Studies on the Interaction of Homologues of Spermine with Deoxyribonucleic Acid and with Bacterial Protoplasts

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Four homologues of the naturally occurring polyamine spermine, of the type $H_2N \cdot [CH_2]_3 \cdot NH \cdot [CH_2]_n \cdot NH \cdot [CH_2]_3 \cdot NH_2$ where $n = 2, 3, 5$ and 6 , have been synthesized. Their ability to stabilize *Escherichia coli* protoplasts against osmotic lysis was compared with that of spermine. All homologues were approximately as effective as spermine. The effect of low concentrations of the homologues on the T_m of calf thymus DNA and of *Aerobacter aerogenes* DNA in 0.03M-sodium chloride–1mM-potassium dimethylglutarate buffer, pH 6.2, was tested. The increase in T_m for a given concentration of amine was found to be $n = 5 > n = 4$ and $n = 6 > n = 3 > n = 2$. When calf thymus DNA in 0.15M-sodium chloride–15mM-sodium citrate was used spermine gave the highest increase in T_m . It is concluded that the stabilization of *E. coli* protoplasts by tetra-amines is a non-specific effect independent of chain length, whereas the elevation of T_m of DNA is a more specific effect which depends on chain length.

Since 1956 new specific methods have been developed for the estimation of spermine and spermidine in biological tissues (Rosenthal & Tabor, 1956; Dubin, 1960; Raina, 1963). The application of these methods has shown that spermine and spermidine occur widely in animals, plants, bacteria and viruses. Although a number of suggestions have been made as to the functions of these amines no very definite conclusions have been reached. Most of the effects attributed to spermine and spermidine have been demonstrated *in vitro* by their addition to the system under investigation, and it is not clear whether these effects are specific for natural polyamines or whether they are non-specific effects of polycations.

To examine two of these effects more closely, namely the interaction of polyamines with DNA to stabilize the double helix, and their ability to stabilize the membranes of bacterial protoplasts, a number of homologues of spermine have been synthesized and then compared with spermine in these two systems. It was thought that if these effects are not simply those of polycations, but also depend upon the spatial arrangement of the nitrogen atoms, then spermine would be expected to show greater activity than its homologues. Guirard & Snell (1964) have demonstrated that, in a strain of *Lactobacillus casei* which shows a stimulation of growth with spermine, certain analogues also show weak stimulatory effects but others antagonize the effect of spermine. No studies have

yet been made with homologues of spermine on possible cellular functions.

This paper describes the effects of homologues of spermine on the melting profile of DNA under various conditions, and also their effects on protoplasts.

MATERIALS AND METHODS

Chemicals. Calf thymus DNA (type 1), spermine tetrahydrochloride, spermidine trihydrochloride and putrescine dihydrochloride were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). *Aerobacter aerogenes* DNA was isolated by the method of Marmur (1963). The hydrochlorides of 3-2-3-tetra-amine,* 3-3-3-tetra-amine, 3-5-3-tetra-amine and 3-6-3-tetra-amine were prepared by adaptation of the method described by Tabor, Tabor & Bachrach (1964) for the preparation of spermine tetrahydrochloride. The following amounts of diamine and acrylonitrile were mixed together with 20ml. of ethanol in 50ml. stoppered test tubes and then allowed to stand at room temperature for 3 days: for 3-2-3-tetra-amine, 2.0ml. of 1,2-diaminoethane and 5.0ml. of acrylonitrile; for 3-3-3-tetra-amine, 2.0ml. of 1,3-diaminopropane and 4.0ml. of

* Abbreviations: 3-2-3-tetra-amine, *NN'*-bis-(3'-amino-propyl)-1,2-diaminoethane; 3-3-3-tetra-amine, *NN'*-bis-(3'-aminopropyl)-1,3-diaminopropane; 3-5-3-tetra-amine, *NN'*-bis-(3'-aminopropyl)-1,5-diaminopentane; 3-6-3-tetra-amine, *NN'*-bis-(3'-aminopropyl)-1,6-diaminohexane; 2-2-2-tetra-amine, *NN'*-bis-(2'-aminoethyl)-1,2-diaminoethane; 2-2-triamine, *N*-2'-aminoethyl-1,2-diaminoethane; T_m (melting temperature), the temperature at which 50% of the total increase in E_{260} has occurred.

