MODE OF ACTION OF SIOMYCIN

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The incorporation of H^3-lysine into *Bacillus subtilis* cells ceased almost immediately after the addition of siomycin, while the continued incorporation of C^{14}-uridine and of C^{14}-thymidine into the trichloroacetic acid-insoluble fraction of the cells was observed for at least a further 20 minutes. In bacterial cell free systems (*Bacillus subtilis* and *Escherichia coli*) siomycin inhibits polyadenylic acid directed polylysine synthesis and polyuridylic acid-directed polyphenylalanine synthesis. However, aminoacyl-transfer RNA synthesis was hardly affected. The "soluble siomycin", monothiomalic acid-siomycin A, was found to have an inhibitory action on bacterial protein biosynthesis, similar to and to roughly the same extent as siomycin A. In contrast to bacterial protein synthesis, C^{14}-leucine incorporation into rabbit reticulocyte hemoglobin was not significantly decreased by the addition of siomycin. Protein biosynthesis in a cell-free system from rabbit reticulocytes was also found to be highly resistant to this antibiotic. These results are in harmony with the previous observation that siomycin showed little toxicity to mammals.

Siomycin was isolated from *Streptomyces sioyaensis* and was found to be a sulfur containing peptide antibiotic by NISHIMURA *et al.*[1] Recent study by EBATA *et al.*[2] indicated that the usual crude siomycin preparation contained three components (98 % of A, 1.2~2 % of B, and 0.5~1 % of C). Precise study of the biological activity of this antibiotic has been hindered owing to its slight solubility in aqueous solution. The situation, however, seemed to be much improved by the recent invention of water soluble siomycin derivatives, for example monothiomalic acid-siomycin A, by EBATA *et al.*[3]

In this study, the effect of siomycin A and a soluble siomycin (monothiomalic acid-siomycin A) on the syntheses of macromolecules in *Bacillus subtilis* cells was investigated. The results indicated that siomycin preferentially inhibits polypeptide synthesis as compared with the biosyntheses of RNA and DNA. This inhibitory effect on protein synthesis was also confirmed by the use of cell-free systems from *Escherichia coli* and *B. subtilis*. On the other hand, this antibiotic hardly showed any inhibitory effect on hemoglobin synthesis in rabbit reticulocytes or on the protein synthesis in the cell-free system from the reticulocytes.

Abbreviations : poly A, polyadenylic acid; poly U, polyuridylic acid.

Methods and Materials

Incorporation of H^3-lysine, C^{14}-uridine and C^{14}-thymidine into *B. subtilis* cells :

B. subtilis (PCI 219) was cultivated in the synthetic medium containing 10 g glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 2.5 g NaCl, 1 g $(NH_4)_2SO_4$, 2 mg $FeSO_4 \cdot 7H_2O$, 2 mg $MnCl_2 \cdot 4H_2O$, and 1 g tryptone per liter. To 100 ml of the fresh medium, 4 ml of an overnight culture was inoculated, then incubation with shaking at 37°C was continued until exponential growth was observed. Then the cell culture was diluted with fresh medium to give an absorbancy at 660 mμ of 0.1. The effect of siomycin on the incorporations of H^3-lysine and C^{14}-uridine or C^{14}-thymidine and H^3-lysine was examined by the use of the cell suspension under the conditions stated in each legend to the figures. For the measurement of the incorporation of these radioactive precursors, 1 ml aliquots of the culture medium were withdrawn and added to 1 ml of cold 10 % TCA (trichloroacetic acid) and kept in an ice bath for 30 minutes. The precipitate formed was collected on a Millipore filter (HA 0.45 μ, 23 mm in diameter), washed with 3×3 ml of 5 % TCA, dried, and then counted in a liquid scintillation spectrometer with conventional toluene scintillation mixture.

Protein synthesis in bacterial cell-free system :

