}, and dG_{12-18} were purchased from Collaborative Research, Inc. ^{14}C-L cell DNA was kindly supplied by Dr. Eduardo Kraiselburd.

Results. Crude extracts of murine spleen were chromatographed on phosphocellulose columns and eluted with a linear gradient from 0 to 0.8 *M* KCl. Column fractions were assayed with four different templates (Fig. 1). Three peaks of DNA polymerase activity were detected with poly d(A-T) in the salt gradient of the chromatogram (Fig. 1, A). DNA polymerase activity which eluted ahead of the salt gradient was probably due to overloading since it responded to all four templates. Peak I eluted at 0.2 *M* KCl and was preferentially stimulated by "activated" DNA (Fig. 1, B). Peak III eluted at 0.45 *M* KCl and was preferentially stimulated by poly rA·poly dT (Fig. 1, B). Activity preferentially responding to poly rA·dT_{12-18} was observed on the trailing edge of peak II, eluting at

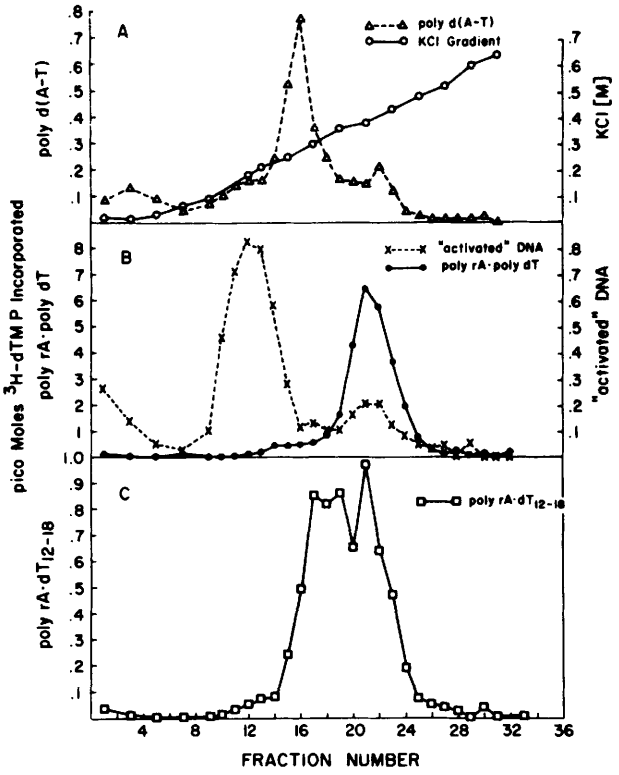

FIG. 2. Phosphocellulose chromatography of normal murine liver extract prepared and assayed as in Fig. 1. Thirty-nine milligrams of crude extract protein were applied to the column.

0.3 *M* KCl (Fig. 1, C). Poly rA·dT$_{12-18}$-stimulated activity reproducibly followed the poly d(A-T)-stimulated activity in the peak II region by at least one fraction. Occasional extracts yielded poly rA·dT$_{12-18}$ activity co-chromatographing with peak III in addition to the activity present in peak II.

Crude extracts of normal mouse liver were chromatographed on phosphocellulose and assayed as described for mouse spleen. Three peaks of DNA polymerase activities were observed (Fig. 2). Peak I, preferentially stimulated by "activated" DNA, eluted at 0.2 *M* KCl, and peak III, preferentially stimulated by poly rA·poly dT, eluted at 0.45 *M* KCl. Activity responding to poly rA·dT$_{12-18}$ eluted in a broad peak from 0.3 to 0.4 *M* KCl and was not coincident with poly d(A-T) activity in the peak II region. In contrast to extracts prepared from spleen, the poly d(A-T) activity eluted in a single major peak at 0.3 *M* KCl.

The distinct template preferences found in eluates from phosphocellulose columns of peak I for "activated" DNA, peak II for poly rA·dT$_{12-18}$, and peak III for poly rA·poly dT may be authentic characteristics of individual enzyme species or the result of certain conditions, *e.g.*, salt concentration in the column eluate. To examine this possibility, pooled fractions from a phosphocellulose column were dialyzed against TEMG or against TEMG containing 0.2, 0.3, or 0.4 *M* KCl and reassayed with all four templates. The response of each peak to a single template was quite sensitive to salt concentration (see Fig. 3). Nevertheless, the preferential stimulation of peak I by "activated" DNA and peak III by poly rA·poly dT was always preserved regardless of salt concentration.

