STUDIES ON THE CONTROL OF DEVELOPMENT. IN VITRO SYNTHESIS OF HPN AND MS NUCLEOTIDES BY RIBOSOMES FROM EITHER SPORULATING OR VEGETATIVE CELLS OF *BACILLUS SUBTILIS**

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1. Introduction

It has been shown by Rhaese et al. [1] that at the beginning of sporulation, defined as the end of logarithmic growth (T_0), two unusual nucleotides, called HPN I and II begin to accumulate within *B. subtilis* cells. Another nucleotide was found to be excreted into the medium at the same time.

Two-dimensional thin layer chromatography revealed that besides by two MS nucleotides (ppGpp, Ms I, and pppGpp, MS II) three other nucleotides (called HPN I, II, III) were present in sporulating cells. The nucleotide excreted into the medium was named HPN IV [2]. The guanosine-tetra and pentaphosphates (MS) accumulate after amino acid starvation in *Bacillus subtilis* [3] as has been observed for *E. coli* [4,5] earlier, and after inhibition of protein synthesis by antibiotics [6]. The HPN nucleotides are produced mainly in response to starvation of carbon sources [3]. Cells growing in rich media, like SYM [6] or NSM [7], begin to accumulate MS I and II at or shortly before T_0 apparently because of exhaustion of amino acids. But both nucleotides begin to decrease approximately 30′ thereafter presumably because protein turnover by protease activity, which increases at this time, causes release from amino acid starvation [2]. Approximately at the time, when the concentrations of MS I and II begin to decrease, HPN III and IV begin to increase. Glucose starvation is apparently responsible for the accumulation of HPN nucleotides, since rigorous deprivation of all carbon sources immediately causes the accumulation of HPN I, II and III as well as the extracellular HPN IV (Rhaese, Dichtelmüller, Grade, unpublished results).

Since *B. subtilis* seems to be able to respond with MS accumulation upon amino acid starvation like *E. coli,* we tried to synthesize MS or HPN or both in vitro according to the method of Haseltine et al. [8].

As will be reported here, ribosomes from vegetative *B. subtilis* cells synthesize MS I and II, but not HPN I and II. Ribosomes isolated from sporulating cells in contrast do not synthesize any or only very little MS nucleotides. They do, however, synthesize HPN I and II, which have been shown to be the tetra-(HPN I) and pentaphosphates (HPN II) of adenosine.

2. Materials and methods

2.1. *Bacterial strain*

B. subtilis strain 60015 (ind$^-$, met$^-$), the transformable Marburg strain (= SB 26 of Nester) was used throughout these studies.

2.2. *Media and growth conditions*

The SYM medium used in this study as well as the conditions for growth and sporulation have been described in detail previously [6].

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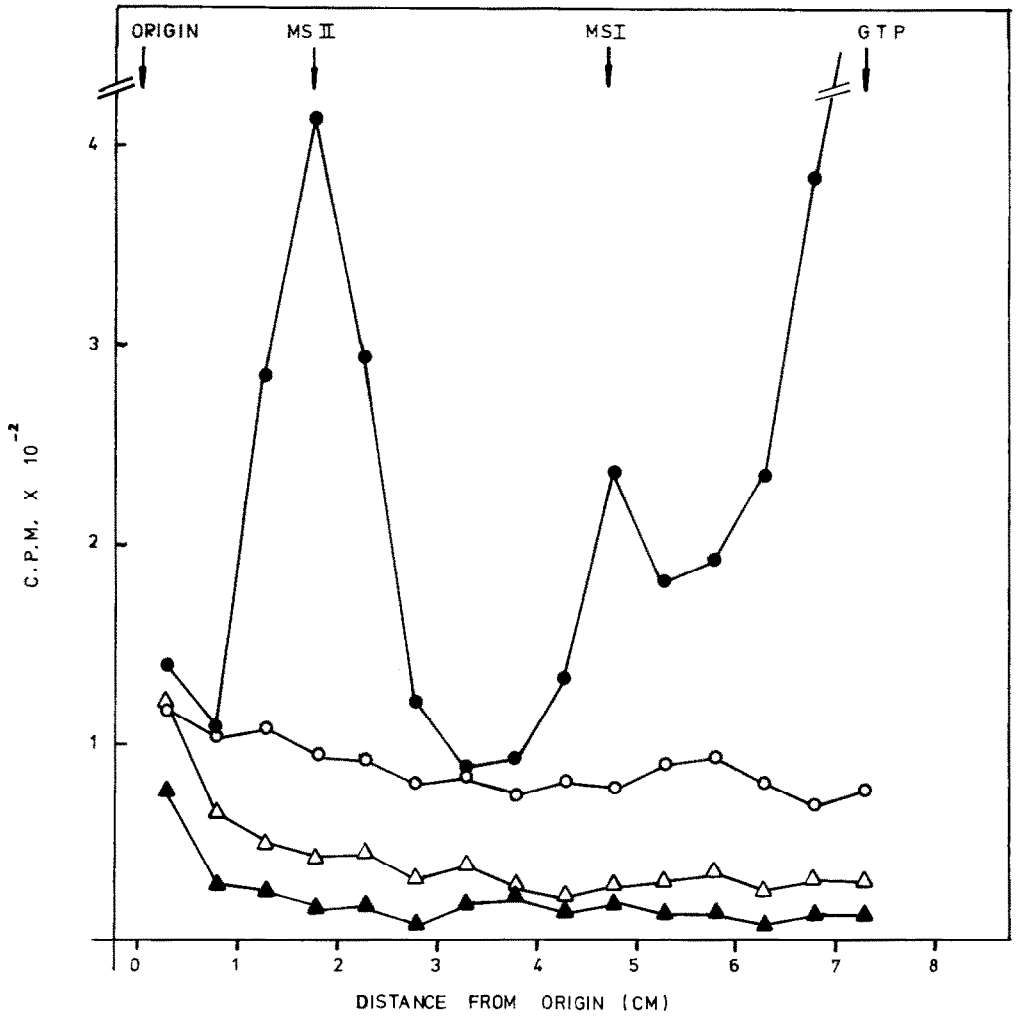

