Antibiotics

Volume VI

Modes and Mechanisms of Microbial Growth Inhibitors

Edited by Fred E. Hahn

With 127 Figures

Springer-Verlag
Berlin Heidelberg NewYork Tokyo 1983

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ISBN-13:978-3-642-68948-2 e-ISBN-13:978-3-642-68946-8
DOI: 10.1007/978-3-642-68946-8

Library of Congress Cataloging in Publication Data. Main entry under title: Modes and mechanisms of microbial growth inhibitors. (Antibiotics; v. 6) Includes bibliographical references and index. 1. Antibiotics. 2. Microbial growth. I. Hahn, F.E. (Fred Ernest), 1916– . II. Series. [DNLM: 1. Growth inhibitors—Pharmacodynamics. 2. Antibiotics—Pharmacodynamics. W1 AN854B v.6 / QV 350 M691] RM267.M616 1983 615'.329 83–393

2131/3130-543210

Preface

It is not certain that the editors of *Antibiotics I* (1967), Drs. GOTTLIEB and SHAW, fully realized that they were laying the foundation for an entire series of which we present here Vol. VI. For some time to come, this will be the last volume of the *Antibiotics* series.

There are several reasons for this. Firstly, the discovery of medicinally useful antibiotics has leveled off, because the number of microbiological products with antimicrobial properties is not infinite. In 1972 some 2500 antibiotic substances were known, of which approximately one per cent are clinically useful. Further search for antibiotics has led to increasing frequency of rediscoveries and drastically decreasing frequency of discoveries of new antibiotics. As the search for antibiotics with a standard methodology in conventional ecological niches has exhausted itself, there is a paucity of new and interesting substances on which to undertake modes/mechanisms of action studies.

Secondly, the mechanism of action field has come of age and its results are now academic knowledge. This also holds true for synthetic chemotherapeutic drugs and becomes the case rapidly for toxic substances with anti-eukaryotic action. The study of mechanisms of action was undertaken for two reasons: one was the basic scientific desire to know how antimicrobial substances interfered with microbial biochemistry; the second one was the hope that such information would be useful in the premeditated design of synthetic antimicrobials. The academic part of the undertaking has been largely satisfied through research of the past three decades. But the practical promise in terms of premeditated drug design has not been fulfilled to the extent originally anticipated. One instance of successful drug design from biochemical principles is that of alafosfalin which is the subject of one of the chapters of this book.

As a consequence of the exhaustion of objects of study and of the near completion of the investigation of known compounds, the source literature on mechanisms of action has decreased to comparatively few new publications. Mechanisms of action are no longer prominent sections of the programs of scientific meetings, dedicated to chemotherapy. The field that was in the forefront of science in the 1950's is now fighting a rearguard action, filling in details of mechanistic knowledge that, in principle, is well established.

This Preface is not the proper place to discuss ways and means by which the hiatus in new drug discovery may be overcome. This editor is confident that new search and research concepts might produce a new wave of discovery or development of chemotherapeutic drugs. If and when this happens, the time may come for yet another volume of Antibiotics.

A few drugs have fallen by the wayside and have not been treated recently or at all in the Antibiotics series, mostly because the scientific authorities on these drugs were not prepared to undergo the labor of writing systematic contributions. This is true, foremost, for the semisynthetic penicillins and cephalosporins despite their great medical importance. Antibiotics, therefore, remains only as complete as the joint efforts of authors and editors could render it, but it is not a scientific dictionary or encyclopedia and, for practical reasons, cannot be expected to be developed to this ideal state of completion.

The usefulness of this volume and of its predecessors lies in the fact that a very large volume of source literature has been considered and critically reviewed in order to make in-depth information on mechanisms of action of chemotherapeutic drugs easily available. This type of treatment broadly exceeds the descriptions of drug actions in academic textbooks. The editor hopes that Vol. VI will be as well received by the scientific community as have been the preceding volumes of this series.

The publisher has supported the assemblage of this book with unflinching interest and also should be thanked for the production of such a handsome and well-appointed book.

Washington, D.C., 1982 FRED E. HAHN

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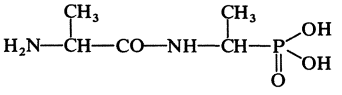
Alafosfalin (Ro 03-7008, Alaphosphin)

C.H. HASSALL

This new antibacterial agent is active in vitro and in vivo against a range of organisms; it is particularly effective against Gram-negative bacteria. Alafosfalin is a phosphonodipeptide (L-alanyl-L-1-aminoethylphosphonic acid, I) which is readily synthesized from simple starting materials. This compound was selected from a group designed as inhibitors of bacterial cell wall biosynthesis (ALLEN et al., 1978).

During the last 4 years the properties and mechanism of action of alafosfalin have been reported. This review provides an outline of the current status of knowledge of this new agent.



L-Ala L-Ala(P)

(I)
Alafosfalin

Toxicology and Safety Evaluation

Studies in the mouse, rat, and baboon (JACKSON and PARKES, unpublished) have established that alafosfalin has a low order of toxicity for animals. The lethal dose of the drug, given orally, is in excess of 10 g/kg for mice and rats and 4 g/kg for baboons. No adverse effects on the general condition of the animals were observed in 90-day studies with the rat (sc and oral) and the baboon (oral) at several dose levels up to 800 mg/kg and 2000 mg/kg, respectively. A small reduction in erythrocyte count was detected in rats after 4 weeks of treatment (sc) with 800 mg/kg and after daily dosing (po) with 4000 mg/kg for 8 weeks or 800 mg/kg for 13 weeks. It was shown with baboons after daily sc or im injection of 400 mg/kg for 8 weeks but not after po dosing of 2000 mg/kg for 13 weeks. The effect never exceeded 15% reduction in erythrocyte count, haematocrit or haemoglobin level. It was readily reversible when dosing was stopped. Similar small effects have been observed with other antibacterial agents (CAPEL-EDWARDS et al., 1977).

These and additional fertility, reproductive, and pharmacokinetic studies in animals (ALLEN et al., 1979a) have provided a good basis for undertaking trials in man.

Antibacterial Studies In Vitro

Spectrum. Alafosfalin is active in vitro against a wide range of bacteria, as illustrated in Table 1. The in vitro activity is significantly reduced in the presence of casein hydrolysate and peptones. The optimum pH for antibacterial activity is 5.5 and it is markedly less at pH 7.5. The antibacterial potency is significantly reduced when the size of the bacterial inoculum is increased from the standard 10^4 to 10^7 colony forming units/plate (ALLEN et al., 1979b). Comparison with commonly used antibiotics, including ampicillin, cephalexin, tetracycline, and cotrimoxazole (ALLEN et al., 1979b), using a range of clinical isolates, indicated that alafosfalin was superior to these agents against common Gram-negative bacteria, in vitro, except for *Proteus* and *Pseudomonas*. However, in general, it was less active against Gram-positive bacteria.

There have been further studies with clinical isolates in Japan (MARUYAMA et al., 1979).

Resistance. Against Gram-negative bacteria from clinical isolates, resistance to alafosfalin was found to be low (0–5%) but it was somewhat higher for Gram-positive genera (Table 2).

