The Binding of Spermine to the Ribosomes and Ribosomal Ribonucleic Acid from *Bacillus stearothermophilus*

By L. STEVENS*

Department of Biological Chemistry, Marischal College, Aberdeen

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1. The total intracellular concentrations of Na^+ , K^+ , Mg^{2+} , spermine, spermidine and RNA were measured in *Bacillus stearothermophilus*. 2. The binding of spermine to ribosomes and to ribosomal RNA from *B. stearothermophilus* was studied under various conditions by using a gel-filtration technique. 3. The affinity of spermine for ribosomes and for ribosomal RNA decreased with increasing ionic strength of the medium in which they were suspended. 4. The extent of spermine binding did not change appreciably in the temperature range 4–60°. 5. Optimum binding occurred at about pH 7.0. 6. The number of binding sites for spermine on either ribosomes or ribosomal RNA was 0.10–0.13/RNA phosphate group. 7. A high proportion of the intracellular spermine is likely to be bound to the ribosomes *in vivo*; spermine competes with Mg^{2+} on equal terms for sites on the ribosomes.

There has been increased interest recently in the biochemical functions of polyamines, stimulated by the suggestion that polyamines are in some way involved in the synthesis of ribosomal RNA (see Cohen & Raina, 1967). To understand the cellular functions of polyamines it is advantageous to know how they are distributed throughout the cell and in particular whether significant proportions of them are associated with the ribosomes and RNA *in vivo*. Unfortunately it is not easy to obtain a direct measure of these proportions, and measurements of polyamines in isolated ribosomes and microsomes (Raina & Telaranta, 1967; Tabor & Kellog, 1967; Khawja & Stevens, 1967) are not very useful in this respect since they depend markedly on the isolation procedures. A preliminary radioautographic approach has been made (Dion & Herbst, 1967), but it suffers from the disadvantage that there is no good specific precursor of spermine or spermidine, and so the labelled polyamines themselves have to be used.

By radioautography and subcellular fractionation it is only possible to locate polyamines within specific organelles; it is not possible to indicate the binding of polyamines to specific macromolecules. An alternative approach, which is adopted in this paper, is to measure binding of spermine to particular macromolecules *in vitro* under conditions that are similar to those of the intracellular environment. Probably the most important feature of the intracellular environment that affects the binding

of polyamines to nucleic acids is its ionic strength. K^+ ions and, to a smaller extent, Na^+ and Mg^{2+} ions are the principal intracellular cations (Lubin & Ennis, 1964; Steinbach, 1962), and, since the intracellular cationic strength cannot be determined directly, in this paper the intracellular K^+ , Na^+ and Mg^{2+} ion concentrations are used to make an approximate estimate of the cationic strength.

In this work the binding of spermine to ribosomes and ribosomal RNA was measured under conditions that were similar to those of the intracellular environment in ionic strength, temperature and spermine concentration. The concentrations of spermine in whole cells and in isolated ribosomes of *Bacillus stearothermophilus* are known (Stevens & Morrison, 1968), so this organism was again used in the present study.

MATERIALS AND METHODS

Chemicals. Spermine tetrahydrochloride, tris and 3,3-dimethylglutaric acid were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. 2,5-Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were obtained from Nuclear Enterprises (G.B.) Ltd., Edinburgh. [^{14}C]-Spermine tetrahydrochloride (specific radioactivity 4.25 mc/m-mole) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A., and [^{131}I]iodinated human serum albumin (30 mc/mg.) from The Radiochemical Centre, Amersham, Bucks. All other chemicals were A.R. grade from British Drug Houses Ltd., Poole, Dorset, where available.

Organism, growth medium and isolation of ribosomes. *Bacillus stearothermophilus* strain 8923 was obtained from

* Present address: Department of Biochemistry, King's College, London W.C.2.

the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. It was grown with aeration on medium consisting of 6.7 g. of tryptone, 5.0 g. of yeast extract, 2.5 g. of K_2HPO_4 , 3.3 g. of NaCl, 0.5 g. of $MgSO_4 \cdot 7H_2O$, 5.0 mg. of $CaCl_2 \cdot 2H_2O$, 5.0 mg. of $FeCl_3 \cdot 5H_2O$ and 5.0 g. of glucose/l. at 65°. It was found that the cells entered the exponential phase more rapidly in this medium of higher Mg^{2+} concentration than in the medium used in the experiments of Stevens & Morrison (1968). Cells were harvested in the exponential phase and the ribosomes were isolated as described by Stevens & Morrison (1968). The crude ribosomal pellet was then washed with 0.35 M- NH_4Cl solution and passed through Sephadex G-200 to remove ribonuclease (Takanami, 1967). The ribosomes were then dialysed against 1000 vol. of M-KCl-0.1 M-tris-HCl buffer, pH 7.6, to remove spermine and spermidine, and then dialysed against 10 mM-sodium cacodylate-HCl buffer, pH 7.0. This procedure also controls the Mg^{2+} content of the ribosomes. Ribosomal RNA was extracted from the ribosomes by using guanidinium chloride (Cox, 1966).

