Mode of action of bottromycin A_2: Effect of bottromycin A_2 on polysomes

Tadahiko Otaka and Akira Kaji

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

Received 30 December 1982

When bottromycin A_2 was added to an in vitro protein synthesis system carried out by naturally occurring polysomes, it inhibited protein synthesis effectively. Examination of the 3 steps of peptide chain elongation revealed that the binding of aminoacyl-tRNA to the polyribosomes was inhibited by bottromycin A_2. In contrast, we concluded that the peptide bond formation and the translocation steps in this system were not inhibited by bottromycin A_2 on the basis of the following observations: (1) The break-down of polysomes, which is dependent on EFG, puromycin and RR (ribosome releasing) factor, was insensitive to bottromycin A_2; (2) The puromycin dependent release of polypeptide from polysomes, with or without EFG, was not inhibited by bottromycin A_2. Thus bottromycin specifically interferes with proper functioning of the A sites of polysomes. This is consistent with the results obtained using the model system with synthetic polynucleotides.

Bottromycin *Polysomes* *Acceptor site* *Protein synthesis*
Aminoacyl tRNA release

1. INTRODUCTION

Bottromycin A_2 is produced by Streptomyces and is a specific inhibitor of bacterial protein synthesis [1,2]. It has been suggested that it is an inhibitor of translocation [3] and of peptide bond formation [4]. In [5,6] we reported that bottromycin A_2 interferes with the ribosomal A site (acceptor site) in the model system. This conclusion was derived from observations that bottromycin A_2 releases oligopeptidyl-tRNAs and aminoacyl-tRNAs from the A site. Because of this releasing reaction, bottromycin A_2 inhibited the binding of aminoacyl-tRNA to the A site [5]. It has also been reported that bottromycin A_2 inhibits the puromycin reaction with ribosome-bound polylysyl- or *N*-acetyl-phenylalanyl-tRNA if puromycin concentration is relatively low. This effect, however, was explained on the basis that bottromycin A_2 interferes with the A site [2], thereby making it more difficult for puromycin to enter [6].

Abbreviations: EFG, elongation factor G; DTT, dithiothreitol; Tris, Tris-(hydroxymethyl) aminomethane; EFTu, elongation factor Tu

One puzzling fact about bottromycin A_2 has been its relative ineffectiveness on the puromycin reaction with nascent polypeptidyl-tRNA on polysomes [7]. In contrast, the antibiotic effectively inhibits phenylalanyl–puromycin or *N*-acetylphenylalanyl–puromycin formation in a polyuridylic acid (poly U)-dependent system [4,6]. To elucidate this puzzle and to extend our hypothesis that bottromycin A_2 acts on the acceptor site of ribosomes, we examined the effect of bottromycin A_2 on polyribosomes. The drug failed to inhibit translocation or peptide bond formation in this system but lessened the binding of aminoacyl-tRNA to the polysomes. Our unified hypothesis that bottromycin A_2 interferes with the A site of ribosomes appears to apply also to naturally occurring polysomes.

2. MATERIALS AND METHODS

The *Escherichia coli* extract, ribosomes and polyribosomes were prepared as in [5,6,8,9]. The source of antibiotics was as in [5,10]. Bottromycin A_2 was kindly provided by Dr N. Tanaka of Tokyo University.

Published by Elsevier Biomedical Press
00145793/83/0000–0000/\$3.00

2.1. *Amino acid incorporation system with polysomes*

The reaction mixture (0.25 ml) for the assay of protein synthesis using intact polysomes contained 20 mM Tris–HCl (pH 7.8), 1 mM DTT, 50 mM NH_4Cl, 8 mM magnesium acetate, 0.2 mM GTP, 7 mM phosphoenolpyruvate, 8.9 μg of pyruvate kinase, 98.7 μg of S-150 [10], 340 μg of a tRNA mixture (containing 1.6×10^5 cpm of [^{14}C]valyl-, [^{14}C]glycyl-, [^{14}C]seryl-, [^{14}C]alanyl tRNA), (other aminoacyl-tRNAs were prepared with ^{12}C-amino acids), and 2.2 A_{260} nunits of polysomes. Where indicated, 10^{-4} M bottromycin A_2 was added. The mixture was incubated at 22°C, and the hot trichloroacetic acid-insoluble radioactivity was measured by the filter disc method [11].

