Fractionation of d(A-T) Polymer from Testes of *Cancer borealis**

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ABSTRACT: A fibrous complex composed of d(A-T) polymer, major deoxyribonucleic acid, ribonucleic acid, and histone has been prepared from the testes of *Cancer borealis* as described previously (Baranowska, B., Baranowski, T., and Laskowski, M., Sr. (1968), *European J. Biochem*, *4*, 345). The complex was treated with massive doses of enzymes (pancreatic ribonuclease, T_1 ribonuclease, and pronase) to remove contaminants; the purified deoxyribonucleic acid (containing only satellite and major deoxyribonucleic acid component) was isolated and chromatographed on methylated albumin kieselguhr by stepwise elution with increasing concentrations of NaCl. Each fraction from methylated albumin kieselguhr was chromatographed on hydroxylapatite with constant salt concentration but varying temperature. This procedure yielded renatured poly d(A-T) which was clearly separated from denatured major component. The relative amount of poly d(A-T) in each methylated albumin kieselguhr fraction increased with increasing concentration of eluting salt, reached a maximum at 0.75 M NaCl, and then decreased. The poly d(A-T) from methylated albumin kieselguhr fractions eluted with lower NaCl concentrations had lower $s_{20,w}$ and higher C + G values than d(A-T) originating from fractions eluted with higher salt concentrations. The reversed procedure in which purified crab deoxyribonucleic acid was first fractionated on hydroxylapatite then on methylated albumin kieselguhr yielded several fractions of renatured poly d(A-T). The $s_{20,w}$ values increased in fractions eluted with higher salt concentrations. A high C + G content was found in the low molecular weight fraction, whereas fractions in the range of 4.8–9.9 S had lower but comparatively constant C + G values. These results strongly support the assumption of a nonuniform distribution of C + G in the d(A-T) polymer. An alternative procedure for the preparation of renatured poly d(A-T) is direct thermal chromatography of the fibrous complex on hydroxylapatite. This step removes ribonucleic acid and histone as efficiently as the more laborious use of enzymes and repeated shaking with chloroform. Hydroxylapatite chromatography may yield a nondenatured major component of crab deoxyribonucleic acid, if upon reaching 80° the temperature is brought back to 60° and the strength of the eluting phosphate buffer increased to 0.5 M.

The use of thermal chromatography on hydroxylapatite as an analytical method for determination of the amount of satellite in a mixture is also described. The method is as simple as the determination of melting temperature profile, and more convenient for quantitative work.

There are essentialy two methods, with modifications, for the preparation of satellite poly d(A-T) free from the major deoxyribonucleic acid component (see the review of Klett and Smith, 1967). In the original method of Sueoka and Cheng (1962b), renatured poly d(A-T) is obtained. Whole DNA[1] from crab testes is subjected to thermal denaturation, cooling, and chromatography on methylated albumin kieselguhr. Upon cooling, the poly d (A-T) renatures and is eluted with 0.7 M NaCl whereas the major component remains denatured and bound to methylated albumin kieselguhr. The second method yields a preparation of native poly d(A-T). It has been described by Davidson *et al.* (1965) and by Nandi *et al.* (1965). Satellite preferentially binds mercuric ion. The mercuric derivative of poly d(A-T) has a higher buoyant density than the major component and is separated by equilibrium density gradient centrifugation in Cs_2SO_4. The mercuric ion is then removed, and native satellite d(A-T) obtained. The disadvantage of this method is its scale, limited by the capacity of an ultracentrifuge. The obvious advantage is the elimination of thermal denaturation.

Our work on crab d(A-T) polymer was initiated by the desire to obtain a substrate for several nucleases that are being studied in this laboratory. Therefore, it was necessary to adjust the method of preparation to a scale yielding hundreds of milligrams of poly d(A-T). Obviously, methods limited by the capacity of the ultracentrifuge had to be excluded, even though it meant sacrificing native conformation.

In a previous paper of this series (Baranowska *et al.*, 1968), a semimacro method of preparation of renatured

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[1] The expression "whole DNA" designates both components of DNA from crab testes and does not imply that the molecules are intact.

poly d(A-T) from crab testes has been described. The preparation was slightly contaminated with RNA and had an unusually low C + G content of 1.5 mole % instead of the accepted 2.6–3.0 mole % (Sueoka and Cheng 1962a,b; Swartz *et al.*, 1962; Goldberg *et al.*, 1962; Smith, 1964; Nandi *et al.*, 1965; Widholm and Bonner, 1966; Skinner, 1967; Hyman and Davidson, 1967).

This preparation of poly d(A-T) has already been used in a study of the inhibitory effect of actinomycin D on the action of micrococcal nuclease (Sulkowski and Laskowski, 1968). It was felt, however, that further work on poly d(A-T) was desirable. It was hoped that a more complete removal of RNA was possible, and that a reason for the discrepancy in C + G values could be found. Both these lines of inquiry were followed.

In the method of Sueoka and Cheng (1962b) thermal denaturation cannot be avoided. In view of this, no attempts were made to avoid scission of DNA. In fact, it was hoped that the previously observed $s_{20,w}$ value of 12 S would be reproduced (Baranowska *et al.*, 1968). Such fragments would be large enough to preserve the character of the DNA and yet small enough to accentuate differences in C + G content, if these two bases are distributed nonuniformally.

The present paper describes a method for preparing crab DNA practically devoid of RNA and histone. The major issue in this paper is the thermal chromatography of crab DNA on hydroxylapatite. The method is recommended as an analytical tool for the determination of satellite poly d(A-T) in a mixture and as a preparative tool for its separation. A combination of chromatography on methylated albumin kieselguhr and on hydroxylapatite leads to fractions that vary with respect to either C + G content or molecular weight, or both.