There was little evidence of cross-resistance between alafosfalin and other antibiotics; these included penicillins and related β-lactams that are effective in inhibiting bacterial cell-wall biosynthesis. The development of resistance to alafosfalin has been studied in vitro. In a typical experiment using *E. coli* the

Table 1. Antibacterial Spectrum of Alafosfalin

Organism	ID_{50} broth	MIC agar		
	Ro 03-7008	Ro 03-7008	PenG	Amp
E. coli 17	0.05	0.06	16	2
E. coli amp R		0.06	>128	>128
K. aerogenes A1	0.05	0.05	32	32
Str. faecalis 5	1.0	1	1.25	1
Micrococcus 7526	1.0	2	<0.12	<0.12
Entrobacter OG2	1.3	4	>128	>128
Ser. marcescens M42	1.4	4	64	8
S. albus 7	1.6	4	<0.12	<0.12
Sal. typhimurium TS6	1.7	8	4	0.5
Citrobacter FT2	2.4	2	16	4
Providence R2	2.6	4	16	32
S. aureus Schoch	3.4	8	<0.12	<0.12
Shigella flexneri		0.25	8	1
H. influenzae H11		16	0.5	0.5
N. gonorrhoea		16	<0.12	<0.12
B. subtilis		16	<0.12	<0.12
Pr. mirabilis M92	11	>128	4	2
Ps. aeruginosa 8295	26	>128	>128	>128
Str. pyogenes		>128	<0.12	<0.12

Table 2. Resistance to alafosfalin among clinical isolates expressed as the percentage of strains with an MIC of >32 μg/ml

Organism		Total no. of strains	% Of strains with an MIC of >32 μg/ml with the following antibiotics:						
			Al	Am	Cx	Me	Tc	Na	Co
Gram-negative	*E. coli*	81	0	27	1	0	12	3	3
	K. aerogenes	41	2	41	0	5	20	10	3
	Enterobacter sp.	41	5	34	34	10	10	5	7
	S. marcescens	14	0	50	64	0	43	0	21
	S. typhimurium	26	0	0	0	0	0	0	0
Gram-positive	*S. aureus*	39	10	0	0	56	3	54	3
	S. albus	37	3	0	0	16	32	14	11
	Micrococcus sp.	36	20	0	0	6	6	100	0
	S. faecalis	41	0	20	100	100	13	100	0

Abbreviations: Al, alafosfalin; Me, mecillinam; Am, ampicillin; Cx, cephalexin; Tc, tetracycline; Co, cotrimoxazole; Na, Nalidixic acid.

organism was cultivated so that populations with resistance to alafosfalin, cephalexin or a combination of these two antibacterials could be selected. Resistance developed to these agents but it was greatly reduced, particularly for alafosfalin, by combination with cephalexin (ATHERTON et al., 1981) or mecillinam (GREENWOOD and VINCENT, 1979). The influence of the emergence of resistance on the duration of effect of alafosfalin on *E. coli* and on *Proteus mirabilis* has been measured in a mechanical model of the infected urinary bladder (GREENWOOD and O'GRADY, 1978). The result was similar to that observed with nalidixic acid and mecillinam (O'GRADY, personal communication). The development of resistance to agents such as nalidixic acid (STAMEY and BRAGONIE, 1976), streptomycin (TSENG et al., 1972) and mecillinam (GREENWOOD and O'GRADY, 1973) in vitro, has not prevented the effective use of these agents, clinically.

Synergy. It has been shown that there is synergy between alafosfalin and other agents, such as D-cycloserine and β-lactams, which have an effect on cell-wall biosynthesis. Over 500 clinical isolates from a wide range of genera have been studied (ALLEN et al., 1979b), using the well-known FICI-isobologram technique. Some results are summarized in Table 3.

The extent of synergy varied greatly with genus and with antibacterial agent. The combination alafosfalin–mecillinam showed good to excellent synergy against more than 80% of *Staphylococcus aureus* strains but less (<30%, FICI <0.5) against intrinsically susceptible Gram-negative organisms. On the other hand, there was little synergy for *S. aureus* with the combination alafosfalin–ampicillin. Further synergy data using Japanese clinical isolates have been reported by MARUYAMA et al. (1979). It is interesting that very small amounts of alafosfalin convert cephalexin from a bacteriostatic into a bactericidal agent (ATHERTON et al., 1981).

Table 3. In vitro synergy between alafosfalin and three β-lactam antibiotics

Organism	β-lactam antibiotic	MIC with β-lactam antibiotic μg/ml	MIC with alafosfalin μg/ml	MIC with combination μg/ml	FICI	% Of all strains with FICI <0.5
E. coli S44	Mecillinam	0.03	0.03	0.0035 + 0.0035	0.23	31
Enterobacter OG3		0.12	0.5	0.024 + 0.096	0.39	16
K. aerogenes KA6		2	1	0.33 + 0.17	0.33	25
S. marcescens SM7		16	16	0.5 + 0.5	0.062	21
S. aureus R1		128	32	13 + 3.2	0.2	83
E. coli EC50	Cephalexin	8	0.25	1.9 + 0.061	0.48	19
Enterobacter OG3		16	1	1.88 + 0.12	0.24	27
K. aerogenes R16		16	2	1.8 + 0.22	0.22	25
S. aureus SAR6		4	64	0.47 + 7.5	0.23	44
Micrococcus MG3		8	64	1.77 + 14.2	0.42	36
E. coli S114	Ampicillin	1	0.06	0.24 + 0.015	0.48	21
Enterobacter OG4		8	4	0.67 + 0.33	0.17	33
K. aerogenes R2		16	8	2.66 + 1.33	0.33	15
S. aureus SA22		1	32	0.24 + 7.76	0.48	15
Micrococcus T14		0.5	16	0.06 + 1.9	0.24	47

Spheroplasts. In the presence of alafosfalin (100, 500 μg/ml) *Proteus mirabilis* cultures showed complete conversion to large spheroplasts after 8 h. These spheroplasts were viable and reverted to rods in alafosfalin-free broth. At lower concentrations the typical penicillin "bow-tie" forms and filaments were observed together with lozenge-shaped forms typical of mecillinam action. Spheroplasts were formed with other Gram-negative rods.

Turbidimetric studies with susceptible Gram-negative strains showed that alafosfalin was bactericidal at 4–8 times the M.I.C., but for Gram-positive organisms it was generally bacteristatic.

Experimental Studies in Animals

The activity of alafosfalin in vivo has been demonstrated (ALLEN et al., 1979b) against *E. coli, K. aerogenes,* and *S. fecalis,* using the mouse septicema model (Table 4). Unpublished investigations have confirmed activity, also, in a mouse model using *Enterobacter cloacae, Salmonella sp., Citrobacter sp.,* and *S. aureus* (CLEELAND, personal communication). The potency, in vivo, reflects the order of antibacterial activity observed in the in vitro studies.

Pharmacokinetic investigations in mice dosed with alafosfalin subcutaneously (ALLEN et al., 1979a) indicated rapid absorption and rapid elimination (half-life 10–11 min). For rats (sc) the half-life was approximately 20 min, for baboons 1 h. Alafosfalin was well absorbed, orally, but there was substantial first-pass metabolism in these animals. Peroral administration of the drug at doses up

Table 4. In vivo activities of alafosfalin and selected β-lactam antibiotics against infections in the mouse septicemia model

Organism	Subcutaneous CD_{50} (mg/kg)[a]			
	Alafosfalin	Ampicillin	Mecillinam	Cephalexin
E. coli 1346	6.2 (5.0–7.5)	0.8 (0.6–1.0)	2.8 (1.2–6.4)	9.2 (7.0–12.2)
E. coli 257	9.7 (7.8–12.1)	ND[b]	ND	ND
E. coli 5152[c]	52.3	>500	>250	ND
K. aerogenes 2	37 (28–49)	162 (123–214)	>400	25 (18–33)
K. aerogenes 9	35 (25–50)	>400	>400	>400
K. pneumoniae 503–988[c]	66.5	12	6	ND
E. cloacae 9456[c]	79	196	27	ND
P. mirabilis 190[c]	939	5	17	ND
P. vulgaris 48[c]	57	ND	0.71	ND
C. freundii 8ASM[c]	176	>500	>500	ND
S. faecalis 404	38 (31–48)	6.5 (3.8–11.1)	ND	>400
S. aureus (Smith)[c]	25	ND	ND	ND
S. aureus (Schoch)	>400	1.3 (0.7–2.3)	>400	26 (14–49)

[a] CD_{50}, 50% curative dose. Numbers in parentheses are 95% confidence limits.
[b] ND, Not determined.
[c] Data were obtained by R. CLEELAND et al. with the mouse septicemia model

to 1 g/kg indicated that the plasma concentration was directly related to the dose. The analysis of rat tissue 1 h after administration established that high levels of [^{14}C]-alafosfalin were in the kidney, plasma, lung and small intestine but relatively low concentrations were in the liver and central nervous system. A significant proportion of the dose was excreted as unchanged drug for all the species investigated. Alafosfalin, po, may be absorbed from the intestine by the action of a facilitated transport system rather than by diffusion. This is suggested by the more efficient absorption of the phosphonodipeptide than L-Ala(P), and evidence that the uptake process could be saturated. Moreover, experiments using alafosfalin in the presence of small natural peptides indicate competition for this transport system (ALLEN, unpublished).