Ribosomes and 105,000×g supernatant fraction were prepared as described by NIRENBERG[4]. The preparations of tRNA were obtained according to VON EHRENSTEIN and LIPMANN[5] and purified by column chromatography on DEAE-cellulose[6]. The incubation mixture for peptide synthesis contained the following components in a final volume of 125 μl (μmoles unless otherwise specified) : 6.25 Tris-HCl (pH 7.8), 2.0 magnesium acetate, 8.75 KCl, 1.25 2-mercaptoethanol, 0.375 ATP, 0.0375 GTP, 1.25 phosphoenol pyruvate, 3.75 μg pyruvate kinase, 50 μg poly A (or poly U), 0.0025 (100 μC/μmole) C^{14}-lysine (or phenylalanine), ribosomes, 105,000×g supernatant fraction and tRNA. After incubation at 37°C for 15 minutes, reaction tubes were dipped into an ice bath and 2.5 μmoles of C^{12}-amino acid corresponding to the labelled amino acid (25 μl) was added. Aliquots of 10~30 μl of the mixture were applied on paper discs and incorporation of C^{14}-amino acid into protein was measured according to MANS-NOVELLI[7]. Owing to the high solubility of the lysine peptide in TCA solution the supernatant of a 1 : 100 mixture of 25 % aqueous sodium tungstate solution and 5 % TCA was employed for the assay of C^{14}-lysine incorporation in place of 5 % TCA used in other systems[8]. The products of polylysine synthesis were analyzed by paper chromatography as described previously[9].

Synthesis of aminoacyl tRNA :

Incubation mixture was the same as that of protein synthesis except that mRNA (poly A or poly U) and ribosomes were omitted. After incubation at 37°C for 15 minutes, 25 μl of the cold amino acid (0.1 M) correspond to the tracer amino acid was added, then 40 μl of the mixture was applied on a paper disc and incorporation of C^{14}-amino acid into cold TCA precipitate was determined by the disc method of MANS-NOVELLI[7] (treatment with hot TCA was omitted). In the case of aminoacyl-tRNA synthesis from reconstituted C^{14}-protein hydrolyzate, 5 μC of the hydrolyzate (Schwarz) was employed in 125 μl of incubation mixture instead of 2.5 μC of C^{14}-lysine or C^{14}-phenylalanine in the synthesis of aminoacyl tRNA of these amino acids, and the mixture of C^{12}-amino acids (0.005 M for each amino acid) was used to dilute out the radioactive amino acids after the incubation.

Incorporation of C^{14}-leucine into rabbit reticulocyte cells :

Reticulocytes were induced in a male rabbit, weighing about 2.8 kg by four daily subcutaneous injections of 2.5 % neutralized phenylhydrazine solution (1 ml/rabbit). Blood was obtained by heart puncture on the 5 th day. The heperinized blood was cooled to 4°C and centrifuged for 10 minutes at 900×g, and the plasma was discarded. The packed cells were washed twice with 5 volume modified GODCHAUX-HERBERT's medium[10], which contained 0.011 M glucose, 10^{-3} M NaH_2PO_4, 0.118 M NaCl, 4×10^{-3} M KCl, 10^{-3} M $CaCl_2$, 1.5×10^{-2} M

$MgCl_2$, 0.026 M $NaHCO_3$, 1.8×10^{-4} M $Fe(NH_4)_2(SO)_2$, 50 units/ml penicillin G and 20 % (v/v) rabbit serum. Incubation medium for C^{14}-leucine incorporation was prepared by the omission of $Fe(NH_4)_2(SO_4)_2$ and rabbit serum and by the addition of C^{14}-leucine (0.24 μC, 160 μC/μmole) and L-amino acids other than leucine in the proportions described by BORSOOK *et al.*[11] The incorporation of C^{14}-leucine was carried out with approximately 7.5×10^8 cells/ml at 37°C for 30 minutes. After the incubation period, the reaction tubes were dipped into an ice bath and added each 0.6 ml of cold saline solution, containing 0.01 M C^{12}-leucine, 0.13 M NaCl, 0.0052 M KCl and 0.0075 M $MgCl_2$. The cells were separated by centrifugation and washed with the same saline solution; 300 μl of cold 0.005 M $MgCl_2$ solution were added to the washed cells which were then homogenized and kept in an ice bath for 15 minutes. The cell-lyzate was centrifuged and radioactivity in the hot TCA-insoluble fraction was assayed in an aliquot of the supernatant by the disc method according to MANS and NOVELLI[7]. The results were expressed in the term of cpm per 100 μg hemoglobin which was calculated from the absorbancies at 540 mμ assuming absorbancy 0.885 for 0.1 % solution of hemoglobin[12].

