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Studies on the selectivity of DNA precipitation by spermine

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ABSTRACT

We have examined the selectivity of the precipitation of DNA by spermine. We have found that the intra- and intermolecular condensation of DNA induced by spermine is highly selective even in the presence of added protein or triphosphates. We have also investigated the influence of buffer components on the threshold concentration of spermine required for DNA precipitation. Representative applications exploiting the selectivity of the precipitation reaction are also described.

INTRODUCTION

The precipitation of DNA and RNA by the tetravalent cation spermine was observed as early as 1959 (1). At a high nucleic acid concentration (2.5 mg/ml), addition of millimolar concentrations of spermine resulted in an insoluble complex. However, the interaction of spermine, or the trivalent spermidine, with DNA has been most thoroughly studied at relatively low concentrations of DNA (less than 100 μg/ml). Addition of spermine to a solution of bacteriophage DNA produced a shear resistant form of the DNA that was suggested to be a compacted form (2). The formation of shear resistant DNA was inhibited by the addition of monovalent or divalent cations. That the addition of spermine to DNA in fact resulted in compaction was demonstrated by spectroscopic techniques (3,4) and by electron microscopy (5). The DNA appears to form compact spherical or toroidal particles at very low DNA concentrations, and intermolecular aggregates at higher concentrations (above 5 μg/ml). Trivalent or more highly charged cations are necessary for intramolecular condensation; this follows from polyelectrolyte theory (6) as condensation requires the neutralization of at least 90% of the phosphate charge (7). That charge neutralization alone is sufficient for condensation was shown by Widom and Baldwin with studies using hexamine cobalt (III), an

inert trivalent metal ion, which produced condensed DNA indistinguishable from that produced with spermine (8). This reaction was also inhibited by monovalent or divalent cations; moreover, Widom and Baldwin also showed that many short DNA molecules could combine to form toroidal particles of similar dimensions to those formed from high molecular weight DNA.

The physical studies described above have led to a detailed understanding of the chemical and structural bases for DNA condensation under simple and well-defined solution conditions. We have extended these studies to solution conditions in which other small molecules and macromolecules were present in order to test the selectivity of the spermine precipitation reaction.

We reasoned that the specificity of the spermine-DNA interaction might exclude most other non-DNA components from the condensed DNA aggregates. Therefore, we investigated the effects of ionic strength, DNA concentration and fragment length, added proteins, and added triphosphates on the spermine precipitation reaction of DNA. We found that the spermine precipitation reaction was highly selective for DNA even under complex solution conditions. We have also established reproducible standard protocols for DNA precipitation and spermine removal that have been very useful in several manipulations in nucleic acid biochemistry.

MATERIALS AND METHODS

Materials Spermine tetrahydrochloride, calf thymus DNA type II (as the sodium salt), and non-radioactive nucleotides were all purchased from Sigma Chemicals and used without further purification. Bovine serum albumin was purchased from Sigma and heated to 65° for 10 minutes to inactivate nucleases before use. [^{14}C]spermine and radioactive nucleotides were purchased from New England Nuclear. Restriction enzymes were from New England Biolabs and Bethesda Research Laboratories, Inc. and were used in buffers suggested by the manufacturers. *E. coli* DNA polymerase I was purified as previously described (9).

Stock Solutions Spermine tetrahydrochloride was dissolved in water to make a 0.10 M solution; the pH of this stock solution was 6.8. All spermine solutions were kept at 4°C for short term storage (weeks) and -20°C for long term storage. Calf thymus DNA was dissolved in 10 mM TrisCl pH 8, 0.1 mM

EDTA. DNA concentrations were determined by absorbance at 260 nm, based on A_{260} = 1 for 50 µg/ml.