Studies in Man

Pharmacokinetics. When the pharmacokinetic studies in the mouse, rat, and baboon were extended to man (ALLEN and LEES, 1980), somewhat similar results were obtained. The drug was well absorbed from the gastro-intestinal tract and approximately half of a 500 mg dose reached the general circulation, the remainder was hydrolyzed to L-alanine and L-aminoethylphosphonic acid. It was found that first-pass metabolism was less than in the case of the animal studies. Unchanged drug and metabolite are mainly excreted. The concentration

of alafosfalin excreted in the urine of healthy volunteers was dose dependant; recovery from urine for doses of 50 mg and 2500 mg increased from $6 \pm 1\%$ to $17 \pm 1\%$. In the case of subjects with impaired glomerular function less alafosfalin was excreted.

The rates of absorption and elimination of alafosfalin have been compared with those of cephalexin to provide a basis for use of combinations. It has been shown in in vitro studies that such combinations reduced the potential for development of resistant strains. When these two antibacterials were co-administered to healthy volunteers, both compounds were absorbed, distributed, and eliminated at closely similar rates. Oral administration of 500 mg alafosfalin with 250 mg cephalexin gave approximately equal concentrations of the drugs in plasma and a fourfold excess of cephalexin in the urine. Such a combination is envisaged for the therapy of urinary tract infections.

Clinical Studies

Clinical trials of alafosfalin administered by im and oral routes have been carried out in several centers in Europe and South America. In all studies, alafosfalin was exceptionally well tolerated. There was a very low incidence of side effects.

In the management of acute enteropathic *E. coli* diarrhoeal disease of infants (HIDALGO, unpublished) intramuscular doses of 10 mg/kg/day resulted in an overall success rate similar to conventional gentamicin therapy; alafosfalin treatment was successful in approximately 80% of cases. Further randomized studies comparing alafosfalin with bactrim and gentamicin (HIDALGO) and with chloramphenicol (BWIBO, unpublished) indicated that the drug was at least as effective as standard agents for the treatment of acute bacterial enterocolitis in infants, children, and adults, and for the treatment of shigellosis in children and adults. (ORTEGA and RAMIREZ; MEYRAN, unpublished results).

Mechanism of Action of Alafosfalin

Investigations by STROMINGER and others had established by the middle 1960's that penicillins and related β-lactams owed their antibacterial activity to inhibition of bacterial cell-wall biosynthesis (TIPPER and STROMINGER, 1965; OSBORN, 1969). At the molecular level this was attributed to a similarity of the molecular conformation of the penicillin to the D Ala D Ala fragment involved in cross-linking, an essential process in the formation of the bacterial cell wall. Penicillin inhibited the bacterial transaminase which accepted as a substrate the terminal $-$D Ala D Ala segment regularly occurring in the pentapeptide precursor of the cross-linked cell-wall structure (Fig. 1).

These studies on the course of biosynthesis of the bacterial cell wall and evidence that effective antibiotics such as D-cycloserine and vancomycin, as

Table 5. Effect of L- and D-Ala(P) on cell-wall enzymes[a]

Enzyme	K_m (mM)	K_i (mM)			Ratio of K_m to K_i		
		L-Ala(P)	D-Ala(P)	D-Cycloserine	L-Ala(P)	D-Ala(P)	D-Cycloserine
Alanine racemase (*E. coli*)	0.93	0.03	0.01	0.65	30	90	1.4
Alanine racemase (*S. faecalis*)	9	Irrev. (0.4)[b]	Irrev. (0.2)	Irrev. (0.1)			
UDP-NAMA-L-Ala synthetase (*S. aureus*)	0.3	3	Inact.[c]	Inact.	0.1		
D-Ala-D-Ala synthetase (*S. faecalis*)	0.5 20	Inact.	0.6 3	0.05 0.1		1 7	10 200
D-Ala-D-Ala adding enzyme (*S. faecalis*)	0.1	Inact.	Inact.	Inact.			

[a] K_m and K_i values were determined by double-reciprocal plots for reversible inhibition. The double values for D-Ala-D-Ala synthetase are for donor and acceptor sites.
[b] Irrev., Irreversible inhibition. Rate constants for inactivation were 4.5×10^{-2} min^{-1} at 1 mM L-Ala(P) and 20×10^{-2} min^{-1} at 5 mM L-Ala(P). Apparent K_i values are shown in parentheses.
[c] Inact., Inactive at 10 mM

well as β-lactams, owed their effect to interference of biosynthesis arising from the action on the utilization of free or combined alanine, encouraged the idea that other compounds might be designed to act as antibacterials in this way. The Roche group in Britain selected the alanine-mimetic L-l-aminoethyl phosphonic acid from a range of alternatives they had investigated, and established that, combined with L-alanine to give the phosphonopeptide, alafosfalin, it inhibited the biosynthesis of the cell walls of various bacteria. In the earliest experiments this was indicated not only by the antibacterial activity of alafosfalin but also by its effect in producing spheroplasts and other aberrant forms.

The mechanism of action of alafosfalin has been elucidated (ATHERTON et al., 1979a). It has been established by experiments involving competition with natural di- and oligopeptides, as well as studies using isolated permease enzymes, that the first stage in the action of alafosfalin involves facilitated transport into the bacterial cell. The phosphonopeptide utilizes normal peptide transport mechanisms for L L-dipeptides (PAYNE, 1976).

The kinetic analysis of uptake of alafosfalin by the permease enzymes was investigated for *E. coli*. The non-linear, double reciprocal plot which was obtained indicated involvement of two non-interacting, saturable permeases. Similar data for Ala_2 and Ala_3 indicated involvement of two permeases in these cases. On the other hand, the double reciprocal plot for Ala Ala Ala (P) transport was consistent with the use of a single permease. It was shown that conversion of the natural peptide into phosphonopeptide caused at least tenfold decrease in affinity (ATHERTON et al., 1979a).