Protein synthesis in reticulocyte cell-free system:

Ribosomes and the soluble enzyme fraction (AS_{70}) were prepared according to ALLEN and SCHWEET[13]. Rat liver tRNA was prepared by the method of ROSENBAUM and BROWN[14] and purified by DEAE-cellulose column chromatography[15]. The incubation mixture contained the following components in a final volume of 70 μl (μmoles unless otherwise specified): 12 μg rat liver tRNA, 5 μg pyruvate kinase, 0.05 ATP, 0.5 phosphoenol pyruvate, 0.0125 GTP, 2.5 KCl, 2.5 Tris-HCl (pH 7.5), 5 μl amino acid mixture, 0.375 $MgCl_2$, 0.625 mμmole C^{14}-leucine (100 μC/μmole), ribosomes (84 μg protein) and AS_{70} enzyme (252 μg protein). The amino acid mixture (except leucine) was a two-fold diluted solution of that described by BORSOOK *et al.*[11] The mixture was incubated at 37°C for 60 minutes, then 10 μl of 0.1 M unlabelled leucine was added. An aliquot (20 μl) of the reaction mixture was applied on a filter disc and incorporation of C^{14}-leucine into hot TCA-insoluble materials was assayed according to MANS and NOVELLI[7].

Materials:

H^3-Lysine, C^{14}-uridine, C^{14}-thymidine, C^{14}-leucine, C^{14}-lysine and C^{14}-phenylalanine were obtained from Schwarz Biological Research Inc. Orangeburg, N. Y. (U. S. A.). Poly A (K salt) and GTP (Na salt) were obtained from Sigma Co., Poly U (K salt) was obtained from Calbiochem, Los Angeles, Calif., (U. S. A.). Siomycin and monothiomalic acid-siomycin were kindly supplied by Dr. M. EBATA and his collaborators in this laboratory.

Results and Discussion

(1) Effect of siomycin on the incorporations of C^{14}-uridine, H^3-lysine and C^{14}-thymidine into cold TCA-insoluble materials in *Bacillus subtilis* cells

As shown in Figs. 1~3, the incorporation of H^3-lysine into *B. subtilis* cells was almost completely inhibited immediately after the addition of siomycin A or its soluble derivative (monothiomalic acid-siomycin A).

The incorporation of C^{14}-thymidine and that of C^{14}-uridine were found to continue for at least 20 minutes after cessation of the incorporation of ^{3}H-lysine. Hence in susceptible bacteria, protein biosynthesis is preferentially inhibited, as compared with that of RNA or DNA, by monothiomalic acid-siomycin A as well as by siomycin A.

(2) Effect of siomycin on peptide synthesis in cell-free extracts from bacterial cells

As shown in Tables 1, 2, and 3, the soluble siomycin, as well as siomycin A, strongly inhibited the poly U-directed polyphenylalanine synthesis and poly A-directed

Fig. 1. Effect of siomycin A on the incorporations of C^{14}-uridine and of H^3-lysine.

The *B. subtilis* (PCI 219) cells harvested at their exponential growth phase were resuspended to give turbidity 0.1 at 660 mμ in 12.5 ml of the fresh medium containing C^{14}-uridine (0.5 μC, 1.02 μmoles) and H^3-lysine (25 μC, 0.12 μmole), then incubated at 37℃. To one half on the incubation medium (5 ml), 50 μl of dioxan solution of siomycin A (202 μg/ml) was added 10 minutes after the start of the incubation. At the indicated intervals the incorporations of the radioactive precursors were determined as described in "Methods and Materials".
●, C^{14}-Uridine; ▲, C^{14}-uridine+siomycin A;
○, H^3-lysine; △, H^3-lysine+siomycin A.

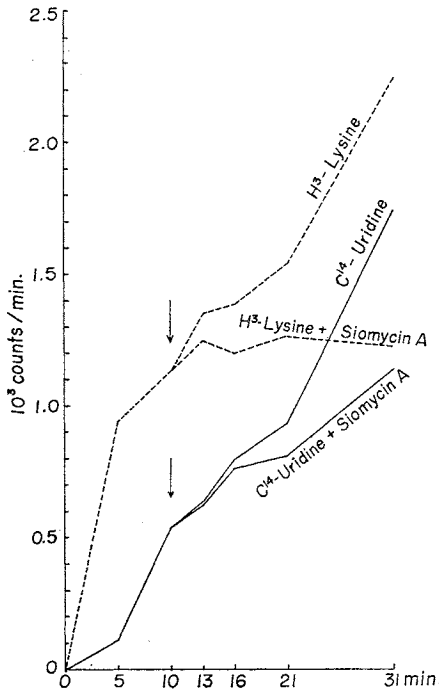
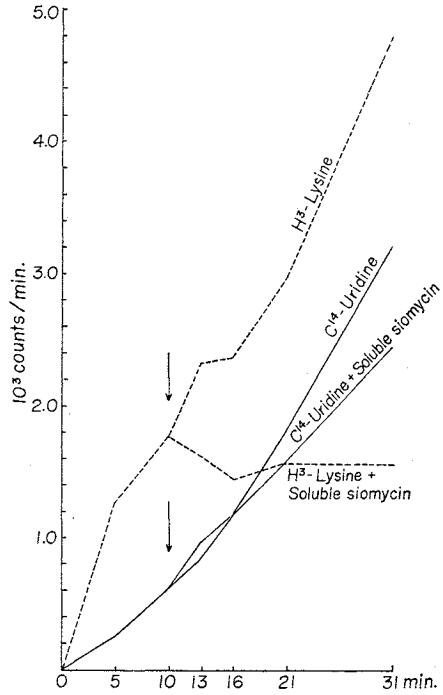


