NOVEL NUCLEOTIDES FROM E. COLI ISOLATED AND PARTIALLY CHARACTERIZED[1]

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SUMMARY Two new nucleotides have been found in the formic acid extracts of Escherichia coli, Clostridium botulinum, Bacillus subtilis and Rhodospirillum rubrum isolated during log phase growth. In E. coli the compounds are present at all times during cell growth but increase in amount during interruption of aeration and transition to stationary phase. They migrate close to ppGpp during one dimensional chromatography on PEI cellulose but are clearly separated from ppGpp by paper chromatography. The compounds are unstable on PEI cellulose and purification was effected by chromatography on A25 Sephadex ion exchange columns. Preliminary characterization indicates that the predominant compound is a dinucleoside polyphosphate and that both compounds contain a modified adenosine nucleoside.

INTRODUCTION Unusual highly phosphorylated nucleotides have appeared frequently in the literature (1-8). Most recently Gallant et al (9) have described yet another new nucleotide present in E. coli and suggest that it is a purine triphosphate. Certain of these nucleotides have been implicated in transcriptional control (8 - 11) and sporulation control (8, 12). This communication describes the isolation and partial characterization of two more unusual nucleotides in E. coli. The predominant new nucleotide has been unambiguously differentiated from ppGpp for which it could easily be mistaken in the most commonly used assay system, thin layer chromatography on PEI cellulose. Evidence is presented that the nucleotides are present throughout the bacterial growth cycle but that the basal levels are affected during growth into stationary phase and upon shift from aerobic to anaerobic growth.

MATERIALS AND METHODS E. coli B was grown at 37°C in minimal medium containing 0.1 M Tris-Cl pH 7.6, 0.16 mM sodium sulfate, 1 mM magnesium sulfate, 10 mM sodium chloride, 7.5 mM ammonium sulfate, 5.5 mM glucose, 0.2 mM calcium chloride, 3 μM ferric chloride and 0.64 mM potassium phosphate. ^{32}P-labelled cells were prepared in 100 ml batches using 10 μCi/ml ^{32}Pi (New England Nuclear). Aliquots of 10 mls were removed at various times and after centrifugation, the pellets were extracted with 0.2 ml of 1 M formic acid at 0°C for 30 minutes. Aeration was by shaking in a 250 ml erlenmeyer flask and transfer to anaerobic conditions was effected by stopping the shaking. One or two dimensional chromatography on PEI cellulose thin layer plates (Brinkmann) was carried out as previously described (13). Paper chromatography was on Orange Ribbon C paper (Schleicher and Schuell) in solvent A con-

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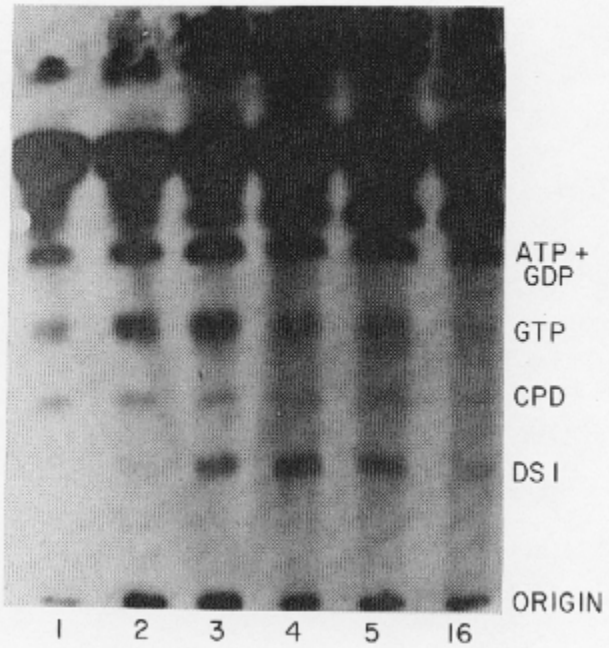

Figure 1. PEI cellulose thin layer chromatography (13) of formic extracts of E. coli isolated at various times during culture growth. The times of extraction (in hours) are indicated below each sample. The relative nucleotide levels are included in Table 1A. The nucleotide CPD is unknown.

taining ammonium sulfate: 0.1 M sodium phosphate pH 7.0: n-propanol; 12 g: 10 ml: 0.2 ml. Purification of the nucleotides was carried out as described in the legend to Figure 2. Clostridium botulinum, Bacillus subtilus, and Rhodospirillum rubrum were grown in similar medium and isolated during mid-log phase growth. UV spectra were obtained using a Beckman ACTA III spectrophotometer. Ribose and phosphate determinations were carried out as previously described (14). Two dimensional analysis of nucleosides and purine bases on cellulose thin layer plates (Brinkmann) was carried out as previously described (15).