Experimental Procedure

Materials. Frozen testes of *Cancer borealis* were purchased from the Marine Biological Supply and Development Corp. (Brunswick, Maine).[2] Crystalline pancreatic RNase and T_1 RNase were purchased from the Worthington Biochemical Corp. and pronase from Calbiochem Corp. Hypatite C (hydroxylapatite) was obtained as a suspension from the Clarkson Chemical Co., Williamsport, Pa., and Carbowax 2000 from the Union Carbide Corp. Lyophilized venom of *Crotalus adamanteus* was purchased from the Miami Serpentarium, Miami, Fla. Water was triple distilled; all other reagents were reagent grade. Thymus DNA was prepared according to the method of Kay *et al.* (1952).

Analytical Methods. The modifications described by Zamenhof (1957) were used to perform the biuret and orcinol reactions. The biuret reaction was followed by measuring the absorbancy difference at 310 and 390 mμ after correcting for the equivalent value of a blank read at the same two wavelengths. The orcinol method as described by Zamenhof (1957) is an adaptation of Mejbaum's (1939) reaction for samples containing large amounts of DNA. Here too, the difference in absorbance at two wavelengths is recorded after correcting for a similar difference in the blank. However, even this precaution does not completely eliminate the absorbance contributed by DNA or mixed deoxyribonucleotides (Figure 1).

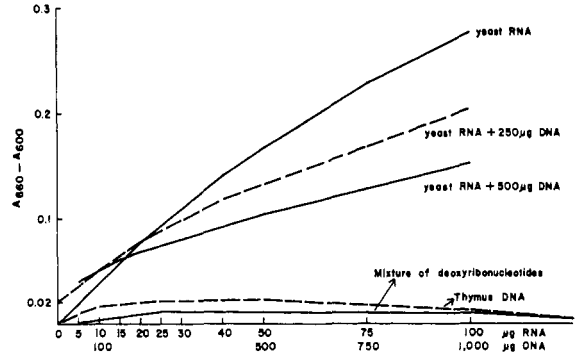

FIGURE 1: Standardization of the orcinol reaction with materials rich in DNA. Dried material was treated with 1 ml of Mejbaum (1939) reagent, heated for 10 min, and diluted with 3 ml of water; absorbancy was read at two wavelengths, 660 and 600 mμ, and corrected for the blank, read at the same wavelengths. The absorbancy difference is plotted *vs.* the amount of RNA (upper scale) valid for the upper three curves. The lower scale applies to the lower two curves.

TABLE I: Composition of Fibrous Complex and of Purified Whole DNA Isolated from Testes of *C. borealis*.[a]

	Orcinol Test[b]	Biuret Test[c]
Fibrous complex	0.260–0.300	0.380–0.450
Purified DNA	0.018–0.021	0.020
Thymus DNA	0.020–0.022	0.018–0.024

[a] Two numbers indicate the range of several determinations. [b] Difference between ($A_{660} - A_{600}$) and a corresponding blank. [c] Difference between ($A_{310} - A_{390}$) and a corresponding blank.

Figure 1 shows that the usefulness of the orcinol reaction is limited to determinations of RNA in DNA preparations of the order of magnitude of 1:50 and higher. When the RNA contamination becomes smaller the orcinol reaction is unreliable. The same applies to the vigorously purified whole DNA of crab (Table I). Table I also shows that the level of RNA contamination of whole crab DNA is similar to that of the presently accepted standard animal DNA from calf thymus.

The base content was determined by two independent methods. In the first method DNA was digested with $HClO_4$ to bases, which were determined according to Wyatt (1952) as described by Bendich (1957). In this method a considerable amount of thymine is destroyed during perchloric acid digestion. With poly d(A-T), T accounts for nearly 50% of the base composition, and a

[2] We are indebted to Mr. Henry Bird for his splendid cooperation.

loss of T of the order of 10% significantly influences the absolute values of C + G (compare Tables IV and VI to see the effect of correcting by setting T = A).

In the second method DNA was digested to nucleosides with crude venom of *Crotalus adamanteus* in the presence of Mg^{2+}. The digestion mixture was then streaked on Whatman No. 3MM paper and chromatographed in ethyl acetate–propanol–H_2O (4:1:2, v/v). This separated A, T, and C + G. The band of C + G was cut out, sewed to another piece of Whatman paper, and chromatographed using isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v). This separated C from G. The recovery of nucleosides was incomplete in this method. About 5–10% of material was not accounted for. Some ultraviolet-absorbing material remained at the origin of the first paper. It may have been due to the incomplete digestion or to the adsorption of nucleosides to the denatured protein of venom which remained stationary. Interpretation of the spectral analysis of this material was difficult because of the presence of large amounts of protein. The absolute values obtained by the two methods did not agree. The enzymatic method gave higher values for T than for A (exactly opposite to the first method) and the absolute values for C + G were significantly lower. However, both methods showed the same trend of values of C + G in consecutive fractions.

Sedimentation coefficients of poly d(A-T) samples in 0.2 M NaCl–1 mM potassium phosphate buffer (pH 6.7) were determined at 20° in a Spinco ultracentrifuge Model E with ultraviolet optics at a concentration of 20 μg/ml at a speed of 50,740 rpm. The 30-mm centrifuge cell used was fitted with a Kel-F centerpiece. Densitometer tracings of the photographs were made on a Spinco Model R Analytrol. Sedimentation coefficients were calculated from the densitometer tracings as described by Schumaker and Schachman (1957).

Melting temperature profiles were determined using a Gilford Model 2000 spectrophotometer with an attached water circulator and temperature programmer. Melting was performed on samples which had been extensively dialyzed against one-tenth standard saline citrate. Temperature was increased at a rate of about 1.0°/min. Adenine hydrochloride was used as a blank.

For analytical purposes a small column of hydroxylapatite (1.25 × 20 cm) was used, with 7 ml of the gel suspension (as obtained from the manufacturer) and 1 mg of DNA; 5-ml fractions were collected at a flow-rate of 1 ml/min. Otherwise the procedure was identical with the preparative hydroxylapatite chromatography described in detail below.