Low salt and moderate salt buffers These buffers approximate typical restriction enzyme assay buffers with added EDTA: LS (low salt) buffer was 10 mM TrisCl pH 8, 10 mM KCl, 6 mM $MgCl_2$, 10 mM EDTA, 1 mM dithiothreitol. MS (moderate salt) buffer is 10 mM TrisCl pH 8, 100 mM KCl, 10 mM $MgCl_2$, 15 mM EDTA and 1 mM dithiothreitol. High salt (HS) buffer was 10 mM TrisCl, 0.5 M NaCl, 0.1 mM EDTA. 10X Buffer 2 used for spermine pellet extraction buffers was 3 M sodium acetate, 0.1 M magnesium acetate. TBE was 90 mM Tris base, 90 mM boric acid, 2.5 mM EDTA, pH 8.3

DNA End Labelling Plasmid DNA (pBR322 with a lac 203 basepair (bp) insert at the EcoRI site) was digested with EcoRI to generate fragments of 4400 and 200 bp. The EcoRI ends were filled in using DNA polymerase I (Klenow fragment) with dATP and [α-^{32}P]TTP. Digestion with endonuclease HhaI generated four end labelled fragments of lengths 145 bp, 60 bp and two 100 bp. The mixture was separated by polyacrylamide gel electrophoresis (the 100 bp fragments were isolated together) and extracted from the gel with phenol (10). The labelled DNA fragments were stored at 4°C in 0.1 mM EDTA.

Spermine Precipitation Precipitations were performed in volumes ranging from 50 microliters (for 100 µg DNA/ml) to 1 ml (1 µg DNA/ml) in 1.5 ml plastic tubes. Spermine was added directly to solutions containing labelled DNA with or without added calf thymus DNA. After vortexing, the solutions were left on ice for 15 minutes. The solutions were then centrifuged for 10 minutes in an Eppendorf microcentrifuge. The percentage of DNA precipitated under the different conditions described was determined by measuring the amount of ^{32}P present in the pellet and in the supernatant (Cerenkov counting). Complete precipitation, as used in the text, corresponded to greater than 95% of the DNA in the pellet. Recovery of radioactivity in the pellet and supernatant was within 3% of the total radioactivity.

Protein Analyses Bacterial cell lysates for the protein precipitation determinations were prepared by a modification of the rapid plasmid DNA preparation procedure of Holmes and Quigley (11) from an Escherichia coli strain containing a pBR322 derivative. The cell suspension buffer contained 20 mM EDTA instead of 50 mM. The nucleic acid containing supernatant obtained after boiling with lysozyme was treated with RNase A for 15 minutes,

diluted with four volumes of water, and precipitated with spermine. Protein was determined using the Bio-Rad Protein Assay, utilizing the microassay procedure with bovine serum albumin as a standard.

RESULTS

DNA precipitation by spermine

Addition of spermine to a solution containing DNA resulted in an aggregation or condensation reaction which at higher DNA concentrations (10 µg/ml) could be followed by measuring the turbidity at 300 nm. The reaction appeared to plateau after 15 minutes at room temperature (unpublished data). When the solution was centrifuged, the DNA formed a pellet. The spermine concentration required to precipitate DNA according to the standard protocol is shown in Fig. 1. Note that the precipitation reaction shows a threshold dependence on polyamine concentration (7) despite the presence of mono and