RESULTS Nucleotide pools were analyzed during shifts from aerobic to anaerobic growth and during growth into stationary phase (Figure 1). Two new nucleotides were visible migrating slower (Rfs 0.36 and 0.24) than GTP (Rf 0.47) after one dimensional chromatography on PEI cellulose thin layer plates. The relative amounts of the nucleotides are included in Table 1. There is a significant increase in one of the compounds both upon cessation of aeration and transition to stationary phase. Gallant et al (9) have observed a similar increase in ppGpp levels after shift to anaerobic growth and from glucose to succinate as carbon source.

Unsuccessful attempts were made to isolate these new compounds from the PEI cellulose thin layer plates. The nucleotides were sufficiently labile that no material could be recovered which would rechromatograph with the same Rf. There-

Table 1. Nucleotide levels from PEI cellulose during growth into stationary phase (A) and during a shift from aerobic to anaerobic growth (B) (expressed as a percent of the total nucleotide pool).

A.	Time*	OD_{450}	ATP +GDP Rf (0.53)	GTP (0.47)	Cpd (0.36)	DS1 (0.24)
	1	0.244	9.3	3.7	0.70	0.26
	2	.472	9.3	2.6	0.74	0.35
	3	.880	6.7	1.7	0.57	0.71
	4	1.45	2.8	1.1	0.55	0.90
	5	1.45	4.6	1.4	0.74	0.94
	16	1.30	3.4	1.8	1.03	1.05
B.	-30	0.215	4.8	1.7	0.37	0.20
	0	.350	5.4	1.6	.42	.23
	+15	.372	2.8	1.3	.61	.88
	+30	.380	2.9	2.0	.71	1.24
	+45	.370	3.0	1.6	.50	1.01
	+60	.378	3.3	1.4	.44	0.90
	+75	.450	4.5	1.4	.41	.17
	+90	.560	6.1	1.9	.45	.22

*In A, Time is expressed in hours after commencement of growth. In B Time is expressed in minutes relative to shift from aerobic to anaerobic growth. Aeration was restarted at +60 minutes in B.

fore, the formic acid extracts of four liters of unlabelled stationary phase E. coli and 100 ml of ^{32}P-labelled stationary phase E. coli were fractionated on two successive A25 Sephadex ion exchange columns. The first column which was eluted with a gradient of triethylammonium bicarbonate pH 8.5 separated the highly phosphorylated nucleotides in peak D from all other nucleotides. The other peaks were identified as peak A: mainly GMP; peak B; ADP and peak C: ATP. The unlabelled peak which eluted after peak D contained protein. A second column was necessary to separate the two components present in peak D. They were labelled DS1 and DS11 (16). Analysis of the two nucleotide components DS1 and DS11 indicated that they were unrelated to ppGpp.

Both of these nucleotides gave a single radioactive and UV absorbing spot after paper chromatography in solvent A. The Rfs were 0.14 and 0.02 respectively for DS1 and DS11 (which can be compared to the following Rfs: A4P, 0.39; ATP, 0.36; ADP, 0.32; AMP, 0.25; G4P, 0.61 and GTP 0.57). Only very faint spots

