ANTIBIOTIC RESISTANCE MECHANISMS IN *MYCOPLASMA* SPECIES

J. L. SCHWARTZ and D. PERLMAN

School of Pharmacy, University of Wisconsin, Madison 53706, U. S. A.

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Examination of the mechanisms of antibiotic resistance of strains of *Mycoplasma fermentans*, *Mycoplasma hominis*, *Mycoplasma laidlawii*, *Mycoplasma orale* II, *Mycoplasma pharyngis*, *Mycoplasma salivarium* and selected species of avian *Mycoplasma* to chloramphenicol, dihydrostreptomycin, and tetracycline showed that in the cultures studied, reduced antibiotic uptake was involved rather than specific degradation of the antibiotic.

Although there have been many studies of the mechanisms of antibiotic resistance in bacteria[3,7,8,11,13,14a,15,16,18,20,21], information is lacking on the antibiotic resistance mechanisms in *Mycoplasma*. We have studied the sensitivity and mechanisms of resistance of chloramphenicol, dihydrostreptomycin, and tetracycline of selected clinical isolates and representatives of 6 species of human *Mycoplasma*, 2 serotypes of avian *Mycoplasma*, and *M. laidlawii* B (from sewage). We have attempted to determine whether resistant cultures have enzymatic mechanisms inactivating these antibiotics, or whether their resistance arises from lack of permeability-uptake of the antibiotic into the resistant cells.

Materials and Methods

Mycoplasma cultures. We are indebted to Dr. S. MADOFF, Massachusetts General Hospital, Boston, and to Mr. Y. E. CRAWFORD, Mycoplasma Research Division, Naval Medical Research Unit No. 4, Department of the Navy, for the clinical isolates used in these studies. The *M. laidlawii* strain B was obtained from Professor H. MORTON, University of Pennsylvania. The avian mycoplasma were obtained from Dr. A. H. HAMDY of The Upjohn Company.

Media. All cultures were grown in Difco PPLO broth fortified with 0.5 % Bacto yeast extract, 0.1 % sodium acetate, and 2 % Bacto PPLO serum fraction. Difco PPLO agar supplemented with 2 % Bacto PPLO serum fraction was used in some experiments for solidified media.

Antibiotics. The tetracycline hydrochloride, erythromycin, chloramphenicol, gentamicin sulfate, kanamycin sulfate, streptomycin sulfate, and dihydrostreptomycin sulfate used in these studies were commercial grade materials. The spectinomycin was a gift from Dr. G. B. WHITFIELD, Jr., The Upjohn Company.

Radioactive antibiotics. Tetracycline-7-T hydrochloride (sp. act. 800 mCi/mM) was obtained from Schwarz BioResearch, Inc. The dihydrostreptomycin-T sesquisulfate (sp. act. 3 Ci/mM) and chloramphenicol (methylene-14C sp. act. 10.1 mCi/mM) were obtained from Amersham/Searle Corporation. All were used without further purification and identification.

Determination of antibiotic sensitivity. Sensitivity to various antibiotics was determined by streaking loopfuls of 72-hour broth culture of the mycoplasma cultures on agar plates containing the antibiotic. The plates were incubated at 37°C in a 5 % CO_2/95 % air atmosphere for up to 10 days and were observed periodically for growth (using a low power microscope).

Antibiotic inactivation studies. Streptomycin adenylylation was studied using the method described by BENVENISTE *et al.*[3] As little as 0.1 nanomole converted to the adenylate could have been easily detected by this assay. Chloramphenicol acetylation was determined by SHAW's method[16]. Microgram amounts of acetylated antibiotic could be detected by this method.

Determination of protein. The protein content of washed mycoplasma cells was determined by suspending an aliquot of cells in LOWRY's reagent[14]. Crystalline bovine serum albumin was the reference standard. Repeated determinations showed that 1×10^{11} cells contained 1 mg of protein.