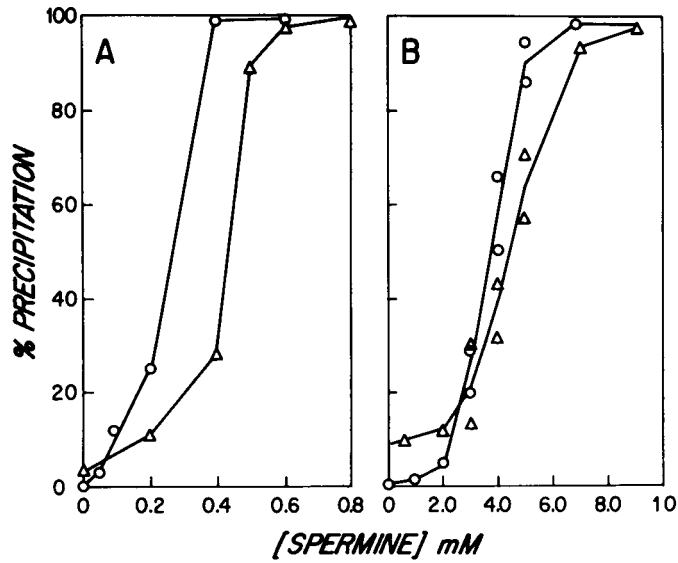

Figure 1. The spermine concentration dependence of DNA precipitation. The percentage DNA precipitated is plotted versus the final spermine concentration. The symbols in both A and B are: ○, 1 µg DNA/ml; △, 100 µg DNA/ml. Panel A: LS buffer was used (total radioactivity was 8000 to 9000 cpm). Panel B: MS buffer was used (total radioactivity was 4000 to 6000 cpm). The 1 µg DNA/ml also contained 0.1 mg/ml bovine serum albumin. Spermine precipitation was done as described in Materials and Methods.

divalent cations, protein and chelating agents in the solution. The precipitation reaction was extremely sensitive to ionic strength, as shown by comparison of Fig. 1A to Fig. 1B. Under moderate salt (MS buffer) conditions (Fig. 1B) the spermine concentration required to completely precipitate the DNA was more than ten times that required under low salt conditions (LS buffer). In addition, the spermine concentration range over which partial precipitation was observed was greatly increased at moderate salt (a 4 mM range as opposed to about 0.3 mM). At both salt concentrations, however, there was very little dependence on DNA concentration (1 - 100 µg/ml). For practical purposes, the important point to note is that 0.8 mM and 10 mM spermine are sufficient to precipitate up to 100 µg DNA/ml under LS and MS buffer conditions respectively.

The contribution of the various solution components to the concentration of spermine required for precipitation was investigated by determining the minimum concentration of spermine required for complete precipitation of the DNA under various salt conditions. As shown in Table 1, the addition of 0.1 M salt increased the concentration of spermine needed for precipitation by more than a factor of 10. $MgCl_2$ at 10 mM or 10 mM $MgCl_2$ with 15 mM EDTA inhibited the precipitation of the DNA, and both conditions required the same higher concentration of spermine for complete precipitation. The inhibition of the precipitation reaction by higher KCl and $MgCl_2$

Additions to Standard Buffer	Final [spermine] for precipitation
none	0.1 mM
0.1 M KCl	1.5 - 2.0 mM
0.1 M KCl + 10 mM $MgCl_2$	7.0 - 10.0 mM
0.1 M KCl + 10 mM $MgCl_2$ + 15 mM EDTA	7.0 - 10.0 mM

Table 1. Dependence of spermine precipitation on buffer conditions. Standard buffer is 10 mM Tris-Cl, pH 8.0; 1 mM dithiothreitol. Spermine precipitation was done as described in Materials and Methods. DNA solutions contained 100 µg/ml calf thymus DNA and 0.01 µg end labelled 100 bp DNA fragment. 100% precipitation was approximately 4500 cpm.

concentrations is easily rationalized. These cations compete with spermine for binding to the DNA, but are unable to condense the DNA themselves (6). However, the inhibition seen with EDTA was surprising. Preliminary ^{13}C-NMR results indicated a complex formation between spermine and EDTA. Changes in the chemical shifts of ^{13}C nuclei of both compounds upon complex formation are consistent with an ionic interaction.

Triphosphates were not coprecipitated with DNA by spermine. Addition of labelled triphosphates (0.2 mM, 90,000 cpm total) to 5 µg DNA/ml in MS buffer resulted in less than 0.5% of the label in the precipitate.

Protein also did not coprecipitate with DNA. Spermine precipitation of a HhaI restriction digest assay buffer containing 29 µg/ml calf thymus DNA resulted in only 4-5% of the bovine serum albumin from the buffer in the DNA pellet. In contrast, more than 12% of the protein coprecipitated with DNA following a standard ethanol precipitation of the same solution. Similarly, spermine precipitation of a cleared bacterial cell lysate (see Materials and Methods) resulted in the coprecipitation of only 6 to 8% of the complex protein mixture.

