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ON THE MODE OF ACTION OF CLADOSPORIN*

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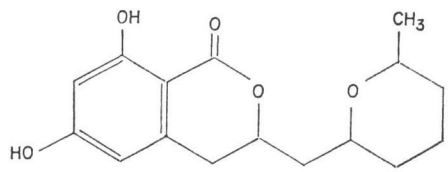
Cladosporin, a fungal isocoumarin derivative, strongly inhibits the uptake and thereby the incorporation of uracil and leucine into cells of *Bacillus brevis* and the incorporation of uridine but not leucine into cells of the ascitic form of EHRLICH carcinoma (ECA) of mice.

Normal uptake was not restored by removal of the antibiotic. In cells of *Escherichia coli* A 19-15 (met$^-$) the inhibition of methionine uptake is associated with the cessation of growth. In a methionine-prototrophic revertant from this organism, the uptake of methionine is still inhibited; growth, however, is hardly affected by cladosporin. *In vitro* no effect on the DNA-dependent RNA polymerase from *E. coli* and on the RNA polymerase II from wheat germ could be detected. The poly(U)-directed poly(Phe) synthesis was also not inhibited by cladosporin. It is concluded that cladosporin inhibits uptake processes which, for the case of essential nutrients, leads to loss of viability.

Cladosporin (3,4-dihydro-6,8-dihydroxy-3-(6-methyltetrahydropyran-2-ylmethyl)isocoumarin), Fig. 1, is a fungal antibiotic which shows a very selective spectrum of activity[2,3].

The growth of aerobic *Bacillus brevis* and anaerobic *Clostridium pasteurianum* is inhibited, whereas *B. subtilis* and Gram-negative bacteria are not affected. Some fungi are sensitive to cladosporin at concentrations a 100-fold higher than the inhibitory concentration for *B. brevis*.

Fig. 1. Cladosporin.



In *B. brevis* the antibiotic strongly inhibits the incorporation of leucine and uracil into the trichloroacetic acid (TCA)-insoluble fraction of cells without inhibiting the incorporation of thymidine[3]. Ochratoxin A, also a fungal isocoumarin derivative, is known to interfere with RNA and protein syntheses in *B. subtilis, B. stearothermophilus,* and *Streptococcus faecalis*[4,5]; this inhibition could be reversed by addition of cyclic AMP. The inhibition by cladosporin, however, was not affected by cyclic AMP, indicating a different mode of action for ochratoxin and cladosporin.

In this paper I wish to define further the effect of cladosporin on bacteria and on mammalian tumor cells.

Parts of the results have been presented at the Annual Meeting of the American Society for Microbiology in Las Vegas, May 14~18, 1978 and at the XIIth International Congress of Microbiology in Munich, September 3~8, 1978.

Materials and Methods

Bacteria and growth conditions

B. brevis ATCC 9999, was grown in nutrient broth (Difco) at 37°C. *E. coli* K 12, *E. coli* A 19,

* This paper is dedicated to Dr. FRITZ LIPMANN on occasion of his 80th birthday.

and *E. coli* A 19-15 were grown at 37°C in a medium containing (per liter): 3 g Lab-Lemco-powder (Oxoid), 5 g peptone (Merck), and 2.5 g NaCl; the pH was adjusted to 7.2. *E. coli* A 19 was isolated and described by GESTELAND[6]. *E. coli* A 19-15, obtained from M. JOKUSCH (Basel), is derived from *E. coli* A 19 (Hfr; met$^-$; λ^+) following selection for increased sensitivity to rifampicin. It is also more sensitive to streptomycin and kirromycin than strain A 19 (E. FISCHER, Tübingen, personal communication); *E. coli* A 19-15 is sensitive to high concentrations of cladosporin[3]. Growth was monitored by following the absorbance of the cultures at 578 nm (A_{578}) in an Eppendorf-photometer.

Assays

Mice bearing EHRLICH ascites carcinomas (ECA) were a gift from H. G. PROBST, Tübingen. Measurement of macromolecular syntheses in ECA cells was performed as described by WEITZEL[7]; nuclei from ECA cells were prepared as reported by PROBST[8].

Wheat germ DNA-dependent RNA polymerase II was obtained from Miles Labs and tested according to JENDRISAK and BURGESS[9]. DNA-dependent RNA polymerase from *E. coli* was kindly provided by W. ZILLIG; the assay is described by FUCHS *et al.*[10].