Antibiotic absorption. Absorption of chloramphenicol, dihydrostreptomycin, and tetracycline by various strains of *Mycoplasma* were carried out as follows: Each culture was grown in 50 ml of broth media for 24 hours and then transferred to 100 ml of fresh broth in 250 ml Erlenmeyer flasks. The inoculated flasks were placed in a 5 % CO_2/95 % air incubator and maintained at 37°C. After 18 hours incubation, radioactive antibiotic was added, an initial sample removed, and the flask replaced in the incubator. The initial time sample (and later samples) was centrifuged for 40 minutes at $3,500 \times g$ on a Sorvall type SP centrifuge at 4°C, and the pellet and the surface rinsed with non-radioactive antibiotic at the same concentration to remove surface absorbed antibiotic. The pellet was then suspended in 50 microliters of water and aliquots of the suspension analyzed for radioactivity and for protein. Radioactivity was determined in a Packard Tri-Carb liquid scintillation counting system (model 2002) with BRAY's solution as counting fluid[5].

Results

Antibiotic Sensitivity

The results of the antibiotic sensitivity tests on the clinical isolates are summarized in Table 1. Although most strains examined were sensitive to 10 mcg/ml of chloramphenicol, gentamicin, kanamycin, and tetracycline, resistant strains of *M. hominis*, *M. pharyngis*, and *M. salivarium* were noted with each antibiotic. As far as these species are concerned, many strains appeared to be resistant to erythromycin, spectinomycin, and streptomycin. In general, *M. pharyngis* was more often resistant to antibiotics than *M. salivarium*. No evidence of streptomycin adenylylation could be demonstrated with 8 cultures of *Mycoplasma* tested (*M. hominis* strains PG 27, 41 TC,

Table 1. Antibiotic sensitivity patterns in *Mycoplasma*

Species	No. strains tested	Tetracycline		Erythromycin		Chloramphenicol		Spectinomycin		Streptomycin		Kanamycin		Gentamicin	
		R	S	R	S	R	S	R	S	R	S	R	S	R	S
M. pharyngis	22	5	17	13	9	4	18	8	14	15	7	6	16	4	18
M. salivarium	22	1	21	9	13	2	20	10	12	9	13	5	17	4	18
M. hominis	3	0	3	2	1	0	3	2	1	2	1	0	3	0	3
M. fermentans	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
M. orale II	1	0	1	1	0	0	1	1	0	0	1	0	1	0	1

Abbreviations: R, resistant. As measured by growth on agar plates containing 10 μg/ml of tetracycline, erythromycin, chloramphenicol, kanamycin and gentamicin, and 20 μg/ml of spectinomycin and streptomycin. S, sensitive.

and No. 7; *M. salivarium* strains 6 and 9; *M. pharyngis* strains 13 and 21; and T-40) using methods giving positive results with *E. coli* carrying an R-factor for streptomycin adenylylation. No inactivation of any type could be demonstrated by incubating streptomycin with mycoplasma cultures for varying lengths of time. No acetylation of chloramphenicol could be demonstrated with two cultures (*M. pharyngis* 24 and *M. salivarium* 6) using methods giving positive results with a *Klebesiella* culture containing an R-factor for acetylating chloramphenicol.

Antibiotic Absorption

Results of experiments on the absorption of dihydrostreptomycin are summarized in Table 2. Sensitive cultures *M. laidlawii* and *M. fermentans* show similar patterns of initial absorption and later efflux of antibiotic. *M. fermentans* shows a slight rise in absorption after 4 hours, while a very slight decrease in absorption is observed in the same time period with *M. laidlawii*. After 4 hours efflux of antibiotic is observed to levels of $1\sim2.5\times10^4$ molecules/cell. A continual pattern of antibiotic absorption is observed with *M. salivarium* No. 19. However initial absorption values are lower with this culture (6~10 times less), and its absorption pattern probably reflects its poorer initial absorption, as final values are closer to the other cultures (3~4 times less).

