Hydroxyapatite Chromatography and Formamide Denaturation of Adenovirus DNA

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Received for publication 9 April 1973

Denatured adenovirus DNA was retained by hydroxyapatite columns under conditions generally used for selective retention of double-stranded DNA, probably due to several partially complementary sequences within single-stranded DNA. It was found that addition of formamide reduced the fraction of sonically treated, denatured adenovirus DNA bound to hydroxyapatite from about 30% to less than 1%. This led to a study of the effect of formamide on the melting temperature (T_m) of double-stranded DNA in solution or bound to hydroxyapatite. The T_m of DNA decreases 0.56 C/1% formamide, a value determined in buffered solutions with purified formamide.

Hydroxyapatite (HA) chromatography has been used extensively for separation of single- and double-stranded DNA (3). It was recently used to estimate the number of viral genome equivalents in adenovirus type 2 (Ad2)-transformed rat cells (18). We observed, however, that denatured human adenovirus DNA was retained on HA columns under conditions (0.12 M phosphate buffer, 65 C) generally employed for passage of single-stranded DNA and quantitative retention of double-stranded DNA. The fraction of denatured Ad2 DNA retained by HA under these conditions was determined as a function of fragmentation, revealing several (4-8) disperse sites for binding of single strands to HA. Addition of formamide (50%) reduced the fraction of denatured, fragmented Ad2 DNA retained on HA, which led to further investigations of hydroxyapatite chromatography of DNA in the presence of formamide.

The use of adequately buffered, purified formamide led to a lower value for the reduction of the melting temperature (T_m) of DNA by formamide than that reported by McConaughy et al. (14).

MATERIALS AND METHODS

Viruses. The sources of virus stocks used in this study are summarized in Table 1. Monolayer cultures of African green monkey kidney (AGMK) cells were infected with simian virus 40 (SV40) at 0.1 PFU/cell. SV40 DNA was extracted directly from infected cells by the Hirt procedure (12). Adenoviruses were propagated in suspension cultures of HeLa-S3 cells with a multiplicity of infection of 2×10^3 particles per cell. After 48 to 72 h the infected cells were harvested by centrifugation, suspended in 0.1 M Tris (pH 8.0),

frozen, thawed, and stored frozen at -70 C. Virus was extracted at 4 C by sonic treatment, followed by centrifugation (10 min at 3,700 rpm) to remove cellular debris. The supernatant fluid was extracted vigorously with an equal volume of trifluorotrichloroethane (Freon 113). Virus in the resulting aqueous phase was purified by three rounds of centrifugation in buoyant CsCl density gradients (12 to 18 h at 40,000 rpm, Ti50 rotor) and stored at 4 C in the buoyant medium buffered with 67 mM Tris, pH 8.0.

Viral DNA. Radioactively labeled adenovirus DNA was prepared by the addition of 1 mCi of ^{14}C -sodium formate (Amersham) or ^3H -thymidine (New England Nuclear Corp.) per liter at 2 to 6 h after infection. Purified adenovirus was diluted with 5 vol of 0.10 M Tris-hydrochloride-0.01 M disodium EDTA (pH 8.5) (TE). Sarcosyl and Pronase B (preincubated 15 min at 37 C) were added to reach concentrations of 5 mg/ml and 1 mg/ml, respectively, followed by 30 min of incubation at 37 C. The lysate was gently extracted three times at room temperature with equal volumes of redistilled phenol (saturated with TE) and one-fifth volume of chloroform-isoamylalcohol (99:1). The phenol was removed from the aqueous phase by ether extraction, and the residual ether was removed with a gentle stream of N_2 . After buoyant equilibrium centrifugation in a CsCl density gradient (36 to 48 h, 15 C, 40,000 rpm, Ti50 rotor) the DNA band was recovered and dialyzed exhaustively against 0.1 $\times$ TE.

