STIMULATION BY ATP OF PROTEIN INITIATION IN A PROKARYOTIC ORGANISM, *B. STEAROTHERMOPHILUS*

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1. Introduction

Initiation complex formation in *E. coli* in vitro systems requires only one nucleotide, GTP [1–3]. It can be partially replaced by GMPPCP, but the 70S initiation complex formed is inactive for peptidyl transfer [2–5]. However, GTP hydrolysis is not needed for translocation of the initiator tRNA, but to permit the release of IF-2** [3,5,6].

It has been shown in some eukaryotic systems that not only GTP but also ATP is involved in polypeptide chain initiation [7–9]. The ATP is apparently needed for efficient binding of mRNA to the ribosomes [7,9] and for conversion of the initiation complex to a form which is reactive with puromycin [8,9]. Recently, Marcus has reported that this last step is preceded by hydrolysis of GTP and ATP, resulting in the formation of ppGpp, and may involve the recycling of initiation factors [7].

Here we report a stimulation by ATP upon initiation complex formation in a prokaryotic in vitro system from *Bacillus stearothermophilus*. The effect, however, does not seem to be strictly analogous to that observed in eukaryotic systems.

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** *Abbreviations:* IF-1, IF-2, and IF-3 : *E. coli* initiation factors; S-IF-2 and S-IF-3: corresponding initiation factors of *B. stearothermophilus*. GMPPCP : β,γ-methylene-guanosine triphosphate; AMPPCP : β,γ-methylene-adenosine triphosphate.

2. Materials and m

B. stearothermophilus and *E. coli* ribosomes, and purified initiation factors were prepared as described previously [10] as were f[^{3}H]Met-tRNA and poly AUG. ppGpp was a gift from Dr Bocquet, C. E. A., Saclay, France. GTP, GDP, ATP and phosphoenol pyruvate were Sigma products. Pyruvate kinase was from Boehringer. GMPPCP and AMPPCP were P. L. Biochemicals (USA) products.

The f[^{3}H]Met-tRNA ribosomal binding assays were carried out as described previously [10].

3. Results and discussion

3.1. *Stimulation of initiation complex formation by ATP*

Even in the absence of any added nucleotides, *B. stearothermophilus* ribosomes and initiation factors can bind some fMet-tRNA (fig.1) (such residual binding has also been noted with *E. coli* systems [3]). The addition of either GTP (fig.1A) or, to a lesser extent, ATP (fig.1B) increases the binding. The addition of both nucleotides results in a large stimulation of initiation complex formation (fig.1A and B). Binding is maximal when both GTP and ATP are present at a concentration of 0.5 mM each. Higher amounts of GTP do not abolish the stimulation by ATP (fig.1A). Non hydrolysable analogs of ATP (AMPPCP) or of GTP (GMPPCP) cannot replace the natural nucleotides.

The effect of ATP does not seem to be trivial. To prevent serious changes in effective Mg^{2+} concentra-

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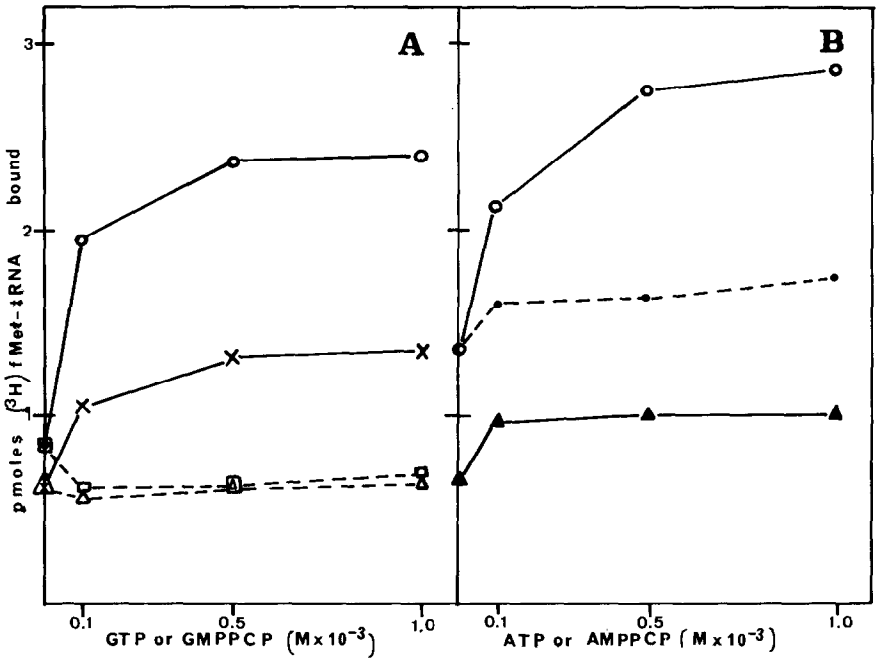