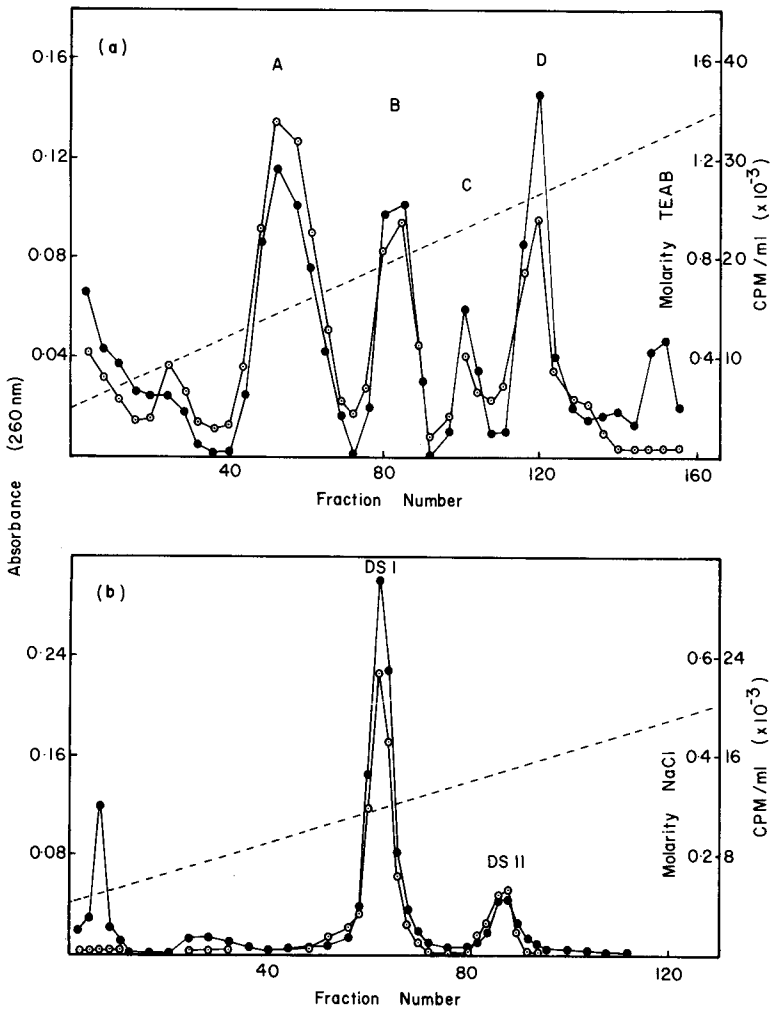

Figure 2. A25 Sephadex ion exchange chromatography of formic acid extracts of cells grown to stationary phase (see RESULTS). Column (a) was 2 x 30 cm equilibrated with 0.2 M triethylammonium bicarbonate (TEAB) pH 8.5. The formic acid extracts were made pH 7.0 with concentrated ammonia and diluted to 0.1 M formate before adding to the column. After washing with 500 ml of 0.2 M TEAB, pH 8.5, a gradient of 500 ml 0.2 M TEAB pH 8.5 and 500 ml 1.5 M TEAB pH 8.5 was applied collecting 6 ml fractions. Absorbance was determined at 260 nm and radioactivity determined by Cherenkov counting of 1 ml aliquots. The peaks were then pooled and concentrated. Peak D was loaded on column (b) equilibrated with 50 mM Tris pH 7.6 and 0.1 M NaCl. A gradient of 250 ml 0.1 M NaCl and 250 ml 0.5 M NaCl in 50 mM Tris pH 7.6 was applied collecting 4 ml fractions. Both peaks were pooled and desalted using a 1 x 100 cm Sephadex G10 column equilibrated with 40 mM TEAB pH 8.0.

(Rf 0.36 and 0.24) running slower than GTP could be found upon chromatography of pure DS1 and DS11 on PEI cellulose plates. Breakdown occurred to a number of products ranging from inorganic phosphate to ATP. This breakdown occurred only on

Table 2. Spectral data of DS1 and DS11 and comparison with literature values for GTP and ATP (18).

Spectral ratios*	DS1	DS1 (changed)	ATP	GTP
$A_{250/260}$	1.09	0.82	0.85	0.96
$A_{280/260}$	0.25	0.16	0.22	0.67
$A_{290/260}$	0.07	0.04	0.03	0.49
λ max (nm)	251	258	257	254
λ min (nm)	227	228	230	228

* All values were taken at pH 7.0 but no significant change was observed at either pH 1.0 or pH 12.0 for DS1. The values for DS11 were the same as those for DS1.

the PEI cellulose plates since rechromatography of DS1 on A25 Sephadex gave a single peak eluting at the same salt concentration as shown in Figure 2b. Also no breakdown was evident during paper chromatography. Dinucleoside monophosphates migrate with a slightly smaller Rf than DS1. (3'-ApT Rf 0.08) Since the Rf of a nucleoside in solvent A increases with increasing phosphate content, the Rf of DS1 is consistent with that of a highly phosphorylated dinucleotide.