Preparative Methods. The methylated albumin kieselguhr column was prepared according to Mandel and Hershey (1960) except that it was much larger. The bed volume was approximately 2000 ml. The effective layer of methylated albumin kieselguhr was 9 × 24 cm in addition to two protective layers of kieselguhr. The sample of DNA (100–180 mg dissolved in 0.05 M potassium phosphate buffer (pH 6.7) made 0.4 M with respect to NaCl) was introduced onto the column. Elution was carried out stepwise, with the same phosphate buffer, and specified volumes and concentrations of NaCl: 500 ml of 0.4 M, 750 ml of 0.5 M, 1000 ml of 0.6 M, 1000 ml of 0.65 M, 1000 ml of 0.7 M, 1000 ml of 0.75 M, 1000 ml of 0.8 M, and finally 3000 ml of 1.0 M. Fractions were pooled and dialyzed against Carbowax 2000. The concentrated sample was either dialyzed against the appropriate buffer or precipitated with ethanol. Chloroform must be present during dialysis and in all subsequent operations when either DNA or poly d(A-T) is kept in solutions of low ionic strength or degradation occurs.

The hydroxylapatite column was used as in the experiments of Miyazawa and Thomas (1965) and Niyogi and Thomas (1968) with varied temperature and fixed concentration of eluting buffer (0.14 M phosphate buffer, pH 6.7). A column (2.5 × 42 cm), fitted with a porous sintered-glass disk and water jacket, was connected to a Haake Model F circulator equipped with a thermoregulator. Hydroxylapatite solution (30 ml) (Hypatite C), diluted 2.5 times with 0.14 M phosphate buffer, was poured into the column at 95° and stirred with a glass rod. Stirring was continued until all air bubbles were removed. The temperature was lowered to 40° and the bed allowed to settle and equilibrate to that temperature. A pressure of 10 psi was applied until the level of buffer was only 1 ml or so above the hydroxylapatite bed. DNA (20–25 mg) in 0.14 M phosphate buffer (concentration of DNA is not critical) was introduced onto the top of the hydroxylapatite bed. After temperature equilibration, a pressure of 10 psi was applied to introduce the sample into the bed. The column was filled with buffer which was previously deaereted and kept under vacuum. The temperature of the buffer was allowed to equilibrate and the elution began. Fractions (15 ml) were collected at a flow rate of 3 ml/min. The pressure was decreased with increasing temperature in order to maintain a constant flow rate. Two 15-ml fractions were collected at 40°, one at 60°, one at 65°, and thereafter at every 3°, with a temperature interval equilibration time of 5 min.

Preparation of Purified Crab DNA. The fibrous complex prepared as described previously by Baranowska *et al.* (1968) was dissolved in one-tenth standard saline citrate and treated with Pronase (250 μg/ml). The mixture was incubated at 40° for 2 hr. The concentration of standard saline citrate was increased 10-fold and the mixture subjected to three consecutive treatments of deproteinization by shaking with a chloroform–isoamyl alcohol mixture (24:1, v/v). The DNA was precipitated as before, dissolved in one-tenth standard saline citrate by stirring overnight at 4°, and treated with 100 μg/ml of pancreatic RNase (previously heated to 80° for 10 min to destroy DNase) and 1000 units/ml of RNase T_1. The amount of pancreatic RNase is twice that recommended by Marmur (1961). The enzymes were allowed to act for 2 hr at 40°. Pronase (100 μg/ml) was next added and the mixture incubated for an additional 2 hr to destroy the nucleases. Deproteinization was again repeated three times and the DNA precipitated with alcohol three times, each time collecting only the fibrous material. The final precipitate was washed in sequence with 66, 75, 85, and 95% alcohol; it was finally washed once with ether and dried at room temperature. The preparation thus obtained is referred to as

TABLE II: Comparison of $s_{20,w}$ Values of Crab DNA Prepared with and without Enzyme Treatment.

	Enzyme Treated $s_{20,w}$ (S)	Untreated $s_{20,w}$ (S)
Whole native crab DNA	12.7	12.5
Renatured d(A-T)[a]	6.3–7.5	6.6

[a] Poly d(A-T) was isolated by thermal chromatography on hydroxylapatite. The $s_{20,w}$ values of several enzyme-treated preparations were determined and indicated as a range.

purified whole crab DNA. This preparation produced absorbancy values with orcinol and biuret reagents that were equal to or lower than those produced with an equivalent amount of thymus DNA (Table I). The preparation gave 46% hyperchromicity at 96°, and had the following spectral ratios: A_{230}/A_{260} of 0.388 and A_{280}/A_{260} of 0.537.

Results

In the previous procedure (Baranowska *et al.*, 1968) the amounts of enzymes used to remove RNA were larger than required for a complete digestion of pure RNA, but rather moderate for the digestion of RNA/DNA hybrids. The reason for this moderation was the fear of introducing DNase as a possible contaminant. In the present work it was decided to disregard this danger and to use massive quantities of all three enzymes (pancreatic RNase, T_1 RNase and pronase) to obtain a preparation of purified whole crab DNA (composed of satellite and major component) that was practically free from contaminants reacting with orcinol and biuret reagents. Results justified this decision (Table II). The values of $s_{20,w}$ are considerably lower than the values of Widholm and Bonner (1965) for poly d(A-T) prepared by their method, but are similar to those of Baranowska *et al.* (1968). Table II shows that degradation occurred prior to the preparation of the fibrous complex. As expected an additional decrease in $s_{20,w}$ occurred after thermal denaturation.

TABLE III: Characterization of Fractions Obtained by Chromatography of Purified Whole Crab DNA on Methylated Albumin Kieselguhr.