The observed template preferences of phosphocellulose-purified enzymes could also be due to the presence of nucleolytic activity in the column fractions which preferentially attacks one or more of the preferred templates. This possibility was examined using radiolabeled polynucleotides.

^{14}C-labeled DNA or ^{3}H-poly rA, mixed with

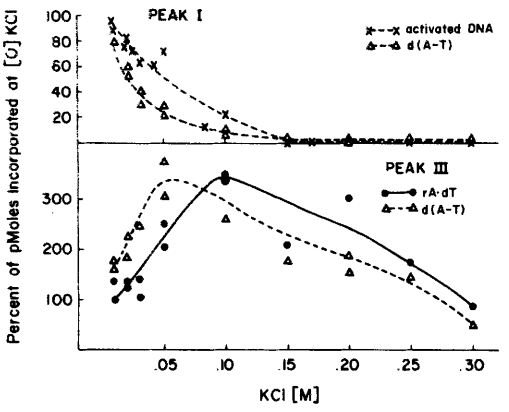

FIG. 3. Effect of monovalent cation concentration on enzymes obtained from phosphocellulose columns. Fractions from phosphocellulose columns were pooled and dialyzed against TEMG. Each peak was assayed with poly d(A-T) and with the preferred template of that peak in the presence of increasing concentrations of KCl. Data represent two experiments using enzymes obtained from two extracts chromatographed on phosphocellulose. The data were normalized to percent of picomoles incorporated at zero KCl.

equimolar quantities of either poly dT or dT_{12-18}, was added to phosphocellulose-purified fractions of each peak. The acid-insoluble radioactivity remaining after 1 hr of incubation at 37° was compared to controls containing buffer instead of column fractions. No reduction in acid-insoluble ^{14}C DNA was observed. Tritiated poly rA mixed with dT_{12-18} was rendered 37% acid soluble by peak I, while no reduction in acid-insoluble radioactivity of ^{3}H-poly rA·dT_{12-18} or ^{3}H-poly rA·poly dT was observed with peaks II and III. Peak I reduced the acid-insoluble radioactivity of ^{3}H-poly rA mixed with poly dT by only 1.8%, suggesting that hydrolysis obtained with ^{3}H-poly rA·dT_{12-18} represents hydrolysis of unhybridized segments of single-stranded poly rA.

The stability of polymerase A varied from one preparation to another. This variable stability, coupled with evidence from phosphocellulose chromatography that peak II from spleen may be contaminated with an additional DNA polymerase activity (Fig. 1), prevent definitive characterization of polymerase A until it can be further purified.

Column fractions representing peaks I and III were pooled and dialyzed against TEMG for determination of the effects of KCl concentration and pH on the activity of each enzyme. The enzyme activity recovered from phosphocellulose as peak I was inhibited by increasing concentrations of KCl (Fig. 3). Optimal activity with both poly d(A-T) and "activated" DNA occurred in the absence of added KCl.

The DNA polymerase activity recovered in peak III was stimulated by low concentrations of KCl. For optimal activity, similar KCl concentrations were required with both poly d(A-T) (0.05 *M* KCl) or poly rA·poly dT (0.1 *M* KCl).

Dialyzed peak fractions from phosphocellulose were also assayed for optimal activity at varying pH. Peak I exhibited maximum activity at pH 6.5–7.0 with both poly d(A-T) and activated DNA templates (Fig. 4). Peak III revealed a broad pH optimum with poly d(A-T) from pH 6.7 to 8.3; poly rA·poly dT stimulated activity was maximal at pH 8.3, exhibiting a broad range of near optimal activity from pH 7.5–8.9.

Pooled, dialyzed fractions representing peaks I and III were assayed for synthetic template preference under conditions of near optimal salt and pH for the "preferred" template (see Materials and Methods). Peak I exhibited a definite preference for deoxyribo templates in the presence of either Mn^{2+} or Mg^{2+} (Table I).