The UV spectrum of freshly prepared DS1 exhibited a λ max at 251 nm at pH 7.0 and the spectral ratios shown in Table 2. After storage for several weeks at -20°C in pH 7.6 Tris buffer including lengthy periods at 5°C, it was found that the spectrum had changed slightly. The λ max had shifted to 258 nm and the A250/260 ratio had changed from 1.07 to 0.89 indicating that some rearrangement had occurred producing a compound with an adenosine-like spectra. Rechromatography of the compound with the modified spectrum both on an A25 Sephadex ion exchange column and paper using solvent A indicated that there was no change in the physical migration of the compound in either system. The UV spectra of DS11 were the same as those of DS1 and are not described. N^1-Methyl adenosine has a spectrum similar to that of DS1 and DS11 and it rearranges at neutral pH to N^6-methyl adenosine (17) which has a spectrum similar to adenosine.

The spectra of both DS1 and DS11 showed a 12% increase in absorbance at 260 nm when incubated for 30 minutes at pH 12.0. This was interpreted as being a hyperchromic shift during either denaturation or cleavage. Using an extinction coefficient for adenosine of 15,300 at 260 nm, the extinction coefficient of DS1 and DS11 was assumed to be approximately 13,500 per base. Ribose and phosphate analyses of the two compounds were then carried out using this assumed extinction coefficient. The ratio of ribose to base was found to be 1.1 for DS1 and 1.0 for DS11. The ratio of phosphate to base was found to be 3.1 for DS1 and 2.6 for DS11.

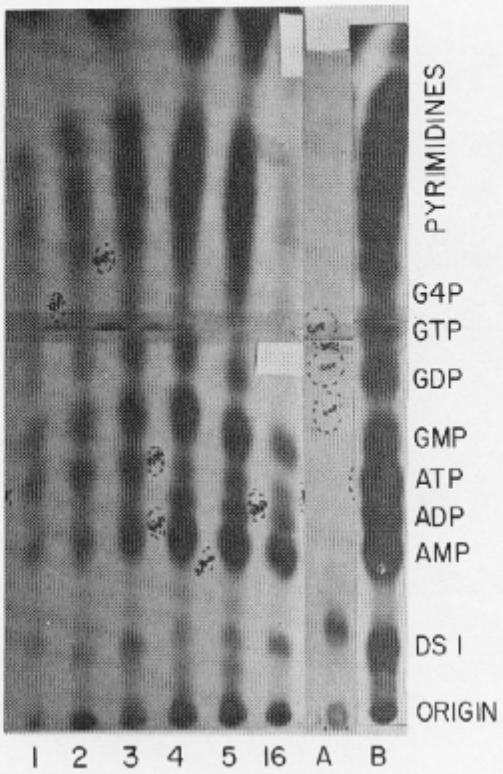

Figure 3. Paper chromatography of the formic acid extracts of E. coli isolated at various times during cell growth. The times are indicated below each sample. Purified DS1 and DS11 were run for comparison in slot A. A formic acid extract of R. rubrum isolated in mid log phase is included in slot B.

Both DS1 and DS11 were hydrolyzed to nucleoside within 10 minutes and to purine base after 30 minutes at 100°C in 1 M HCl. The same nucleoside and purine base were obtained from DS1 and DS11 as determined by two dimensional thin layer chromatography on cellulose (15). The nucleoside had Rfs of 0.81 and 0.22 in the first and second dimension respectively as compared to Rfs of 0.74 and 0.21 for adenosine. The purine base had Rfs of 0.77 and 0.25 which are different from those of adenine (Rfs 0.70 and 0.22). The same results were obtained both before and after the spectrum had changed. The only modified adenosines which migrate faster than adenosine in the first dimension are the N-6 modified compounds (15).

In order to relate the DS nucleotides isolated by ion exchange chromatography to those observed during nucleotide analysis, the formic acid extracts were analyzed by paper chromatography (Figure 3). Using solvent A all of the purine nucleotides were clearly separated. In both of the experiments described in Figure 1 and Table 1 the same results were obtained except that the levels of DS1 were three fold