Fig.1. Nucleotide requirements for initiation complex formation on *B. stearothermophilus* ribosomes. The incubation mixture (final vol 100 μl) contained : 20 mM MOPS (pH 7.2); 5.5 mM magnesium acetate; 75 mM NH_4Cl; 2.5 A_{260} units f[^{3}H]Met-tRNA (19 pmol f[^{3}H]Met); 0.15 A_{260} units poly AUG; and 26.5 pmol *B. stearothermophilus* ribosomes; initiation factors and nucleotides as indicated. Incubation was for 15 min 50°C and the amount of f[^{3}H]Met-tRNA bound in the absence of initiation factors (about 0.15 pmol in all cases) was subtracted. (A) 6 pmol S-IF-2; 50 pmol S-IF-3; 0.5 mM ATP; and GTP or GMPPCP at the concentrations indicated (B) 7.5 pmol S-IF-2; 50 pmol S-IF-3; 0.5 mM GTP; and ATP or AMPPCP as indicated. (×——×)GTP; (▲——▲)ATP; (○——○)GTP + ATP; (△- - -△)GMPPCP; (□- · -□)ATP + GMPPCP (●- - -●)GTP + AMPPCP.

tions, stoichiometric amounts of Mg^{2+} were always added with the nucleotides [11]. The action of ATP cannot be explained by regeneration of GTP from GDP, as has been suggested for a eukaryotic system [12]. When GDP is substituted for GTP, the addition of ATP has no effect on the binding of fMet-tRNA, except when a GTP regenerating system (pyruvate kinase and phosphoenol pyruvate) is added (fig.2). Therefore, it appears that GTP has to be present for ATP to have an effect and the stimulation is not due to the phosphorylation of GDP into GTP.

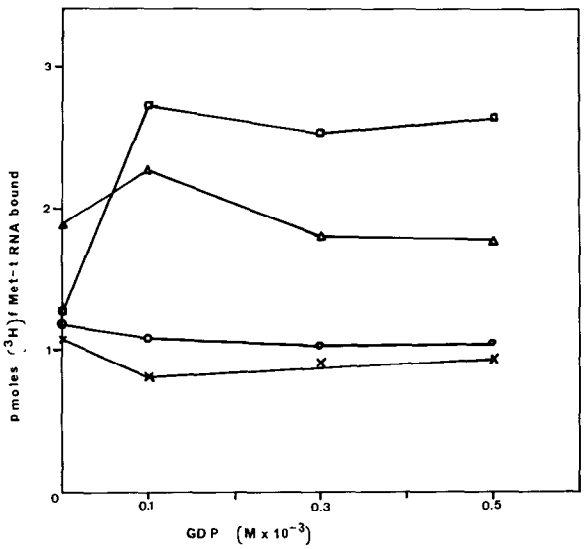

Fig.2. Initiation complex formation in the presence of GDP. Same incubation mixture as in fig.1 with 9 pmol S-IF-2; 50 pmol S-IF-3; and, where added, 0.5 mM ATP; 3 μg pyruvate kinase; 1 mM phosphoenol pyruvate. GDP as indicated. (×——×)GDP; (○——○)GDP + ATP; (△) GDP + pyruvate kinase + phosphoenol pyruvate; (□——□)GDP + ATP + pyruvate kinase + phosphoenol pyruvate.