Measurement of binding of spermine to ribosomes and ribosomal RNA. The method used was that introduced by Hummel & Dreyer (1962) and subsequently applied by Fairclough & Fruton (1966). A column (internal diam. 0.5 cm., overall length 115 cm., and surrounded by a water jacket of 3.8 cm. diam. through which water was circulated) was filled with Sephadex G-25 to a height of 100 cm. The column was equilibrated with a solution containing 10 mM-sodium cacodylate-HCl buffer, pH 7.0, and the appropriate final concentration of KCl and spermine tetrahydrochloride. [^{14}C]Spermine tetrahydrochloride was diluted with unlabelled spermine tetrahydrochloride so that the final solution always contained 6.25 $\mu C/l$. The flow rate through the column was 1.3-1.6 ml./hr. at 4°, 3.0-4.0 ml./hr. at 37° and 6.0-7.0 ml./hr. at 60°. After equilibration, a sample of ribosomes or ribosomal RNA, equivalent to about 0.7 μ mole of RNA phosphate, dissolved in 0.2 ml. of the same medium as used in equilibration, was applied to the column. Each sample was eluted with the same medium until the peak and the trough of spermine had emerged (see Fairclough & Fruton, 1966): fractions (0.8 ml.) were collected, and their radioactivities were determined and their absorption spectra plotted. A 0.2 ml. sample of each fraction was mixed with Bray's (1960) scintillation liquid and counted in a Packard Tri-Carb liquid-scintillation counter at 75-80% efficiency. The area of the trough was measured and from this the fraction of bound spermine was determined.

Measurement of the extracellular space in bacterial pellets and the intracellular Na^+ , K^+ and Mg^{2+} concentrations. The procedure for determining the extracellular space was essentially that of Schultz & Solomon (1961) with [^{131}I]iodinated albumin. In place of cytocrit tubes, small graduated centrifuge tubes, made from short lengths of 2 ml. graduated pipettes sealed at the bottom, were used. The bacteria were harvested by centrifugation at 11000g for 30 min. Each bacterial pellet was thoroughly resuspended in an approximately equal volume of the supernatant growth medium, to which a suitable amount of [^{131}I]iodinated albumin had been added. The suspension was pipetted with a Pasteur pipette into a small graduated centrifuge tube and then centrifuged at 3000g for 30 min. at 20° in a swing-out head. The supernatant was carefully removed. Samples (0.1 ml.) of the growth medium containing [^{131}I]iodinated albumin, obtained both before and

after mixing with the bacterial pellet, were used for measurement of radioactivity. Then 10.0 ml. of Bray's scintillation liquid was added to each of these samples and they were counted in a Packard Tri-Carb liquid-scintillation counter.

The Na^+ , K^+ and Mg^{2+} ions were extracted from the dried bacterial pellet with ammonium oxalate as described by Schultz & Solomon (1961). Then Na^+ and K^+ were determined in an Evans Electro-selenium flame photometer. The Mg^{2+} was determined colorimetrically after conversion into magnesium ammonium phosphate (Alcock & MacIntyre, 1966).

Determination of polyamines, RNA and phosphate. Polyamines were extracted in cold 0.5 M- $HClO_4$, separated by paper electrophoresis and stained with ninhydrin (Raina & Cohen, 1966). RNA was extracted in 0.5 M- $HClO_4$ for 10 min. at 75° and determined as described by Ceriotti (1954). Phosphate was determined as described by Ames & Dubin (1960).