A consistent decrease in absorbed antibiotic with time occurred with the dihydrostreptomycin resistant cultures. Absorption levels of antibiotic per cell were 5~10 fold lower than with those seen with the antibiotic sensitive cells. The efflux rate

Table 2. Dihydrostreptomycin absorption by *Mycoplasma*

		Exposure time	$STMH_2$ molecules per cell	% remaining of initial absorption
A. Sensitive cultures	*M. fermentans* Pg 18	10 min.	1.9×10^4	100
		4 hrs.	2.4×10^4	126
		8 hrs.	1.0×10^4	53
		24 hrs.	1.3×10^4	68
	M. laidlawii B	10 min.	3.8×10^4	100
		4 hrs.	3.6×10^4	95
		8 hrs.	1.6×10^4	42
		24 hrs.	2.2×10^4	58
	M. salivarium 19	10 min.	3.4×10^3	100
		4 hrs.	3.7×10^3	109
		24 hrs.	4.6×10^3	135
B. Resistant cultures	*M. hominis* 7	10 min.	3.5×10^3	100
		4 hrs.	1.5×10^3	43
		24 hrs.	1.2×10^3	34
	M. salivarium 9	10 min.	5.1×10^3	100
		4 hrs.	2.4×10^3	47
		24 hrs.	1.0×10^3	20
	M. pharyngis 21	10 min.	6.4×10^3	100
		4 hrs.	2.1×10^3	33
		24 hrs.	1.4×10^3	22
	M. pharyngis 6	10 min.	6.4×10^3	100
		4 hrs.	1.7×10^3	26
		24 hrs.	1.7×10^3	26
	M. orale II	10 min.	2.9×10^3	100
		4 hrs.	2.1×10^3	72
		8 hrs.	2.2×10^3	76
		24 hrs.	1.5×10^3	51

Dihydrostreptomycin was added to each flask at a concentration of 20 μg/ml.

Table 3. Tetracycline absorption by *Mycoplasma*

		Concentration mcg/ml	Exposure time	Molecules absorbed/cell	% remaining of initial
A. Sensitive cultures	*M. salivarium* 19	10	10 min.	2.6×10^5	100
			4 hrs.	8.3×10^4	31
			8 hrs.	4.6×10^4	19
	M. salivarium 20	10	10 min.	2.2×10^5	100
			4 hrs.	3.3×10^4	13
			10 hrs.	1.6×10^4	6
			24 hrs.	3.7×10^4	17
	M. hominis 41TC	10	10 min.	8.4×10^4	100
			4 hrs.	2.7×10^4	32
			10 hrs.	1.9×10^4	23
			24 hrs.	1.2×10^4	15
	M. salivarium 17	10	10 min.	8.0×10^4	100
			4 hrs.	2.8×10^4	36
			10 hrs.	2.0×10^4	25
			24 hrs.	1.5×10^4	19
	M. laidlawii B	10	10 min.	1.2×10^5	100
			4 hrs.	4.2×10^4	33
			8 hrs.	1.8×10^4	15
			24 hrs.	1.6×10^4	13
	M. laidlawii B	0.6	10 min.	7.6×10^3	100
			4 hrs.	2.1×10^4	277
			8 hrs.	2.7×10^3	36
			24 hrs.	4.6×10^3	61
	M. pharyngis 25	2.0	10 min.	1.1×10^4	100
			2 hrs.	6.5×10^3	57
			4 hrs.	3.5×10^3	31
			24 hrs.	4.7×10^3	41
B. Resistant cultures	*M. laidlawii* B	10	10 min.	1.9×10^5	100
			2 hrs.	2.4×10^4	13
			4 hrs.	2.3×10^4	12
			24 hrs.	1.4×10^4	7
	M. laidlawii B	0.6	10 min.	1.0×10^4	100
			4 hrs.	3.2×10^3	32
			8 hrs.	2.7×10^3	27
			24 hrs.	1.5×10^3	15

observed with *M. orale* II was quite a bit lower than the other resistant cultures but again this can be attributed to a lower initial absorption. Final values of all resistant cultures are almost the same—$1 \sim 2 \times 10^8$ molecules of antibiotic/cell.