Table 1
Dependence of ATP effect upon ribosomes and initiation factors

Origin of ribosomes	Initiation factor additions	f[^{3}H]Met-tRNA bound		GTP + ATP
		+ GTP	+ GTP + ATP	GTP
B. stearothermophilus	IF-2 + S-IF-3	0.70	1.19	1.70
	IF-2 + S-IF-3 + IF-1	1.66	2.38	1.44
	S-IF-2 + S-IF-3	0.64	0.90	1.40
	S-IF-2 + S-IF-3 + IF-1	1.39	1.63	1.18
E. coli	IF-2 + S-IF-3	2.96	3.03	1.02
	IF-2 + S-IF-3 + IF-1	4.40	4.51	1.03
	S-IF-2 + S-IF-3	1.97	2.03	1.03
	S-IF-2 + S-IF-3 + IF-1	3.39	3.65	1.07

With *B. stearothermophilus* ribosomes, same incubation mixture as in fig.1 with *E. coli* ribosomes the incubation mixture (100 μl) contained : 50 mM Tris–HCl (pH 7.5); 50 mM NH_4Cl; 5 mM magnesium acetate; 0.15 A_{260} units poly AUG; 18 pmoles f[^{3}H]Met-tRNA (S. A. 2553 cpm/pmol). In both cases incubation was for 20 min at 37°C. S-IF-2 (4.5 pmol); S-IF-3 (33 pmol); IF-2 (5.6 pmol); IF-1 (22 pmol); GTP (1 mM); and GTP + ATP (0.5 mM each) were added where indicated.

Another, though remote possibility for an indirect effect of ATP would be that it is involved in recharging initiator tRNA. However, discharge is slight (5–10%) during incubation with the complete system, and addition of ATP has little effect upon the level of charged fMet-tRNA present, ATP, therefore, appears to exert a direct rather than an indirect effect upon the initiation process.

3.2. *Requirements for stimulation by ATP*

The effect is species specific and depends upon the origin of the ribosome and not of the initiation factors (table 1). fMet-tRNA binding to *B. stearothermophilus* ribosomes is stimulated by ATP when either *B. stearothermophilus* or *E. coli* initiation factors are used; but ATP has no effect when *E. coli* ribosomes are used. It should be noted that ATP

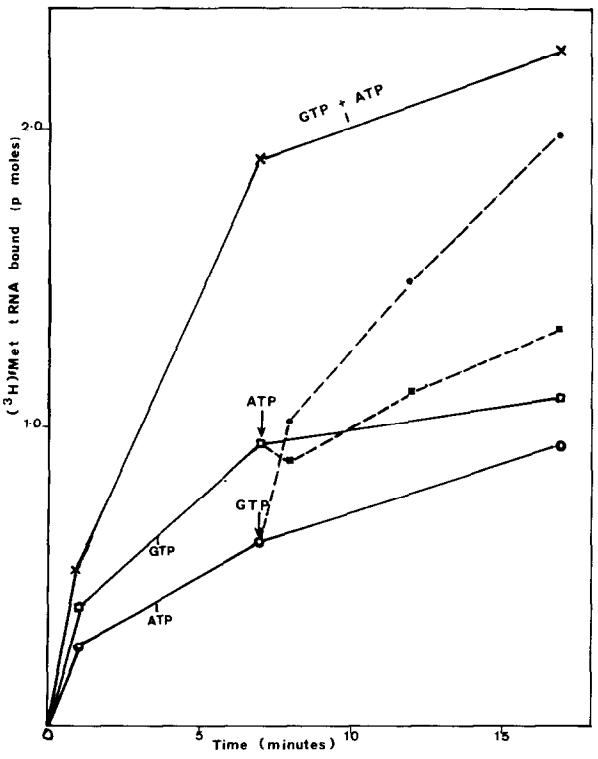

Fig.3. Kinetics of initiation complex formation. Incubation as in fig.1A for the times indicated. Nucleotides (0.5 mM each) were added with equivalent amounts of magnesium either at zero time (solid lines) or after several minutes (indicated with arrows and followed by dotted lines).