RESULTS

The total intracellular concentrations of spermine, spermidine, Na^+ , K^+ , Mg^{2+} and RNA phosphate (Table 1) are calculated from measurements of their total concentrations in the bacterial pellet, their extracellular concentrations and the volume of the extracellular space within the bacterial pellet. The intracellular concentrations of Na^+ , K^+ and Mg^{2+} ions are greater than the concentrations of these ions in the growth medium. The extracellular Na^+ and K^+ concentrations do not appreciably alter during

Table 1. *Intracellular and extracellular concentrations of cell constituents of B. stearothermophilus*

Cells were harvested in the exponential phase. The intracellular and extracellular concentrations of constituents and the extracellular space of the bacterial pellet were determined as described in the Materials and Methods section. The results are given either as means $\pm$ s.d. with the numbers of determinations in parentheses, or, where small samples were taken, as means with the individual results in parentheses.	
Intracellular concentration	
Spermine	1.70 mM (1.85, 1.42, 1.83)
Spermidine	1.22 mM (1.11, 1.13, 1.43)
K^+	173 $\pm$ 31 mM (8)
Na^+	190 $\pm$ 59 mM (8)
Mg^{2+}	35 $\pm$ 17 mM (11)
RNA phosphate	102 mM (154, 94, 88, 74)
Initial extracellular concentration	
K^+	38 mM
Na^+	85 mM
Extracellular concentration at time of harvesting	
K^+	37 $\pm$ 3 mM (9)
Na^+	85 $\pm$ 7 mM (9)
Mg^{2+}	2.76 mM (2.60, 2.92)
Extracellular space in bacterial pellet	0.31 $\pm$ 0.04 ml./g. (7)

Table 2. *Binding of spermine to ribosomes and ribosomal RNA from B. stearothermophilus at various temperatures and potassium chloride concentrations*

Binding experiments were carried out as described in the Materials and Methods section with a final spermine concentration of 0.1 mM, and three different concentrations of KCl (0.1, 0.2 and 0.4 M).

	Concn. of KCl ... Temperature	Spermine bound ($\mu\text{mole}/\mu\text{mole}$ of RNA phosphate)		
		0.1 M	0.2 M	0.4 M
Ribosomes	4°	0.085	0.035	0.007
	37	0.078	0.028	0.018
	60	0.066	0.037	—
Ribosomal RNA	4	0.145	0.055	0.014
	37	0.143	0.050	0.012
	60	0.140	0.050	0.006

Fig. 1. Effect of pH on the binding of spermine to ribosomal RNA from *B. stearothermophilus*. Binding was measured as described in the Materials and Methods section with 0.1 M-spermine and 10 mM buffer: Δ , 3,3-dimethylglutaric acid-NaOH; $\bullet$, sodium cacodylate-HCl; $\blacksquare$, tris-HCl.

growth of the bacteria. This is expected when the final yield of bacteria is 1–2 g./l.

Binding experiments were first carried out to see how spermine binding was affected by (i) ionic strength, (ii) temperature and (iii) pH. Increases in ionic strength (Table 2) cause marked decreases in the extent of binding of spermine to both ribosomes and ribosomal RNA, whereas changes in temperature in the range 4–60° have little effect on binding. Optimum binding occurs at pH 7.0 (Fig. 1), and binding decreases slowly with increasing acidity, presumably owing to the amino groups of

the nucleotides of RNA acquiring a positive charge. The binding decreases more rapidly in alkaline buffer, when the amino groups of spermine begin to lose their positive charges. The pK values for the primary and secondary amino groups of spermine are reported by Rogers, Ulbricht & Szer (1967) to lie between pH 7.7 and 9.7, and by Hirschman, Leng & Felsenfeld (1967) to lie between pH 8.19 and 10.99 at 25°. The highest binding of spermine to RNA occurs in the presence of cacodylate buffer (Fig. 1). The latter has been shown (Hirschman *et al.* 1967) not to form a complex appreciably with spermine, and it was therefore used in all subsequent binding experiments.

The binding of spermine to ribosomes and ribosomal RNA was measured at various concentrations of free spermine and the results were plotted as $\log c$ against r , where c is the free spermine concentration in mM and r is the number of moles of spermine bound/mole of RNA phosphate. In 0.1 M-potassium chloride, ribosomal RNA and ribosomes bind the saturating amount of spermine when the concentration of spermine is raised to 0.1 mM (Figs. 2a and 2b). In 0.3 M-potassium chloride the amount of spermine bound continues to increase when the concentration of free spermine is raised to 1 mM (Fig. 2c).

If it is assumed that all the spermine molecules bind to equivalent and non-interacting sites on the ribosome or on ribosomal RNA, then from the plots of c/r against r the values of k , the intrinsic association constant, and n , the number of binding sites for spermine/RNA phosphate group, may be determined (Scatchard, 1949). Lines were drawn through the points by the method of least-squares best fit and the values for k and n are given in Table 3. The intrinsic association constant, and hence the affinity of spermine for ribosomal RNA, is significantly decreased in 0.3 M-potassium chloride. The number of binding sites on the ribosomes and ribosomal RNA in 0.1 M-potassium chloride and on ribosomal RNA in 0.3 M-potassium chloride do not vary significantly.