The results of a study of tetracycline absorption by *Mycoplasma* are collected in Table 3. At concentrations ten to fifty times higher than the minimum inhibitory concentrations (M. I. C.) of these organisms, a pattern of rapid uptake followed by a sharp decrease in absorbed tetracycline occurred. At levels closer to the M. I. C. of each culture, varying patterns of absorption are seen. *M. laidlawii* B (M. I. C. of 0.5 mcg/ml) at a tetracycline concentration of 0.6 mcg/ml shows a large increase at 4 hours of absorbed tetracycline. By 8 hours, efflux of tetracycline had occurred and a slight absorption increase takes place through the final sampling at 24 hours. Much slower rates of efflux are observed at 2 mcg/ml of tetracycline with *M. pharyngis* No. 25 (compared with rates at 10 mcg/ml with the other cultures). After 4 hours, a rise in absorption is observed. Such a rise in final absorption is also observed with avian *Mycoplasma* U-10 (Fig. 1) at a concentration just at its M. I. C. of 0.2 mcg/ml.

A resistant laboratory developed mutant of *M. laidlawii* B* showed a strikingly

* This culture selected by Mr. CARL FRATERRIGO had M. I. C. greater than 60 mcg/ml.

Fig. 1. Absorption and efflux of tetracycline from avian *Mycoplasma* culture U-10

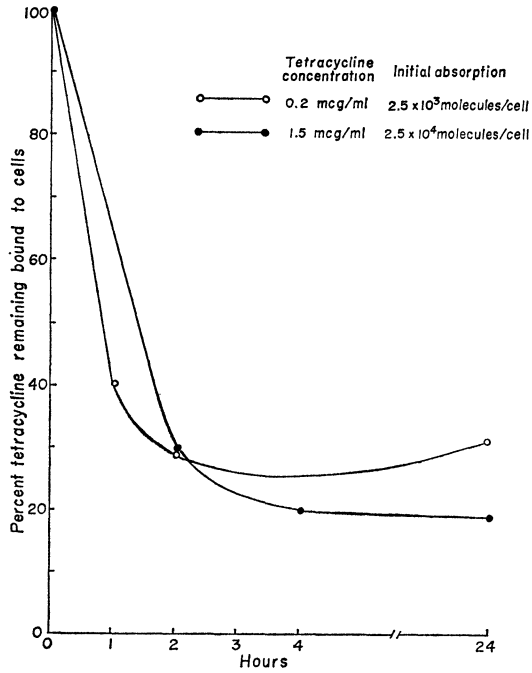


Fig. 2. Initial absorption of tetracycline by avian *Mycoplasma* U-1

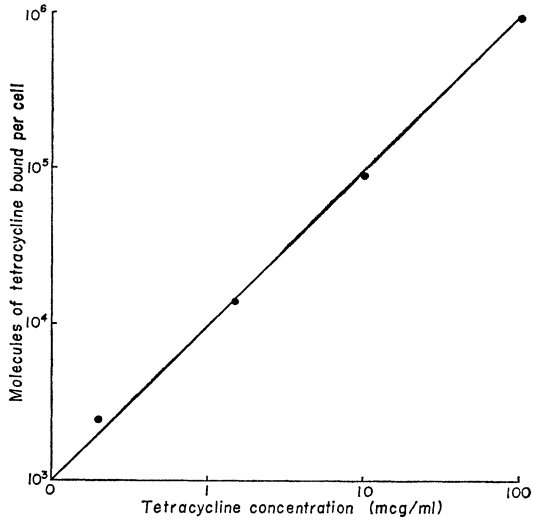
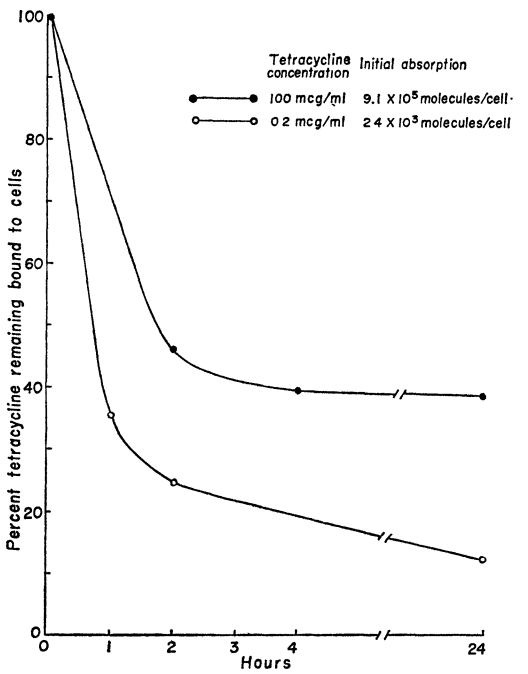