Tritiated thymidine was added to SV40-infected AGMK monolayers 18, 36, and 60 h after infection in increments of 10 μCi per plate (2 mCi/liter). Viral DNA was extracted by the Hirt procedure (12) 5 to 7 days after infection and banded in CsCl density gradients containing 0.25 mg of ethidium bromide per ml (19). Monomeric, closed circular SV40 DNA was purified by velocity sedimentation in self-generating CsCl density gradients (5). The resulting DNA was rebanded in a CsCl buoyant density gradient contain-

ing ethidium bromide as above. This material was stored at -20°C in the buoyant medium and was used to generate nicked circular SV40 DNA by photochemical reaction (8). Ethidium bromide was removed by passage over small Dowex 50 columns equilibrated with 5 M NaCl. The resulting DNA was dialyzed exhaustively against $0.1 \times \text{TE}$.

Purification of formamide. Commercial formamide from several sources was observed to have high conductivity and optical density (260 to 320 nm) and also led to alkaline pH in unbuffered aqueous solution. The following procedure was developed to remove contaminants of the formamide. Commercial formamide was stirred for 1 to 2 h at room temperature with 30 to 50 g of Amberlite MB1 mixed bed resin per liter and 10 g of Norite A activated charcoal per liter. The suspension was filtered twice through hard paper to remove all traces of resin and charcoal. The filtrate was extracted three times with anhydrous ether. Residual ether and the ammonia contaminant were removed by exhaustive purging with N_2 gas (3 to

5 h of bubbling at room temperature with vigorous stirring). Table 2 presents physical data comparing the original, intermediate, and purified formamide from two commercial lots. Figure 1 presents the pH of buffered and unbuffered solutions of one lot of formamide before and after purification. Unpurified formamide gives an increase in pH both in neutral water and in standard saline citrate buffer (SSC), but 0.12 M phosphate buffer (NaP or KP) can control the pH of the formamide. NaP and KP refer to an equimolar ratio of mono- and di-basic phosphates of sodium and potassium, respectively, with pH generally between 6.8 and 7.0.

Hydroxyapatite chromatography. Hydroxyapatite was purchased from Clarkson Chemical Co. (Hypatite C stored in 1 mM NaP, 4 C). The hydroxyapatite suspension was boiled in loading buffer (0.12 M NaP, 0.4% sodium dodecyl sulfate [SDS] or 0.12 M KP) prior to packing each column. Columns were packed and washed with 0.48 M NaP or 0.50 M KP prior to equilibration with the loading buffer.

Liquid scintillation counting. Samples (0.1 to 0.5 ml) were diluted approximately 40 times with 50% toluene, 50% methanol containing 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-2-(5-phenyloxazoly)benzene per liter. Radioactivity was determined in a Beckman model LS-233 or a Packard model 3375 spectrometer. Appropriate standards were prepared to assess quenching with different sample buffers.

Spectrophotometry. (i) Optical density measurements were made with a Zeiss PMQII single-beam spectrophotometer. Buffer and sample solutions were compared by successive measurements in the same quartz cell by using an open-path blank for 100% transmittance.

(ii) The concentration of purified adenovirus was assessed by optical density at 260 nm in $0.1 \times \text{SSC}$ with 5 mg of SDS per ml. A value of $A_{260} = 1.0$ was assumed to correspond to a concentration of 10^{12}

TABLE 1. *Virus stocks*

Virus	Strain	Source
Ad2	Adenoid 6	R. J. Huebner, NIH, Bethesda, Md.
Ad3	Sutherland	A. Svedmyr, Stockholm, Sweden
Ad5	Adenoid 75	A. Svedmyr, Stockholm, Sweden
Ad7	Gomen	A. Svedmyr, Stockholm, Sweden
Ad12	Huie	W. Doerfler, Cologne, W. Germany
Ad18	D.C.	A. Svedmyr, Stockholm, Sweden
SV40		P. A. Sharp, Cold Spring Harbor, N.Y.

TABLE 2. *Selected parameters of formamide during different stages of purification*

Lot	Parameters of formamide					
	Purification step ^a	pH (50% aq) ^b	A_{330}	A_{270}	25 ^c	μS^d
Lot A	I	9.2	0.03	0.26	1.4463	310
	II	8.6	0.01	0.11	1.4440	7
	III	6.6	0.00	0.09	1.4440	7
Lot B	I	7.4	0.02	0.26	1.4471	400
	II	7.4	0.00	0.09	1.4434	8
	III	6.4	0.00	0.09	1.4434	8
Double-distilled water		6.4	0.00	0.00	1.3330	4

^a Roman numerals indicate the three purification steps: I, commercial stocks; II, filtered formamide, which was stirred 60 to 90 min with 10 g of charcoal and 30 to 50 g of Amberlite/liter of formamide prior to filtration; III, ether extraction and N_2 purging for 3 h of step II formamide.

