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Polyamine Stimulation of Nucleic Acid Synthesis in an Uninfected and Phage-Infected Polyamine Auxotroph of *Escherichia coli* K12

(arginine/agmatine ureohydrolase/putrescine/spermidine/lysine/cadaverine)

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ABSTRACT **The addition of arginine to cultures of *Escherichia coli* K12 deficient in agmatine ureohydrolase (EC 3.5.3.7) results in polyamine depletion and a striking inhibition of nucleic acid accumulation and growth. The omission of lysine from these cultures leads to a further decrease in growth rate and nucleic acid synthesis. In arginine-inhibited cells the addition of putrescine or spermidine, in the presence or absence of lysine, restores the control rate of growth and nucleic acid accumulation. Under the same conditions of arginine inhibition in the absence of lysine, the addition of cadaverine alone stimulates growth rate and RNA synthesis. The addition of lysine to polyamine-depleted cultures results in cadaverine production and in the appearance of a new spermidine analogue, containing lysine carbon. The new compound has been identified as *N*-3-aminopropyl-1,5-diaminopentane.**

Infection of this arginine-inhibited, polyamine-depleted mutant with T_4D results in markedly decreased amounts of DNA accumulation, as compared to infected cells uninhibited by arginine. Supplementation of arginine-inhibited infected cells by putrescine or spermidine restores DNA synthesis to the uninhibited level.

Two constitutive pathways function in the synthesis of putrescine in *Escherichia coli* (1, 2). In the *E. coli* K12 mutant deficient in agmatine ureohydrolase (EC 3.5.3.7) (3), the conversion of agmatine to putrescine is blocked. The addition of arginine prevents putrescine formation in this mutant by inhibition of the synthesis of ornithine from glutamate. Such a polyamine auxotroph has permitted us to investigate the dependence on polyamines of RNA and DNA synthesis in uninfected cells and the possible requirement for polyamines for DNA synthesis in phage-infected cells.

In 1957, Hershey (4) described two low molecular weight ninhydrin-positive compounds in purified T-even phage preparations; these amines were also demonstrated to be present in the uninfected host. These compounds were shown to be derived from arginine, and were concomitantly injected with phage DNA upon infection; they were identified as putrescine and spermidine by Ames and coworkers (5, 6), who showed that the polyamine content of the T-even phage could neutralize 40–50% of the DNA phosphates. Cohen and Raina (7) have demonstrated that the net synthesis of polyamines after infection parallels DNA synthesis. The uniqueness of the phage system, i.e., DNA synthesis without concomitant accumulation of RNA, permitted us to investigate whether in this system DNA synthesis controls polyamine synthesis or vice versa, or neither. Cohen and Dion (8) have reported that polyamine synthesis is independent of phage DNA synthesis. The present study indicates that phage DNA synthesis is dependent on the availability of polyamines in polyamine-depleted mutant cells. Studies of the synthesis of nucleic acids and polyamines in the uninfected depleted mutant have also revealed an unsuspected role of cadaverine in the synthesis of a new spermidine analogue and in RNA synthesis.

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MATERIALS AND METHODS

Some properties of *E. coli* K12 (MA159) (His^-, Leu^-, Thr^-) defective in agmatine ureohydrolase activity have been described (3). The characterization of bacteria bearing this block has been described (3, 9). The mutant that was used in this study, MA159, was kindly given to us by Dr. W. Maas. The organism is derived from a *thy*$^-$ derivative (MA145) of MA135 (3) by transduction into a *ser A* strain, PA260. In this study, two methods of polyamine depletion were used. In method 1 (J. Poindexter, personal communication), an overnight broth culture is chilled 5 hr at 4°C and diluted 1:20 in AFA medium (AF medium, see ref. 3, supplemented with 100 μg of arginine/ml). 0.1-ml aliquots are plated on AFA agar plates, and after overnight growth at 37°C, bacteria are scraped from the agar surface with small aliquots of AFA medium. These harvested bacteria are then centrifuged at 4080 $\times$ *g* for 15 min, and resuspended in Davis–Mingioli medium (9) without glucose. After an additional wash, aliquots of the suspension are added to various AF media containing the designated supplements.

In method 2, an overnight broth culture is chilled 5 hr at 4°C and diluted 1:40 in AFA medium (final concentration of arginine = 100 μg/ml). Overnight growth at 37°C results in polyamine-depleted cells. These are centrifuged and washed in Davis–Mingioli salts medium without glucose. Aliquots of a 4-fold concentrated suspension are added to AF media containing the designated supplements.