acrilonitrile; for 3-5-3-tetra-amine, 2.0g. of 1,5-diaminopentane and 3.4 ml. of acrilonitrile; for 3-6-3-tetra-amine, 2.0g. of 1,6-diaminohexane and 2.4 ml. of acrilonitrile. The hydrochlorides of the dinitrile adducts of the form $\text{NC}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NH}\cdot[\text{CH}_2]_n\cdot\text{NH}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CN}\cdot 2\text{HCl}$ were separated out by addition of 6N-HCl at 0° until a dense white solid appeared. These were recrystallized in a mixture of ethanol and HCl. The nitriles were reduced to tetra-amines as described by Tabor *et al.* (1964), except that in place of bubbling H_2 through the solutions they were shaken for 24 hr. in a closed system consisting of a conical flask attached to a rubber bladder filled with about 2l. of H_2 . The hydrochlorides of the tetra-amines were recrystallized from ethanol and HCl and further purified on Dowex 50 (X2) with an HCl gradient. The yields of the dinitrile adducts were about 55% of theoretical and the reduction step gave about 20% of theoretical.

Polyamine estimation. The reaction with fluorodinitrobenzene as described by Dubin (1960) was used.

Chloride estimations. Chlorides were estimated by using a Sigma modification of the method of Schales & Schales (1941). The method is described in Sigma Technical Bulletin no. 830.

Paper chromatography. The solvent system was ethylene glycol monomethyl ether-propionic acid-water (14:3:3, by vol.) saturated with NaCl, and the chromatograms were stained for polyamines with Amido Black (Raina, 1963).

T_m determinations. These were made in the Unicam SP.800 spectrophotometer with the SP.870 and SP.875 electrical heating coil and control unit respectively. The settings on the control unit were calibrated against a National Physical Laboratory thermometer, which was placed in one of the cells. Correction was made for the exposed stem. Four melting profiles were determined on each DNA sample in 0.15M-NaCl-15mM-sodium citrate; their average standard deviations were $\pm 0.5^\circ$. Duplicate estimations only were made of the melting profiles of DNA in 0.03M-NaCl-1mM-dimethylglutarate buffer, pH6.2, since the changes in T_m on addition of polyamines were much greater at this ionic strength.

Preparation of protoplasts. *Escherichia coli* type B was grown in a basal salts medium (Monod, Cohen-Bazire & Cohn, 1951) and the protoplasts were prepared by the

method of Repaske (1958). Samples (2ml.) of the cell suspensions were harvested, incubated with lysozyme in the presence of EDTA and then suspended in 1 ml. samples of distilled water or a solution of the polyamine, and their turbidities were measured at 650m μ .

RESULTS

Table 1 summarizes some of the properties of the homologues of spermine which were prepared. It will be noted that their hydrochlorides all decompose before melting, and they all have similar R_F values when run in ethylene glycol monomethyl ether-propionic acid-water, a solvent used for separating diamines from triamines and tetra-amines (Herbst, Keister & Weaver, 1958). It is interesting that there is a change in the E_{350}/E_{390}

Fig. 1. Calibration curves: 3-2-3-tetra-amine, $\square$; 3-3-3-tetra-amine, $\bullet$; 3-5-3-tetra-amine, $\blacksquare$; 3-6-3-tetra-amine, $\circ$ (estimated as their dinitrophenyl derivatives; Dubin, 1960).

Table 1. *Properties of homologues of spermine*

The R_F values were determined in ethylene glycol monomethyl ether-propionic acid-water (14:3:3, by vol.). The spectra of the dinitrophenyl derivatives were determined in solution in dioxan (Dubin, 1960). The data from Dudley, Rosenheim & Rosenheim (1924) and Dubin (1960) for spermine are included for comparison.						
Compound	Melting point	Theoretical chloride (%)	Estimated chloride (%)	R_F	Dinitrophenyl derivatives	
					$\lambda_{\text{max.}}$ (m μ)	E_{350}/E_{390}
3-2-3-Tetra-amine, 4HCl	275° (decomp.)	45	44.8	0.25	359	1.47
3-3-3-Tetra-amine, 4HCl	280 (decomp.)	42.5	40.3	0.23	360	1.35
3-5-3-Tetra-amine, 4HCl	295-300 (decomp.)	39.7	37.9	0.25	361	1.26
3-6-3-Tetra-amine, 4HCl	275-282 (decomp.)	38.3	38.6	0.32	361	1.24
Spermine	310 (decomp.)			0.23	360	1.24

Table 2. Effect of homologues of spermine on the stability of *Escherichia coli* protoplasts

E. coli protoplasts were prepared by the method of Repaske (1958) and then suspended in the solutions indicated. All amines were 1mM and were present as the hydrochlorides.