^b pH measurements were carried out with a Radiometer model 26 pH meter and GK2301 C combined reference and glass electrode at ambient room temperature.

^c Refractive index.

^d Conductivity was measured by using a Philips model PR9501 conductivity meter and a PW9513 cell at ambient room temperature.

FIG. 1. pH measured by a glass electrode at different concentrations of formamide in neutral distilled water (A), standard saline citrate (SSC) (B), 0.12 M potassium phosphate buffer, pH 7.0 (KP) (C). Symbols: ○, unpurified formamide; ●, formamide purified by the procedure described in Materials and Methods. The influence of formamide on pH measurements with a glass electrode was not assessed.

particles/ml (13). The concentration of purified DNA was estimated by assuming $A_{260} = 1.0$ for 50 $\mu\text{g/ml}$.

(iii) A thermostated 3-cell holder with a calibrated thermistor immersed in the center cell was used to follow the hyperchromic transition at 260 nm on melting of Ad2 DNA.

Sedimentation of DNA. Samples were sedimented through linear 5 to 20% sucrose gradients containing 1 M NaCl, 0.01 M Tris (pH 7.4), and 0.001 M EDTA. Native duplex adenovirus DNA was used as a marker and assumed to have a value of $s_{20,w} = 31S$ (11). Sedimentation coefficients for samples were estimated by the ratio of average displacements of the sample and marker DNA from the bottom of the sample lamella. Molecular weights were calculated for native duplex or neutral denatured DNA from sedimentation coefficients and the relations given by Studier (20).

RESULTS

Detection of breaks in adenovirus DNA. Samples of adenovirus DNA were examined for single- or double-strand scissions by sedimentation of denatured DNA through neutral (pH 7.4) sucrose gradients ($s_{\text{neut, den}}$) (Fig. 2). Assuming a value of $s_{20,w} = 31S$ for native duplex DNA (11), intact denatured DNA was recovered as a symmetrical peak at a position correspond-

ing to $s_{\text{neut, den}} = 72S$. This value corresponds to DNA with a duplex molecular weight of about 2.0×10^7 , according to the relationship determined by Studier (20).

Hydroxyapatite chromatography of degraded viral DNA. Denatured Ad2 DNA was retained on HA columns under conditions (0.12 M phosphate, 65 C) generally employed for the passage of single-stranded DNA and selective retention of duplex or partially duplex DNA (Table 3). Intact Ad2 DNA was denatured in 0.12 M NaOH at room temperature and degraded by heating from 3 to 48 min at 100 C, chilled to 4 C, and neutralized with an equal volume of 0.24 M NaH_2PO_4 . The size distribution of the single-strand fragments was deter-

FIG. 2. Two superimposed neutral sucrose gradients centrifuged at 55,000 rpm for 60 min in the SW56 rotor of an L2-65B centrifuge. Native (○) and denatured (●) Ad2 DNA were layered on the gradients in sample volumes corresponding to the bar (S) at the top of the gradient. The sedimentation coefficient of the native DNA was assumed to be 31S from which the denatured DNA was estimated to have $s_{\text{neut, den}} = 72S$. The latter DNA was denatured in 0.2 M NaOH for 5 min at 20 C prior to centrifugation.

TABLE 3. Degradation of Ad2 DNA and retention of single-strand fragments by HA columns

Sample	Determination			
	Time at 100 C (min)	Concn NaOH (M)	$s_{\text{neut, den}}$ (S)	Retained by HA in 0.12 M NaP, 65 C (%)
1	0	0.12	72	98
2	3	0.12	69	95
3	6	0.12	49	88
4	12	0.12	32	79
5	24	0.12	19	73
6	48	0.12	10	47
7	48	0.24	7	10
8	15 ^a	0.00	9	32

^a Sonically treated before denaturation (see Fig. 4A).