Fig. 1. Distribution of radioactivity within a one-dimensional thin-layer chromatogram. 20 μl of the reaction mixture (see Materials and methods) containing unwashed ribosomes from vegetative cells were applied and the chromatogram developed and further processed as described in Materials and methods. The reaction mixture contained either [^{3}H]GTP (—●—), [^{3}H]ATP (—○—), [^{3}H]UTP (—△—) or [^{3}H]CTP (—▲—).

2.3. *Preparation of ribosomes and ribosomal wash*

Ribosomes and ribosomal wash were prepared exactly as described [8].

2.4. *Reaction mixture and analysis of reaction products*

The reaction mixture for in vitro synthesis of unusual nucleotides of Haseltine et al. [8] was slightly modified. GTP was used at a concentration of 1.1 mM for the synthesis of MS by vegetative ribosomes, but no GTP was necessary for the synthesis of HPN by sporulation ribosomes. The concentration of ribosomes was 150–200 μg/50 μl reaction mixture. 40 μg of 0.5 M NH_4Cl-ribosomal wash and 5 μCi and [^{3}H]ATP or [^{3}H]GTP were used.

The reaction mixture was analysed either by one-dimensional thin layer chromatography (TLC) according to Cashel et al. [9] using 1.5 M potassium phosphate, pH 3.4 or two-dimensional TLC using 3.3 M formate, 4.2% borate, pH 7.0 in the first dimension

and 1.5 M potassium phosphate, pH 3.4 in the second dimension.

The amount of unusual nucleotides formed was measured by cutting the one-dimensional chromatogram into 0.5 cm wide and 1.0 cm long strips which were placed in scintillation vials containing 10 ml Bray's [10] solution and counted with a Packard-Tricarb Scintillation spectrometer. To detect the nucleotides in a two-dimensional chromatogram, the entire chromatogram was first cut into 0.5 cm wide stripes, then cut again every 0.5 cm to give 0.5 X 0.5 cm sized pieces, which were counted as described above.

3. Results

When washed ribosomes isolated from vegetative cells and 0.5 M NH_4Cl-ribosomal wash were incubated as described in Materials and methods in the presence of 5 μCi [^{3}H]GTP and unlabeled ATP, it was found that mainly MS II, but also MS I is synthesized (see fig. 1). Any other tritium labeled triphosphate, like ATP, UTP or CTP is not converted to substances migrating slower than GTP in a one-dimensional TLC system (fig. 1). Further analysis of the products produced by vegetative ribosomes in the presence of [^{3}H]GTP in a two-dimensional thin layer chro-