Lysates of T_4r^+D were prepared by infection of *E. coli* B at a multiplicity of infection (MOI) of 0.1 and incubation overnight. Purified phage stocks were obtained by two cycles of low (4080 $\times$ *g*)- and high (27,300 $\times$ *g*)-speed centrifugation of overnight broth lysates.

Polyamines were estimated (10) by *in situ* scanning of fluorescent dansyl polyamine derivatives separated on silica gel by thin layer chromatography (TLC) plates (Analtech, Newark, Del.). For determinations of polyamine content of cells and media, Millipore filters containing the filtered cells were extracted in 1.0 ml of 0.2 N perchloric acid. To the filtrate

(medium) was added 2.0 N perchloric acid to a final concentration of 0.2 N. The acid extracts were dansylated as described (10).

For the estimation of agmatine, acid extraction, and dansylation were performed as above. Aliquots were spotted on Avicel TLC plates (Analtech, Newark, Del.) and subjected to thin layer electrophoresis in 8% formic acid (pH 2.0) for 120 min at 15 V/cm. Direct scanning of the fluorescent spots was performed on a Farrand scanning spectrofluorometer (Farrand Optical Co., Inc., N.Y.) with primary excitation at 335 nm and secondary emission at 520 nm.

The nucleic acids of uninfected cells were extracted in 5% Cl_3CCOOH at 80°C for 15 min and analyzed for DNA by the Burton method (11) and for RNA by the orcinol procedure (12). The diphenylamine reaction was used to estimate DNA accumulation after infection (13).

Putrescine and spermidine hydrochloride salts and dansyl chloride were obtained from Calbiochem (Los Angeles, Calif.). The hydrochloride salt of cadaverine was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio). The free base of *N*-3-aminopropyl-1,5-diaminopentane was obtained from Dr. E. F. Elslager (Parke–Davis, Ann Arbor, Mich.), converted to the hydrochloride salt, and recrystallized from absolute ethanol. All of the above compounds were homogeneous after chromatography and ninhydrin staining, or when chromatographed as dansyl derivatives.

RESULTS

Identification of cadaverine and a new spermidine analogue

We have reported (8) preliminary results correlating polyamine-depletion and supplementation on growth rate and nucleic acid synthesis in the agmatine ureohydrolase mutant of *E. coli* K12 and the wild-type parent *E. coli* K12 MA160. We had noted the absence of putrescine in the polyamine-depleted mutant and that the amount of spermidine was approximately one-half that observed in the wild strain. However, on closer examination, by use of appropriate dansyl standards, we have now observed the presence of cadaverine and the anomalous behavior of the compound previously throught to be dansyl spermidine. Our evidence (Dion and Cohen, manuscript in preparation) indicates the presence of cadaverine and a new spermidine analogue, namely, *N*-3-aminopropyl-1,5-diaminopentane, in polyamine-depleted cultures. Dansylated $HClO_4$ extracts of polyamine-depleted cells contained two fluorescent derivatives that cochromatographed with authentic dansyl cadaverine and dansyl *N*-3-aminopropyl-1,5-diaminopentane in ethyl acetate–cyclohexane 30:60 and in benzene–triethylamine 80:16. The derivatives from these cells did not cochromatograph with dansyl putrescine and dansyl spermidine.

A pellet of 6.8×10^{11} putrescine-depleted cells was extracted at 4°C twice with 20 ml of 5% Cl_3CCOOH. After removal of Cl_3CCOOH with ether, the solution was made strongly alkaline and extracted with butanol (14). After acidification, butanol was removed under reduced pressure. The residue, containing about 20 μmol of polyamine, was dissolved in 2 ml of water and fractionated with an HCl gradient (15) on a column of Dowex-50 (H^+) (0.8×8.0 cm). Polyamines were monitored by dansylation and cadaverine was eluted between 156–186 ml, whereas material that eluted between 276 and 300 ml cochromatographed, after dansylation, with dansyl *N*-3-aminopropyl-1,5-diaminopentane. Pooled fractions were dried, dissolved in 0.01 M HCl, and converted to crystalline picrates according to the method of Beer and Kosuge (16). The melting points of picrates of peak 1 and peak 2 were 215–217°C and 182–183°C, respectively, as reported for the picrates of cadaverine (221°C) (17) and *N*-3-aminopropyl-1,5-diaminopentane (182°C) (18); melting points for the picrates of putrescine and spermidine prepared by this method were found to be 250°C and 208–209°C, respectively, essentially as reported earlier (17).