Peak III previously showed a strong preference for poly rA·poly dT in the presence of Mn^{2+}. The sensitivity of detection of peak III with poly rA·poly dT and Mn^{2+} is demonstrated in Table I. As Robert *et al.* (10) have suggested, DNA polymerase activity in the presence of Mn^{2+} is relatively nonspecific for differentiation of ribo as opposed to deoxyribo template preferences. We verified this observation by the apparent preference of peak III for poly rA·poly dT > poly dA·dT_{12-18}, > poly rA·dT_{12-18} > poly dA·poly dT. However, in the presence of Mg^{2+}, peak III revealed a distinct preference for deoxyribo templates.

None of the phosphocellulose purified fractions were active in the presence of poly rC·dG_{12-18} and Mg^{2+}; minimal activity was detected with poly rC·dG_{12-18} and Mn^{2+}. Little or no activity was detected in the presence of single-homopolymer templates with any of the enzyme preparations.

Discussion. Murine lymphoid cell extracts yield three peaks of DNA polymerase activity when column eluates are assayed with poly d(A-T). Each of the peaks can be preferentially stimulated by a specific template: peak I by "activated" DNA, peak II by poly rA·dT_{12-18}, and

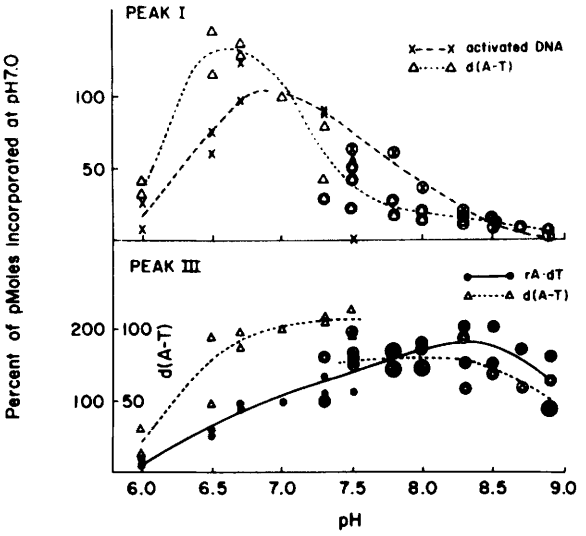

FIG. 4. Effect of pH on enzymes obtained from phosphocellulose. Fractions from phosphocellulose columns were pooled and dialyzed against TEMG. Each peak was assayed with poly d(A-T) and with the preferred template of that peak, at varying pH. 50 m*M* MES buffer (2 *N*-morpholino ethansulfonic acid) was used from pH 6.0 to 7.5 (naked symbols) and 50 m*M* Tris–HCl was used from pH 7.3 to 8.9 (enclosed symbols). Data represent two experiments using enzymes obtained from 2 phosphocellulose columns of individually prepared extracts. Data are expressed as percent of picomoles incorporated at pH 7.0.

peak III by poly rA·poly dT. These template preferences are not solely the result of monovalent cation effects on the template; however, total activity observed with a given template can be strongly influenced by salt concentration (Fig. 3). Hydrolysis of specific templates by nucleases in the column fractions did not contribute to the observed template preferences. These studies provide evidence that under controlled conditions preferential template utilization may be used to differentiate enzymatic species of DNA polymerase.

Polymerase C has been observed by several other workers in extracts from a variety of mammalian tissues (1, 2). Peak I obtained from phosphocellulose columns of murine lymphoid cells is now firmly identified as Polymerase C. This conclusion is supported by the following

TABLE I. Synthetic Homopolymer Template Preferences of Phosphocellulose Purified Enzymes of Murine Spleen.