The mixture (0.35 ml) for protein synthesis using puromycin-pretreated polysomes was essentially the same as above except that it contained 6 mM magnesium acetate, 480 μg of soluble enzymes free of RR factor [14,15] in place of S-150, 1.37 A_{260} nunit of puromycin-pretreated polysomes [10] and 230 μg of a tRNA mixture containing 1.7×10^5 cpm of [^{14}C]valyl-, [^{14}C]alanyl-, [^{14}C]seryl- and [^{14}C]glycyl-tRNA (other aminoacyl-tRNAs were prepared with ^{12}C-amino acids). Where indicated, 10^{-4} M bottromycin A_2 was added and the reaction mixture was incubated for 10 min at 30°C.

2.2. *Binding of aminoacyl-tRNA to polysomes*

The formation of the EFTu aminoacyl-tRNA–GTP ternary complex was carried out as in [12]. The reaction mixture (0.35 ml) for the binding of 15 [^{14}C]aminoacyl-tRNAs (made with uniformly ^{14}C-labeled amino acid mixture from New England Nuclear) contained 200 μl of puromycin-treated polysomes (11.5 A_{260} nunits/ml) and 4.6×10^4 cpm of 15 [^{14}C]aminoacyl-tRNA–EFTu–GTP ternary complexes. Where indicated, 10^{-4} M bottromycin A_2 was added. After preincubation of polysomes with bottromycin A_2 for 3 min at 37°C, the binding reaction was carried out for 5 min and 15 min at 37°C. The reaction mixture (300 μl) was then placed on 4.8 ml of a sucrose gradient (15–30% in 10 mM Tris–HCl (pH 7.4), 10 mM magnesium acetate, 50 mM NH_4Cl and 6 mM β-mercaptoethanol). The tubes were centrifuged for 55 min at 38000 rev./min with an SW 50.1 rotor. Fractions (4 drops each) were collected from the bottom of the tube and the ribosome-bound cold (4°C) and hot (95°C) trichloroacetic acid-insoluble radioactivities were measured by the filter disc method [11]. The values of hot trichloroacetic acid-soluble radioactivities (cold trichloroacetic acid-insoluble radioactivities minus hot trichloroacetic acid-insoluble radioactivities) were regarded as bound [^{14}C]aminoacyl-tRNA. This value is sensitive to RNase.

2.3. *The assay of EFG-dependent puromycin reaction using polysomes with bound nascent polypeptides*

Polyribosomes containing nascent peptides labeled with ^{14}C amino acids were prepared as follows: The reaction mixture (2 ml) contained 1.7 ml polysomes (11.5 A_{260} nunits/ml) and 2.6×10^8 cpm of 15 [^{14}C]aminoacyl-tRNA–EFTu–GTP ternary complexes. The incubation was carried out for 15 min at 37°C, and labeled polysomes were isolated by sucrose density gradient centrifugation. The reaction mixture (0.35 ml) for the puromycin reaction with polysomes contained 310 μl polysomes carrying the [^{14}C]peptidyl-tRNA obtained above and 0.1 mM puromycin. Where indicated, 10^{-4} M bottromycin A_2, 4.7 μg EFG and 0.1 mM GTP were added. The incubation was carried out for 3 min at 37°C. The mixture (300 μl) was analyzed by sucrose gradient centrifugation as in section 2.2. Hot (95°C) trichloroacetic acid-insoluble radioactivity bound to the ribosomes was measured by the disc filter method [11].

3. RESULTS

3.1. *Effect of bottromycin A_2 on protein synthesis by naturally-occurring polysomes*

In fig.1, naturally-occurring polysomes with nascent peptide chains were isolated and their protein synthetic activity was measured. It is clear from fig.1 that bottromycin A_2 has an inhibitory effect on protein synthesis by polyribosomes. However, it was noted that the inhibition was less at the beginning of the reaction.