Arginine-inhibited, polyamine-depleted cells were grown in the presence of uniformly labeled [^{14}C]lysine (1.1 μmol/ml; 3.25×10^5 cpm/μmol). The cells were extracted with Cl_3CCOOH, and the polyamines were extracted into butanol and fractionated on Dowex-50 (H^+) as described above. Two peaks of radioactivity were obtained. Material from the first peak was dansylated and this derivative cochromatographed with dansyl cadaverine. Nevertheless, the cadaverine (88% of the radioactivity in the first peak) was further purified from a trace of lysine by electrophoretic separation on Avicel plates in 0.1 M citrate buffer (pH 3.5) for 2 hr at 17.5 V/cm. After dansylation, radioactive material in the second peak cochromatographed with dansyl *N*-3-aminopropyl-1,5-diaminopentane. The specific activity (cpm/μmol) through the second peak was essentially constant and slightly less (2.24×10^5 cpm/μmol) than five-sixths of the specific activity of the exogenous [^{14}C]lysine. Since the bacteria had been depleted in the presence of nonradioactive lysine, this slight dilution of the labeled lysine is not unexpected. We conclude that the new polyamine was derived from this amino acid, as is cadaverine. The appearance of these compounds prompted us to examine the effect of lysine supplementation.

Polyamines and growth rates

Polyamine-depleted cells, grown in the absence of arginine, exhibited similar mass doubling times in the presence or absence of lysine, i.e., 68 and 64 min, respectively. However, the growth rates of similarly treated cells grown in the presence of arginine were prolonged to 135 min in the presence of lysine plus arginine and 183 min in cultures grown in the presence of arginine without lysine. The addition of putrescine to arginine-inhibited cells, with or without lysine, enhanced the growth rates to control values, i.e., 66 min in both instances. The addition of cadaverine to arginine-inhibited cells supplemented with lysine decreased the mass doubling time only slightly (from 135 to 115 min). In contrast, the mass doubling time of arginine-inhibited cultures not containing lysine was significantly reduced by the addition of cadaverine, namely, from 183 to 105 min; however, in no instance was cadaverine as effective as putrescine.

Effect of polyamines on nucleic acid synthesis

Concurrent with the above growth rate studies, samples were removed from each culture at identical cell densities of about 1.5, 3.0, and 4.5×10^8 cells/ml, corresponding to turbidities of 60, 120, and 180 Klett units (420 filter), for analyses of DNA and RNA accumulation. These are shown in Fig. 1. The increases in nucleic acid, especially RNA, paralleled the changes in turbidity. Omission of arginine, allowing the synthesis of putrescine and spermidine from ornithine, resulted in the control rate of RNA and DNA synthesis, with or without lysine. The addition of arginine inhibited nucleic acid synthesis, as well as growth; these functions were further

inhibited by the omission of lysine. Polyamine analyses, as shown in Fig. 2, or arginine-inhibited cultures (analysis 2) grown in the presence of lysine revealed no detectable amounts of putrescine; however, the presence of cadaverine and a new spermidine analogue, presumably derived from cadaverine through lysine, were apparent. A similarly treated culture (analysis 6) grown in the absence of lysine revealed no detectable amounts of putrescine or cadaverine, but low levels of the spermidine analogue were observed. This culture has the longest mass doubling time and lowest rate of RNA synthesis.

Putrescine addition to arginine-inhibited cultures enhanced the rates of RNA and DNA synthesis to control levels. Polyamine analyses of these cultures (analyses 3 and 7) revealed increases in intracellular putrescine and spermidine, but no detectable amounts of cadaverine or spermidine analogue, whether grown with or without lysine, indicating the possible inhibition of lysine decarboxylase by putrescine and/or spermidine. No effect of cadaverine was apparent on synthesis of RNA or DNA in lysine-supplemented cultures. However, the addition of cadaverine stimulated the rate of RNA synthesis in arginine-inhibited cultures grown in the absence of lysine (Fig. 1), while no effect was obtained on DNA synthesis. Polyamine analyses of these cells (Fig. 2, analyses 4 and 8) revealed increased intracellular concentrations of both cadaverine and the spermidine analogue, but no detectable putrescine or spermidine.

No detectable amounts of agmatine were observed in any of the above cultures except for trace amounts in arginine-inhibited cultures unsupplemented with polyamines.