Template and primer	pMoles dNMP Incorporated/hr[a]			
	Peak I		Peak III	
	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}
rA·dT (36 μM)	0.69	0.01	25.6	0.75
dA·dT (12 μM, 15 μM)	1.57	0.26	1.57	2.18
rA·dT_{12-18} (12 μM, 14 μM)	0.20	0.01	5.52	0.32
dA·dT_{12-18} (12 μM, 14 μM)	0.68	0.84	13.1	8.37
rA (24 μM)	0.03	0.06	0.09	0.08
dA (24 μM)	0.08	0.10	0.15	0.13
dT_{12-18} (28 μM)	0.03	0	0.99	0.01
dG_{12-18} (26 μM)	0.25	0	0.88	0
rC·dG (47 μM)	0.16	0	0.17	0
d(A-T) (20 μM)	—	—	0.30	0.43

[a] Each assay represents activity of 15 μl of pooled fractions from a phosphocellulose column, dialyzed against TEMG.

characteristics:(1) elution from phosphocellulose at 0.2 *M* KCl (Fig. 1, and 2, Refs. 1, 4, 11, 12); (2) inhibition by increasing concentrations of KCl (Fig. 3, Refs. 12, 13); (3) neutral pH optimum (Fig. 4, Refs. 4, 12, 13); and (4) a preference for deoxyribonucleic acid templates (Table I, Refs. 12, 14).

Enzyme N has also been observed in a variety of mammalian tissues (1, 4, 5). We now confirm the identity of peak III obtained from phosphocellulose columns of murine lymphoid cell extracts as Polymerase N. This conclusion is supported by the following observations: (a) elution from phosphocellulose at 0.4–0.45 *M* KCl (Fig. 1 and 2, Refs. 1, 2, 12); (b) stimulation by 0.1 *M* KCl (Fig. 3, Ref. 15); (c) alkaline pH optimum (Fig. 4, Refs. 4, 12, 13); and (d) preferential use of deoxyribonucleotide templates in the presence of Mg^{2+} (Table I, Refs. 12, 13). Chang and Bollum (5) have also reported the stimulation of Polymerase N by a poly rA·poly dT template. Use of this template provides a highly sensitive method for determination of enzyme *N* activity. Assay of peak III from phosphocellulose with "activated" DNA detects only 1–4% of the activity detectable with poly rA·poly dT, while poly d(A-T) detects 5–6% of the activity detectable with poly rA·poly dT.

One fact emerges from the assay of phosphocellulose-purified DNA polymerases. Poly rA·poly dT-stimulated DNA polymerase activity in the presence of Mn^{2+} can be specific for a single enzyme and, contrary to previous reports (16–20), represents Polymerase N. This activity must be classified as DNA-directed according to current criteria for differentiating RNA-directed from DNA-directed DNA polymerases.

Phosphocellulose chromatography of extracts of murine spleen and liver also reveals the presence of Polymerase A (1) (poly rA·dT_{12-18}-stimulated), (Fig. 1 and 2, C). As mentioned in the Results, peak II (Fig. 1, C) and peak II (Fig. 1, A) are reproducibly separated by one fraction. Two alternatives have been considered: (a) they are two enzymes, (b) only one enzyme is present, but an impurity interferes in a different manner with the 2 templates (poly d(A-T) and poly rA·dT_{12-18}). Data obtained in a study of optimal ionic requirements do not rule out the presence of two enzymes in the peak II region of the phosphocellulose column, one of which may be RNA-directed (10, 14, 21). We have not rigorously excluded the presence of an RNA tumor virus "reverse transcriptase" in our murine spleen extracts. However, the enzymes obtained from phosphocellulose and identified by us as Polymerases C, A, and N differ from murine tumor virus RNA-directed DNA polymerase (1) in that they were not stimulated to incorporate ^{3}H-dGTP in the presence of poly rC·dG_{12-18} regardless of whether Mg^{2+} or Mn^{2+} was the divalent cation supplied.

Summary. Murine DNA polymerases C, A, and N have been separated on phosphocellulose columns. Polymerase C is preferentially stimulated by "activated" DNA, and polymerase N is preferentially stimulated by poly rA·poly dT. The template preferences of polymerases C ("activated" DNA) and N (poly rA·poly dT) recovered from phosphocellulose columns were retained regardless of salt concentration and were not due to contaminating nucleases in the column fractions. The optimal salt and pH, as well as synthetic template preferences, of peaks I and III recovered from phosphocellulose firmly identify them as polymerase C and polymerase N, respectively. The number of polymerases and the identity of polymerase activities in peak II has not been resolved, but the presence of polymerase activity stimulated by poly rA·dT_{12-18} is suggestive of an RNA-directed DNA polymerase.

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