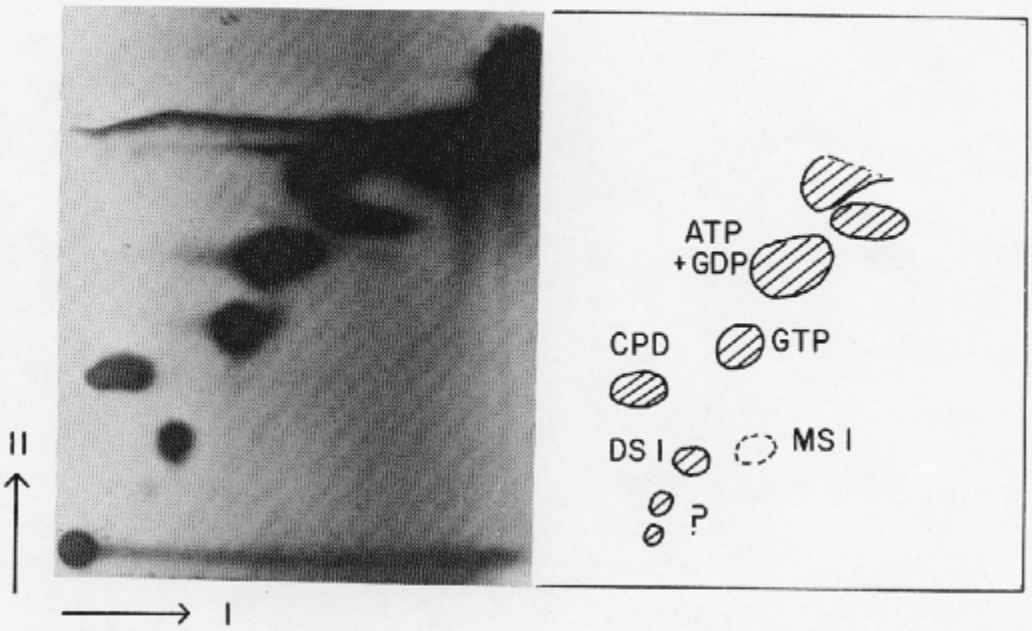

Figure 4. Two dimensional PEI cellulose thin layer chromatogram (13) of formic acid extracts from E. coli and R. rubrum. The schematic diagram labels the nucleotides. The nucleotide CPD is unknown as are the two faint spots below DS1.

higher. DS1 therefore breaks down even in the formic acid extracts on PEI cellulose but not as extensively as when it is pure. The position of ppGpp (Rf 0.61) slightly overlaps with the pyrimidine mono- and dinucleotides but when these were removed by fractionation on A25 ion exchange columns there was no ppGpp visible on the chromatogram. DS11 was obscured by material at the origin and could not be quantitated. There were other faint radioactive spots observed on paper running between DS1 and AMP (Rf 0.17) and between DS1 and DS11 (Rf 0.07). The identities of these compounds were not determined but one of them may be related to the faster of the two new nucleotides observed on PEI cellulose. Unfortunately the relationship of DS11 to the nucleotides observed on PEI cellulose remains obscure. There are some very faint spots which are sometimes observed on PEI cellulose (Rf 0.11 in dimension II in Figure 4) and one of these may correspond to DS11. As pointed out by Gallant et al (9) there are several unidentified highly phosphorylated nucleotides in E. coli which should be investigated.

The relation of these compounds to the rel gene was investigated using the isogenic pair of CP78 (rel^+) and CP79 (rel^-). Under conditions of amino acid starvation both of the spots migrating slower than GTP were visible although a slightly lower level of DS1 was observed in the rel^- strain. The same two spots

were also observed in the formic acid extracts of B. subtilis, R. rubrum and Clostridium botulinum. These new compounds migrate differently in two dimensions from all of the nucleotides reported to be in B. subtilis by Rhaese (12). An extract of E. coli was cochromatographed in two dimensions on PEI cellulose with extracts from B. subtilis and R. rubrum. The two spots migrating slower than GTP in both dimensions cochromatographed at the same positions in all extracts. The chromatogram of E. coli and R. rubrum extracts is shown in Figure 4. In addition a crude extract from R. rubrum was chromatographed on paper in solvent A. A strong spot, Rf 0.14, corresponding to a DS1-like compound was visible (Figure 3).

DISCUSSION Highly phosphorylated nucleotides have been implicated in the control of sporulation (8, 12) and transcription (8, 10, 11). The large amounts of DS1 in several species of bacteria and the change of DS1 levels during the slow down of growth of E. coli suggest that DS1 may play some metabolic role as well. The precise role has not been determined. The structure of the compound must be determined before any firm conclusions regarding a metabolic function can be drawn. The data described here are preliminary but they clearly show that both DS1 and DS11 are different from all previously described nucleotides. Both compounds contain a labile modification on the adenosine ring and while the structure of DS11 is more complex, DS1 would seem to be a dinucleoside hexaphosphate. Further work is in progress to fully characterize the nucleotides.

It is surprising that a nucleotide present in as large amounts as DS1 has not previously been observed. Two explanations can be given. The first is that the compound breaks down on PEI cellulose and artificially low levels or none may be observed. The second is that DS1 may very easily be mistaken for ppGpp during one dimensional analysis on PEI cellulose.

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