In [5] we reported that oligopeptidyl-tRNA was released by bottromycin A_2 in the poly(U)-dependent polyphenylalanine synthesis system. In the polysomal system shown in fig.1, however, no appreciable release of peptidyl-tRNA took place

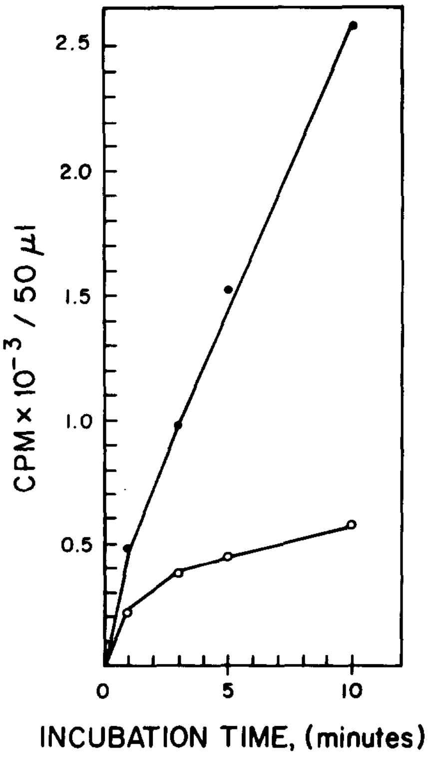

Fig.1. Time course of protein synthesis by polyribosomes in the presence of bottromycin A$_2$. Experimental conditions are described in the text. Bottromycin was added 3 min prior to the addition of labeled aminoacyl-tRNA because we suspected that the antibiotic might have difficulty in binding to polysomes as erythromycin does [13]: (●) no addition; (○) 10^{-4} M bottromycin was added.

from the polysomes under conditions where bottromycin A$_2$ had a very strong inhibitory effect on protein synthesis (not shown).

3.2. *Effect of bottromycin A$_2$ on puromycin-pretreated polyribosomes*

In [7] the puromycin reaction with polyribosomes was insensitive to bottromycin A$_2$. This observation, together with the relative ineffectiveness of bottromycin A$_2$ on initial protein synthesis by polyribosomes, suggests that the ineffectiveness is due to the nascent peptidyl group which might cause steric hindrance between the ribosomes and bottromycin A$_2$. It is possible to study protein biosynthesis using polyribosomes with their nascent chains removed because one can specifically eliminate ribosome-releasing (RR) factor which releases ribosomes from mRNA [10,14,15] from the crude soluble extract [10]. Taking advantage of such an RR-factor-free extract, the effect of bottromycin A$_2$ was studied on puromycin-treated polysomes. Over 99% inhibition (from 7000 cpm to 42 cpm) was observed under conditions otherwise similar to those in fig.1. The inhibition by bottromycin A$_2$ was much stronger than in the case using untreated polysomes with bound nascent polypeptides (fig.1).

3.3. *Lack of inhibitory effect of bottromycin A$_2$ on translocation or the peptidyl transferase reaction*

In table 1, polysomes isolated from growing *E. coli* were first incubated with a mixture of [^{14}C]aminoacyl-tRNA–EFTu and GTP complexes so that the binding of aminoacyl-tRNA could take place at the A sites of polysomes. The peptidyl transferase present in the polysomes would then catalyze the peptide bond formation between the nascent polypeptide chain and the newly bound [^{14}C]aminoacyl-tRNA. The radioactive polypeptide chains thus made will react with puromycin if they are located at the D (donor) or P (peptidyl) site. On the other hand, the peptidyl-tRNA which was bound at the A (acceptor) site does not react with puromycin in the absence of EFG and GTP. The results in table 2 show that the puromycin

Table 1

Lack of inhibitory effect of bottromycin A$_2$ on the puromycin reaction with nascent polypeptides in the presence and absence of EFG