Fig. 3. Absorption and efflux of tetracycline from avian *Mycoplasma* U-1



different pattern of absorption and efflux than its sensitive counterpart. At concentrations of 10 mcg/ml and 0.6 mcg/ml a rapid efflux of tetracycline was observed after initial absorption. This is in contrast to the large rise in absorption observed with the sensitive *M. laidlawii* B at 0.6 mcg/ml. Even the rate of efflux at 10 mcg/ml was considerably greater with the resistant *M. laidlawii* B as compared to the sensitive organism.

With culture U-1, an avian *Mycoplsma* Serotype D, 4 different concentrations were used in uptake studies. In Fig. 2, initial uptake is plotted against tetracycline concentration. Absorption increased linearly with increased antibiotic concentration. In general, this holds true with the other cultures as well.

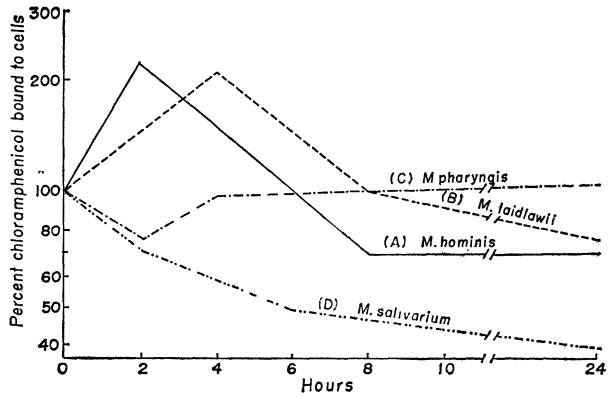
Absorption and efflux patterns of U-1 (serotype J) are seen in Fig. 3 for tetracycline concentrations of 100 and 0.2 mcg/ml. At the higher concentration a greater percentage of antibiotic is retained in the cell.

The experiments on chloramphenicol uptake are summarized in Fig. 4. At concentrations just above the minimum inhibitory concentration of *M. laidlawii* B (M.I.C.

8 mcg/ml) and *M. hominis* 41 TC (M. I. C. 2 mcg/ml) an increase in absorption is observed after 4 hours. After 24 hours, absorption levels have decreased to about 70% of initial values. *M. pharyngis* No. 25 (M. I. C. 2 mcg/ml), after an initial decrease, also shows a pattern of increased absorption. With *M. salivarium* 6, a culture resistant to more than 10 mcg/ml of chloramphenicol, the amount of antibiotic absorbed dropped with increase incubation.

Fig. 4. Absorption and efflux of chloramphenicol from *Mycoplasma* cultures.

(A) *M. hominis* 41TC ; Chloramphenicol concentration 3 mcg/ml; initial absorption 3.7×10^4 molecules/cell
(B) *M. laidlawii* B ; Chloramphenicol concentratlon 10 mcg/ml; initial absorption 2.1×10^5 molecules/cell
(C) *M. pharyngis* 25 ; Chloramphenicol concentration 4 mcg/ml; initial absorption 5.7×10^4 molecules/cell
(D) *M. salivarium* 6 ; Chloramphenicol resistant : Chloramphenicol concentration 10 mcg/ml ; initial absorption 6.8×10^4 molecules/cell



Discussion

A review of the reports on the antibiotic sensitivity patterns of various species of *Mycoplasma*[1,2,4,6,9,10,12,17,19] shows that *M. fermentans*, *M. hominis*, *M. pneumoniae* and *M. salivarium* are generally sensitive to less than 6.25 mcg/ml of chloramphenicol, gentamicin, kanamycin and tetracycline, although resistant strains (resistant to at least 10 mcg/ml) are not uncommon. For example, in 84 strains of *M. salivarium*, STEWART and co-workers[17] noted 28 resistant to chloramphenicol, 30 resistant to kanamycin, and 26 resistant to tetracycline. Erythromycin and streptomycin are generally found to be ineffective in inhibiting mycoplasma with the exception of erythromycin on *M. pneumoniae* which is effective at concentrations between 0.1~1 mcg/ml.