mined by sedimentation of the denatured DNA in neutral (pH 7.4) sucrose gradients. Figure 3 shows that boiling in alkali reduced the weight average $s_{neut, den}$ from 72S to 10S in 48 min. The degraded DNA samples (0.5 ml) were also prepared for HA chromatography by addition of 0.1 ml of 2.0 M NaOH. After 5 min at room temperature the samples were neutralized with HCl and adjusted to 0.12 M NaP, 0.4% SDS (final DNA concentration 6.7 μ g/ml). The samples were passed over an HA column at 65 C within 1 min. The extent of fragmentation and the fraction of each sample retained by HA under these conditions are summarized in Table 3. It can be concluded that intact single strands are quantitatively retained by the HA columns and as much as 50% of the DNA is retained after degradation of the DNA to a weight average sedimentation coefficient $s_{neut, den} = 10$.

Sonic treatment was next used as an alternative method to generate duplex DNA fragments with molecular weight of about 4×10^5 ($s_{neut, den} = 9$ gradient not shown). Passage of sonically treated denatured Ad2 DNA mixed with sonically treated marker duplex DNA over HA in 0.12 M NaP, 65 C, led to retention of 32% of the DNA (Fig. 4A). It was observed, however, that the same phosphate buffer with 50% formamide, 23 C, led to quantitative passage of the sonically treated, denatured Ad2 DNA and selective retention of sonically treated duplex DNA (Fig. 4B). The duplex DNA was eluted quantitatively with 0.48 M NaP. This result led to a more thorough investigation of the effect of

Fig. 3. Superimposed patterns from several sucrose gradients of Ad2 DNA degraded in 0.12 M NaOH at 100 C for different time periods. The sucrose gradients were centrifuged as described in Materials and Methods and in the legend of Fig. 2. The samples applied were boiled for the following times: ●, 0 min; ○, 3 min; ■, 6 min; □, 12 min; ▲, 24 min; △, 48 min. For the undegraded DNA $s_{neut, den} = 72S$, and after 48 min at 100 C, $s_{neut, den} = 10S$.

Fig. 4. Hydroxyapatite chromatography of sonically treated, denatured Ad2 DNA with and without formamide in the elution buffer. A, 3H -DNA, sonically treated, (●) was denatured by heating to 100 C for 15 min before chromatography at 65 C with a ^{14}C -labeled sonically treated, double-stranded DNA marker (○). Loading buffer was 0.12 M NaP, 0.4% SDS, and 0.48 M NaP was added at the arrow to elute double-stranded DNA. B, Hydroxyapatite chromatography was performed at 23 C with 50% formamide in the 0.12 M NaP, 0.4% SDS. The same denatured (●) and native DNA (○) were used as in experiment A.

formamide on denaturation and hydroxyapatite chromatography of viral DNA.

The retention of sonically treated, denatured DNA of other viruses on HA columns in 0.12 M NaP, 65 C, was also investigated. Samples were prepared and passed over HA columns in the manner described above (Fig. 4). The retention of a significant fraction (15–25%) of sonically treated, denatured DNA by HA under these conditions is a rather common feature of the DNA from different adenovirus serotypes (Table 4). The corresponding fraction of SV40 DNA was much lower (2–3%). The concentration of DNA used in these experiments is substantially lower than would allow intermolecular hybridization during HA chromatography (see Fig. 8). When similarly prepared DNA samples were subjected to HA chromatography at room temperature in the presence of 50% formamide, the fraction of retained, denatured DNA was generally 1% or less.

Although intact single strands of Ad2 DNA were retained by HA at 65 C in 0.12 M NaP (Table 3), we have been able to pass up to 70% of applied, intact, single-stranded Ad2 DNA at 23 C in 0.12 M NaP with 50% formamide. Intact duplex Ad2 DNA simultaneously applied was quantitatively retained under these conditions.

TABLE 4. Retention of sonically treated, denatured viral DNA by HA columns at 65 C, 0.12 M NaP

DNA type	DNA retained (%)
SV40	2
Ad2	18 ^a
Ad5	18
Ad3	17
Ad7	14
Ad12	5
Ad18	22

^a This value differs from that presented in Table 3 (18% vs. 32%). It seems likely that this is due to the variable extent of fragmentation achieved by sonic treatment. The fraction of denatured Ad2 DNA retained by HA changes very rapidly as a function of fragmentation in the region corresponding to sonically treated DNA (see Fig. 10).