Bottromycin A$_2$ (10^{-4} M)	EFG, GTP	Puromycin derivative formed (cpm)
+	–	–1412
+	+	–1486
+	–	+2059
+	+	+2093

The experimental conditions are described in the text. The values represent the puromycin-dependent decrease of radioactive peptide bound to the polysomes. The radioactive peptide bound to polysomes was made in vitro as described in the text. The ribosome-bound radioactivity in the absence of puromycin was 2698 cpm reaction, thus measured, was significantly stimulated by EFG, indicating that some of the peptidyl-tRNAs were located at the A site while the other peptidyl tRNAs were already translocated to the D site. It is clear from this table that the EFG-dependent puromycin reaction which is an indication of the combined reaction of peptide bond formation and translocation was not influenced by bottromycin A_2.

The fact that the puromycin reaction with polysomes was insensitive to bottromycin A_2 was further supported by the experiment in fig.2. As reported earlier, the breakdown of polysomes takes place due to the release of ribosomes from mRNA in the presence of puromycin, EFG, GTP and ribosome-releasing (RR) factor [10,14–16]. This reaction was dependent on peptide bond formation between the nascent polypeptide and puromycin. It is clear from fig.2 that bottromycin A_2 did not prevent the loss of polysomes in this system. One can conclude from this experiment that bottromycin A_2 inhibits neither EFG action nor peptidyltransferase activity. A control experiment showed that bottromycin A_2 alone did not break down polysomes into monosomes in the absence of puromycin. As an additional control, a peptide synthetase inhibitor, sparsomycin, effectively inhibited breakdown of the polysomes.

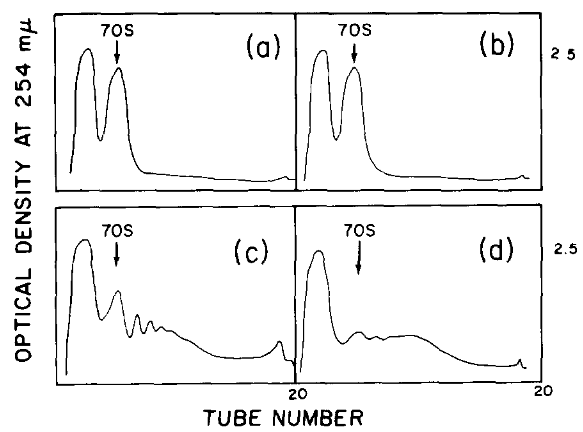

Fig.2. Lack of effect of bottromycin A_2 on puromycin-dependent polysome breakdown. The experimental conditions for polysome breakdown were essentially the same as those in [10]. The reaction mixture (0.25 ml) contained 10 mM Tris–HCl (pH 7.4), 8.2 mM $MgSO_4$, 50 mM NH_4Cl, 8.5 mM $(NH_4)_2SO_4$, and 6 mM β-mercaptoethanol. In addition, it contained 3.2 mM phosphoenolpyruvate, 0.16 mM GTP, 16 mg of pyruvate kinase, 0.05 mM puromycin, 3.3 A_{260} nunits of polysomes, 98.7 μg of S-150 and the antibiotics where indicated. The mixture was incubated at 37°C for 10 min, cooled to 0°C and layered onto a 5 ml linear sucrose gradient (15–30%) in a buffer containing 10 mM Tris–HCl (pH 7.4), 10 mM Mg-acetate, 50 mM NH_4Cl and 6 mM β-mercaptoethanol. After centrifugation at 38000 rev./min for 60 min in a Beckman SW50.1 rotor at 4°C, the distribution of the ultraviolet-absorbing materials in the gradient was monitored with an ISCO gradient analyzer at 254 nm: (a) no addition; (b) 10^{-4} M bottromycin A_2; (c) 10^{-4} M sparsomycin; (d) no antibiotic and no incubation. Sedimentation was from left to right. The arrows represent the position of 70 S ribosomes.