Eluting NaCl (M)	$s_{20,w}$ of the Fraction (S)	% of Total DNA Charged	% of d(A-T)[a] in the Fraction	% of Total d(A-T)	$s_{20,w}$ of Renatured d(A-T) (S)	C + G[b] in Poly d(A-T) (mole %)
0.6	9.7	18.6	13.7	8.4	4.6, 4.8	4.05
0.65	11.6	39.5	24.0	31.4	5.6, 6.3	2.72
0.7	12.7	25.3	55.0	46.0	7.1, 8.0	2.09
0.75	14.9	5.5	80.0	14.0	7.1, 9.0	2.17
0.8		2.2	<10.0	Trace		
1.0		2.0	Trace	Trace		

[a] Columns 4–7 refer to the d(A-T) portion which has been separated from each of the methylated albumin kieselguhr fractions by thermal chromatography on hydroxylapatite. [b] As free bases, corrected for the loss of thymine, assuming T = A.

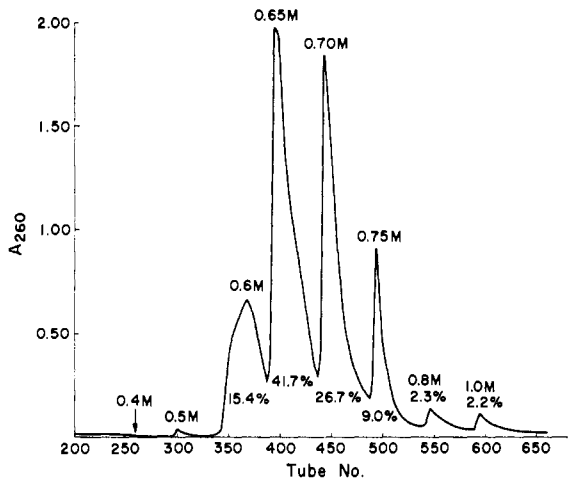

FIGURE 2: Elution pattern obtained from column chromatography of purified crab DNA on methylated albumin kieselguhr. Purified crab DNA (2800 A_{260} units) in 0.4 M NaCl and 0.05 M phosphate buffer (pH 6.7) was charged on a column (9.6 × 21.5 cm). Elution proceeded in a stepwise manner using the following concentrations of NaCl: 0.5, 0.6, 0.65, 0.7, 0.75, 0.8, and 1.0 M. The flow rate was 6.5 ml/min, 19.6 ml/tube. The molarity of NaCl and the amount of DNA expressed in per cent of charged material are indicated for each peak. Each peak contains both poly d(A-T) and the major component in different proportions (see text and Figure 3).

The enzyme-treated preparation of whole crab DNA was analyzed by CsCl equilibrium density gradient centrifugation.[3] The centrifugation was performed using DNA from *Sarcina lutea* as density marker (ρ 1.731 g/cm^3), and the following buoyant densities were obtained for the two components of crab DNA: poly d(A-T) 1.680 g/cm^3, major component 1.702 g/cm^3. We have performed equilibrium density gradient centrifugation on other samples of crab DNA used in this study with DNA from *Micrococcus lysodeikticus* as the

[3] We are indebted to Professor Waclaw Szybalski for this determination.