Thermal chromatography on hydroxyapatite. Samples of SV40 DNA, Ad18 DNA, and Ad2 DNA were sonically treated in 0.12 M KP and applied to HA columns at 70 C. The temperature was increased stepwise, and after 3 to 5 min at a given temperature the columns were washed with 4 to 5 bed volumes of 0.12 M KP. The amount of DNA eluted after each temperature increment was plotted cumulatively as the fraction of total DNA applied to the column (Fig. 5). The T_m of the DNA bound to HA was estimated from the intersection of the curve drawn through the elution profile and the line representing 50% of the DNA applied to the column.

A similar experiment was performed with the same DNA preparations except that elution from the HA columns was effected by a stepwise gradient of formamide while the temperature was maintained at 70 C (Fig. 6). This allowed a determination of the concentration of formamide required to reduce the T_m of each DNA to 70 C.

Potassium phosphate was chosen for these experiments because of the reduced solubility of the sodium salt at high concentrations of formamide. Preliminary experiments with 0.12 M NaP (65 C–75 C) led to incomplete recovery of DNA at high concentrations of formamide (>50%), although the DNA could be eluted by subsequent application of a 0.12 M NaP solution lacking formamide. In this work we have employed 0.12 M NaP and 0.12 M KP in the presence of no more than 50% and 75% formamide, respectively, which is within the solubility range for these buffers. At 0.12 M phosphate there was no indication of elution of duplex DNA at temperatures up to 70 C and formamide concentrations up to 60%.

For comparison with the T_m determined by

HA chromatography the thermal denaturation of sonically treated Ad2 DNA in 0.12 M KP solution was investigated at different formamide concentrations. Optical density measurements were made at 270 nm over a temperature range of 25 C to 95 C. The T_m in the absence of formamide was 89.5 C, within 1 C of the value of T_m observed for Ad2 DNA bound to HA (Fig. 5).

The effect of formamide on the T_m of duplex DNA in solution and on HA is presented in Fig. 7. In 0.12 M KP the T_m of duplex DNA in solution or on HA decreases by 0.56 C/1% formamide. This is significantly lower than the value of 0.72 C/1% formamide presented by McCaughy et al. (14).

FIG. 5. Cumulative thermal elution of sonically treated viral DNA from hydroxyapatite with 0.12 M KP. Sonically treated native DNA of SV40 form-II (■), Ad18 (▲), and Ad2 (●) were adsorbed on hydroxyapatite at 70 C in 0.12 M KP and eluted by denaturation with a stepwise temperature gradient.

FIG. 6. Cumulative elution of sonically treated viral DNA from hydroxyapatite with a stepwise formamide gradient in 0.12 M KP. Sonically treated native DNA of SV40 form-II (■), Ad18 (▲), and Ad2 (○, ●, two columns) was attached at 70.2 C in 0.12 M KP. When the formamide concentration reached 60%, all DNA applied to the column was eluted. No further DNA was eluted on shifting to 0.5 M KP without formamide.

FIG. 7. Relationship between formamide concentration and decrease of T_m of viral DNA in 0.12 M KP. The relationship was established with SV40 form-II DNA (■), Ad18 DNA (▲), and Ad2 (●) bound to hydroxyapatite at 70 C as well as for Ad2 DNA (○) in solution. The unbroken line corresponds to ΔT_m of -0.56 C/1% formamide, and the broken line corresponds to a ΔT_m of -0.72 C/1% formamide.

Renaturation of denatured Ad2 DNA. After thermal denaturation of sonically treated Ad2 DNA in 0.12 M KP, 70% formamide ($T_m = 50$ C), the temperature was rapidly dropped from 70 C to 27 C. Reassociation of the DNA was observed by decrease in optical absorbance at 270 nm, and the kinetics were analyzed as described by Britten and Kohne (6). The course of the reassociation reaction follows the theoretical curve predicted for second-order kinetics (Fig. 8). The product of initial DNA concentration and time (C_0t) at 50% reassociation was 6.4×10^{-2} mol s/liter which corresponds to a total duplex genome size of about 2×10^7 molecular weight.