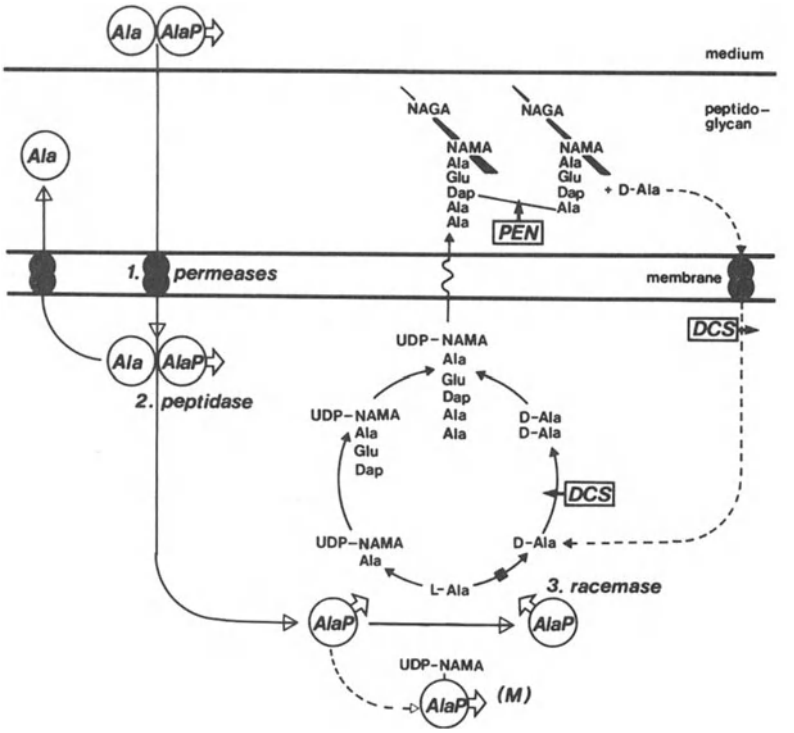


Fig. 1. Diagrammatic scheme for the mechanism of action of alafosfalin. *Abbreviations: Ala, AlaP,* alafosfalin; *DCS,* D-cycloserine; *PEN,* penicillin; *NAGA,* Nacetylglucosamine; *M,* metabolite M of Ala(P)

Differences in sensitivity and development of resistance of bacteria can be attributed largely to this first stage in the mechanism of action. As a consequence of this action of peptide permeases, alafosfalin is accumulated in the cytosol of the bacterium; the intracellular concentration may reach more than 1000 times that of the alafosfalin in the medium.

In the second stage the alafosfalin is converted, intracellularly by peptidases to L-1-amino-ethylphosphonic acid (L-Ala(P)) and L-alanine. [^{14}C]-labelling studies indicated that the L-Ala residue was not accumulated as for L-Ala(P) in the presence of chloramphenicol, presumably as a result of the preferential reversible action of the active transport on the natural amino acid. It was shown that L-Ala(P) was not transported into the cell to a significant degree. It has been established that the Ala(P) which is released acts at two points in the pathway of cell-wall peptidoglycan biosynthesis. It inhibits alanine racemase (LAMBERT and NEUHAUS, 1972) which is required for the conversion of L- to D-alanine, which is incorporated in the *terminal* residues -D Ala D Ala of the muramyl pentapeptide (Table 5).

Secondly, it interacts with UDP-NAMA in the presence of ligase (ITO et al., 1966), simulating L-alanine; further muramyl peptide chain extension is precluded since the phosphonic, unlike the carboxylic, function cannot form a

peptide bond. Similarly, this limitation ensures that Ala(P) does not act as a false substrate in protein biosynthesis.

Figure 1 summarizes the current knowledge of the mechanism of action of alafosfalin. This mechanism also provides an explanation of some of the characteristics of the agent. The greater sensitivity of Gram-negative than Gram-positive bacteria to alafosfalin is probably due to the combined effects of higher cytosol pool levels of L-alanine (TEMPEST and MEERS, 1970) and, normally, reduced intracellular peptidase activity for the latter group.

Structure–Activity Relationships

A number of analogues of alafosfalin have been investigated as antibacterial agents. Apart from phosphonopeptides incorporating Ala(P), only those based

Table 6. Effect of chain length on the in vitro antibacterial activity of phosphonopeptides having the formula (L-Ala)$_n$-L-Ala (P)

Organism	MIC of (L-Ala)$_n$-L-Ala(P) (μg/ml)				
	$n=1$	$n=2$	$n=3$	$n=4$	$n=5$
E. coli NCIB8879	0.5	0.5	0.12	0.5	1.0
E. coli 33[a]	0.12	0.5	0.12	0.5	0.12
S. aureus (Oxford)	8	8	32	>128	>128
S. aureus O-G SAR1[a]	8	8	64	>128	>128
Enterobacter sp. 1398	4	8	>128	>128	>128
Serratia marcescens ATCC14756	8	4	4	16	>128
K. aerogenes O-G KA1	0.25	0.12	0.5	0.5	0.5
S. faecalis O-G FS5	2	0.06	0.25	0.25	0.25
H. influenzae NCTC4560	4	0.06	0.06	0.06	0.5
S. typhimurium S12	4	32	128	>128	>128

[a] Ampicillin-resistant strains.

Table 7. Antibacterial activity of alanine phosphonopeptides with mixed stereochemistry [(Ala)$_n$-Ala(P)][a]

Organism	MIC (μg/ml) of compounds with the following stereochemistry:							
	LLL	LLD	DLL	DLLL	LLLD	LDLL	DLLLL	LDLLL
E. coli 33	0.5	>512	256	2	>512	>512	4	16
E. coli NCIB8879	0.5	>512	256	2	>512	>512	4	64
K. aerogenes O-G KA1	0.12	>512	>512	32	>512	>512	16	>128
K. aerogenes Typ 33BA	8	>512	>512	32	>512	>512		
S. faecalis O-G FS1	0.12	16	128	32	32	64		>128
S. faecalis O-G FS5	0.06	64	256	32	32	128	>512	4
H. influenzae OGHI-1	0.06	128	128	64	32	64	<0.5	4

[a] C-terminal L or D refers to L- or D-Ala(P). All other L and D refer to alanine

Table 8. Antibacterial activities of phosphonodipeptides having the formula X-L-Ala P

Organism	MIC (μg/ml) of the compounds having the following N-terminal amino acids (X) in the dipeptide X-L-Ala(P)[a]							
	Met	Phe	Ala	Val	Lys	Pro	Gly	γ-Glu
E. coli NCIB8879	0.12	0.5	0.5	0.5	4	64	16	>256
K. aerogenes KA1	0.12	1	0.5	0.25	4	128	16	32
Enterobacter sp. 1398	1	2	1	2	16	>256	128	>256
S. faecalis FS5	1	2	2	8	16	64	>256	32
S. aureus NCIB8625	1	4	8	8	16	>256	>256	>256
S. marcescens ATCC14756	2	4	8	8	32	>256	>256	>256
S. typhimurium S12	8	32	64	64	>256	>256	>256	>256
H. influenzae NCTC4560	16	32	32	64	128	16	>256	>256
Proteus mirabilis 92	32	128	>256	>256	>256	>256	>256	>256

[a] Abbreviations: Met, L-methionyl; Phe, L-phenylalanyl; Ala, L-alanyl; Val, L-valyl; Lys, L-lysyl; Pro, L-prolyl; Gly, glycyl; γ-Glu, γ-glutamyl.

on Gly(P) had significant antibacterial properties, but these were generally less interesting than for the Ala(P) series. (ALLEN et al., 1979b). Variation in the length of the oligopeptide chain with same or different amino acid residues and with variations in the L- or D- character of the residues produced differences in the antibacterial properties (ATHERTON et al., 1979b). These differences, in vitro, could be attributed, generally, to the effect of the changes in molecular structure on permease-facilitated transport into the bacterial cell (PAYNE, 1975), or on the release by peptidases, intracellularly, of Ala(P). Tables 6, 7, 8 illustrate the effect of the change in molecular structure (ATHERTON et al., 1979b) of Ala(P) phosphonopeptides on the antibacterial activity in vitro. Evidently, in vivo antibacterial activity of phosphonopeptides will be determined also by such factors as tissue distribution and metabolism in the host.