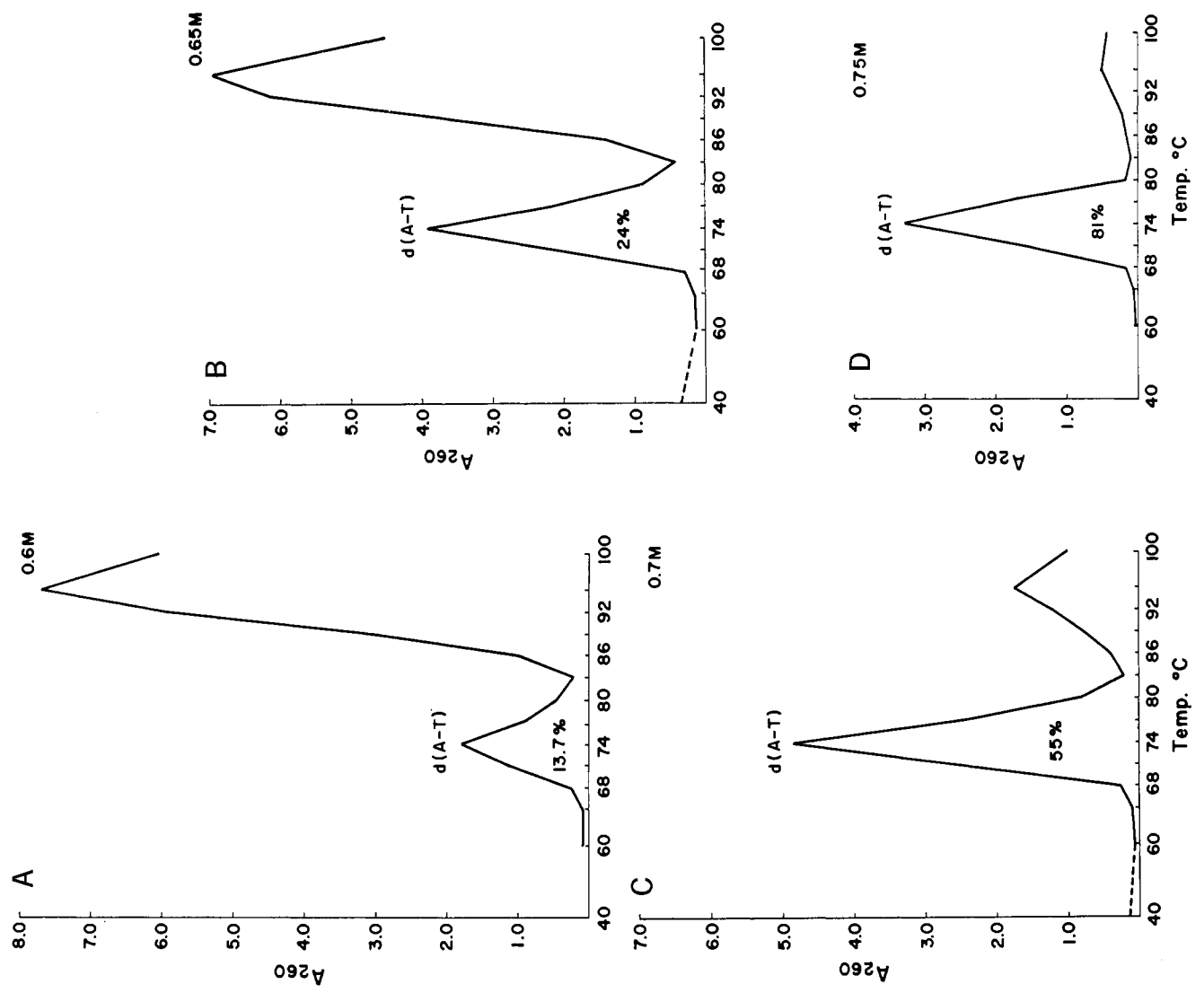

FIGURE 3: Thermal chromatography on hydroxylapatite of DNA fractions obtained from methylated albumin kieselguhr chromatography. (A) The fraction eluted with 0.6 M NaCl (Figure 2) was pooled, and the DNA precipitated with two volumes of ethanol, dissolved in distilled water, and dialyzed against distilled water for 12 hr. The DNA was again precipitated with alcohol, dissolved in a small volume of water, dialyzed against 0.14 M phosphate buffer (pH 6.7) for 24 hr, and charged on an hydroxylapatite column. Thermal chromatography proceeded by stepwise increase of temperature and elution with 0.14 M phosphate buffer (pH 6.7). After the temperature was adjusted to 40° and allowed to equilibrate for 5 min the column was washed with 20 ml of buffer. After the temperature was raised to 60°, then 65°, the procedure was repeated using a temperature equilibration time of 5 min. The flow rate was 3 ml/min, 15 ml/tube. Only the fractions collected at 71, 74, and 77° were pooled to represent the peak of poly d(A–T). Fractions eluted at 80 and 86° were discarded. The fractions collected between 89 and 100° represent major component. The per cent of poly d(A–T) in the 0.6 M methylated albumin kieselguhr fraction is expressed in terms of the total charge on the hydroxylapatite column and is indicated in the figure. (B) Fraction eluted from methylated albumin kieselguhr with 0.65 M NaCl. (C) Fraction eluted from methylated albumin kieselguhr with 0.70 M NaCl. (D) Fraction eluted from methylated albumin kieselguhr with 0.75 M NaCl. (E) Fraction eluted from methylated albumin kieselguhr with 0.80 M NaCl.

TABLE IV: Two Independent Methods of Analysis of Poly d(A-T) Fractions Obtained from Methylated Albumin Kieselguhr Chromatography (Mole Per Cent).[a]

Eluting NaCl (M)	A	T	G	C	C + G
By Enzymatic Hydrolysis with Venom to Nucleosides					
0.60	48.0	49.5	1.24	1.23	2.47
0.65[b]	48.7	49.4	0.91	0.98	1.89
	48.3	49.8	0.91	0.94	1.85
0.70	49.0	49.4	0.88	0.76	1.64
Unfractionated[c]	47.2	51.1	0.87	0.80	1.67
d(A-T)	47.5	51.1	0.76	0.71	1.47
	47.0	51.4	0.81	0.82	1.63
By $HClO_4$ Degradation, Uncorrected for the Loss of T					
0.60[d]	50.4	45.6	2.28	2.00	4.28
0.65[e]	54.9	42.6	1.20	1.23	2.43
	53.3	43.4	1.86	1.51	3.37
	55.3	41.7	1.68	1.37	3.05
0.70[e]	52.4	45.4	1.10	1.20	2.30
	48.3	49.6	1.01	1.01	2.02
	51.5	46.3	1.07	1.11	2.18
0.75	53.3	43.4	1.08	1.29	2.37

[a] Poly d(A-T) was obtained from each methylated albumin kieselguhr fraction by thermal chromatography on hydroxylapatite. [b] The same methylated albumin kieselguhr column, two different hydroxylapatite columns. [c] Purified whole DNA was fractionated directly on hydroxylapatite columns. [d] Combined from three different methylated albumin kieselguhr columns, one hydroxylapatite column. [e] A separate chromatography on methylated albumin kieselguhr and on hydroxylapatite.

density marker (ρ 1.731g/cm^3). The values for buoyant density agreed well with those of Dr. Szybalski.

Figure 2 illustrates the elution pattern of native crab DNA on methylated albumin kieselguhr. The pattern was reproducible. Some batches of methylated albumin showed a somewhat lower retaining ability, which resulted in a shift of the pattern to the left by 0.05 M NaCl. However, for the same preparation of methylated albumin, reproducibility was quite good. In agreement with Pochon *et al.* (1966) and Baranowska *et al.* (1968), each fraction contained poly d(A-T) and major component. The relative amount of poly d(A-T) increased in fractions eluted with higher salt concentrations, reached a maximum at 0.75 M, and then decreased (Table III). In earlier experiments, the per cent of poly d(A-T) in each peak was measured by determining the melting temperature profile. In subsequent fractionations it was determined by thermal chromatography on hydroxylapatite; this method has been used for calculating the results shown in Table III. The values of $s_{20,w}$ increase and the content of C + G decreases for fractions eluted with higher concentrations of salt.

The detailed distribution of poly d(A-T) and major component in each of the methylated albumin kieselguhr fractions is shown in Figure 3A–E. Examination of the symmetry of each poly d(A-T) peak suggests that qualitative differences also exist. More than half of the poly d(A-T) is eluted at 74° in the 0.60 M fraction (Figure 3A), while less than half is eluted at 74° in the 0.75 M fraction (Figure 3D). The analysis of bases and $s_{20,w}$ values definitely establishes the nonidentity of these fractions (Table III).