Precipitation of different sized DNA molecules showed that there was a threshold of DNA fragment lengths precipitated at a given spermine concentration. As shown in Table 2, this effect was important primarily under moderate salt conditions. There was apparently little or no effect of DNA fragment length using the standard protocol on precipitation for fragments larger than 200 bp, as demonstrated by a single inflection point for precipitation of a mixture of labelled 4400 and 200 bp fragments as a function of spermine concentration (data not shown). However, below this length there is a striking length dependence in moderate salt. More than twice the concentration of spermine was required to precipitate a 100 bp fragment from MS buffer than that required for the 4400 bp-200 bp mixture, even in the presence of high molecular weight carrier DNA. A fragment 60 bp long with carrier DNA did not precipitate from MS buffer even at 15 mM spermine. This particular DNA fragment length appears to be near the transition point for precipitation under MS buffer conditions. Polyacrylamide gel analysis of a HpaII restriction endonuclease digest (brought to 100 mM KCl) of a pBR322 derivative precipitated with 10 mM spermine demonstrated that all fragments 67 bp and larger were present in the redissolved pellet

Length of labelled fragment	Buffer	Final [spermine] for precipitation		
		with carrier		without carrier
		100µg/ml	1µg/ml	0.1µg/ml
4400 + 200	MS	3 mM	2 mM	ND
100	MS	7 - 10 mM	7 mM	5 mM
60	MS	*	ND	ND
4400 + 200	LS	0.15 mM	0.4 mM	ND
100	LS	0.6 mM	0.4 mM	0.6 mM
60	LS	1.5 mM	ND	1.0 mM**

Table 2. Dependence of spermine precipitation on DNA fragment length. Spermine precipitation was done as described in Materials and Methods, with solutions containing 0.01 µg end labelled DNA fragments with or without calf thymus DNA as a carrier. Total radioactivity per tube ranged from 3000 to 6000 cpm. ND - not determined. *Does not start to precipitate even at 15 mM spermine. **90% appeared to be the maximum precipitation possible under these conditions.

and were undetectable in the supernatant. Fragments 34 bp and smaller appeared in the supernatant and pellet.

The DNA length dependence at low salt was much less pronounced than at moderate salt. A small effect was seen with the 100 bp fragment. The 60 bp fragment required more spermine for precipitation and was 90% precipitable at the lowest DNA concentration tested (0.1 µg/ml). For DNA fragments larger than 200 bp, then, spermine precipitated the DNA under a wide variety of solution conditions. For fragments smaller than this, there was a dependence on solution conditions, or possibly on kinetic factors (see Discussion).

Table 2 also demonstrates that the presence of high molecular weight DNA was not necessary for precipitation of the short fragments, as these fragments precipitated in the absence of carrier DNA at spermine concentrations appropriate for the DNA concentration and buffer being tested.

The DNA pellets dissolved readily in buffer containing 0.5 M salt, or in 5 - 10X TBE for gel electrophoretic analysis. The presence of spermine in the redissolved pellet did not alter the electrophoretic mobility of the DNA for polyacrylamide gel electrophoresis, even for complex mixtures of DNA fragments, i.e., bacteriophage DNA or plasmid DNA digested with various restriction endonucleases (data not shown).

Spermine Removal

Spermine was removed from the redissolved DNA pellet by dialysis against buffer containing 0.5 M NaCl. After 24 hours, 99% of [^{14}C]spermine was gone, leaving less than 0.002 mole spermine per mole DNA phosphorous. Spermine was removed from the DNA pellet directly with an extraction procedure. The spermine associated with the DNA was exchanged with other cations (Na^{+}, Mg^{++}) in the presence of 75% ethanol. The two buffers successfully used in this way were 75% ethanol, 1 X Buffer 2; and 75% ethanol, 1 X Buffer 2, 25 mM EDTA. The spermine precipitate was dispersed in extraction buffer and left on ice for one hour. After centrifugation, up to 97% of the [^{14}C]spermine was found in the extraction solution; all of the DNA remained in the pellet. These data are shown in Table 3. Pellets extracted with the solution containing EDTA did not digest quite as readily with restriction enzymes as those extracted with 75% ethanol, 1 X Buffer 2 (unpublished observations).