Table 2
Effect of pre-incubation with, and then removal of, ATP

Additions	f[^{3}H]Met-tRNA bound		GTP + ATP
	+ GTP	+ GTP + ATP	GTP
	(pmoles)		
Ribosomes + S-IF-2 + S-IF-3	2.25	3.69	1.64
(Ribosomes) + S-IF-2 + S-IF-3	1.91	3.34	1.75
(Ribosomes) + (S-IF-2 + S-IF-3)	0.61	0.99	1.63
(Ribosomes + ATP) + (S-IF-2 + S-IF-3)	0.69	1.05	1.53
(Ribosomes + S-IF-2 + S-IF-3)	1.84	3.01	1.64
(Ribosomes + S-IF-2 + S-IF-3 + ATP)	1.92	2.86	1.49
(S-IF-2 + S-IF-3 + ATP) + ribosomes	0.74	1.11	1.51

Incubation mixture as in fig.1 with 9 pmol S-IF-2; 50 pmol S-IF-3; 0.5 mM GTP (where indicated) and 0.5 mM ATP (where indicated). The components in parentheses were pre-incubated 15 min at 50°C, in the presence (0.5 mM) or absence of ATP, and then dialyzed overnight against the incubation buffer.

has a greater observable effect when purified, rather than crude (high salt ribosomal wash), initiation factors are used (not shown), perhaps because of ATPase activity in the latter.

3.3. *Effect of nucleotide sequence of addition*

The effect of ATP was followed in time (fig.3). The addition of ATP affects both the initial and final level of initiation complex formation. Also, the sequence of addition of the nucleotides is important. If ATP is added after formation of initiation complex in the presence of GTP alone is almost complete (fig. 3), then it exerts little effect. On the other hand, if the system is first incubated with ATP, then, upon addition of GTP, there is a rapid increase in initiation complex formation and the final level of fMet-tRNA bound is similar to that observed when both GTP and ATP are present at time zero. This shows that GTP is necessary for initiation complex formation in agreement with the results of fig.2, and that ATP has to be present at the time of formation in order to have an effect. It also confirms that the role of ATP involves neither the regeneration of GTP nor the recharging of initiatior tRNA.

A possible explanation is that ATP increases activity by phosphorylation of the ribosome or the initiation factors. However, when the different components of the initiation system are pre-incubated, either independently or together, with ATP which is then removed by extensive dialysis, we still find a stimulatory effect of ATP in the subsequent assays, and no evidence of increased activity of the components (table 2). If phosphorylation occurs, and is responsible for the increased activity observed in the presence of ATP, it is easily reversible. It should be noted that the presence of ATP in the pre-incubation mixture does not result in an increased inactivation of *B. stearothermophilus* initiation factors, as is the case of *E. coli* initiation factors [13].

3.4. *Effect of ppGpp on initiation complex formation*

Marcus has reported that wheat germ systems utilise ATP during initiation complex formation to synthesize ppGpp [7]. *B. stearothermophilus* can also synthesize this compound [14] and highly phosphorylated nucleotides appear to play a regulatory role in *Bacilli* [15]. We looked at the effect of directly adding ppGpp to our system. At low concentrations (0.01 nM to 0.01 mM), ppGpp had no effect on initiation complex formation (fig.4). At higher concentrations (0.1 to 1 mM), initiation complex formation in the presence of GTP alone was only slightly inhibited (less than in *E. coli* system [16]), but the stimulation by ATP was completely abolished at 1 mM ppGpp. This is not inconsistent with the ATP effect being mediated by ppGpp formation, which is inhibited by the addition of excess ppGpp.