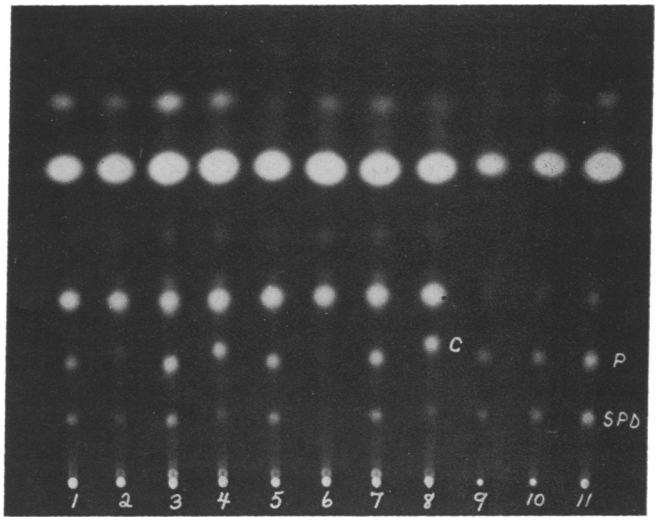

FIG. 2. Thin layer chromatogram of dansyl polyamine derivatives of perchloric acid extracts of cell cultures at T = 180, chromatographed twice in ethyl acetate–cyclohexane (30:60, v/v). 1 + 5, *AF*; 2 + 6, *AFA*; 3 + 7, *AFAP*; 4 + 8, *AFAC*. Designations as in legend to Fig. 1. *1–4*, with lysine; *5–8*, without lysine. *9–11*, dansyl putrescine and spermidine standards at concentrations of 75, 150, and 300 pmol/spot for dansyl putrescine and 25, 50, and 100 pmol/spot for dansyl spermidine. *P*, putrescine; *C*, cadaverine; *SPD*, spermidine. The dansyl spermidine analogue has a slightly higher R_f value than dansyl spermidine in this solvent system.

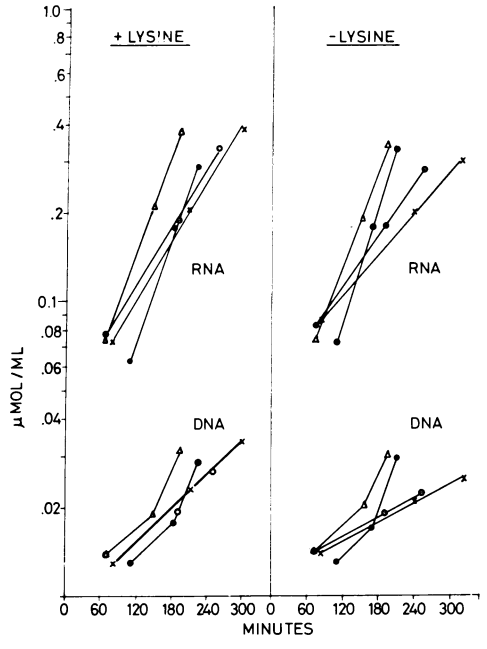

FIG. 1. Effect of polyamine depletion and supplementation on RNA and DNA synthesis in the agmatine ureohydrolase mutant of *E. coli* K12. The mutant was depleted of polyamines as described in method 2 in *Methods*. Washed cells were added to the designated media at an initial turbidity of 40. Aliquots for nucleic acid analyses were withdrawn from each culture at identical turbidities, i.e., T = 60, 120, and 180. *AF* (●—●), arginine-free; *AFA* (×—×), arginine added; *AFAP* (Δ—Δ), arginine and putrescine added; *AFAC* (○—○), arginine and cadaverine added. Arginine and lysine HCl added at concentrations of 100 and 200 μg/ml, respectively. Putrescine and cadaverine dihydrochlorides added to a final concentration of 1.0 μmol/ml.