Effect of pH on formamide denaturation of DNA. The following experiment demonstrates that the denaturation of duplex DNA in the presence of formamide is markedly affected by pH. Examination of the thermal denaturation profiles of Ad2 DNA on HA (Fig. 4) or in solution (not shown) revealed the need to raise the temperature from 5 C to 10 C above the T_m in order to completely denature and subsequently separate intact strands of DNA. Denaturation of all but a small region of the DNA would be likely to lead to a rapid reannealing on reducing the temperature. Samples of intact Ad2 DNA were adjusted to 71% formamide and 0.12 M potassium phosphate of specified pH. After 10 min at 40 C or 55 C, corresponding to 9 C below and 6 C above the T_m in 0.12 M KP, respectively, the samples were sedimented through neutral (pH 7.4) sucrose gradients (Fig.

9). At 40 C complete denaturation takes place between pH 10 and 11, leading to rapidly sedimenting (72S), single-stranded DNA. At 55 C the complete denaturation transition occurs between pH 8 and 9.

DISCUSSION

A major application of HA chromatography in nucleic acid research has been the effective separation of single- and double-stranded DNA (3). This is generally achieved when samples are applied to HA columns with between 0.10 M and 0.18 M phosphate buffer. Under these conditions double-stranded DNA is selectively retained by the HA column until the phosphate buffer concentration is raised to 0.2 M to 0.4 M. In some cases, however, a fraction of denatured DNA has been retained by HA in a manner resembling the behavior of double-stranded DNA (3). Interstrand cross-linking (1, 2, 7) and highly repetitive sequences (6, 21) have been proposed to account for the properties of the native-like fraction of denatured DNA by very rapid interstrand reassociation. We have observed that denatured adenovirus DNA binds to HA (Tables 3 and 4) with 0.12 M phosphate buffer. Yet adenovirus DNA is free of interstrand cross-links (11) and, as seen in Fig. 8, contains no (highly) reiterated sequences. An alternative explanation for the behavior of denatured adenovirus DNA on HA is that single-stranded molecules of adenovirus DNA may contain complementary or partially complementary sequences. Intramolecular reannealing would presumably be rapid and would lead to regions within single-stranded DNA that would interact with HA as duplex DNA. At least two such structures are implied from the inverted

FIG. 8. Reassociation of sonically treated, denatured Ad2 DNA in 0.12 M KP-70% formamide. Optical density measurements were recorded at 270 nm after changing water baths from 70 C to 27 C ($T_m = -23$ C). Extent of reassociation was evaluated as $(A_{270}^{70C} - A_{270}^{27C}) / (A_{270}^{70C} - A_{270}^{25C})$ where A_{270}^{25C} was taken as the optical density of the DNA at 25 C prior to thermal denaturation. DNA concentration was 32 μ g/ml. The arrow indicates 50% reassociation at $C_0t = 0.064$ mol s/liter.

FIG. 9. The effect of pH on complete denaturation of adenovirus type 2 DNA. Intact Ad2 DNA was analyzed in neutral sucrose gradients as described in Fig. 2 after incubation in 50% formamide at 40 C or at 55 C. These temperatures correspond to 9 C below and 6 C above the T_m in 50% formamide and 0.12 M KP. The incubations were performed in 0.12 M potassium phosphate buffers of different pH.

terminal redundancy of adenovirus DNA (10, 23) and from the nucleotide sequence of the so-called virus-associated (VA) RNA, determined by Ohe and Weissman (17) and shown to be transcribed from adenovirus DNA (16).

The passage of single-stranded DNA through HA columns (0.12 M phosphate, 65 C) is independent of molecular weight (15). With denatured adenovirus DNA, however, we observed retention on HA columns with a striking dependence on the extent of fragmentation of the DNA (Table 3). Weight average molecular weights ($\bar{M}_w$) were estimated for fragmented Ad2 DNA from the average sedimentation coefficients (Fig. 3, Table 3) and Studier's relation (20). Intact Ad2 single strands are totally retained by HA (0.12 M phosphate, 65 C), and the fraction retained decreases slowly until reaching $\bar{M}_w/M_0 = 0.1$ (Fig. 10). With more extensive fragmentation the fraction of HA-retained DNA drops precipitously. It has recently been reported that Ad2 DNA contains a small, inverted terminal repetition which allows intact single strands to circularize (10, 23). The termini can come together to form a small double-stranded region, which may contribute to retention of intact single-stranded Ad2 DNA on HA. The data presented in Fig. 10 show that this cannot be the major cause for HA-retained DNA, because the fraction of intact strands remaining in a partially degraded sample would drop immediately and very rapidly with decreasing