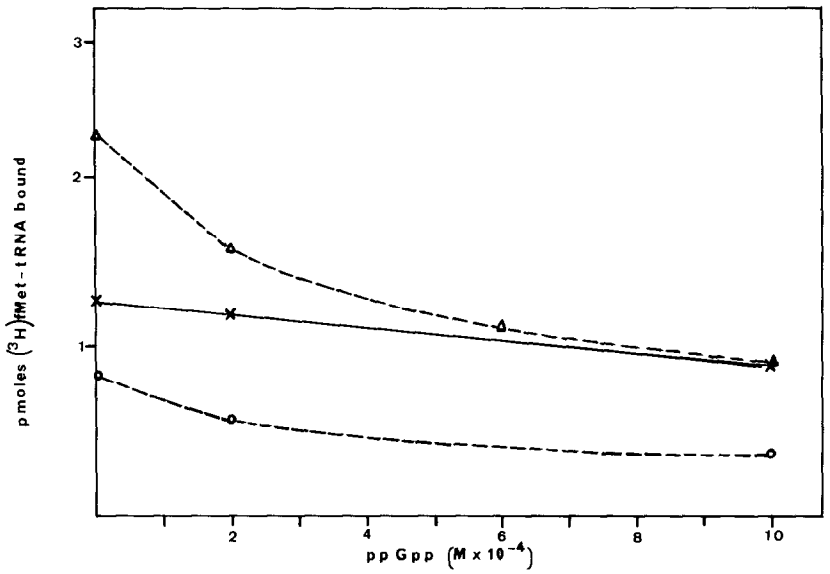

Fig.4. Effect of ppGpp upon initiation complex formation. Incubation as in fig.1B. GTP and ATP (when present) were both 0.5 mM; ppGpp was added as indicated. (X——X)GTP; (○- - -○)ATP; (△- - -△)GTP + ATP.

3.5. *Effect of ATP on puromycin reactivity of fMet-tRNA bound and on the recycling of initiation factors*

However, the situation in *B. stearothermophilus* is not analogous with the wheat germ system in several respects. As we have shown, ATP has little effect if it is added after initiation complex has been formed in the presence of GTP alone. Also, unlike eukaryotic systems the initiation complex formed in the presence of GTP alone reacts with puromycin, as does that formed in the presence of both GTP and ATP (table 3). Binding of mRNA (labelled poly AUG) does not depend significantly upon the presence of either GTP or ATP (not shown).

To test other analogies with eukaryotic systems, we tried initiation with purified unformylated initia-

Table 3
Puromycin reactivity of initiation complex

Additions	+ GTP		+ ATP		+ GTP	+ ATP
	fMet-Puro reaction	fMet-tRNA bound	fMet-Puro reaction	fMet-tRNA bound	fMet-Puro reaction	fMet-tRNA bound
	(pmoles)		(pmoles)		(pmoles)	
S-IF-2	0.12	0.08	0.06	0.03	0.16	0.15
S-IF-2 + S-IF-3	1.56	1.39	1.11	0.80	3.03	2.49

Same incubation mixture as in fig.1 with 0.5 mM of GTP and/or ATP, 4 pmol S-IF-2 and, where indicated, 50 pmol S-IF-3. For fMet-tRNA binding assay, incubation was for 12 min at 50°C. For puromycin assay, after 12 min at 50°C, 10 μl puromycin (5 mg/ml) were added, and incubation continued for 5 min; the reaction was stopped with 1 ml sodium acetate, 0.1 M (pH 5), and 1.5 ml of ethyl acetate were added. After stirring, 1 ml of the organic layer was counted. The values obtained in the puromycin reaction were multiplied by 1.5 to bring them to the same vol as for the binding assay. The amount of fMet-tRNA bound (0.15) or reactive with puromycin (0.5 pmol) in the absence of initiation factors has been subtracted.

Table 4
Effect of *E. coli* IF-1 and of ATP

Temperature (°C)	*E. coli* IF-1	f[^{3}H]Met-tRNA bound (pmoles) + GTP	+ GTP + ATP	GTP + ATP/GTP
60	–	0.59	1.12	190
	+	1.47	2.34	159
50	–	0.89	1.47	165
	+	2.19	3.19	141
37	–	0.84	1.29	154
	+	2.53	2.96	118
25	–	0.88	1.14	1.30
	+	2.14	2.41	1.13

Same incubation mixture as in fig.1 with 6 pmol S-IF-2; 13 pmol S-IF-3; 42 pmol *E. coli* IF-1, where indicated; and 0.5 mM each of ATP and/or GTP, where indicated. Incubation was 5 min at 60°C, 15 min at 50°C, and 25 min at 37°C or 25°C. The amount of fMet-tRNA bound in the absence of initiation factors has been subtracted (from 0.05 pmol at 25°C to 0.15 pmol at 60°C).

tor tRNA. Initiator tRNA in eukaryotes is normally not formylated [17], and also, in some circumstances, in *Streptococcus faecalis* [18]. Some initiation complex formation took place, and could be stimulated by ATP, but it was only 5–10% of that obtained with purified formylated initiator tRNA.