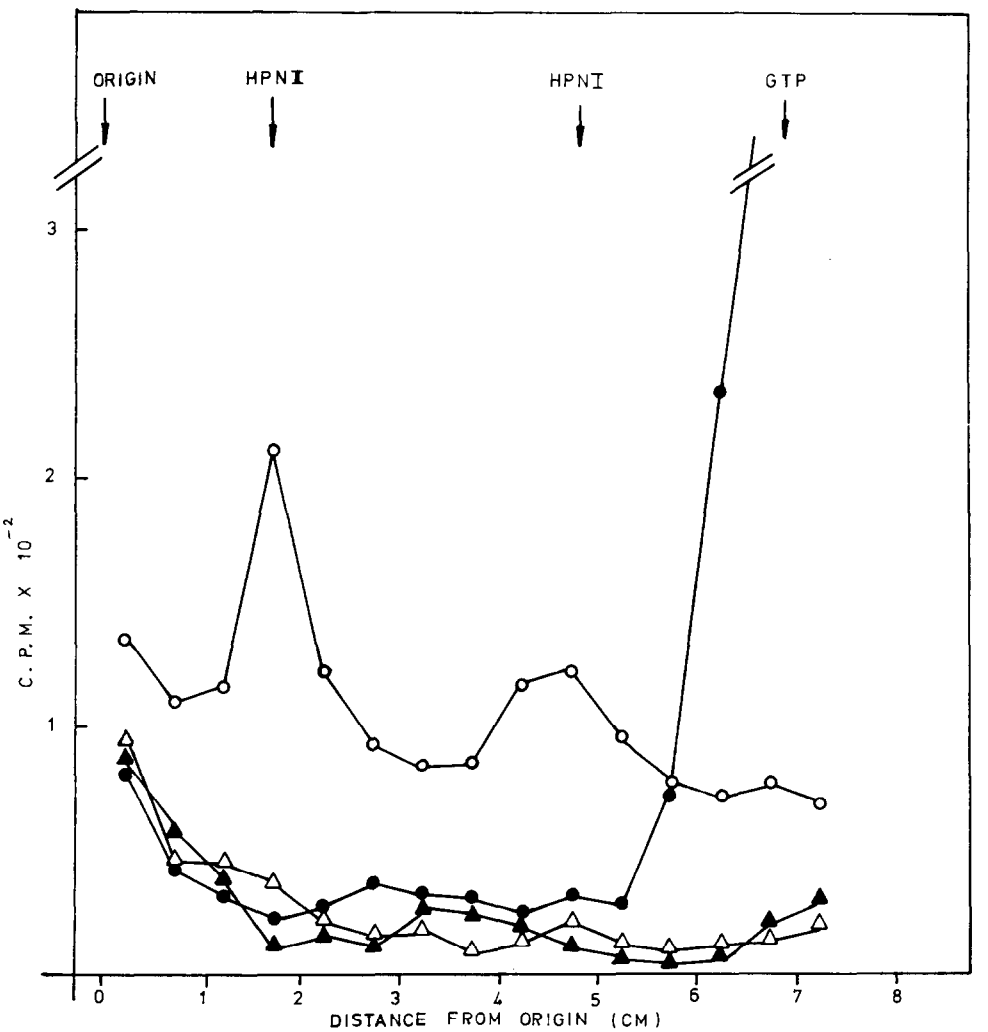

Fig. 2. Conditions as described in fig. 1, but the reaction mixture contained ribosomes from sporulating cells (T_2). [^{3}H]GTP (–●–), [^{3}H]ATP (–○–), [^{3}H]UTP (–△–), [^{3}H]CTP (–▲–).

Table 1
Synthesis of MS nucleotides by ribosomes from vegetative cells

Complete system*		MS II cpm	MS II nmoles/ml	MS II % Activity	MS I cpm	MS I nmoles/ml	MS I % Activity
	wr	411	980	100	263	660	100
	uwr	405	970	100	245	610	100
– ATP	wr	76	190	19	43	130	19
	uwr	68	170	17	42	105	17
– GTP	wr	0	0	0	0	0	0
	uwr	0	0	0	0	0	0
– wash	wr	115	290	29	65	160	24
	uwr	–	–	–	–	–	–
– Rib.	wr	62	160	16	58	145	22
	uwr	40	100	12	45	112	18
– Mg^{2+}	wr	92	230	23	72	180	27
	uwr	102	255	27	63	158	26
– DTT	wr	370	930	95	243	610	93
	uwr	364	910	92	228	580	95
+ Poly-U + tRNA	wr	385	960	96	255	640	96
	uwr	393	965	98	243	610	100
+ Poly-U + tRNA + Phe	wr	390	965	98	243	610	93
	uwr	384	950	97	235	590	97

* 'Complete system' is the reaction mixture described in Materials and methods, wr are washed, and uwr are unwashed ribosomes.

matogram using purified unlabeled MS I and II of *E. coli* as marker revealed the identity of the *B. subtilis* vegetative ribosomal products with ppGpp and pppGpp. It should be noted that ATP is not a substrate for the phosphorylation reaction of ribosomes isolated from vegetative cells but is required in the reaction mixture (see below).