Incorporation and uptake

B. brevis was grown in nutrient broth to an A_{578} of 0.5. The antibiotics were added followed after 5 minutes by the radioactive precursors and incubation was continued for another 5 minutes. For uptake studies the cells were immediately collected by vacuum filtration through presoaked (saline containing 1 mM precursor) membrane filters (0.45 μm pore size; Sartorius) and washed twice with 5 ml saline and dried. Scintillator fluid (5 ml of toluene containing 4 g PPO per liter) was added and the radioactivity was determined in a Mark III (Nuclear Chicago) liquid scintillation counter. Under these conditions the uptake of uridine is linear for at least 9 minutes as shown by KUPKA *et al.*[11]. The uptake of uracil (1 μCi/0.1 μmol) and leucine (1 μCi/0.02 μmol) was linear for 20 minutes when measured under the conditions described, which are identical to the conditions used for the evaluation of the antibacterial activity of cladosporin. For incorporation studies the cells were suspended in an equal volume of cold 10% TCA containing unlabeled precursor at 1 mM. The TCA-insoluble material was collected on membrane filters and washed twice with 5 ml of cold 5% TCA and dried. The radioactivity retained on the filters was determined. *E. coli* strains were grown in Lab-Lemco-medium to an A_{578} of about 1.5 the cultures were diluted with prewarmed (37°C) synthetic medium[12] to an A_{578} of 0.4 and reincubated. At $A_{578}=0.5$ the antibiotic and 5 minutes later the precursors were added. After 10 minutes the cells were rapidly vacuum filtered through presoaked (0.1 M LiCl) membrane filters, rinsed twice with 5 ml of 0.1 M LiCl and dried. The radioactivity retained on the filters was determined. For uptake studies usually one control mixture was kept at 0°C.

Chemicals

The following radioactive compounds were purchased from Amersham Buchler: [8-^{14}C]ATP (48 Ci/mol); α-amino-[1-^{14}C]isobutyric acid (51.6 Ci/mol); 2-deoxy-D-[1-^{3}H]glucose (19 Ci/mmol); L-[U-^{14}C]leucine (59 Ci/mol); L-[methyl-^{3}H]methionine (11 Ci/mmol); [2-^{14}C]uracil (62 Ci/mol); [2-^{14}C]uridine (53.2 Ci/mol); [2-^{14}C]thymidine (61 Ci/mol). Unlabeled ribonucleoside triphosphates and calf thymus DNA were purchased from Boehringer and Soehne. Cladosporin was isolated from *Eurotium glabrum* and methylated with dimethyl sulfate as described before[3]. Rifampicin was obtained from Ciba-Geigy AG; all other chemicals were reagent grade from Merck.

Results and Discussion

Earlier *in vivo* experiments with *B. brevis* on the inhibition of incorporation of leucine, uracil, and thymidine into the 5% TCA-insoluble fraction of cells (protein, RNA, DNA) showed that the "uptake" of precursors into RNA and protein stopped immediately after the addition of 0.5 μg cladosporin per ml of culture. In fact, the incorporation of uracil was inhibited at significantly lower concentrations than the incorporation of leucine (Fig. 2).

When we tested the effect of cladosporin on the incorporation of radioactive precursors into cells

Fig. 2. Effect of cladosporin on the macromolecular syntheses in cells of *B. brevis*. *B. brevis* was grown to an A_{578} of 0.6; the indicated amounts of cladosporin were added and 5 minutes later the radioactive precursors. After 20 minutes of incubation the incorporation into TCA-precipitable material was determined. The incorporation was calculated in % of the control containing no antibiotic.

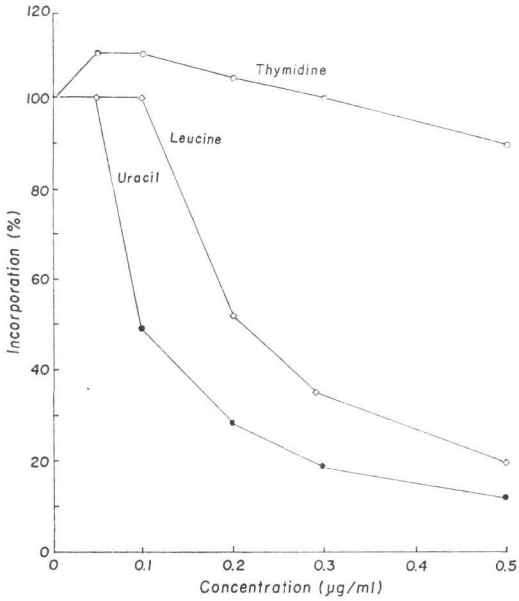


Fig. 3. Inhibition of the incorporation of uridine into TCA-precipitable material in ECA cells by cladosporin and dimethyl cladosporin. 3×10^6 cells were incubated with the antibiotics at 37°C in buffered saline containing heparin for 10 minutes. The radioactive uridine was added and the incubation continued for 20 minutes. The incorporation was calculated in % of the control containing no antibiotic.