Polyamines and phage synthesis

Fig. 3 illustrates the effect of polyamines on phage DNA synthesis in the T_4D-infected wild strain and the agmatine

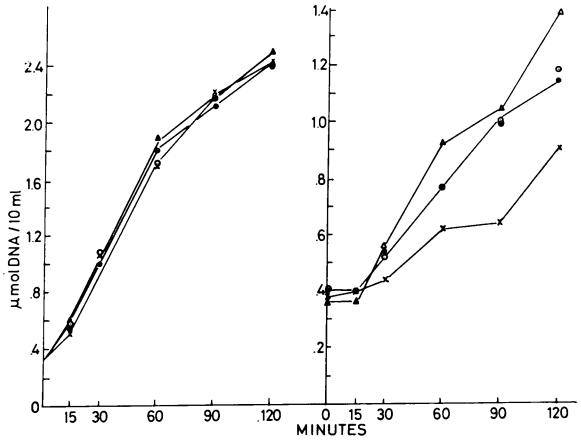

FIG. 3. The effect of polyamines on DNA synthesis in T_4D-infected *E. coli* K12 (wild, *left*) and *E. coli* K12 (Agmatine UH$^-$, *right*). Experimental conditions for polyamine depletion are described as method 1 in *Methods*. Washed cells were added to *AF* and *AFA* media at an initial turbidity of 40 Klett units. When the turbidity increased to 80, putrescine (*AFAP*) or spermidine (*AFASPD*) was added, and infection with T_4D ensued 15 min later at an MOI of 5. Final concentrations of arginine, putrescine, and spermidine were 100 μg, 1.0 μmol, and 0.5 μmol/ml, respectively. ●—●, − arginine; ×—×, + arginine; Δ—Δ, + arginine and putrescine; ○—○, + arginine and spermidine.

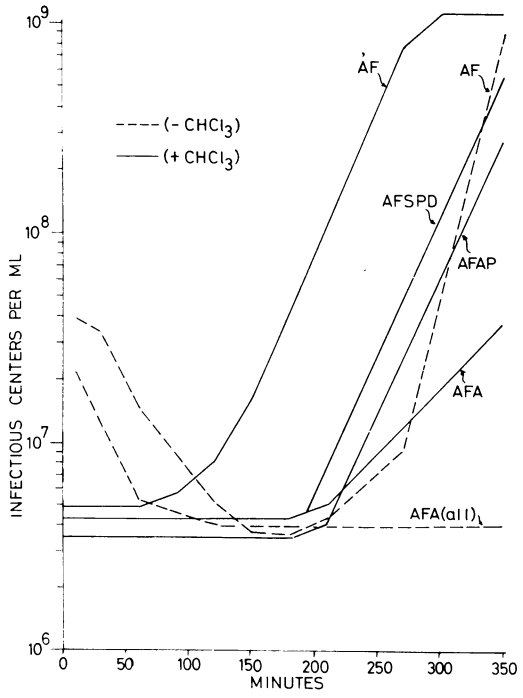

FIG. 4. Effect of polyamines on one-step growth of T_4D-infected *E. coli* K12 (Agmatine UH^-). Conditions for polyamine depletion and growth before infection were exactly as described in Fig. 3. After infection with T_4D at an MOI of 0.1, each culture was diluted 10^{-4}. Assays for infectious centers on *E. coli* B and chloroform lysis were performed according to Adams (19).

ureohydrolase mutant of *E. coli* K12 grown in the complex medium described by Hirshfield *et al.* (3). Infection of polyamine-depleted cells, released from arginine-inhibition, results in the initiation of DNA synthesis at about 15 min after infection, permitting a 3-fold increase in DNA accumulation by 120 min. Maintenance of arginine inhibition not only produces a later time of DNA initiation, but also a slower rate of DNA accumulation. The addition of putrescine or spermidine to similarly treated cultures 15 min before infection restores both the time of DNA initiation and the rate of DNA accumulation to control values. No differences were observed when the wild parent was infected under this range of conditions.

In one-step growth experiments (19) at low multiplicity (MOI = 0.1) in these media, we observed a 90% loss of infectious centers upon infection, as shown in Fig. 4. Phage release, without the use of chloroform lysis, was observed only in the infected culture grown in the absence of arginine, and only after a rather extended period of time after the initiation of DNA synthesis. However, when infected cultures were lysed with chloroform, the release of infectious particles was observed, indicating that these mature phage particles are maintained in an inactive state, perhaps through association with a membrane component. Despite the addition of chloroform, arginine-inhibition is characterized by a prolonged latent period, even in presence of chloroform. Nevertheless, polyamines increased the production of phage to approximately normal levels.

An electron microscopic study (kindly performed by Dr. Margit Nass) of multiply infected (MOI = 5) cultures under these conditions revealed the relative absence of intracellular phage in arginine-inhibited cells, as well as the stimulatory effects of exogenous polyamines on phage synthesis in such systems. In addition, we have noted that the mutant under all of the above condition appears extremely fragile after infection.