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Arabinosylcytosine

W.A. CREASEY

Introduction

Arabinosylcytosine (1-β-D-arabinofuranosylcytosine; cytosine arabinoside; cytarabine; ara C) belongs to a class of nucleosides with D-arabinose as the pentose sugar moiety. The inversion of the 2′-hydroxyl group as compared with ribonucleosides (Fig. 1) conveys markedly different properties, and in many respects the arabinosides are biologically more akin to 2′-deoxyribonucleosides. Several arabinosides, including arabinosylthymine and arabinosyluracil (ara U), occur naturally in the sponge *Cryptotethya crypta* (BERGMANN and FEENEY, 1951; BERGMANN and BURKE, 1955), and COHEN (1963) has reviewed the possible biological significance of such nucleosides. The arabinosides that have shown antitumor activity, however, have been obtained synthetically, beginning with ara C in 1959 (WALWICK et al., 1959). Ara C is now the most active antimetabolite for inducing remissions in nonlymphocytic leukemia (ELLISON, 1968), and in combination with anthracyclines, such as daunorubicin, leads to complete remission rates in the range of 60% to 70% (KREMER, 1975). Arabinosyladenine (vidarabine; ara A) was originally synthesized as an anticancer drug (LEE et al., 1960), but has since acquired a more important role as an antiviral agent, particularly for viruses of the herpes group (PAVAN-LANGSTON, 1975).

The basic (CREASEY, 1975; MALEY, 1977) and clinical (HO and FREIREICH, 1975) pharmacology of ara C, and its usefulness in treating leukemia (CLARYSSE et al., 1976), have been described earlier, and ara A has been reviewed more recently (CASS, 1979). In this chapter, we shall summarize the available information, stressing later data that may shed more light on the action of ara C and its derivatives.

Physicochemical Properties

Ara C has pK_a values of 3.85 and >13, melts at 212–213.5°, and shows absorption maxima of 279 nm (E = 13,400) at pH 2 and 272 nm (E = 9,500) at pH 12 (COHEN, 1966).

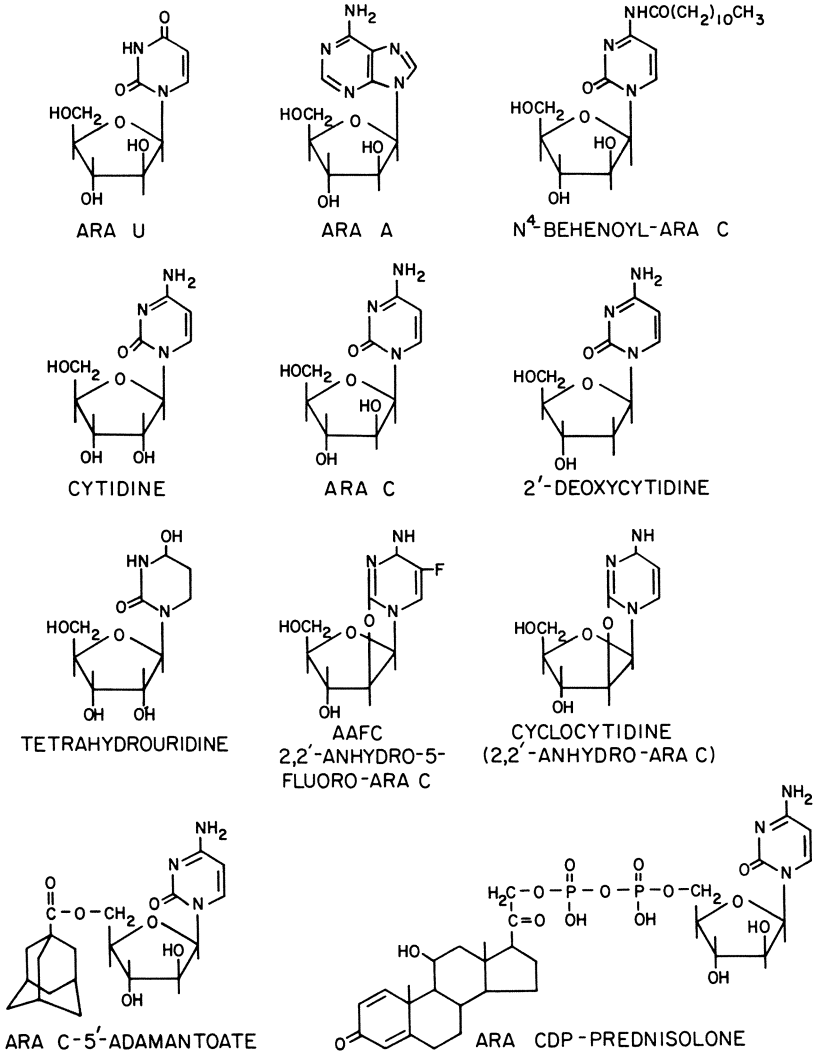

Fig. 1. Structures of ara C and related compounds

Synthesis

A variety of approaches have been used to synthesize arabinosides (COHEN, 1966; SUHADOLNICK, 1970). Among the early synthetic routes we might mention treatment of tritylribofuranosylthymine with methanesulfonyl chloride, followed by reaction with aqueous methanol and ammonia, and then acid hydrolysis to give arabinosylthymine (FOX et al., 1957), and phosphorylation of ribonucleosides with polyphosphoric acid, and subsequent hydrolysis of the cyclized prod-

ucts to give arabinoside diphosphates (WALWICK et al., 1959; ROBERTS and DECKER, 1967). The N^4-substituted derivatives of ara C and 5-fluoro-ara C were prepared by thiation with phosphorus pentasulfide, alkylation, and reaction with nucleophiles (WEMPEN et al., 1968). Substituted 1-β-D-xylofuranosyl nucleosides blocked at the 3′- and 5′-positions have served as starting materials; a 2′-O-alkylsulfonyl group was introduced, and further transformation to arabinosides occurred via the 2′,3′-epoxy derivatives (HUNTER, 1965). Some of the earlier routes to ara C gave rather low yields; subsequently more efficient syntheses were devised (e.g., HESSLER, 1976). The 5′-O-methyl derivatives of ara C and ara U (GIZIEWICZ et al., 1972), as well as 2,2′-O-cyclocytidine (KANAI et al., 1970), were prepared from appropriate precursors by reaction with partially hydrolyzed phosphorus oxychloride. Methylation with dimethylsulfate (DARZYNKIEWICZ et al., 1972) and acylation with acid anhydrides (WARNER et al., 1972; MONTGOMERY and THOMAS, 1972) have been used to prepare various substituted arabinosides. Among the derivatives that have been synthesized more recently are conjugates obtained by condensing steroid-21-monophosphates with ara CMP-morpholidate (HONG et al., 1980), and N^4-acyl compounds such as behenoyl-ara C (KATAOKA and SAKURAI, 1980; YAMADA et al., 1980). Ara A was prepared initially in quantity by condensing 2,3,5-tri-O-benzyl-D-arabinofuranosylchloride with N-benzoyladenine (GLAUDEMANS and FLETCHER, 1963).

Structure–Activity Relationships

As might be expected, structural modifications may greatly alter the antitumor, antiviral, and immunosuppressive actions of ara C and other arabinosides. Thus, the L-enantiomer of ara C has no antitumor activity (TOLMAN and ROBINS, 1971), while both deamination of ara C to ara U (CHU and FISCHER, 1962), and N^4-butyrylation of di-O-esters of ara C (MONTGOMERY and THOMAS, 1972) or N^4-methylation or hydrazination of ara C itself (DOLLINGER et al., 1967) lead to inactive compounds. On the other hand, other N^4-reactions that yield N^4-hydroxy derivatives (BURCHENAL et al., 1967) or N^4-acyl compounds, such as behenoyl ara C (KATAOKA and SAKURAI, 1980), do not lead to loss of activity. However, both certain N^4-substituted compounds and 5′-esters such as the adamantoate of ara C (NEIL et al., 1970), as well as other long-chain esters (MONTGOMERY and THOMAS, 1972), may lose the schedule dependence characteristic of ara C and function in some respects as depot forms of this nucleoside. Substitutions at the 5-position of the cytosine ring do not generally lead to loss of activity (LENAZ et al., 1969). Other active derivatives include the 3-N-oxide of ara C (PANZICA et al., 1971), the steroid conjugates (HONG et al., 1980), and thioarabinosylcytosine (BREMERSKOV et al., 1970). Cyclocytidine, like the N^4-derivatives, is resistant do deamination, and through its slow conversion to ara C achieved a better therapeutic index than the latter against L1210 (HOSHI et al., 1971). Analogous to this is 2,2′-anhydro-1-β-D-arabinofuranosyl-