In the wheat germ system, the addition of ATP may favorise recycling of initiation factors [8], as does IF-1 and IF-3 in *E. coli* systems [5,19]. In our *B. stearothermophilus* system, we have been unable to find a factor corresponding to IF-1 [10], and the effect of ATP may be related to this. In the presence of limiting amounts of S-IF-3, the addition of *E. coli* IF-1 can stimulate fMet-tRNA binding to *B. stearothermophilus* ribosomes [10]. The addition of optimal amounts of IF-1 does not abolish stimulation by ATP and vice-versa (table 4). At optimum temperature for *B. stearothermophilus* ribosomes (50–60°C) the effects of ATP and IF-1 do not appear to be equivalent. In our system, *E. coli* IF-1 possibly acts by stabilizing the initiation complex on the *B. stearothermophilus* 30S subunit, which is structurally and functionally similar to that of *E. coli* [20,21], while ATP acts when the 50S subunit, where the GTPase and ATPase activities are located [22], joins the complex. Also, the stimulatory effect of ATP decreases with the incubation temperature (table 4) and may therefore be related to the high temperature at which *B. stearothermophilus* normally grows.

4. Conclusion

In contrast to *E. coli* ribosomes, with *B. stearothermophilus* ribosomes initiation complex formation is stimulated by ATP as well as GTP, but maximum stimulation occurs when both the nucleotides are present; and their terminal phosphate must be hydrolysable. In the presence of ATP and GTP, *B. stearothermophilus* ribosomes synthesize a highly phosphorylated guanine derivative, ppGpp, and the role of ATP in initiation might be related to this synthesis. We discarded the role of ATP as being trivial and corresponding solely to the well-known effect on eukaryotic systems.

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References

[1] Lucas-Lenard, J. and Lipmann, F. (1967) Proc. Nat. Acad. Sci. USA 57, 1050–1057.
[2] Anderson, J. S., Bretscher, M. S., Clark, B. F. and Marcker, K. A. (1967) Nature 215, 490–492.
[3] Dubnoff, J. S., Lockwood, A. M. and Maitra, U. (1972) J. Biol. Chem. 247, 2884–2894.
[4] Ohta, T., Sarkar, S. and Thach, R. E. (1967) Proc. Nat. Acad. Sci. USA 58, 1638–1644.
[5] Benne, R. and Voorma, M. O. (1972) FEBS Lett. 20, 347–353.
[6] Grunberg-Manago, M., Dondon, J. and Graffe, M. (1972) FEBS Lett. 22, 217–221.
[7] Marcus, A. (1970) J. Biol. Chem. 245, 955–961.
[8] Anderson, W. F., Bosch, C., Gros, F., Grunberg-Manago, M., Ochoa, S., Rich, A. and Stachelin, T. (1974) FEBS Lett. 48, 1–6.
[9] Schreier, M. N. and Staehelin, T. (1973) in: Regulation of Transcription in Eukaryotes (Bautz, Fartson and Feosten, eds.) 24th Mossbach Colloquium. Springer Verlag, Berlin 335–349.
[10] Kay, A. C., Graffe, M. and Grunberg-Manago, M., Biochimie France, in the press.
[11] Kaempfer, R. (1972) J. Mol. Biol. 71, 583–598.
[12] Watson, G. M. and Gill, G. N. (1975) Biochim. Biophys. Acta 390, 231–245.
[13] Legault-Demarre, L., Jeantet, C. and Gros, F. (1973) Mol. Gen. Genet. 125, 301–318.
[14] Richter, D. and Osono, K. (1974) FEBS Lett. 44, 270–273.
[15] Rhaese, M. J. and Groscurth, R. (1974) FEBS Lett. 44, 87–93.
[16] Legault, L., Jeantet, C. and Gros, F. (1972) FEBS Lett. 27, 71–75.
[17] Smith, A. E. and Marcker, K. A. (1970) Nature 226, 607–610.
[18] Samuel, C. E., Murray, C. C. and Rabinowitz, J. C. (1972) J. Biol. Chem. 247, 6856–6865.
[19] Kay, A. C. and Grunberg-Manago, M. (1972) Biochem. Biophys. Acta 277, 225–230.
[20] Isono, S. and Isono, K. (1975) Eur. J. Biochem. 50, 483–488.
[21] Higo, K., Held, W., Kahan, L. and Nomura, M. (1975) Proc. Nat. Acad. Sci., USA 70, 944–948.
[22] Horne, J. R. and Erdmann, V. A. (1973) Proc. Nat. Acad. Sci. USA 70, 2870–2873.