Extraction Buffer	%[^{14}C]spermine in Extract		%[^{32}P]DNA
	30 min	1 hr	recovered
75% EtOH, 1 X Buffer 2	87	93	99.8
75% EtOH, 12 mM $MgCl_2$	51	57	ND
75% EtOH, 1 X Buffer 2, 25 mM EDTA	86	97	99.9

Table 3. Spermine pellet extraction. 50 µg of calf thymus DNA was precipitated from LS buffer with 1 mM [^{14}C]spermine (1.1×10^6 cpm/µmol spermine) as described in Materials and Methods. The resulting DNA pellet contained 0.2 mole spermine per mole DNA phosphorous as determined from total [^{14}C]spermine in the pellet, the specific activity of the [^{14}C]spermine, and the 50 µg of DNA assumed to be precipitated. 200 µl of extraction buffer was added to the DNA pellet, vortexed to disperse the pellet, then left on ice. For the [^{14}C]spermine experiments, the extraction solutions were centrifuged for 5 minutes in a microfuge and 50 µl of the solution was removed to an aqueous scintillation cocktail for counting. Total [^{14}C]spermine originally in the pellet was taken to be the sum of the ^{14}C extracted and that remaining in the pellet after extraction. Total ^{14}C in the pellets was 30,000 to 40,000 cpm. For the [^{32}P]DNA experiments, the same protocol was followed except that nonradioactive spermine was used for precipitation and radioactivity (Cerenkov) in the entire extraction solution and pellet were determined after one hour. Total ^{32}P was 5400 to 7400 cpm.

Representative Applications

The selectivity of the spermine precipitation reaction can be exploited to purify DNA from impurities and buffer components. Some representative applications follow.

Precipitation of restriction endonuclease digests. Spermine precipitation of DNA digested with restriction endonucleases separated the DNA from buffer salts and proteins prior to gel electrophoresis. The precipitation reaction does not require large volumes or depend on temperature and was complete within 15 minutes. When fragments smaller than 100 bp were to be precipitated, we typically diluted the solutions to low salt conditions. For analytical purposes, the pellet was redissolved in one fifth final volume of 5X TBE without spermine removal. For larger quantities of DNA (preparative digests), we extracted the spermine before gel electrophoresis, because the DNA dissolved more readily in TBE after spermine removal.

Separation of fragments from triphosphates. The failure of triphosphate precipitation by spermine was exploited during end labelling reactions to purify labelled DNA fragments from triphosphates without resorting to column chromatography.

Purification of fragments from polyacrylamide gels. After grinding gel slices in high salt buffer and extracting the solution with phenol (10), DNA can be precipitated from the aqueous phase with ethanol, then redissolved in an appropriate buffer and dialyzed. However, DNA fragments isolated by this procedure contain UV absorbing and light scattering contaminants unless further purified on Sephadex A25 (12). Spermine, unlike ethanol, does not precipitate these impurities with the DNA. For isolation of DNA fragments from gels with spermine, the aqueous phase was diluted to 0.1 M salt (the DNA concentration was then about 0.1 µg/ml). Spermine was added to 3 mM and the DNA precipitated. The DNA pellet was redissolved in HS buffer, dialyzed against HS buffer and then dialyzed into TE. This method produced fragments with much improved UV spectra. The absorbance ratios A_{260}/A_{280} and A_{260}/A_{235} were 1.88 and 1.98 for the spermine precipitated fragments; this can be compared with values of 1.85 and 1.28 for ethanol precipitated fragments and 1.84 and 2.28 given for purified bacteriophage DNA (13). The removal of polyacrylamide-derived impurities by spermine precipitation was

also apparent by a visible decrease in the size of the DNA pellet. The ill-defined impurities that accompany ethanol precipitation complicate many manipulations in DNA chemistry and enzymology. Their selective removal with spermine precipitation should find wide application.