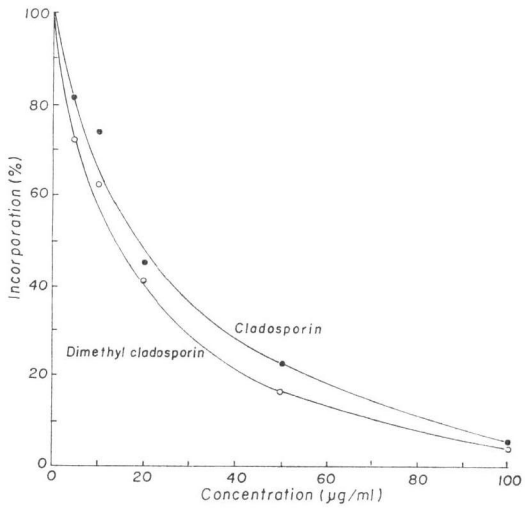


Table 1. Effect of cladosporin and dimethyl cladosporin on the incorporation of thymidine, uridine, and leucine into TCA-precipitable material in ECA cells.

Cells (3×10^6) were preincubated with the antibiotics in buffered saline[13] for 10 minutes. The radioactive precursors (0.1 μCi) were added and after 20 minutes the cells were centrifuged, the pellet was suspended in 5% TCA. The acid-insoluble material was collected on membrane filters and washed twice with 5 ml TCA. After drying 5 ml scintillator fluid were added to the filters and the radioactivity was determined.

Antibiotic added	μg/ml	Incorporation (pMol)		
		Thymidine	Uridine	Leucine
Control	0	100	360	65
Cladosporin	10	84	300	57
	100	88	32	55
Control	0	45	300	37
Dimethyl cladosporin	15	40	150	34
	100	41	31	34

of the ascitic form of EHRLICH carcinoma (ECA), we found that the incorporation of uridine was strongly inhibited whereas the incorporation of leucine and thymidine was hardly affected (Table 1). The concentration dependence of the inhibitory effect of cladosporin and dimethyl cladosporin on the incorporation of uridine into ECA cells is shown in Fig. 3. The dimethyl derivative is slightly more

active than cladosporin itself. The concentrations needed for 50% inhibition of uridine incorporation are 6×10^{-5} M for cladosporin and 4×10^{-5} M for dimethyl cladosporin. It is interesting that the dimethyl derivative of cladosporin showed the same inhibitory effect since when the hydroxyl groups are methylated, the antibiotic activity is drastically decreased: For example, no inhibition of fungi by dimethyl cladosporin was observed and the concentration required to inhibit growth of *B. brevis* was a 100-fold higher[3]. Because of these findings we first assumed that the incorporation of the appropriate precursor into RNA might be the target of the action of cladosporin in pro- and eukaryotes, and that the inhibition of the incorporation of leucine in *B. brevis* might be a secondary effect.

In vitro experiments with DNA-dependent RNA polymerase from *E. coli*, however, showed that cladosporin and dimethyl cladosporin had no effect on the enzyme activity (Table 2). No inhibition of the incorporation of UTP in isolated nuclei from ECA cells was observed (data not shown); and purified RNA polymerase II from wheat germ was also not inhibited by either compound. Furthermore, no effect was observed on the polypeptide elongation and $tRNA^{Phe}$-charging reactions in bacteria as measured by poly(U)-directed poly(Phe) synthesis (the author is grateful to H. WOLF, Tübingen, for conducting this experiment). Cladosporin has also no effect on the energy supply of the cells as indicated by the ongoing of DNA synthesis in *B. brevis* and ECA cells.

Table 2. Effect of cladosporin and dimethyl cladosporin on DNA-dependent RNA polymerase from *E. coli*. Assay according to FUCHS *et al.*[10]

Antibiotic added	μg/ml	AMP incorporated nMol/ml
Control	0	137
Cladosporin	1	135
	10	140
	100	136
Dimethyl cladosporin	1	123
	10	137
	50	139
Rifampicin	5	5

Since inhibition of the uptake of the appropriate precursors into cells would also result in lower incorporation of these precursors into macromolecules we investigated the uptake of uracil, uridine, and other metabolites into cells of ECA and *B. brevis*. The results are given in Tables 3~5.