Table IV shows the results of several experiments designed to establish the composition of poly d(A-T) in the fractions obtained from methylated albumin kieselguhr chromatography. The tendency seen in Table III is visible in all experiments; fractions eluted with lower salt concentration have a higher C + G content. The C + G content reaches a minimum in fractions eluted with 0.70 and 0.75 M NaCl. Table IV also shows the discrepancy in values obtained by the two methods of analysis. It is well known that thymine is partly destroyed by treatment with $HClO_4$. In spite of this we believe that the most reliable results are obtained by this method, corrected by setting T = A. Only the corrected values for C + G are shown in Table III.

Thermal chromatography of crab DNA on hydroxylapatite results in the separation of renatured poly d(A-T) and denatured major component. However, a small modification of the procedure leads to the isolation of the native form of the major component. After the d(A-T) polymer has been removed from the hydroxylapatite column by continual elution with increasing temperature up to 80°, the temperature is lowered to 60° and the major DNA eluted with 0.5 M phosphate buffer. This salt-eluted DNA is not denatured as shown by the results of Figure 4. The melting curve for the preparation eluted at 60° with 0.5 M phosphate buffer is compared with that for the preparation eluted with 0.14 M phosphate buffer at temperatures between 85 and 100°.

TABLE V: Nucleoside Composition of Fractions Collected at Indicated Temperatures from a Hydroxylapatite Column Charged with Purified Whole Crab DNA.[a]

Temp of Collection (°C)	% of Total d(A-T)	Mole % Nucleosides of				
		A	T	G	C	C + G
71	29.4	47.5	51.0	0.71	0.77	1.48
74	45.5	47.5	51.0	0.96	0.97	1.93
77	25.1	48.3	49.3	1.17	1.16	2.33

[a] Three parallel runs on columns of 30 ml of hydroxylapatite were made. Each was charged with 490 A_{260} units of purified whole crab DNA. In each experiment three fractions of 15 ml were collected at indicated temperatures. Fractions obtained at the same temperature in parallel runs were pooled and analyzed for nucleosides after digestion with crude venom of *C. adamanteus*.

TABLE VI: Characterization of Fractions Obtained by Chromatography of Renatured Poly d(A-T) on Methylated Albumin Kieselguhr.[a]

Eluting NaCl (M)	% of Total d(A-T)	Mole % of the Free Base					$s_{20,w}$ (S)
		A	T	G	C	Av G + C	
Starting[b] material	100	48.7	48.7	1.49	1.15		
		48.7	48.7	1.51	1.15	2.63	
		48.7	48.7	1.45	1.14		
0.4 + 0.5	5.5	46.3	46.3	3.85	3.56	7.41	
0.6[c]	37.4	48.9	48.9	1.21	0.92		
		49.0	49.0	1.20	0.86	2.16	4.8
		48.8	48.8	1.31	0.98		
0.65[c]	31.0	48.9	48.9	1.19	0.98		
		49.0	49.0	1.22	0.86	2.13	7.0
0.7[c]	22.0	48.8	48.8	1.20	1.19		
		48.8	48.8	1.17	1.17	2.36	9.9
0.75	2.5						11.4

[a] Purified whole crab DNA was subjected to chromatography on large hydroxylapatite columns as described in the text; 12 separate runs were made; 1200 A_{260} units of d(A-T) in 0.2 M NaCl–0.05 M phosphate (pH 6.7) were accumulated and charged on a large 8.5 × 15 cm methylated albumin kieselguhr column; elution was by stepwise gradation of NaCl as indicated in the first column of this table. Base composition was determined after digestion with $HClO_4$; all values corrected for the loss of thymine by assuming T = A. [b] Each sample from a different hydroxylapatite column. [c] Parallel samples of the same fraction.

The results of Figure 4 also show that a second thermal denaturation of d(A-T) polymer is totally reversible.

In the experiments described so far native crab DNA was chromatographed on methylated albumin kieselguhr and fractions so obtained were subjected to thermal chromatography on hydroxylapatite, at which time denaturation occurred. To obtain a control it was decided to reverse the procedure. The experiment was intended to show that renaturation is essentially complete and that renatured poly d(A-T) fractionates on methylated albumin kieselguhr in the same manner as the native poly d(A-T).

When purified whole crab DNA is subjected to thermal chromatography on hydroxylapatite without previous chromatography on methylated albumin kieselguhr, and three fractions corresponding to the poly d(A-T) peak: 71, 74, and 77°, are collected separately and analyzed for their C + G content, the results of Table V are obtained. No significance is attached to the absolute values, because we are inclined to believe that digestion with the crude venom leads to low values. However the trend is obvious: the lower the eluting temperature the lower is the C + G content of the fraction. This control experiment demonstrates that the composition of fractions observed in Figure 3A–E and Table III is a reflection of the previous separation on methylated albumin kieselguhr.