3.4. *Inhibitory effect of bottromycin on the binding of aminoacyl-tRNA to polyribosomes*

The data in section 3.3 eliminate the possibility of all but one step in the peptide elongation reaction being the target of bottromycin A_2; i.e., the binding of aminoacyl-tRNA to polysomes. This possibility was directly supported by the experiment indicated in table 2. In this experiment, puromycin-treated polysomes were mixed with [^{14}C]aminoacyl-tRNA in the presence of EFTu but in the absence of EFG. This table shows that bottromycin A_2 inhibits binding of [^{14}C]aminoacyl-tRNA to the polysomes. It should be noted from this table that a significant effect of bottromycin A_2 could be observed after 15 min incubation but not after 5 min incubation. A similar effect of bottromycin was observed with binding of phenylalanyl-tRNA to the complex of poly(U) and ribosomes [5]. This is consistent with our original proposal that bottromycin A_2 releases aminoacyl-tRNA rather than inhibits the binding itself.

Table 2

Effect of bottromycin A_2 on the binding of [^{14}C]aminoacyl-tRNAs to polysomes

Bottromycin A_2 (10^{-4} M)	[^{14}C]Aminoacyl-tRNA bound (cpm)	
	5 min	15 min
–	974	1337
+	837	524

The experimental conditions are described in the text

3.5. *Effect of bottromycin A₂ on the run-off of ribosomes from polysomes*

In the experiment shown in fig.3 polysomes were isolated from growing *E. coli*, and protein synthetic activity as well as sedimentation behavior of the polysomes were followed in the presence and absence of bottromycin A₂. It is clear from this figure that in the absence of antibiotics, protein synthesis takes place, accompanied by the disappearance of the polysomes as a result of run-off of ribosomes. When bottromycin A₂, tetracycline, or sparsomycin was added, protein synthesis was inhibited. In addition, as a result of inhibition of protein synthesis by the antibiotics, more polysomes remained intact because fewer ribosomes ran off from the polysomes.

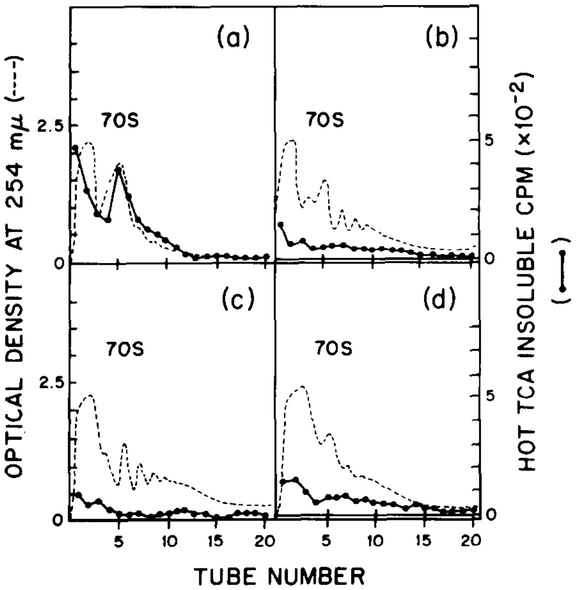

Fig.3. Bottromycin A₂ inhibits run-off of ribosomes from polysomes. The experimental conditions for protein synthesis by polyribosomes was essentially the same as that described in the text. The mixture (0.25 ml) contained, in addition to 2.2 A_{260} nunits of polysomes and other components, 210 μg tRNA containing 2.1 × 10^4 cpm [^{14}C]valyl-tRNA and 19 [^{12}C]aminoacyl tRNAs and 435 μg *E. coli* soluble proteins. After incubating for 10 min at 37°C, the analysis of polysomal distribution was performed as in [10]. Hot trichloroacetic acid-insoluble radioactivity of each fraction was measured by the filter disc method: (a) no antibiotic; (b) 10^{-4} M bottromycin A₂; (c) 10^{-4} M sparsomycin; (d) 5 × 10^{-4} M tetracycline. Sedimentation was from left to right.