Suspending medium	Turbidity (E_{650})	
	Expt. 1	Expt. 2
(A) Whole cells		
Water	0.72	0.77
(B) Protoplasts		
Water	0.07	0.09
Putrescine	0.12	0.3
Spermidine	0.24	0.48
3-2-3-Tetra-amine	0.6	0.76
3-3-3-Tetra-amine	0.64	0.75
Spermine	0.55	0.75
3-5-3-Tetra-amine	0.63	0.72
3-6-3-Tetra-amine	0.56	0.83

Fig. 2. Change of T_m of calf thymus DNA with varying concentrations of added amines: putrescine, $\circ$; spermidine, $\square$; 3-2-3-tetra-amine, $\blacksquare$; 3-3-3-tetra-amine, $\bullet$; spermine, Δ ; 3-5-3-tetra-amine, $\blacktriangle$; 3-6-3-tetra-amine, $\bullet$. The calf thymus DNA was initially dissolved in 0.03M-NaCl-1mM-potassium dimethylglutarate buffer, pH 6.2, and had a final concentration of 60 $\mu\text{g./ml}$. T_m of calf thymus DNA in the absence of added amine was 73.5°.

of the dinitrophenyl derivatives of the amines having different chain length. The ratio characteristic of a primary amine is about 2.1 and that of a secondary amine about 0.62, whereas an amine having equal numbers of primary and secondary groups lies approximately midway between (Dubin, 1960). It is possible that the secondary amino groups in the shorter chain tetra-amines do not react quantitatively with fluorodinitrobenzene, thus giving a ratio nearer to that of a primary amine as Dubin (1960) found with 2-2-2-tetra-amine and

Fig. 3. Comparison of the abilities of different tetra-amines to increase the T_m of DNA under different conditions. Calf thymus DNA ($G+C=42$ moles/100 moles): in 0.15M-NaCl-15mM-trisodium citrate, pH 7.0 $\pm$ 0.3, $\bullet$; in 0.03M-NaCl-1mM-potassium dimethylglutarate buffer, pH 6.2, $\blacksquare$. *A. aerogenes* DNA ($G+C=57.5$ moles/100 moles): in 0.03M-NaCl-1mM-potassium dimethylglutarate buffer, pH 6.2, $\blacktriangle$. The added tetra-amines had the general formula: $\text{H}_2\text{N}\cdot[\text{CH}_2]_3\cdot\text{NH}\cdot[\text{CH}_2]_n\cdot\text{NH}\cdot[\text{CH}_2]_3\cdot\text{NH}_2$, where $n=2, 3, 4, 5$ and 6, and their final concentration was 20 μM . T_m values in the absence of added amines: calf thymus DNA in 0.15M-NaCl-15mM-trisodium citrate, 87.1°; calf thymus DNA in 0.03M-NaCl-1mM-potassium dimethylglutarate, 73.5°; *A. aerogenes* DNA in 0.03M-NaCl-1mM-potassium dimethylglutarate buffer, 80.7°.

2-2-triamine. This is not borne out by Fig. 1, however, which shows that 3-2-3-tetra-amine and 3-3-3-tetra-amine give as high extinction coefficients for the equivalent number of amino groups as do the higher homologues. The closer proximity of the four aromatic rings may cause a change in the absorption spectrum.

Table 2 shows that the stabilizing effect on protoplasts of the tetra-amines appears to be independent of chain length and therefore is probably a non-specific effect.

The changes in the T_m as a function of concentration and of chain length between the two secondary amino groups are shown in Figs. 2 and 3 respectively. The upper limit of the amine concentration which could be used was governed by aggregation and precipitation of DNA. With calf thymus DNA in 0.03M-sodium chloride-1mM-potassium dimethylglutarate buffer, pH 6.2, precipitation occurred when the amine concentration was increased to 40 μM with the 3-3-3-tetra-amine and 3-5-3-tetra-amine, to 50 μM with the 3-6-3-tetra-amine and spermine, and to 80 μM with the 3-2-3-tetra-amine. Fig. 3 shows that 3-5-3-tetra-amine produced the greatest elevation in T_m and that

3,2,3-tetra-amine is appreciably less effective than the other four amines when measured at low ionic strength. Spermine causes the greatest increase in T_m when measured at high ionic strength. The effect on the T_m thus appears to depend on chain length.