The experiment was then performed in which large amounts of purified crab DNA were passed through hydroxylapatite and the fractions eluted at 71, 74, and

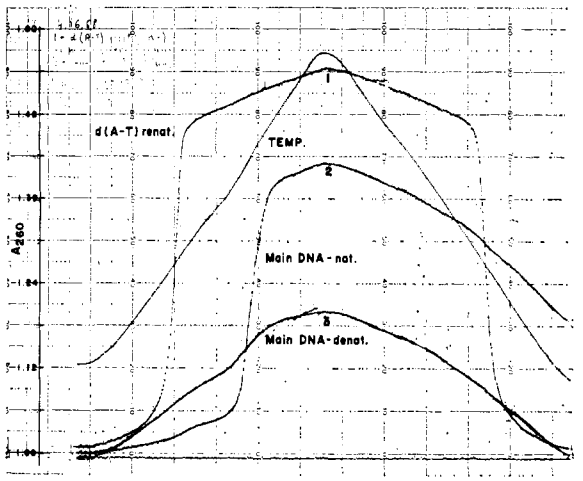

FIGURE 4: Melting temperature profiles of crab DNA components. Melting was performed using a Gilford Model 2000 spectrophotometer with an attached water circulator and temperature programmer. All samples were extensively dialyzed against one-tenth standard saline citrate before melting. Temperature was increased at a rate of 0.9°/min. Adenine hydrochloride was used as the blank at a concentration of 0.995 A_{260}/ml in one-tenth standard saline citrate. (1) Renatured poly d(A-T) was isolated by thermal chromatography on hydroxylapatite as described in the text, and exhaustively dialyzed against one-tenth standard saline citrate for 72 hr. (2) Native DNA was separated from poly d(A-T) using thermal chromatography on hydroxylapatite, by first eluting poly d(A-T) in the temperature range 70–80°. The temperature was then lowered to 60° and native main DNA eluted with 0.5 M phosphate buffer (pH 6.7). The DNA was exhaustively dialyzed against one-tenth standard saline citrate. (3) Denatured main DNA was prepared by thermal chromatography on hydroxylapatite. Fractions were collected between 91 and 97°, pooled, and exhaustively dialyzed against one-tenth standard saline citrate.

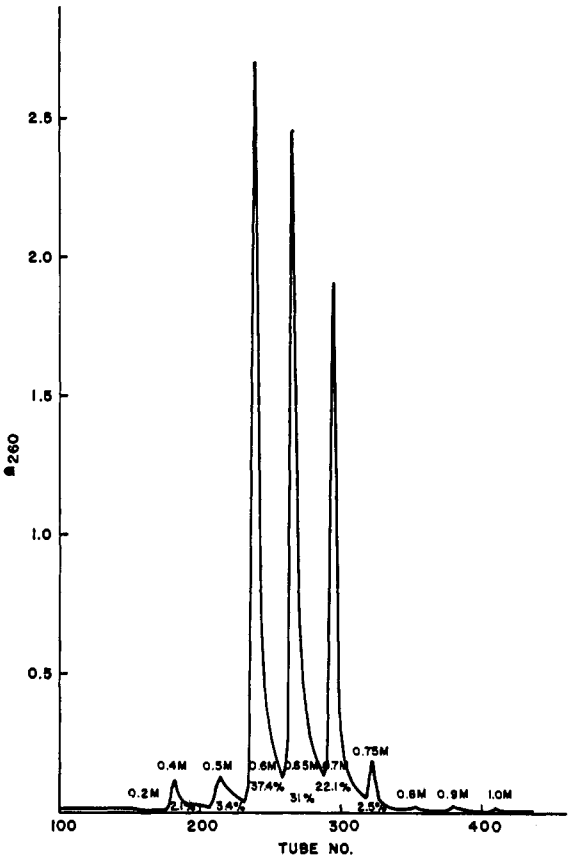

FIGURE 5: Elution pattern obtained from chromatography of renatured poly d(A-T) on methylated albumin kieselguhr. Renatured poly d(A-T) was isolated from purified crab DNA by thermal chromatography on hydroxylapatite as described in the text; 12 separate runs were made. Runs 1, 6, and 12 were used for the determination of base composition of the starting material (Table V). The remaining d(A-T) fractions were pooled and made 0.05 M with respect to phosphate buffer, pH 6.7, and 0.2 M with respect to NaCl. This poly d(A-T) (1200 A_{260} units) was charged on a methylated albumin kieselguhr column (8.5 × 15 cm). The flow rate was 7 ml/min, 21 ml/tube. Elution proceeded in a stepwise manner using the following concentrations of NaCl: 0.2, 0.4, 0.5, 0.6, 0.65, 0.7, 0.75, 0.8, 0.9, and 1.0 M. The molarity of eluting NaCl and per cent d(A-T) in each peak are indicated in the figure.

77° representing renatured poly d(A-T) were pooled. The material was chromatographed on methylated albumin kieselguhr (Figure 5). The pattern was quite similar to that presented in Figure 2 with native DNA, and the recovery was equally good. Fractions were analyzed and the results are summarized in Table VI. The same tendency is seen that was observed in experiments with native DNA. The early fractions eluted with lower salt concentration are richer in C + G and have smaller molecular weights. The content of C + G stabilizes early and becomes practically constant in the range from 0.6 to 0.70 M, indicating that in this range fractionation is mostly dependent upon size. The 0.75 M fraction was too small to analyze.

For methodological considerations, an experiment was then performed in which fibrous DNA complex was subjected to thermal chromatography (Figure 6, solid line). The elution pattern is compared with that of purified crab DNA (dashed line). The purified DNA separates into two components, poly d(A-T) and the major component. The complex separates into three components. The first peak, which is eluted at 40°, contains RNA and histone, as indicated by a positive reaction with orcinol and biuret reagents. The other peaks contain no orcinol- or biuret-reacting material. This experiment demonstrates a comparatively simple method of preparing renatured poly d(A-T) on a fairly large scale directly from the fibrous complex. The modification described above can be applied for preparing renatured d(A-T) and native major component.

Another observation dealing with methodology required additional controls of purity of poly d(A-T). The melting profile of d(A-T) (Figure 4) shows a very slow increase in absorbance in the range of temperature from 55 to 95°. This slow and uniform increase accounts for 16% of the total hyperchromicity. There is no inflection in the region from 65 to 80° corresponding to cooperative melting of the "main native DNA." The slope of the melting curve of d(A-T) in the region 55–95° is identical with the slope of the "main native DNA" melting curve above 80°, but differs in length; the latter extends only from 80 to 95°.