Table 1. Cytotoxic activity of ara-C derivatives in vitro

Cell type	Reference	Concentration for 50% inhibition (ID_{50})							
		ara C	ara A	ara FC[a]	ara FU[b]	AAFC	ara CMP	ara CMP[c] -pred	ara CMP[d] -cort
L5178Y	2	1×10^{-7}							
L5178Y	1	1.2×10^{-7}		1.2×10^{-7}	7.6×10^{-6}	1.2×10^{-7}			
L5178Y/AraC	1	1.2×10^{-4}		1.2×10^{-4}	2.3×10^{-5}	3.3×10^{-6}			
P815	1	2.5×10^{-8}		1.2×10^{-8}	7.6×10^{-6}	2.5×10^{-8}			
P815/AraC	1	2.5×10^{-5}		4×10^{-5}	7.6×10^{-6}	2×10^{-6}			
L1210	3	1×10^{-7}					5×10^{-8}	3×10^{-8}	8×10^{-8}
Human myeloid precursor	4	4.1×10^{-8}	3×10^{-7}						

References: (1) CHU and FISCHER, 1962; (2) BURCHENAL et al., 1975; (3) HONG et al., 1980; (4) NISSEN et al., 1978.
[a] 5-fluoro-ara C,
[b] 5-fluoro-ara U,
[c, d] Prednisolone and cortisol conjugates of ara CMP

Table 2. Comparative activity of ara C derivatives against L1210 in vivo

Compound	ILS[a] (%)	Reference
AraC	70	HOSHI et al., 1971
Cyclocytidine	>300	
AraC-3-N-oxide	167	PANZICA et al., 1971; NEIL et al., 1970
AraC-5′-adamantoate	300	
AraC	80	
3,5-Di-O-butyryl-araC	70	MONTGOMERY and THOMAS, 1972
2,3,5-Tri-O-butyryl-araC	174	
Ara CMP-prednisolone	116	HONG et al., 1980
Ara CMP-cortisol	86	
AraC	65	
N^4-Behenoyl-araC	300	KATAOKA and SAKURAI, 1980
AraC	89	
N^4-Stearoyl-araC		
solution	150	AOSHIMA et al., 1977
suspension	>200	

[a] Increase in life span of treated compared with control mice

5-fluorocytosine (AAFC), which is of interest because of its apparent lack of cross-resistance to ara C (BURCHENAL et al., 1975).

Other arabinosides that do not have cytosine derivatives as the base also may be active. Ara A is important for its antiviral action, but it has antitumor activity (CASS, 1979); it is less active than ara C as a suppressor of myeloid precursor cells (NISSEN et al., 1978). Arabinosyl-6-mercaptopurine (KIMBALL et al., 1966), and the arabinosides of guanine, hypoxanthine, and 5-fluorouracil (COHEN, 1966) are also active inhibitors of tumor growth. Some information on the activity of various analogs appears in Tables 1 and 2.

Assay Methods

Early pharmacokinetic studies of ara C utilized tritium-labeled drug. After preliminary determination of the levels of radioactivity in body fluids, ara C was separated chromatographically, usually on paper, from its metabolite ara U. An example of this type of approach is the study of CREASEY et al. (1966).

Bioassay has been used extensively in determining ara C in body fluids. For example, actinobolin-resistant strains of *Streptococcus faecalis* (HUNT and PITILLO, 1968) have proved to be sensitive organisms for this assay; tetrahydrouridine may be added to the assay system to inhibit deamination of ara C, bringing the limit of detection down to 0.1 μg/ml (HANKA et al., 1970). L cells growing in culture have also been used for bioassay of arabinosides (BORSA et al., 1969).

Biochemical assays include on the one hand measurement of the inhibition of incorporation of tritiated thymidine into DNA by L cells or mouse spleen cells in suspension, which is sensitive to as little as 0.04 μg/ml (BAGULEY and FALKENHAUG, 1971), or rat bone marrow cells, reported to be sensitive to 0.003 μg/ml (VAN PROOIJEN et al., 1976), and on the other hand, phosphorylation of ara C with ATP-γ-[^{32}P] and isolation of ara CMP by thin-layer chromatography on DEAE cellulose. This method could detect ara C concentrations down to 0.6 μg/ml (MOMPARLER et al., 1972).

Radioimmunoassay has employed a sheep antiserum to an ara CMP-ovalbumin conjugate. The methodology involved no need for prior extraction of plasma, urine or cerebrospinal fluid samples, was completely cross-reactive to ara CMP, ara CTP and ara C, but only 0.008% cross-reactive to ara U. Plasma levels down to 1 ng/ml could be detected (PIALL et al., 1979).

For gas-chromatographic separation, initial deproteinization and extraction of interfering materials with ether and isoamyl alcohol was necessary. Both acetylation and methylation were employed to give suitable derivatives. For plasma samples, gas chromatography with a nitrogen-sensitive detector had a lower limit of 40–70 ng/ml, whereas gas chromatography–mass spectrometry could be sensitive to 1 ng/ml (BOUTAGY, 1978).

High-pressure liquid chromatography of deproteinized plasma using ammonium formate elutants and a Partisil column was capable of detecting as little as 20 ng/ml of ara C (BURY and KEARY, 1978).

Pharmacokinetics

Although in aqueous solution ara C rather readily undergoes deamination to ara U in a reaction catalyzed by such anions as bisulfite, lactate, and phosphate (NOTARI et al., 1972), this spontaneous process does not appear to be important in vivo. Predominantly, ara C is deaminated enzymatically by a pyrimidine nucleoside deaminase, also known as cytidine deaminase. This enzyme was first described in dog liver (SCHMIDT, 1932) and mouse kidney (GREENSTEIN et al., 1947). Subsequently it was identified in *Escherichia coli* (PIZER and COHEN, 1960) and a wide range of animal and human tissues (CREASEY, 1963; CAMIENER and SMITH, 1965; HALL and LEVINE, 1967; STOLLER et al., 1978). Both the mouse kidney (CREASEY, 1963) and human liver (CAMIENER, 1967) enzymes are sulfhydryl-dependent, have virtually no activity for free bases, and deaminate 5-halogenated derivatives more rapidly than unsubstituted compounds. The K_m of the human enzyme was measured with ara C as 1.2 to 1.6×10^{-4} M, and a 2-keto group, an unsubstituted N^3, free 3′-α-hydroxyl groups, and a nonphosphorylated 5′-hydroxyl were necessary for activity. The activity of inhibitory analogs was increased by N^4 substitution in the order: methylamino; keto; hydrazino; thio; amino; hydroxyamino. In the search for effective inhibitors of cytidine deaminase, the most promising compound developed was tetrahydrouridine (Fig. 1), formed by catalytic reduction of cytidine in the presence of rhodium and subsequent hydrolysis (HANZE, 1967). The compound is an inhibitor of the partial or regulatory type and preincubation is needed for optimal action (CAMIENER and SMITH, 1968).