values of $\bar{M}_w/M_0$. The actual data are more suggestive of several sets of proximal complementary or partially complementary sites dispersed through the single strands of Ad2 DNA. A single-stranded DNA fragment containing such a site would be retained by the HA column. A crude estimate of the number of sites for HA retention can be made from the data in Fig. 10 in the region of maximum slope and most extensive fragmentation. There appear to be four to eight such sites, or clusters of sites, spread throughout the Ad2 genome.

At 23 C, 50% formamide was found to reduce the HA-retained, single-strand fraction of adenovirus DNAs to background levels (<1%). If the sites leading to HA retention of single strands indeed have double-strand DNA structure, the apparent T_m (without formamide) is less than 51 C (using $T_m = -0.56 \text{ C}/1\% \text{ formamide}$, Fig. 7). Yet the observed HA retention of single-stranded DNA was determined at 65 C without formamide. This suggests that the formamide may play its role by either preventing the single-strand intramolecular interactions or by interfering with their retention on HA. Whichever the case, this study shows that formamide and hydroxyapatite can be used as compatible reagents for the study of DNA.

Pettersson and Sambrook (18) have employed 0.14 M NaP, 0.10 M NaCl, 0.4% SDS, 65 C as conditions for passage of single-stranded fragments (about 500 nucleotides) of adenovirus DNA through HA columns with less than 5% retention. Under these conditions, but lacking NaCl, we have observed significant retention of

FIG. 10. The relationship between molecular weight of fragmented adenovirus type 2 DNA and the fraction of denatured DNA retained on hydroxyapatite in 0.12 M NaP, 0.4% SDS, 65 C. Adenovirus type 2 DNA was degraded as described in Fig. 3 and Table 4. The percentage retained on hydroxyapatite is plotted against the relative size of the genome (weight average).

sonically treated, denatured adenovirus DNA on HA, although less than with chromatography using 0.12 M NaP. Furthermore, sonically treated, duplex DNA is not quantitatively retained by HA columns with 0.14 M NaP in the presence of formamide, particularly at elevated temperatures.

Thermal chromatography with hydroxyapatite and formamide revealed that the ability of formamide to reduce the T_m of double-stranded DNA was significantly less than previously reported. We observed $\Delta T_m = -0.56$ C/1% formamide compared to a $\Delta T_m = -0.72$ C/1% formamide reported by McConaughy et al. (14). Their result was recently challenged by Blüthmann et al. (4), who found a $\Delta T_m = -0.60$ C/1% formamide. The latter investigators treated their formamide by a procedure similar to ours, but without the final purging of dissolved ammonia with N_2 . Both groups employed SSC to buffer solutions containing formamide, but it is clear from Fig. 1 that SSC cannot buffer unpurified formamide. Data from Wetmur and Davidson (Table 5 of reference 22) suggest that the T_m of DNA begins to drop precipitously in the interval pH 9 to 10 (about -20 C decrease in T_m over this range of pH). These considerations suggest that the pH of poorly buffered, alkaline formamide solutions contributed to the greater reduction in the thermal stability of double-stranded DNA by formamide reported by McConaughy et al. (14). We have consistently used purified formamide, effectively buffered with 0.12 M KP or NaP (Fig. 1). The enhancement of T_m reduction of DNA in formamide solutions by moderately elevated pH was also evident in the results shown in Fig. 9.

The purified formamide has in addition been found to be satisfactory for use in electron microscopy of nucleic acids by the modified Kleinschmidt procedure described by Davis et al. (9).

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Cancer Society, the Wallenberg Foundation, Stockholm, Sweden, and the Jane Coffin Childs Memorial Fund for Medical Research. Clark Tibbetts is a postdoctoral fellow supported by grants from the Jane Coffin Childs Memorial Fund for Medical Research.

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