DISCUSSION

The results show that although tetra-amines are able to reduce osmotic lysis of bacterial protoplasts, the effect seems to be relatively non-specific since all five tetra-amines stabilize to approximately the same extent. Tabor's (1962) results with 1mM-2,2,2-tetra-amine, which stabilizes only slightly, suggested that it might have been a specific effect. The amines may form bridges having electrostatic bonds between some of the anionic groups of the protoplasts, possibly phosphate and carboxyl groups. It seems unlikely, however, in view of the high concentration of amine required for stabilization when compared with the amounts present in the cell, together with the lack of specificity, that stabilization of the cell membrane is a natural function of spermine.

The effect of tetra-amines on the T_m of double-stranded DNA on the other hand does seem to depend on chain length, maximum stabilization occurring with the natural polyamine, spermine or the homologue having one carbon atom more than spermine. These results are similar to those of Mahler & Mehrotra (1963) with diamines, where they found that diaminopentane gave maximum stability, though higher concentrations of diamines were required than of tetra-amines to produce the same change in the T_m . The mechanism of stabilization is not fully understood. Mahler & Mehrotra (1963) suggest that diamines probably form a bridge across the double helix with a site of attachment on each of the polynucleotide chains, one to a phosphate residue, and the other possibly to the adenine or thymine. They suggest this because of the dependence of the change in the T_m with added amine on the base composition of the DNA. Tsuboi (1964), on the other hand, proposes for spermine that interaction with DNA produces four bonds between the phosphates of DNA and the amino groups of spermine, the secondary amino groups making a bridge across the shallow groove in the double helix and the primary amino groups bonding to the adjacent nucleoside phosphate along the helices. He suggests that the strength of amine binding to DNA decreases with increasing temperature and that the dependence of stabilization on base composition is an apparent effect only, in that DNA having a high guanine and cytosine content will have a higher initial T_m and thus give a lower change in T_m when amine is added.

The results with spermine analogues at low ionic strength reported in this paper seem to fit Tsuboi's (1964) hypothesis. The polyamines raise the T_m to a similar extent when two DNA samples (calf thymus and *A. aerogenes*) of markedly different base compositions are used. With spermine, 3-5-3-tetra-amine and 3-6-3-tetra-amine the hydrocarbon chain is long enough to cross the shallow groove of the double helix; with the higher homologues it may even do so more obliquely and attach to a phosphate group further away. With the 3-2-3-tetra-amine and to a smaller extent with the 3-3-3-tetra-amine the hydrocarbon chain between the secondary amino groups may not be long enough to span the groove, though other arrangements may be possible involving only three of the four amino groups and giving rise to stabilization of the same order as that given by spermidine. The type of stabilization in 0.15M-sodium chloride-15mM-sodium citrate may be somewhat different as much higher concentrations of amine are necessary and their contribution to the total ionic strength is about 5-10% of the total, whereas in 0.03M-sodium chloride-1mM-potassium dimethylglutarate buffer it is about 0.5-1.0% of the total. The maximum increase in T_m when measured in 0.03M-sodium chloride-1mM-potassium dimethylglutarate buffer occurs with about 40 μ M concentrations of tetra-amine. The DNA concentration used was 60 μ g./ml., which means that the ratio of tetra-amine amino groups to DNA phosphate groups is about 1:1. When 0.15M-sodium chloride-15mM-trisodium citrate is used the maximum increase in T_m occurs with amine concentrations greater than 3.2mM: the ratio of amino groups to phosphate groups is now greater than 60:1. Thus the effects of amines may be twofold, a non-specific effect of a cation shielding the phosphate groups and a specific effect in forming the suggested bridge structure. The former would make a greater contribution at higher ionic strength.

The absorption spectra of DNA were examined in 0.03M-sodium chloride-1mM-potassium dimethylglutarate buffer, pH 6.2, with 20 μ M concentrations of each of the tetra-amines and they were found to be unchanged when compared with the spectrum measured in the absence of the amines. This suggests that it is unlikely that the amines react with the bases of DNA.

Although in this paper only the results of binding polyamines to double-stranded DNA have been measured, it is likely that polyamines *in vivo* may have a wider role in stabilization involving polynucleotides and oligonucleotides. Polyamines are not confined to the nucleus (Stevens, 1966) and substantial amounts may be present in the ribosomes (Cohen & Lichsteinstein, 1960). There is evidence that they may be involved in the synthesis

of messenger RNA on a DNA template (Krakow, 1963) and also that they may be involved in codon-anticodon stabilization (Ochoa & Weinstein, 1965).

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