DISCUSSION

From the measurements of the intracellular concentrations of Na^+ , K^+ and Mg^{2+} it appears that the intracellular cationic strength is about 0.5. This is likely to be a maximum value for two reasons. First, the method used here for calculating intracellular concentrations would include in the estimate any ions bound to the outer surface of the bacteria, and these would not contribute to the intracellular cationic strength. Secondly, the value of 0.5 is based on the assumption that all ions measured are free, whereas there is good evidence for Mg^{2+} ions that as many as 80% of the Mg^{2+} ions are bound

Fig. 2. Binding of spermine to ribosomes and ribosomal RNA from *B. stearothermophilus* at various concentrations of spermine. Binding experiments were carried out as described in the Materials and Methods section. (a) Ribosomal RNA in 0.1 M-KCl; (b) ribosomes in 0.1 M-KCl; (c) ribosomal RNA in 0.3 M-KCl.

Table 3. *Intrinsic association constants and the number of sites for binding spermine to ribosomes and ribosomal RNA from B. stearothermophilus.*

The intrinsic association constants (k) and the number of binding sites (n) for spermine were determined from graphs of c/r against r (c and r are defined in Fig. 2). Lines were drawn by the method of least-squares best fit, and the s.d. for the number of binding sites and the intrinsic association constant were obtained from the regression line of c/r on r .

	$10^{-4}k$	n (spermine/RNA phosphate)
Binding of ribosomal RNA in 0.1 M-KCl	8.2 ± 0.9	0.133 ± 0.029
Binding of ribosomal RNA in 0.3 M-KCl	0.54 ± 0.08	0.102 ± 0.012
Binding of ribosomes in 0.1 M-KCl	5.9 ± 0.9	0.127 ± 0.030

(Edelman, Ts'o & Vinograd, 1960; Lusk, Williams & Kennedy, 1968): the proportions of ions bound are likely to be much lower for Na^+ and K^+ . Thus a more realistic estimate of the intracellular cationic strength is between 0.3 and 0.4.

The binding of spermine to ribosomes and ribosomal RNA shows a small negative temperature coefficient in the range 4–60°, and this suggests that the extent of binding is not likely to be appreciably different in psychrophils, mesophils and thermophiles, provided that their intracellular ionic strengths are similar.

It is interesting to compare the nature of polyamine binding to ribosomes and ribosomal RNA with that of Mg^{2+} binding, since the latter is considered important in maintaining ribosomes in the 70S form (see Petermann, 1964), and polyamines seem partially able to replace Mg^{2+} in the cells (Hurwitz & Rosano, 1967). In 0.1 M-potassium chloride, ribosomes and ribosomal RNA become saturated with

spermine when the concentration of the latter is raised to 1 mM, whereas Mg^{2+} ions saturate at about 10 mM (Goldberg, 1966; Choi & Carr, 1967). A low concentration of spermine (0.3 mM) completely displaces Mg^{2+} ions from ribosomes when the concentration of the Mg^{2+} ions is 5 mM (Choi & Carr, 1967). Hurwitz & Rosano (1967) claimed that in *Escherichia coli* the free intracellular Mg^{2+} ion concentration is approximately the same as that in the extracellular environment provided that the latter is in the range 4 μM –10 mM. In contrast with this, Lusk *et al.* (1968) claimed that *E. coli* can accumulate Mg^{2+} ions by active transport. The latter also appears to be the case in the present study involving *B. stearothermophilus*, unless over 90% of the intracellular Mg^{2+} is bound. The total intracellular Mg^{2+} concentration is about 20 times that of spermine, whereas the binding constant for Mg^{2+} is about one-tenth that of spermine: thus the two cations are likely to compete on equal terms for sites on the ribosome.

The number of sites for spermine binding on isolated ribosomal RNA does not significantly exceed the number of sites on the intact ribosomes, and similar results have been obtained for Mg^{2+} binding (Goldberg, 1966). This suggests that the binding sites for both Mg^{2+} and spermine are present on the RNA moiety, and that few sites are unmasked by the removal of ribosomal protein.

It is noteworthy that the association constant for spermine and DNA measured by equilibrium dialysis (Hirschman *et al.* 1967) is similar to that for spermine and ribosomal RNA reported here, although the number of sites for spermine/DNA phosphate group is about half the number of sites/RNA phosphate group.

In conclusion, it seems likely from these results that a high proportion of the spermine is bound to the ribosomes *in vivo*, and this may be important in understanding how the ribosomes function *in vivo*.

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