In the earliest studies of ara C disposition in humans, plasma levels were undetectable within 20 min (CREASEY et al., 1966; TALLEY et al., 1967), and the relatively rapid urinary excretion (up to 91% in 24 h) was largely (86%–96%) in the form of ara U. Subsequent, more definitive, studies have described biphasic plasma clearance curves with initial t 1/2 values of 3 to 9 min (BAGULEY and FALKENHAUG, 1971), 12 min (MULLIGAN and MELLETT, 1968; HO and FREI, 1971), and 15 min (MOMPARLER et al., 1972). The secondary t 1/2 is more prolonged, as much as 111 min according to HO and FREI (1971). A more recent study (VAN PROOIJEN et al., 1976) has, however, reported initial and secondary t 1/2 values of <2 and 9–16 min, respectively. During continuous intravenous infusions at the commonly used therapeutic dose of 200 mg/m^2 for 5 days, plasma levels were around 0.15 μg/ml (BODEY et al., 1969). When administered into the lateral ventricles of patients with implanted Ommaya reservoirs, ara C was cleared very slowly with a t 1/2 of 5 to 11 h (CREASEY et al., 1968b). A value for the t 1/2 of about 2 h was reported by HO and FREI (1971) for ara C when administered intrathecally. The same workers measured concentrations of ara C in cerebrospinal fluid that were 40% of those in the coincident plasma after systemic administration of drug. MORRISON et al. (1975) described a computer-based multicompartment model for levels of ara C and ara CTP that included predictions of the degree of inhibition of DNA synthesis that might be achievable.

As regards the comparative pharmacokinetics of ara C in other species, the t 1/2 in mice has been estimated as 16 min (BAGULEY and FALKENHAUG,

1971) and 20 min (BORSA et al., 1969). In the report of MULLIGAN and MELLETT (1968), the half-lives of ara C in plasma ranged from 95 min in dogs, through 43 min in rats, 35 min in hamsters, to no detectable levels of ara C in monkeys. Studies undertaken in dogs and monkeys have also shown that pretreatment with tetrahydrouridine greatly prolonged the plasma t 1/2 of ara C and increased the amount of unmetabolized drug in the urine (MULLIGAN and MELLETT, 1968).

Among the derivatives whose pharmacokinetics have been studied are AAFC (KREISS, 1975), cyclocytidine (HO et al., 1975) and N^4-behenoyl-ara C (YAMADA et al., 1980). In the case of AAFC administered to humans, the bulk of the material was excreted unchanged in the urine, and the plasma t 1/2 was about 1 h. Cyclocytidine was also quite rapidly excreted (60% in 5 h), and nearly half of the urinary material was unchanged drug in dogs. The primary and secondary half-lives in this species were 40 min and 2.0 to 2.5 h. In humans the plasma t 1/2 values for behenoyl-ara C were 60 and 180 min, while in monkeys the corresponding figures were 40 and 120 min.

Toxicity

A. Hematologic

Leukopenia and thrombocytopenia are the limiting toxic effects of ara C (BERNARD et al., 1966; HENDERSON and BURKE, 1965; LIVINGSTON and CARTER, 1968; TALLEY and FREI, 1968). The major depression among the white cell elements occurs in the granulocyte series; reticulocytopenia is more often seen with more prolonged infusions and higher dosages. The leukocyte nadir usually occurs within one week, and recovery is complete in two, provided the bone marrow is not compromised initially. In the bone marrow itself, a megaloblastic state is seen that was first reported by TALLEY and VAITKEVICIUS (1963). This situation is readily reversed after therapy is stopped. It is generally considered to result from inhibition of DNA synthesis in the face of continued synthesis of RNA and proteins. Eventually, this imbalanced growth situation leads to loss of the ability to proliferate; a similar situation apparently holds true in KB cells (KARON et al., 1966).

B. Gastrointestinal

Mucositis, nausea, vomiting, and occasional mild transient elevation of liver enzymes in the blood, occur during therapy with ara C. These symptoms are not usually pronounced enough to justify discontinuation of therapy (LIVINGSTON and CARTER, 1968). Histologically, the cells of the LIEBERKÜHN's crypts show imbalanced growth and partial synchronization during recovery (VERBIN et al., 1972).

C. Miscellaneous

Ara C has been reported to lead to motor dysfunction, arachnoiditis, and altered mental status after intrathecal administration (BAND et al., 1973). A variety of nuclear and chromosomal abnormalities have been described in cells treated with ara C. They include nuclear blebs and vacuoles (AHEARN et al., 1967; HENEEN and NICHOLS, 1967), chromosome breaks (KIHLMAN et al., 1963; BREWEN, 1965; BENEDICT et al., 1970) and deletions (FAHMY et al., 1966; HISS and PRESTON, 1977). Since exposure to UV light, which decreased incorporation of thymidine into DNA, also reduced the incidence of chromatid breaks (BENEDICT and KARON, 1971), there is probably a relation between the formation of such breaks and DNA synthesis and repair. As might be expected, such lesions lead to teratogenic effects in rat embryos (CHAUBE et al., 1968; RITTER et al., 1971), and ara C has been reported to be mutagenic and oncogenic (BENEDICT and JONES, 1979). These findings could be significant in terms of long-term toxicity.

Cyclocytidine can produce similar toxicity to ara C but, in addition, it causes parotid pain and postural hypotension quite frequently (BURGESS et al., 1977).

Experimental Antitumor Activity

Ara C is active against a number of experimental tumors carried in rodents (Table 3). Although the drug is active against established as well as newly im-

Table 3. Experimental tumors reported responsive to ara C

	Tumor		Reference
Mouse	Ehrlich ascites	L5178Y	EVANS et al., 1964
	S180 ascites	T4lymphoma	
	S180 solid	L1210	
	L1210	P388	DIXON and ADAMSON, 1965
	P288	K1964	
	L1210	C1498	WODINSKY and KENSLER, 1965
	L1210/MTX	P815 (4 strains)	
	L1210/38280	P329	
	P388 (5 strains)	RCS	
	P1534	Mecca lymphosarcoma	
	P1534J	Lymphoma 2	
	L5178Y	Lymphoma 4	
	P288	P1798	
	AK4	Ca755	
	P1081	S180	
Rat	Walker 256	Novikoff hepatoma	

Tumors are listed that showed >50% increase in survival time or >50% inhibition of solid tumor growth at optimum dose schedule.

planted tumors, its efficacy does decline with the age of the neoplasm (GRISWOLD et al., 1970). Studies carried out in vitro have shown that *Escherichia coli* (SLECHTA, 1961), L5178Y (CHU and FISCHER, 1962), HeLaS-3 (KIM and EIDINOFF, 1965), and Don C cells (YOUNG and FISCHER, 1968) are sensitive to ara C, and this inhibition of growth may be prevented or reversed by 2′-deoxycytidine. Evidence for imbalanced growth, discussed above, included the formation of giant cells, accumulation of RNA and protein, and partial synchronization after recovery.

As an inhibitor of DNA synthesis, ara C appears to affect primarily those cells in S phase of the cell cycle, but there is also evidence of an effect at the late G_1-S phase transition (KARON and SHIRAKAWA, 1969; TOBEY, 1972). On the basis of its S-phase selectivity, SKIPPER et al. (1970) developed experimental treatment protocols for L1210 leukemia. The thymidine labeling index may serve as a useful guide to optimal retreatment intervals (STRAUS and MORAN, 1978).