4. DISCUSSION

From the preceding work on the effect of bottromycin A₂ on in vitro polypeptide synthesis using synthetic polynucleotides, it was postulated that bottromycin A₂ interferes with the interaction of oligo-peptidyl- or aminoacyl-tRNA with the A site (acceptor site) of ribosomes [5,6]. This causes either release of oligo-peptidyl- and aminoacyl-tRNA or inhibition of aa-tRNA binding. We extended our study to polysomes and examined whether or not the above hypothesis could also be applied to the action of bottromycin A₂ on naturally-occurring polysomes. As in the system with synthetic-polynucleotides, bottromycin A₂ exerted a strong inhibitory effect on protein synthesis by isolated polysomes. However, polysomes with nascent peptides were less sensitive than those without. Thus, nascent peptidyl groups attached to polysomes appear to retard bottromycin A₂'s action. It should be pointed out that polysomes without nascent peptides would ordinarily be very unstable in cell extracts because of the presence of ribosome-releasing factor [10,14–16]. However, we have developed a method which eliminates RR factor from the extract leaving all other factors for protein synthesis [15]. In this RR factor-free extract, polysomes without nascent chains are stable. We therefore tested the effect of bottromycin A₂ on such polysomes and found that the inhibition of protein synthesis was almost complete.

We examined the effect of bottromycin A₂ on all three steps involved in chain elongation; i.e., binding of aminoacyl-tRNA, peptide bond formation, and translocation. It was found that, in accordance with our previous results with systems involving synthetic polynucleotides, bottromycin A₂ did not inhibit translocation or peptide bond formation by polyribosomes. On the other hand, the aminoacyl-tRNA binding to the polyribosomes was inhibited. It should be noted that, in contrast to the system using synthetic polynucleotides, bottromycin A₂ did not release bound peptidyl-tRNA from polysomes. This is understandable in view of the fact that oligopeptidyl- and aminoacyl-tRNA, but not polypeptidyl-tRNA, were released from complexes of ribosomes and synthetic polynucleotides [5]. Bottromycin A₂ may not release peptidyl-tRNA from polysomes because most of the nascent peptides attached to them are relatively large. In fact, large nascent peptidyl-tRNA binds tightly to the 50 S ribosomal subunit and is not released even at 10^{-3} M Mg^{2+} [17].

From the results of our preceding communications and these data we conclude that a similar mode of action of bottromycin A_2 applies to both the polysome system and the synthetic polynucleotide system. This antibiotic inhibits protein synthesis by interfering with the correct positioning of aminoacyl-tRNA in the A-site. This would explain the release of aminoacyl-tRNA reported here and in [5] since the distorted binding of this ligand would probably make it less strongly held in the A-site. Since erythromycin releases oligopeptidyl-tRNA from ribosomes [8] and polysomes [18], the antibiotics are analogous in this respect. However, one striking difference is that bottromycin A_2 releases aminoacyl-tRNA from the A site of ribosomes while erythromycin releases oligopeptidyl-tRNA from a site *near* the D (donor) (peptidyl) site [19]. In this respect, bottromycin A_2 is similar to tetracycline [20–22] because both antibiotics act at the A (acceptor) site. On the other hand, bottromycin A_2 is different from tetracycline in that it releases bound aminoacyl-tRNA, while tetracycline does not [20]. Yet another antibiotic, micrococcin, a translocation inhibitor [4], is similar to bottromycin A_2 in that it acts at the A site of ribosomes. However, bottromycin A_2 differs from micrococcin because micrococcin inhibits, like tetracycline, binding of aminoacyl-tRNA [9] and does not release aminoacyl-tRNA [5] once bound. In addition, bottromycin A_2 does not inhibit translocation while micrococcin does [4,9]. These considerations suggest that an antibiotic which appears to have multiple effects on various steps of protein synthesis, such as bottromycin A_2, erythromycin [8,18,23,24], fusidic acid [25–30] or micrococcin [9], may actually exert its effects through a single action: binding to a particular site of ribosomes. Determination of the exact site of the binding of these antibiotics by extending already available information [31,32] would therefore shed light on the mode of ribosome function.