As mentioned previously, no mechanisms of antibiotic resistance have been proposed for *Mycoplasma*. In bacterial systems, SHAW's demonstration of the acetylation of chloramphenicol by R-factor carrying bacteria[16], and the phosphorylation and acetylation of kanamycin[13,15] as well as the adenylylation of streptomycin[3] in similar inactivation studies encouraged us to investigate for the possibility of similar mechanisms in antibiotic resistant *Mycoplasma*. To date we have found no inactivation or degradation of antibiotics by *Mycoplasma*, and the present study suggests that differences in antibiotic uptake-permeability are responsible for the resistance noted. In the case of streptomycin, resistant organisms absorb significantly less antibiotic than the sensitive cultures. Patterns of efflux after initial absorption are also markedly different, and seem to be closely related to the amount of antibiotic initially absorbed.

The results with tetracycline are similar to what was found by FRANKLIN and co-workers[7,8] with tetracycline absorption studies on *E. coli*. FRANKLIN found that at 10 mcg/ml, accumulation of drug ceased after 10 minutes, and that initial drug absorption was linearly proportional to the concentration of tetracycline in the media. The finding that at much higher tetracycline concentrations (100 mcg/ml) efflux levels off at a higher level than at lower concentration is also similar to FRANKLIN's finding. FRANKLIN also demonstrated that a resistant *E. coli* accumulated significantly less tetracycline than a sensitive culture. In the one resistant organism studied here, the accumulation patterns at M.I.C. tetracycline levels of the sensitive *M. laidlawii* clearly indicated a substantial difference in the amount of antibiotic absorbed.

In comparing the two avian cultures at the same low concentration of 0.2 mcg/ml of tetracycline, it will be noted that U-10 (M. I. C. 0.2 mcg/ml) absorption patterns indicate

a leveling off of efflux and final absorption of tetracycline (7.9×10^2 molecules/cell), while U-1 (M. I. C. 1.0 mcg/ml) shows a continuous decrease in efflux throughout the sampling record (final value of 2.9×10^2 molecules/cell). The levels of absorption and efflux being indicative of their differences in sensitivity. This pattern is similar to that observed by CORCORAN and co-workers[18], where it was noted that at the same concentration, a resistant bacterial culture will accumulate less erythromycin than a sensitive culture.

The same patterns of higher drug accumulation in sensitive cultures and differential efflux patterns between resistant and sensitive cultures are also observed in the absorption studies with chloramphenicol. The only direct comparison can be made between *M. laidlawii* B and *M. salivarium* 6, both assayed at 10 mcg/ml. A final accumulation of 1.6×10^4 molecules per cell was noted for the sensitive *M. laidlawii* culture, while that of the resistant *M. salivarium* was 2.7×10^4 molecules per cell. The general pattern of an increase in absorption somewhere in the sampling period for the sensitive cultures, and a continuous decrease for the resistant culture was also the same as the other antibiotics studied.

The conculusion then, that resistance in *Mycoplasma* to dihydrostreptomycin, tetracycline, and chloramphenicol is related to some permeability-uptake mechanism seems to be well supported by the data presented.