In an identical experiment described above, ribosomes isolated from sporulating cells (T_2 or T_5) do not produce any significant amount of MS I or II as judged from the incorporation of [^{3}H]GTP into material migrating slower than GTP on PEI–thin-layer plates (see fig. 2). Neither tritium-labeled UTP or CTP in the presence of unlabeled ATP are phosphorylated. However, [^{3}H]ATP is converted to substances migrating similarly to MS I and MS II on PEI–thin-layer plates developed one-dimensionally. These substances are identical with HPN I and II found in sporulating cells after exhaustion of glucose in our NSM or SYM medium or after starvation for carbon sources [2].

The above described results are obtained with washed ribosomes and ribosomal (0.5 M NH_4Cl) wash as well as with unwashed ribosomes.

We then tried to determine whether or not ribosomes or ribosomal factors alone are sufficient to synthesize either MS or HPN nucleotides. As can be seen in table 1, washed ribosomes without ribosomal wash synthesize only little MS I and II. Ribosomal wash itself without ribosomes also shows no synthesizing activity (complete system minus ribosomes). In addition, the system depends on the presence of ATP, GTP, and magnesium.

Table 1 also shows that synthesis of MS I and II does not seem to depend on the presence of uncharged tRNA, mRNA nor protein synthesis as has been initially described for the *E. coli* system by Haseltine et al. [8]. However, we have not purified ribosomes and ribosomal wash according to Haseltine and Block [11].

Table 2
Synthesis of HPN nucleotides by ribosomes from sporulating cells

Complete system*		HPN II			HPN I		
		cpm	nmoles/ml	% Activity	cpm	nmoles/ml	% Activity
	wr	210	84	100	124	50	100
	uwr	200	80	100	135	54	100
– ATP	wr	0	0	0	0	0	0
	uwr	0	0	0	0	0	0
– GTP	wr	215	86	100	118	48	96
	uwr	202	81	100	125	50	93
– wash	wr	41	16	19	25	10	20
	uwr	–	–	–	–	–	–
– Rib.	wr	22	9	11	18	7.5	15
	uwr	25	10	12	20	8.0	15
– Mg^{2+}	wr	56	22	27	27	11	22
	uwr	46	18	23	32	13	24
– DTT	wr	205	79	100	110	44	88
	uwr	197	78	97	124	50	93
+ Poly U + tRNA	wr	218	87	100	132	53	100
	uwr	200	80	100	130	52	96
+ Poly U + tRNA + Phe	wr	208	83	100	123	49	100
	uwr	208	83	100	132	53	100

* 'Complete system' is the reaction mixture described in Materials and methods, wr are washed, uwr are unwashed ribosomes.

These authors were able to show a dependence of *E. coli* ribosomes and ribosomal wash on the presence of tRNA and mRNA to synthesize MS I and II after further purification. The absolute requirement of ATP and GTP agrees well with the mechanism of MS synthesis described by Sy and Lipmann [12]. ATP seems to be the pyrophosphate donor for GDP and GTP also in the in vitro synthesis of MS I and II by *B. subtilis* ribosomes.

In contrast, ribosomes isolated from sporulating cells of *B. subtilis* do not require any GTP as is shown by the incorporation of almost the same amount of [^{3}H]ATP into substances shown to be HPN I and II in the absence of GTP (see table 2). However, ATP is absolutely necessary as is magnesium and ribosomal wash when washed ribosomes were used. Increasing the ATP concentration results in an increase in the incorporation of [^{3}H]ATP into HPN I and II spots (Rhaese and Groscurth, unpublished data).

Likewise, in this unpurified preparation of ribosomes and ribosomal wash we could not detect any dependence of the synthesizing activity on the presence of tRNA, mRNA or on protein synthesis (table 2). Similar to MS synthesis, more HPN II is synthesized than HPN I.