Fig. 2. Effect of "soluble siomycin" on the incorporation of C^{14}-uridine and of H^3-lysine.

Experimental conditions were the same as described in the legend of Fig. 1 except that the water solution of monothiomalic acid-siomycin A, in place of siomycin A, was added to give a final concentration of 8.5 μg/ml.
●, C^{14}-Uridine; ▲, C^{14}-uridine+soluble siomycin;
○, H^3-lysine; △, H^3-lysine+soluble siomycin.



polylysine synthesis in the cell-free system from *E. coli* and *B. subtilis*.

Paper chromatographic analysis of the reaction product of polylysine synthesis demonstrated that no significant amount of small lysine peptide soluble in TCA-tungstate reagent was synthesized in the presence of an excess of siomycin.

(3) Effect of siomycin on aminoacyl tRNA synthesis

As shown in Table 4, aminoacyl tRNA synthesis was found to be very tesistant to this antibiotic.

Table 1. Effect of siomycin A on peptide synthesis in *Escherichia coli* cell-free system

Incubation mixture (125 μl) contained 434 μg (protein, *ca.* 13.6 O.D.$_{260}$ units) of *E. coli* K-12 ribosomes, 192 μg (protein) of 105,000×*g* supernatant of *E. coli* K-12, and 2.8 O.D.$_{260}$ units of tRNA from *E. coli* K-12 in addition to the components described under "Methods and Materials". After the incubation, 25 μl of the C^{12}-amino acid (0.1 M) corresponding to the used C^{14}-amino acids used was added and then an aliquot (10 μl) of the mixture was applied on a disc for the assay.

Siomycin A added	C^{14}-Lysine incorporation directed by Poly A		C^{14}-Phenylalanine incorporation directed by Poly U	
μM	cpm/disc	(%)	cpm/disc	(%)
0	4,675	(100)	12,407	(100)
4.6	2,547	(54.5)	8,958	(60)
0	4,498	(100)		
11.5	227	(5.1)		

From these results it may be concluded that, in the presence of the siomycin, aminoacyl tRNA synthesis is not inhibited but a later step of the protein synthesis of the bacterial system is blocked.

(4) Effect of siomycin on hemoglobin synthesis in reticulocytes

As shown in Table 5, siomycin showed no inhibitory effect on the hemoglobin synthesis in reticulocytes cells in contrast to the protein synthesis in bacterial cells.

(5) Effect of siomycin on the hemoglobin synthesis in cell-free system

Effect of this antibiotic on the hemoglobin synthesis was also tested in a cell-free system. As shown in Table 6, no appreciable effect of the antibiotic was observed at a concentration where puromycin showed a strong inhibitory effect.

These findings demonstrated that the protein synthesis of mammalian system, represented by hemoglobin synthesis, was hardly effected by this antibiotic.

Fig. 3. Effect of "soluble siomycin" on the incorporations of C^{14}-thymidine and of H^3-lysine.

Experimental conditions were the same as described in the legend to Fig. 2 except that C^{14}-thymidine (5 μC, 0.207 μmole), H^3-lysine (20 μC, 0.095 μmole) and deoxyadenosine 2.5 mg were added in place of the radioactive precursors used in the experiment cited in Fig. 2.
●, C^{14}-Thymidine; ▲, C^{14}-thymidine+soluble siomycin; ○, H^3-lysine; △, H^3-lysine+soluble siomycin.