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Literaiure Cited

1) ARAI, S.; K. YOSHIDA, A. IZAWA, K. KUMAGAI & N. ISHIDA : Effect of antibiotics on growth of *Mycoplasma pneumoniae* Mac. J. Antibiotics 19 : 118～120, 1966
2) ARAI, S.; K. YURI, A. KUDO, M. KIKUCHI, K. KUMAGAI & N. ISHIDA : Effects of antibiotics on the growth of various strains of *Mycoplasma*. J. Antibiotics 20 : 246～253, 1967
3) BENVENISTE, R.; T. YAMADA & J. DAVIES : Enzymatic adenylylation of streptomycin and spectinomycin by R-factor resistant *Escherichia coli*. Infection and Immunity 1 : 109～119, 1970
4) BRAUN, P.; J. O. KLEIN & E. H. KASS : Susceptibility of genital mycoplasmas to antimicrobial agents. Appl. Microbiol. 19 : 62～70, 1970
5) BRAY, G. A. : A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1 : 279～285, 1960
6) CHELTON, E. T. J. : The *in vitro* resistance to antibiotics of *Mycoplasma mycoides* var. *capri*. J. Appl. Bact. 29 : 213～216, 1966
7) FRANKLIN, T. J. & A. GODFREY : Resistance of *Escherichia coli* to tetracycline. Biochem. J. 94 : 54～60, 1965
8) FRANKLIN, T. J. & B. HIGGINSON : Active accumulation of tetracycline by *Escherichia coli*. Biochem. J. 116 : 287～297, 1970
9) HAMDY, A. H. & C. J. BLANCHARD : *In vitro* activity of lincomycin and spectinomycin against serotypes of avian Mycoplasma. Appl. Microbiol. 20 : 26～30, 1970
10) JAO, R. L. & M. FINLAND : Susceptibility of *Mycoplasma pneumoniae* to 21 antibiotics *in vitro*. Am. J. Med. Sci. 36 : 639～650, 1967
11) KISER, J. S.; G. O. GALE & G. A. KEMP : Resistance to antimicrobial agents. Adv. Appl. Microbiol. 11 : 77～100, 1969
12) KÖHLER, W. : Antibiotikaempfindlichkeit humaner PPLO-Stämme. Zent. Bakt. (Orig.) 185 : 355～366, 1962
13) KONDO, S.; M. OKANISHI, R. UTAHARA, K. MAEDA & H. UMEZAWA : Isolation of kanamycin and paromamine inactivated by *E. coli* carrying R-factor. J. Antibiotics 21 : 22～29, 1968
14) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL : Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 : 265～275, 1951

14a) OKAMOTO, S. & Y. SUZUKI : Chloramphenicol-, dihydrostreptomycin- and kanamycin inactivating enzymes from multiple drug-resistant *Escherichia coli* carrying episome "R". Nature 208 : 1301～1303, 1965

15) OZANNE, B.; R. BENVENISTE, D. TIPPER & J. DAVIES : Aminoglycoside antibiotics : Inactivation by phosphorylation in *Escherichia coli* carrying R-factors. J. Bact. 100 : 1144～1146, 1969

16) SHAW, W. V. : The enzymatic acetylation of chloramphenicol by extracts of R factor-resistant *Escherichia coli*. J. Biol. Chem. 242 : 687～693, 1967

17) STEWART, S. M.; M. E. BURNET & J. E. YOUNG : *In vitro* sensitivity of strains of *Mycoplasmas* from human sources to antibiotics and to sodium aurothiomalate and tylosin tartrate. J. Med. Microbiol. 2 : 287～292, 1969

18) TAUBMAN, S. B.; F. E. YOUNG & J. W. CORCORAN : Antibiotic glycosides. IV. Studies on the mechanisms of erythromycin resistance in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U. S. 50 : 955～962, 1963

19) TAYLOR-ROBINSON, D. : Mycoplasma of various hosts and their antibiotic sensitivities. Postgrad. Med. J. 43 (March suppl.) : 100～104, 1967

20) UMEZAWA, H.; S. TAKEZAWA, M. OKANISHI & R. UTAHARA : Adenylylstreptomycin, an inactivated product of streptomycin by *Escherichia coli* carrying R factor. J. Antibiotics 21 : 81～82, 1968

21) YAMADA, T.; D. TIPPER & J. DAVIES : Enzymic inactivation of streptomycin by R factor-resistant *Escherichia coli*. Nature 219 : 288～291, 1968