Polyamines and synthesis of phage DNA

The stimulatory effects of exogenous polyamines on synthesis of phage DNA have been revealed more strikingly through use of a simplified medium containing only supplements necessary for growth, i.e., histidine, leucine, and threonine. Under these conditions, as shown in Fig. 5, phage DNA synthesis begins at 15 min, resulting in a 2- to 3-fold accumulation of DNA in T_4D-infected cultures not containing arginine. Addition of arginine delayed the inception of DNA synthesis for 1 hr, and only a 60% increment had been formed after 3 hr. Exogenous putrescine or spermidine added 15 min before infection decreases the time necessary for the initiation of DNA synthesis, and almost restores the control level of DNA accumulation.

DISCUSSION

The polyamine requirements of *Hemophilus parainfluenza* were reported by Herbst and Snell (20) in 1948; however, until recently few pliable polyamine auxotrophs have been available for the investigation of the mode(s) of action of these ubiquitous organic bases. Although much evidence has ac-

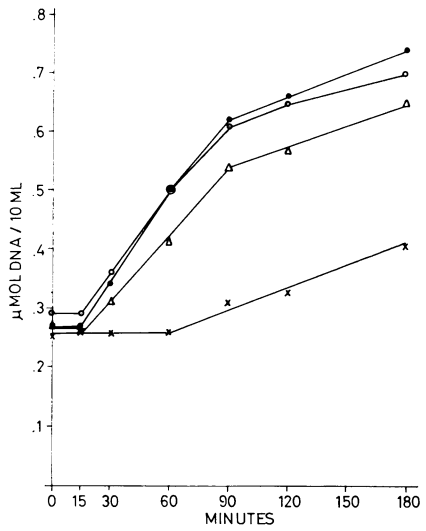

FIG. 5. Effect of polyamines on DNA synthesis in T_4D-infected *E. coli* K12 (Agmatine UH^-) in minimal medium (MIN, see below). A 6-hr culture in MIN was chilled overnight at 4°C; the cells were washed twice and resuspended in MIN and an aliquot was added to MIN containing arginine (100 μg/ml). This culture was depleted by growth for 6 hr and chilled overnight at 4°C. Aliquots of washed cells were added to either MIN (●—●) or MIN + A (×—×) at a cell density of about 1×10^8/ml. (T = 40). At T = 80 or about 2×10^8 cells/ml., putrescine or spermidine was added to MIN + *A* cultures at final concentrations of 1.0 and 0.5 μmol/ml. After 15 min cultures were infected with T_4D at an MOI = 5 after the addition of L-tryptophan (25 μg/ml). MIN, minimal salts medium of Davis and Mingioli (9) supplemented with glucose, L-histidine, L-leucine, and L-threonine at concentrations of 2 mg, 30 μg, 50 μg, and 50 μg/ml, respectively. *A*, arginine; *P*, putrescine; *SPD*, spermidine. Δ—Δ, MIN + *AP*; O—O, MIN + *ASPD*.

cumulated concerning polyamine enhancement of RNA synthesis *in vitro* and *in vivo* (21), *in vivo* studies have been exclusively concerned with organisms capable of endogenous polyamine synthesis. It may be mentioned that polyamine effects on *in vitro* DNA synthesis, by utilization of purified DNA polymerase I, have generally been inhibitory (22, 23), except for one study that used nucleohistone as primer (23). Brewer and Rusch (24) have reported a stimulation by spermine of the DNA polymerase activity of *Physarum* nuclei.

That the polyamines can play a significant role in phage morphogenesis has been amply demonstrated in the case of polyamine reversal of abortive infections of *E. coli* K12 (λ) with rII mutants. Both the diamines (25), and spermidine and spermine (26) are active in this respect that has been ascribed to an effect on the bacterial membrane (27). Other effects of polyamines on transcription and morphogenesis after infection have been described (28, 29).

The present study has demonstrated that polyamine addition to polyamine-depleted cells permits the development of rates of nucleic acid synthesis and growth similar to these activities observed in the same cells in which endogenous polyamine synthesis is not inhibited. In addition we have observed the appearance of cadaverine and a new spermidine analogue when cells are depleted of polyamines in the presence of lysine. Cadaverine at least appears to possess some of the biological activity of putrescine and spermidine, perhaps via the formation of the "spermidine-analogue".

Investigations of phage DNA synthesis, without the concomitant accumulation of RNA, have indicated that, although polyamine synthesis is not dependent upon DNA synthesis (8), phage DNA synthesis is markedly dependent upon polyamine synthesis.

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