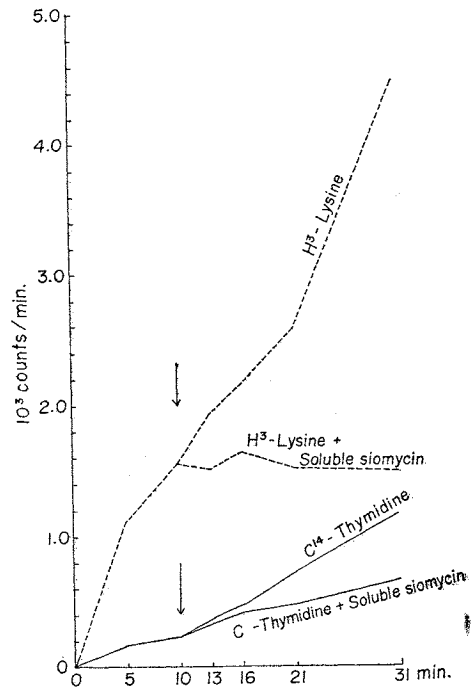


Table 2. Effect of "soluble siomycin" on peptide synthesis in *Escherichia coli* cell-free system

Incubation mixture(125 μl)contained 6.0 O.D.$_{260}$ units of preincubated *E. coli* Q 13 ribosomes, 196 μg (protein) of preincubated *E. coli* Q13 105,000×*g* supernatant fraction, and 3.0 O.D.$_{260}$ units of *E. coli* Q 13 tRNA in addition to the components described under "Methods and Materials". After the incubation, 25 μl of the C^{12}-amino acid (0.1 M) corresponding to the C^{14}-amino acid used was added, and then an aliquot (30 μl) of the mixture was applied on a disc for the assay.

"Soluble siomycin" added μM	Poly A-directed C^{14}-lysine incorporation		Poly U-directed C^{14}-phenylalanine incorporation	
	cpm/disc	(%)	cpm/disc	(%)
0	14,216	(100)	40,666	(100)
5	13,568	(95.4)	32,272	(79.4)
10	12,028	(84.5)	14,816	(36.5)
20	1,311	(9.2)	640	(1.6)
40	1,116	(7.8)	481	(1.2)

Table 3. Effect of "soluble siomycin" on peptide synthesis in *Bacillus subtilis* cell-free system

Incubation mixture(125 μl)contained 9.1 O.D.$_{260}$ units of preincubated *B. subtilis* ribosomes, 136 μg (protein) of *B. subtilis* preincubated 105,000×*g* supernatant fraction and 2.8 O.D.$_{260}$ units of *B. subtilis* tRNA in addition to the components described under "Methods and Materials". After the incubation, 25 μl of nonlabelled amino acid (0.1 M) corresponding to the C^{14}-amino acid used was added, and then 30 μl of the mixture was applied for the assay.

"Soluble siomycin" added μM	Poly A-directed C^{14}-lysine incorporation		Poly U-directed C^{14}-phenylalanine incorporation	
	cpm/disc	(%)	cpm/disc	(%)
0	3,454	(100)	1,710	(100)
2.1	2,758	(80)	1,378	(81)
10.4	680	(19.6)	157	(9.2)
52	224	(6.4)	113	(6.6)
260	249	(7.2)	110	(6.4)

Table 4. Effect of "soluble siomycin" of the synthesis of aminoacyl tRNA

Incubation mixture contained 196 μg (protein) of *E. coli* Q13 105,000×g supernatant, 3 0 O D $_{260}$ units of tRNA from *E. coli* Q13, in addition to the components described under "Methods and Materials"

"Soluble siomycin" added μM	Aminoacyl-tRNA synthesis from					
	Reconstituted C^{14}-protein hydrolyzate		C^{14}-Phenylalanine		C^{14}-Lysine	
	cpm/disc	(%)	cpm/disc	(%)	cpm/disc	(%)
0	48,608	(100)	5,999	(100)	4,687	(100)
8	47,316	(97)	6,187	(103)	4,394	(94)
40	47,297	(97)	6,074	(101)	4,372	(93)

Table 5. Effect of siomycin on synthesis of hemoglobin (Hb) in rabbit reticulocytes

Antibiotics added mM	C^{14}-Leucine incorporation into hemoglobin fraction in the presence of					
	"Soluble siomycin"				Cycloheximide	
	Exp. I		Exp. II			
	cpm/100 μg	Hb (%)	cpm/100 μg	Hb (%)	cpm/100 μg	Hb (%)
0	1,896	(100)	1,019	(100)		
7.14×10^{-5}	1,986	(105)	935	(91.8)	983	(96.5)
7.14×10^{-4}	1,584	(83.5)	964	(94.6)	407	(39.9)
7.14×10^{-3}	1,913	(101)	724	(71.1)	156	(15.3)
7.14×10^{-2}	1,855	(97.8)	1,103	(108)	195	(19.1)
7.14×10^{-1}	1,697	(89.5)	1,001	(98.2)	72	(7.1)