Comparing the synthesizing activity of both systems (from vegetative and sporulating cells) it can be seen that approximately 10 times more MS than HPN nucleotides are synthesized (900 nmoles/ml versus 80 nmoles/ml). The results shown in tables 1 and 2 clearly indicate that not ribosomes themselves but ribosomes together with factors associated with ribosomes synthesize MS and HPN nucleotides. Some activity present in washed ribosomes (see complete systems minus wash, tables 1 and 2) is probably due to some factors not removed by high salt washing.

The almost identical behaviour of ribosomes from sporulating cells in incoporating ATP or ADP into

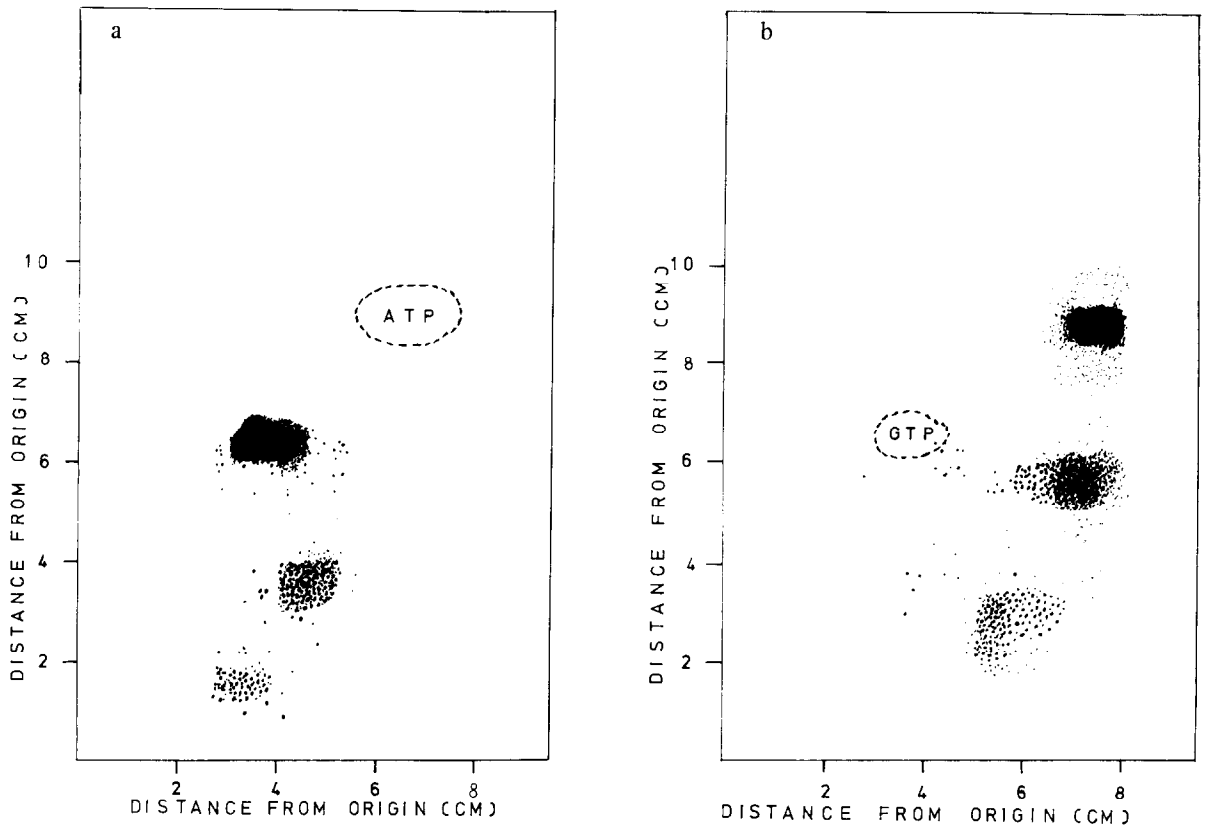

Fig. 3. Two-dimensional thin-layer chromatograms (see Materials and methods) of reaction mixtures containing vegetative ribosomes and [^{3}H]GTP (fig. 3b) and sporulation ribosomes and [^{3}H]ATP (fig. 3a). Unlabeled ATP (fig. 3a) and GTP (fig. 3b) were used as markers. The chromatograms show only the different positions of MS I (fig. 3a, close to GTP), and MS II (fig. 3a, close to the origin) compared to HPN I (fig. 3b, close to GTP and ATP), and HPN II (fig. 3b, close to the origin). Quantitative data are given in tables 1 and 2.