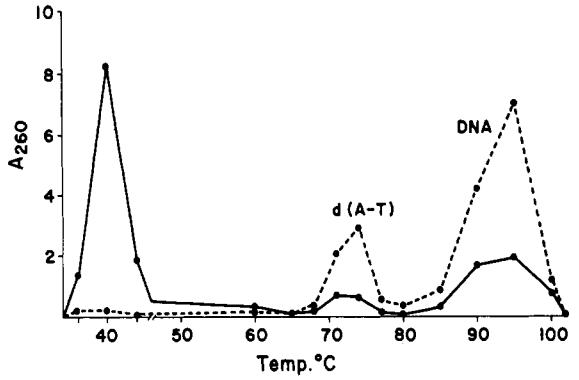

FIGURE 6: Thermal chromatography of fibrous DNA–RNA complex on hydroxylapatite. Fibrous complex was prepared according to Baranowska *et al.* (1968). After the last precipitation with ethanol, the complex was dissolved in 0.14 M phosphate buffer, pH 6.7. A total of A_{260} units was charged on a small column (1.25 × 20 cm) and 5-ml fractions were collected at a flow rate of 1 ml/min. Temperature was increased in a stepwise manner while eluting with 0.14 M phosphate buffer (pH 6.7). (●—●—●) Fibrous DNA–RNA complex and (●..●..●) purified whole crab DNA.

This slow increase in absorbance arose suspicion as to the purity of d(A-T) with respect to possible contamination by the major DNA component. Two types of experiment were performed. First, attempts were made to detect contamination by equilibrium density gradient centrifugation in CsCl. To this end two cells were centrifuged simultaneously. The first contained thermally denatured whole crab DNA and DNA of *M. lysodeikticus* as a density marker. Three bands were observed: 1.731, 1.717, and 1.680 g per cm^3. The second cell contained only renatured d(A-T) obtained by thermal chromatography on hydroxylapatite and was purposely overloaded (8 μg). Only one symmetrical band with a peak at 1.680 g/cm^3 was observed, and no band corresponding to denatured or native main component could be detected.

It is possible that contamination was not detected by cesium gradient centrifugation because it was below the level of sensitivity of this method. Therefore an experiment of the type shown in Table V was performed. Fractions eluted at 71, 74, and 77° from a hydroxylapatite column were collected separately. They differ significantly in C + G content (Table V). If part of the C + G content is due to contamination by the major DNA component, the three fractions of d(A-T) should vary in amount of contamination. All three samples were melted simultaneously in one-tenth standard saline citrate and showed slow hyperchromic regions above 55° that were essentially identical with respect to slope and length.

The reason for the slow hyperchromic region of the d(A-T) preparations extending from 55 to 95° still remains obscure. Since the contamination with major component, if it exists, is below the level of detection by either of the two methods used, causes other than contamination seem more probable.

Unpublished results from this laboratory suggest that this phenomenon of slow hyperchromicity at high temperature may be correlated with the molecular weight of the polymer.

Discussion

Thermal chromatography on hydroxylapatite is a convenient analytical method for determination of the amount of poly d(A-T) satellite in whole crab DNA. The method is as simple as melting temperature determination and more reliable quantitatively. It suffices to measure the total absorbancy of the pooled poly d(A-T) fraction to have a direct percentage of d(A-T) in the sample. Another advantage is that contaminants which interfere with the melting of poly d(A-T) are eliminated at lower temperatures.

Thermal chromatography on hydroxylapatite also offers several advantages as a preparative method. The separation of poly d(A-T) and major DNA component is excellent, with no overlap. The method may be adjusted to almost any scale provided the width of the column is kept within limits allowing thermal equilibration. An additional advantage which has not yet been fully exploited is the possible utilization of comparatively crude preparations of DNA. This may reduce the time required for isolation and eventually improve the quality of the preparation.

Two different types of chromatography have been used in this work. Table VII compares the properties that are responsible for the preferential binding of DNA to hydroxylapatite or methylated albumin kieselguhr. Only one similarity exists. Shorter fragments are eluted earlier from both columns. With respect to other properties the two columns act in an opposite manner. The combination of the two allows for a more efficient fractionation of crab DNA than was previously possible.

TABLE VII: Preferential Binding of Crab DNA to Hydroxylapatite and Methylated Albumin Kieselguhr.[a]

DNA Property	Hydroxylapatite	Methylated Albumin Kieselguhr
Conformation	Double stranded	Single stranded
Base composition	G-C rich	A-T rich
Molecular weight	High	High

[a] Hydroxylapatite chromatography is used with constant molarity of eluting buffer and varying temperature. Methylated albumin Kieselguhr chromatography is used at constant temperature and varying molarity of eluting salt.

As used in this work hydroxylapatite releases fractions according to their melting temperatures. This means that the A + T content is the major basis for fractionation. Undoubtedly, some effect is exerted by the chain length of a fragment. Niyogi and Thomas (1968) working with a similar type of column have shown pronounced differences in melting points of a

series of homologs from di- to hexanucleotides. It would be expected, however, that the relative differences would decrease with increasing chain lengths, particularly with fragments in the range from 100 to 500 nucleotides. Within the thermal range corresponding to the appearance of d(A-T) polymer, the method is sensitive to small changes in C + G content (Table V and Figure 3A–E).

The major conclusion from all presented experiments is that subfractions of d(A-T) polymer have been obtained. Some of these subfractions differ in their C + G content, which suggests a nonuniform distribution of C + G throughout the molecule. The conclusion is valid regardless as to whether the separated fractions represent different portions of an identical original molecule or homologous portions of originally different molecules.

No satisfactory explanation can be offered for the relatively high content of C + G in short fragments. A possible contamination with the major component has been considered. However, this assumption is inconsistent with the fact that identical distribution is found for both native (Table IV) and renatured poly d(A-T) (Table VI). Upon heating, the major component is irreversibly denatured and firmly bound to methylated albumin kieselguhr.

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