The results presented here are in harmony with the fact that siomycin is highly active against gram positive bacteria and mycobacteria but exhibits very low toxicity to mammals[1]. Peptide synthesis in the cell-free system from gram negative *E. coli* was also inhibited. at a concentration where inhibition of the growth of the cells was hardly observed. Hence the difference in susceptibility of gram negative and gram positive bacteria to siomycin may be due to a difference in factor(s) other than those contained in the protein-synthesizing system.

Table 6. Effect of siomycin on protein synthesis in the cell-free system from rabbit reticulocytes

Antibiotics added mM	C^{14}-Leucine incorporation in the presence of			
	"Soluble siomycin"		Puromycin	
	cpm/disc	(%)	cpm/disc	(%)
0	2,393	(100)		
7.14×10^{-5}	2,552	(107)	2,367	(98.9)
7.14×10^{-4}	2,265	(94.7)	1,199	(91.9)
7.14×10^{-3}	2,537	(106)	984	(41.1)
7.14×10^{-2}	2,422	(101)	142	(5.9)

References

1) Nishimura, H.; S. Okamoto, M. Mayama, H. Ohtsuka, K. Nakajima, K. Tawara, M. Shimohira & N. Shimaoka : Siomycin, a new thiostrepton-like antibiotic. J. Antibiotics, Ser. A 14 : 255 ~263, 1961

2) Ebata, M.; K. Miyazaki & H. Otsuka : Studies on siomycin. I. Physicochemical properties of siomycin A, B and C. J. Antibiotics 22 : 364~368, 1969

3) Ebata, M ; K. Miyazaki & H. Otsuka : Studies on siomycin. V. Derivatives of siomycin A prepared with thiolcarboxylic acid. J. Antibiotics 22 : 451~456, 1969

4) Nirenberg, M. W. : Cell free protein synthesis directed by messenger RNA. "Method in Enzymology" ed. by S. P. Colowick & N. O. Kaplan, Academic Press Inc., New York, vol. VI, pp. 17~23, 1963

5) VON EHRENSTEIN, G. & F. LIPMANN : Experiments on hemoglobin biosynthesis. Proc. Natl. Acad. Sci., U. S. A. 47 : 941～950, 1961
6) HOLLEY, R. W.; J. APGAR, B. P. DOCTOR, J. FARROW, M. A. MARINI & S. H. MERRILL : A simplified procedure for the preparation of tyrosine- and valine- acceptor fractions of yeast "soluble ribonucleic acid". J. Biol. Chem. 236 : 200～202, 1961
7) MANS, R. J. & G. D. NOVELLI : Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. Arch. Biochem. Biophys. 94 : 48～53, 1961
8) GRIFFIN, A. C.; V. WARD, L. C. CANNING & B. H. HOLLAND : Effect of precipitating reagent upon amino acid incorporation by *in vitro* mammalian systems. Biochem. Biophys. Res. Commun. 15 : 519～524, 1964
9) TANAKA, K. & H. TERAOKA : Effect of erythromycin on polylysine synthesis directed by polyadenylic acid in an *Escherichia coli* cell-free system. J. Biochem. 64 : 635～648, 1968
10) GODCHAUX, W. & E. HERBERT : The effect of chloramphenicol in intact erythroid cells. J. Mol. Biol. 21 : 537～553, 1966
11) BORSOOK, H.; E. H. FISCHER & G. KEIGHLEY : Factors affecting protein synthesis *in vitro* in rabbit reticulocytes. J. Biol. Chem. 229 : 1059～1070, 1957
12) GROLLMAN, A. P. : Inhibitors of protein biosynthesis. II. Mode of action of anisomycin. J. Biol. Chem. 242 : 3226～3233, 1967
13) ALLEN, E. H. & R. S. SHWEET : Synthesis of hemoglobin in a cell-free system. I. Properties of the complete system. J. Biol. Chem. 237 : 760～767, 1962
14) ROSENBAUM, M. & R. A. BROWN : Simplified preparation and properties of undegraded rat liver transfer ribonucleic acid. Anal. Biochem. 2 : 15～22, 1961
15) TAKANAMI, M. : Preparation of ribosome and sRNA. Tanpakushitsu Kakusan Koso 11 : 490～500, 1966 (in Japanese)