HPN I and II and of ribosomes from vegetative cells in incorporating GTP and GDP into MS I and II together with similarities in the chromatographic behaviour of both kinds of nucleotides on a two-dimensional thin layer chromatogram (see fig. 3a and b) suggests that HPN I is the tetraphosphate and HPN II the pentaphosphate of adenosine. Preliminary studies using α-^{32}P-labeled ATP (Rhaese and Groscurth, unpublished results) also seem to indicate that HPN I differs from MS I (ppGpp) only in so far as adenine instead of guanine is the base moiety in this nucleotide. The same could apply to HPN II. However more work is needed to determine exactly the structure of HPN I and II.

4. Discussion

We have proposed previously [1] that HPN I and II accumulation by sporulating but not vegetative cells of *B. subtilis* is somehow related to sporulation. If this is true then some components of sporulating cells should be able to synthesize HPN. On the other hand since Gallant and Margason [3] have shown that MS I and II accumulate in amino acid starved cells of *B. subtilis*, some components of vegetative cells should be able to synthesize MS. The discovery of Haseltine et al. [8] that *E. coli* ribosomes are able to accumulate MS nucleotides as a product of an idling step in protein synthesis, prompted us to try to synthesize

MS with ribosomes from vegetative cells. Indeed ribosomes from vegetative cells of *B. subtilis* behave like *E. coli* ribosomes. However, ribosomes isolated from sporulating cells (T_2) do not synthesize any ppGpp or pppGpp. Instead, ATP is incorporated into material comigrating with HPN I and HPN II found in cells starved for glucose and to some extent in sporulating cells of *B. subtilis* depending on the medium used. The results obtained so far all seem to indicate that HPN I is adenosine tetraphosphate and HPN II is adenosine pentaphosphate. Since ATP is required for the synthesis of MS nucleotides by vegetative ribosomes of *B. subtilis* and also for the synthesis of the HPN nucleotides by ribosomes of sporulating cells, whereas GTP can be omitted in the latter case, we conclude that the synthesis of both classes of regulating nucleotides, MS and HPN, is via the same mechanism proposed by Haseltine et al. [8] and by Sy and Lipmann [12]. Further evidence in favour of this conclusion will be published elsewhere [14].

The remarkable and hitherto unknown difference in the synthesizing activity of ribosomes of the same organism to synthesize different regulatory nucleotides (MS and HPN) depending only on the stage of their development, seems to indicate that the MS nucleotides regulate some functions in vegetative cells of *B. subtilis* as proposed for *E. coli* [15,16] and the HPN nucleotides regulating functions in sporulating cells, even might be the trigger for sporulation [1]. More work, however, is needed before the function of HPN synthesized by ribosomes of sporulating cells is known. Since ribosomes from both vegetative and sporulating cells synthesize regulatory nucleotides, it is unlikely that proteolysis accounts for the described changes.

References

[1] Rhaese, H. J., Dichtelmüller, H. and Giesel, F. (1972) in: Spores V, p. 176.
[2] Rhaese, H. J., Dichtelmüller, H. and Grade, R., Eur. J. Biochem. submitted for publication.
[3] Gallant, J. and Margason, G. (1972) J. Biol. Chem. 247, 2289.
[4] Cashel, M. and Gallant, J. (1969) Nature 221, 838.
[5] Cashel, M. (1969) J. Biol. Chem. 245, 2309.
[6] Rhaese, H. J., Grade, R. and Dichtelmüller, H., Eur. J. Biochem. submitted for publication.
[7] Freese, E. and Portnagel, P. (1967) J. Bacteriol. 94, 1957.
[8] Haseltine, W., Block, R., Gilbert, W., Weber, K. (1972) Nature 238, 381–384.
[9] Cashel, M., Lazzarini, R. A. and Kalbacher, B. (1969) J. Chromatog. 40, 103.
[10] Bray, G. A. (1960) Analyt. Biochem. 1, 279–285.
[11] Haseltine, W. A. and Block, R. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1564–1568.
[12] Sy, J. and Lipmann, F., (1973) Proc. Natl. Acad. Sci. U.S. 70, 306.
[13] Swanton, M. and Edlin, G. (1972) Biochem. Biophys. Res. Commun. 46, 583.
[14] Rhaese, H. J. and Groscurth, R. manuscript in preparation.
[15] Cashel, M. (1970) Cold Spring Harbor Symp. Quant. Biol., 35, 407.
[16] Young, H. L., Zubay, G., Urm, E., Reiness, G. and Cashel, M. (1974) Proc. Natl. Acad. Sci. U.S. 71, 63.