In ECA cells (Table 3), uptake of uridine was inhibited to the same extent as incorporation into

Table 3. Effect of cladosporin on the uptake and incorporation of uridine, α-aminoisobutyric acid, and deoxyglucose in ECA cells. 6×10^6 cells were preincubated with the antibiotic for 10 minutes, the precursors (0.2 μCi) were added and after 10 minutes the mixture was divided into equal parts and centrifuged. For the uptake one half of the cells was resuspended in saline containing the precursor (1 mM); the cells were immediately collected on membrane filters, rinsed twice with 5 ml saline and after drying the radioactivity was determined (uptake). The other half of the cells was suspended in 5% cold TCA and treated as described in Table 1 (incorporation).

Cladosporin (μg/ml)	°C*	Uridine		α-Aminoisobutyric acid		Deoxyglucose	
		uptake (pMol)	incorporation (pMol)	uptake (pMol)	incorporation (pMol)	uptake (pMol)	incorporation (pMol)
0	0	17	12	11	0.6	0.3	0.04
0	37	270	157	65	3.9	1.4	0.08
10	37	142	86	53	2.6	1.2	0.10
20	37	80	48	55	2.8	1.2	0.10
50	37	40	21	57	3.3	1.5	0.09

*: Incubation temperature.

Table 4. Inhibition of the uptake and incorporation of uracil, uridine, leucine, and α-aminoisobutyric acid into cells of *B. brevis*. *B. brevis* was grown to an A_{578} of 0.5. The antibiotics were added. After 5 minutes the radioactive precursors (0.1 μCi) were added and after another 5 minutes the uptake and the incorporation into TCA-insoluble material was determined. As a positive control for a RNA polymerase inhibitor the effect of rifampicin was also tested.

Antibiotic (μg/ml)		°C	Uracil		Uridine		Leucine		α-Amino-isobutyric acid	
			uptake	incorp.	uptake	incorp.	uptake	incorp.	uptake	incorp.
			(pMol/5×10⁷cells)		(pMol/5×10⁷cells)		(pMol/5×10⁷cells)		(pMol/5×10⁷cells)	
	0	0	65	41	0.5	0.21	16	5.5	0.8	0.8
	0	37	430	240	1.5	1.35	52	16.0	17.1	0.7
Rifampicin	5	37	340	16	1.7	0.38	51	15.7	—	—*
Cladosporin	0.1	37	330	178	1.2	0.9	48	13.0	14.0	1.0
	0.5	37	116	57	0.8	0.55	22	7.0	9.2	1.0
	1.0	37	55	29	0.7	0.32	16	4.9	8.5	0.6
	5.0	37	38	30	0.5	0.30	8	4.0	6.0	0.6

*: not done

TCA-precipitable material. Uptake of leucine or α-aminoisobutyric acid (which cannot be incorporated into protein) was not affected by cladosporin. The uptake of 2-deoxy-D-glucose also remained unchanged when cladosporin was added to the cell suspension. The inhibition of uptake of uridine was very pronounced and seemed to be rather specific in ECA cells since the incorporation and the uptake of thymidine was unchanged.

Table 5. Effect of the substrate concentration on the inhibition of the uptake of uracil in *B. brevis*. Cladosporin concentration: 3.4×10^{-6} M.

Uracil (M)	Substrate/inhibitor	Inhibition (%)
9.0×10^{-6}	2.6	84
2.2×10^{-5}	6.5	83
4.4×10^{-5}	13.0	80
9.0×10^{-5}	26.4	81

In *B. brevis* the uptake of deoxyglucose, thymidine, thymine, and adenosine was unchanged in the presence of cladosporin, but the uptake of uracil and to a lesser extent the uptake of uridine, leucine, and α-aminoisobutyric acid (Table 4) was inhibited. The concentrations needed for the inhibition of uracil uptake compared favorably to the minimal inhibitory concentrations (MICs) needed for the prevention of growth of *B. brevis*. The inhibitory effect of cladosporin was unchanged when different concentrations of uracil were used for uptake studies (Table 5); thus a competitive inhibition of uptake can be ruled out. This result is in good agreement with our finding that the MIC for *B. brevis* was not dependent on the concentrations of uracil and leucine in the assay medium. The uptake of uracil and leucine in *B. brevis* could not be restored to control levels when the antibiotic was removed after 10 minutes of preincubation*, indicating an irreversible damage of a cell function involved in the transport of uracil and leucine by cladosporin.