Fragment isolation. The poor precipitation of very short DNA fragments under moderate salt conditions can be an extremely useful characteristic of the spermine precipitation reaction. For example, we have digested a pBR322 derivative containing the lac 203 operator-promoter region with EcoRI; the fragments were end labelled with DNA polymerase I and digested with PvuII to generate two fragments over 2000 bp, a 180 bp fragment (which was desired for sequencing purposes), and a 20 bp fragment. The two large fragments were precipitated with polyethylene glycol (Fisher Carbowax 6000) (14). Addition of spermine to the polyethylene glycol supernatant, after dilution of the high salt buffer to 0.10 M NaCl, resulted in precipitation of the 180 bp fragment only, leaving the 20 bp fragment in solution with the [α-^{32}P]TTP employed in the original labelling reaction.

DISCUSSION

In principle, each of the factors affecting the selectivity of spermine precipitation that we have examined could be studied in the absence of all of the others. Properly done, the result would be a set of phase diagrams that rigorously described the equilibrium properties of the condensation reaction. Even if such a comprehensive study were carried out, it is not likely that the selectivity features of this reaction that we have described would change appreciably. This conclusion is based on the fact that when spermine neutralizes the DNA charge, the complex has a very high preference for intra- and intermolecular association. It was nevertheless striking that the resulting precipitates did not include a large fraction of other solution components or even macromolecules as coprecipitates. Some additional aspects of the reaction deserve further comment.

Although the spermine concentration necessary for DNA precipitation increased with increasing ionic strength as expected, the reaction was probably affected in part by kinetic variables which we have not examined (see below). However, all the spermine precipitation reactions we have investigated show a typical threshold dependence on the concentration of

spermine required. Therefore, the standard conditions we have described, which were designed to examine the extremes of conditions encountered in laboratory work, can serve as guidelines for the minimum concentration of spermine required for precipitation under many intermediate solution conditions.

The precipitation reactions we have described were deliberately limited to 15 minutes for condensation, and to a convenient set of centrifugation conditions. Kinetic effects of fragment length and salt dependence, or the effect of differences in centrifugation times required, would not be evident with this approach. Kinetic effects, may, in fact, be largely responsible for the spermine concentration dependence seen in some cases. Widom and Baldwin (8) found that longer times were required for condensation of higher concentrations of DNA and for shorter fragment lengths using hexamine cobalt (III). From a practical standpoint, this distinction is unimportant except in the cases where we see no precipitation (i.e., a 60 bp DNA fragment in MS buffer). Incubating longer than 15 minutes in these cases may allow precipitation to occur.

Complexation of the spermine by EDTA may be responsible for the higher concentrations of spermine necessary for DNA condensation when EDTA was present. This may also explain the slightly higher spermine extraction efficiency observed with an extraction buffer containing EDTA (Table 3). The similar effects of $MgCl_2$ and EDTA on the precipitation reaction also illustrates the fact that when a single component was varied in our experiments, several other solution interactions were undoubtedly occurring. For example, the addition of EDTA to a DNA solution containing $MgCl_2$ would be expected to lower the spermine concentration required for precipitation by preventing the Mg^{++} competition for DNA sites. However, EDTA also complexed spermine; thus the net result was that the addition of EDTA to chelate Mg^{++} had little effect on the spermine concentration required to precipitate DNA. However, we emphasize that the selectivity and spermine concentration dependence of the precipitation reactions were both highly reproducible even under these complex solution conditions.

Spermidine condenses DNA as readily as does spermine, although a higher concentration is required (4). We would expect that the characteristics of the spermidine reaction would be identical to the spermine precipitation

reaction. However, since a trivalent cation is necessary for condensation, spermine should precipitate DNA more reproducibly over a wider range of temperatures and pH's, because dissociation of a single proton from spermidine would prevent it from effecting DNA condensation.

Particularly useful characteristics of this reaction are the lack of precipitation of triphosphates, most protein, and short fragments of DNA. Applications exploiting these characteristics have been described; however additional applications suitable for special systems can be envisaged. For example, K. Backman (personal communication) utilized spermine precipitation to separate a restriction endonuclease generated DNA fragment to which EcoRI linkers had been ligated from the short linkers still present in the ligation reaction mixture.

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