ACKNOWLEDGEMENT

This work was supported by US PHS grant GM 12053.

REFERENCES

[1] Tanaka, H., Sashikata, K., Yamaguchi, H. and Umezawa, H. (1966) J. Biochem. (Tokyo) 60, 405–410.

[2] Lin, Y.C. and Tanaka, N. (1968) J. Biochem. (Tokyo) 63, 1–7.

[3] Pestka, S. (1970) Arch. Biochem. Biophys. 136, 89–96.

[4] Pestka, S. and Brot, N. (1971) J. Biol. Chem. 246, 7715–7722.

[5] Otaka, T. and Kaji, A. (1976) J. Biol. Chem. 251, 2299–2306.

[6] Otaka, T. and Kaji, A. (1981) FEBS Lett. 123, 173–176.

[7] Pestka, S. (1972) J. Biol. Chem. 247, 4669–4678.

[8] Otaka, T. and Kaji, A. (1975) Proc. Natl. Acad. Sci. USA 72, 2649–2652.

[9] Otaka, T. and Kaji, A. (1974) Eur. J. Biochem. 50, 101–106.

[10] Hirashima, A. and Kaji, A. (1973) J. Biol. Chem. 248, 7580–7587.

[11] Mans, R.J. and Novelli, G.D. (1961) Arch. Biochem. Biophys. 94, 48–53.

[12] Weissbach, H., Redfield, B. and Brot, N. (1971) Arch. Biochem. Biophys. 145, 676–684.

[13] Pestka, S. (1974) Antimicrob. Agents Chemother. 5, 255–267.

[14] Ryoji, M., Karpen, J.W. and Kaji, A. (1981) J. Biol. Chem. 256, 5798–5801.

[15] Ryoji, M., Berland, R. and Kaji, A. (1981) Proc. Natl. Acad. Sci. USA 78, 5973–5977.

[16] Kung, H., Treadwell, B.V., Spears, C., Tai, P. and Weissbach, H. (1977) Proc. Natl. Acad. Sci. USA 74, 3217–3221.

[17] Monro, R. (1969) Nature 223, 903.

[18] Otaka, T. and Kaji, A. (1982) Arch. Biochem. Biophys. 214, 846–849.

[19] Tanaka, S., Otaka, T. and Kaji, A. (1973) Biochim. Biophys. Acta 331, 128–140.

[20] Kaji, A. and Ryoji, M. (1979) Antibiotics VI: Mechanism of action of antibacterial agents (Hahn, F.E. ed) pp.304–328, Springer Verlag, Berlin, Heidelberg.

[21] Sarkar, S. and Thach, R.E. (1968) Proc. Natl. Acad. Sci. USA 60, 1479–1486.

[22] Suarez, G. and Nathans, D. (1965) Biochem. Biophys. Res. Commun. 18, 743–750.

[23] Mao, J.C.H. and Robishaw, E.E. (1972) Biochemistry 11, 4864–4872.

[24] Tanaka, K. and Teraoka, H. (1968) J. Biochem. (Tokyo) 64, 635–648.

[25] Otaka, T. and Kaji, A. (1973) Eur. J. Biochem. 38, 46–53.

[26] Richman, N. and Bodley, J.W. (1971) Proc. Natl. Acad. Sci. USA 69, 686–689.

[27] Cabrer, B., Vazquez, D. and Modolell, J. (1972) Proc. Natl. Acad. Sci. USA 69, 733–736.

[28] Miller, D.L. (1972) Proc. Natl. Acad. Sci. USA 69, 752–755.

[29] Richter, D. (1972) Biochem. Biophys. Res. Commun. 46, 1850–1856.

[30] Modolell, J. and Vazquez, D. (1973) J. Biol. Chem. 248, 488–493.

[31] Langlois, R., Lee, C.C., Cantor, C.R., Vince, R. and Pestka, S. (1976) J. Mol. Biol. 106, 297–313.

[32] Langlois, R., Cantor, C.R., Vince, R. and Pestka, S. (1977) Biochemistry 16, 2349–2356.