In order to substantiate further that the inhibition of transport components is correlated to the antibiotic activity of cladosporin, different strains of *E. coli* were tested (Table 6). For *E. coli* K 12 and *E. coli* A 19 neither growth nor uracil uptake was affected by cladosporin. In *E. coli* A 19-15, a mutant sensitive to high concentrations of cladosporin (the MIC being 100 μg/ml) uracil uptake was almost

* The cells were centrifuged at room temperature, washed twice with prewarmed medium and resuspended in the original volume of prewarmed medium containing the radioactive precursors.

completely inhibited at this concentration. Inhibition of uracil uptake, however, cannot explain an inhibition of growth because *E. coli* A 19-15 does not depend on uracil. Since *E. coli* A 19 and *E. coli* A 19-15 are methionine auxotrophs, the effect of cladosporin on the uptake of this amino acid was investigated. The results are given in Fig. 4. *E. coli* A 19 is not sensitive to cladosporin and no inhibition of methionine uptake was observed; but in the cladosporin-sensitive strain the uptake of methionine was completely inhibited at 100 μg cladosporin per ml—which would explain the inhibition of growth. A methionine-prototrophic revertant (*E. coli* A 19-15-I) obtained from *E. coli* A 19-15 by selection on minimal agar was no longer sensitive to cladosporin, while the methionine uptake was still inhibited. The sensitivity of this revertant to rifampicin, kirromycin, and streptomycin was unchanged, pointing out that no change in the permeability of the cells had taken place. Table 7 summarizes the effect of cladosporin on the four strains of *E. coli*. The inhibition of methionine uptake resulted in the inhibition of growth only in the methionine-auxotroph organism A 19-15, whereas in the methionine-prototrophic strain A 19-15-I no antibiotic effect was detected, while the uptake of methionine was still sensitive.

Table 6. Uptake of uracil by different strains of *E. coli* in the presence of cladosporin.

E. coli was grown to an $A_{578}=0.5$ and incubated with the antibiotic for 5 minutes then the radioactive uracil (0.1 μCi/ml) was added and after 10 minutes the uptake was measured (for details see Methods).

Cladosporin (μg/ml)	Uracil uptake (pMol/ml of *E. coli* culture)		
	K 12	A 19	A 19-15
0	842	821	844
1	841	838	856
5	890	848	897
50	884	822	794
100	866	824	110

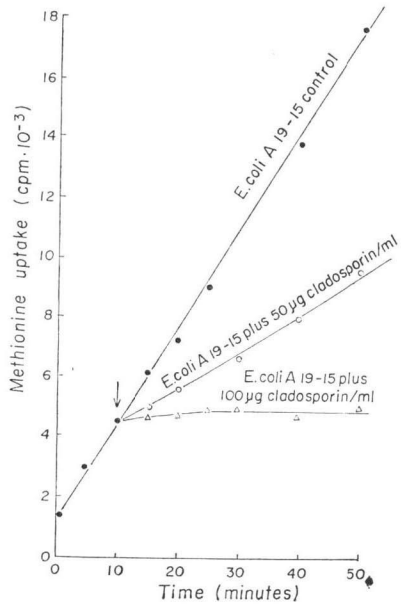
Table 7. Effect of cladosporin on different strains of *E. coli*.

Organism	Methionine auxotrophy	Sensitivity of methionine uptake to cladosporin	Inhibition of growth by cladosporin
E. coli K 12	−	−	−
E. coli A 19	+	−	−
E. coli A 19-15	+	+	+
E. coli A 19-15-I (methionine-prototrophic revertant)	−	+	−

Fig. 4. Inhibition of methionine uptake in *E. coli* A 19 and A 19-15 by cladosporin.

The cultures were grown and diluted as described in Materials and Methods.

At $A_{578}=0.5$ the radioactive methionine (0.05 μCi/ml) was added. After 10 minutes the cultures were divided in three equal parts. Cladosporin (10 μg/ml and 100 μg/ml) was added to two of the batches. At the indicated time intervals 1.0 ml samples were withdrawn and the methionine uptake was determined. The arrow indicates the addition of cladosporin. All curves obtained with *E. coli* A19 were identical to the control curve for *E. coli* A19-15.



B. brevis, which is highly sensitive to cladosporin, does not grow on synthetic media containing only glucose and salts. The inhibition of uptake of essential nutrients like amino acids or nucleosides

from complex media prevents growth in this organism.

These results show that in sensitive prokaryotic cells cladosporin interferes with the uptake of uracil, uridine, leucine, and other amino acids resulting in the decreased incorporation of these precursors into RNA or protein. This inhibitory effect is irreversible: after exposure to cladosporin the uptake of uracil and leucine could not be restored following removal of the antibiotic. This offers a good explanation for our finding that cladosporin is bactericidal rather than bacteriostatic. Whether this irreversibility is due to a covalent binding of cladosporin to the cells has still to be examined.

In ECA cells the inhibition of uptake prevents uridine from being incorporated into RNA. Whether this decrease in uridine uptake is due to an inhibition of uridine kinase remains to be investigated. In ECA cells the uptake of uridine follows the kinetics of facilitated diffusion[14], and inhibition of uridine kinase would therefore result in a lower uptake of uridine.

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References

1) BRECHT-FISCHER, A.; H. ZÄHNER & H. LAATSCH: Stoffwechselprodukte von Mikroorganismen. 183. Imacidin, ein neues Acylpeptidantibioticum aus *Streptomyces olivaceus*. Arch. Microbiol, in press.
2) SCOTT, P. M.; W. VAN WALBEEK & W. M. MACLEAN: Cladosporin, a new antifungal metabolite from *Cladosporium cladosporioides*. J. Antibiotics 24: 747~755, 1971
3) ANKE, H.; H. ZÄHNER & W. A. KÖNIG: Metabolic products of microorganisms. 170. On the antibiotic activity of cladosporin. Arch. Microbiol. 116: 253~257, 1978
4) HELLER, K. & R. RÖSCHENTHALER: Some results on the mode of action of the mycotoxin ochratoxin A in bacteria. *In*: Internatl. Sympos.: Design and mechanism of action of antimetabolites, Varna, pp. 273~284, Publishing House of the Bulgarian Academy of Sciences, Sofia, 1976
5) BUNGE, I.; G. DIRHEIMER & R. RÖSCHENTHALER: *In vivo* and *in vitro* inhibition of protein synthesis in *Bacillus stearothermophilus* by ochratoxin A. Biochem. Biophys. Res. Commun. 83: 398~405, 1978
6) GESTELAND, R. F: Isolation and characterization of ribonuclease I mutant of *Escherichia coli*. J. Mol. Biol. 16: 67~84, 1966
7) WEITZEL, G.; F. SCHNEIDER, A. M. FRETZDORFF, J. DURST & W. D. HIRSCHMANN: Untersuchungen zum cytostatischen Wirkungsmechanismus der Methylhydrazine. II. Z. Physiol. Chem. 348: 433~442, 1967
8) PROBST, G.: Verschiebungen von DNA Polymerase Aktivität zwischen Kern und Plasma im Verlauf des Zellzyklus von EHRLICH Ascites Tumorzellen. Doctoral Dissertation, University of Tübingen, GFR, 1973
9) JENDRISAK, J. J. & R. R. BURGESS: A new method for the large-scale purification of wheat germ DNA-dependent RNA polymerase II. Biochemistry 14: 4639~4645, 1975
10) FUCHS, E.; R. L. MILLETTE, W. ZILLIG & G. WALTER: Influence of salts on RNA synthesis by DNA-dependent RNA-polymerase from *Escherichia coli*. Eur. J. Biochem. 3: 183~193, 1967
11) KUPKA, J.; T. ANKE, F. OBERWINKLER, G. SCHRAMM & W. STEGLICH: Antibiotics from basidiomycetes. VII. Crinipellin, a new antibiotic from the basidiomycetous fungus *Crinipellis stipitaria* (FR.) PAT. J. Antibiotics 32: 130~135, 1979
12) DAVIS, B. D. & E. S. MINGIOLI: Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. 60: 17~28, 1950
13) MARTIN, E. M.; J. MALEC, S. SVED & T. S. WORK: Studies on protein and nucleic acid metabolism in virus-infected mammalian cells. 1. Encephalomyocarditis virus in KREBS II mouse-ascites-tumor cells. Biochem. J. 80: 585~597, 1961
14) JACQUEZ, J. A.: Transport and enzymic splitting of pyrimidine nucleosides in EHRLICH cells. Biochim. Biophys. Acta 61: 265~277, 1962