Clinical Antitumor Activity

The only disease for which ara C is a preferred drug is acute myelocytic leukemia (CLARYSSE et al., 1976). Ara C is most effective when given by continuous intravenous infusions or 8-hourly injections or infusions for up to 120 h, with rest periods of 9 or more days between courses to permit recovery of normal marrow cells. This is in accord with its primary action on S-phase cells; long exposure would extend the cytotoxic action to a larger percentage of the cell population (MOMPARLER, 1974). High-dose therapy (up to 7.5 g/m^2) with ara C has been used in refractory leukemias, with moderate success (RUDNICK et al., 1979), but in general, the nucleoside is now used primarily in combination with other drugs. For example, it forms one component of the COAP regimen, which consists of cyclophosphamide, vincristine, ara C, and prednisone (WHITECAR et al., 1972). With this treatment schedule, remissions were obtained in 56% of patients with acute myelocytic leukemia. In the CAM regimen, ara C is combined with cyclophosphamide and methotrexate; the latter drug apparently serving to increase and prolong the levels of ara CTP that are achieved intracellularly (ROBERTS et al., 1979). The CAM regimen, however, may be no more effective, and is certainly more toxic, than the combination of ara C with 6-thioguanine (SKEEL et al., 1980). This combination initially gave in excess of 50% remissions (GEE et al., 1969), and has continued to be a standard element in the chemotherapeutic approach to acute myelocytic leukemia. Studies of the combination of ara C and 6-thioguanine have shown on the one hand that there is a schedule-dependent interaction, in which increased incorporation of the thiopurine into DNA may occur (LE PAGE et al., 1972), and on the other that 6-thioguanine is a competitive inhibitor of cytidine deaminase in cultured leukocyte samples from leukemic patients (ROBINS, 1978). At the present time a combination of ara C with either of the anthracyclines adriamycin or daunorubicin (KREMER, 1975) is used very widely, giving remis-

sion rates of up to 70%. The combination with daunorubicin has proved very successful (more than 70% complete remissions) for reinduction therapy (STEIN, 1979), and apparently may also be used in elderly patients without dose reduction (REIFFERS et al., 1980).

Ara C does find some use as an alternate therapeutic agent for acute lymphoblastic leukemia (RIVERA et al., 1980; *The Medical Letter,* 1980) and histocytic lymphoma (KREMER, 1975). The drug has also been tested for activity against solid tumors with limited success. As a result of studies that showed sensitization of cells to acute ara C death by 5-fluoro-2′-deoxyuridine, the combination has been given in Phase I clinical trial with evidence of responses in breast, head, and neck cancer and carcinoid syndrome (CUMMINGS et al., 1979).

Immunosuppression

Like other inhibitors of DNA synthesis, ara C is immunosuppressive, affecting both primary and secondary immune responses (BUSKIRK et al., 1965; FISCHER et al., 1966; MITCHELL et al., 1969). This action may complicate the antitumor (BALDINI et al., 1968; HEPPNER and CALABRESI, 1972) and antiviral (GRAY, 1975) action of ara C; the 5′-acyl derivatives may be more potent than ara C itself as immune suppressors (GRAY, 1975). Attempts to use ara C to treat certain diseases belived to be of autoimmune origin, such as multiple sclerosis (GORE et al., 1979), have not proved successful.

Antiviral Action

The usefulness of ara C as an antiviral agent was identified as early as 1962, when it was shown to be effective in treating herpes simplex keratitis of the eye (UNDERWOOD, 1962). Since then the list of known sensitive viruses has increased to include adenoviruses 2, 7, and 12 (FELDMAN and RAPP, 1966), vaccinia (RENIS and JOHNSON, 1962), pseudorabies, swine and fowl pox, and B viruses (BUTHALA, 1964), Rous sarcoma (BADER, 1966), avian sarcoma (TEMIN, 1967), Rauscher and Maloney murine leukemia viruses (HIRSCHMAN et al., 1969). The apparently more pronounced antiviral action of ara A, together with its lesser myelosuppressive action (CASS, 1979), have diverted attention away from ara C for clinical use in treating virus disease.

Biochemistry of Ara C

The biochemistry of ara C will be dealt with under the three major areas of activation, mechanism of action, and resistance mechanisms.

A. Uptake and Phosphorylation

Ara C may enter cells by some type of facilitated diffusion and requires phosphorylation for activity. As a result, there generally appears to be a correlation between response of cells to drug and their ability to form and maintain intracellular levels of ara C in phosphorylated form, principally as ara CTP (KESSEL et al., 1967; RUSTUM et al., 1978). 2′-deoxycytidine inhibits the phosphorylation of ara C (CHU and FISCHER, 1965), because both nucleosides are converted to monophosphates by the same enzyme deoxycytidine kinase. This enzyme has been purified from calf thymus in which it is present in high levels. The K_m values were 4×10^{-5} M for ara C compared with 1.4×10^{-5} M for deoxycytidine (DURHAM and IVES, 1969; MOMPARLER and FISCHER, 1968), which explains the efficacy of deoxycytidine reversal of the action of ara C. There was little or no specificity for phosphate donors, and the enzyme was markedly inhibited by dCTP, and to some extent dCDP and dCMP (DURHAM and IVES, 1969). Since deamination may occur both at the level of the nucleoside, and at the monophosphate level through the action of deoxycytidylate deaminase (MALEY and MALEY, 1972), the balance of these catabolic enzymes with respect to deoxycytidine kinase activity will determine just what the cytotoxic potential of ara C is for any particular cell type.

Conversion of ara CMP to ara CDP is the function of a specific enzyme, deoxycytidylate kinase (SUGINO et al., 1966). There is evidence that ara CMP is a better substrate for this kinase than is dCMP (HANDE and CHABNER, 1978). The final step in the phosphorylative pathway is catalyzed by a nonspecific nucleoside diphosphate kinase (NAKAMURA and SUGINO, 1966).

B. Mechanism of Action

There is still much uncertainty regarding the precise mechanism of action of ara C (CREASEY, 1975; MALEY, 1977). Early studies on deoxycytidine reversal suggested that ara C, after phosphorylation, may function as an inhibitor of ribonucleotide reductase (CHU and FISCHER, 1962), and this appeared to be consistent with experiments on the incorporation of precursors into DNA (SILAGI, 1965; KIM and EIDINOFF, 1965; CREASEY et al., 1966; CHU and FISCHER, 1968). However, indirect evidence such as the existence of an irreversible component to the damage (SILAGI, 1965), direct measurements of intracellular nucleotide pools (SKOOG and NORDENSKJÖLD, 1971; INAGAKI et al., 1969) which showed only transient effects at best on dCTP pools, and experiments on inhibition of partially purified ribonucleoside diphosphate reductase from Novikoff ascites tumor (MOORE and COHEN, 1967), effectively ruled out this enzyme as a significant site of ara C action.

Ara CTP is a competitive inhibitor of mammalian DNA polymerases (KIMBALL and WILSON, 1968; INAGAKI et al., 1969; FURTH and COHEN, 1968; GRAHAM and WHITMORE, 1970; MOMPARLER, 1972). Values for the K_i were in the range of 1 to 9 μM for ara CTP, and were thus essentially identical to the K_m values for dCTP (3 to 9 μM). As regards the different forms of polymerase, reverse transcriptase was found to be 200 times more sensitive

to ara CTP than the DNA-dependent enzyme (MÜLLER et al., 1972; TUOMINEN and KENNEY, 1972). Bacterial polymerases are generally less sensitive to ara CTP than the corresponding mammalian enzymes, and it appears that the bacterial replicase is much more sensitive than other forms that are involved in repair synthesis (RAMA REDDY et al., 1971; MASKER and HANAWALT, 1974). The same differential sensitivity has also been reported in mammalian cells, such as hepatocytes (STEINSTROM et al., 1974). However, the repair process is inhibited in mammalian cells at the higher ara CTP levels associated with toxicity, and in the presence of agents such as hydroxyurea, and interestingly the incorporated analog does not seem to be a determining feature (DUNN and REGAN, 1979).

Chain elongation, rather than initiation, is the process that is apparently affected by ara CTP (HELLGREN et al., 1979; WIST, 1980), but although evidence obtained as early as 1972 suggested that incorporated analog leads to termination of chain growth (MOMPARLER, 1972), this is by no means an established fact. Indeed, there is other evidence that enhanced release of nascent single-strand DNA occurs through preferential inhibition of polymerization rather than a termination process (DIJKWEL and WANKA, 1978). Other experimental data indicate that when DNA synthesis resumes after inhibition by ara C, there is replication of DNA segments that were already replicated before exposure to drug occurred, and this may lead directly to induction of chromosome lesions (WOODCOCK et al., 1979).

The extent to which ara C is incorporated into nucleic acids, and the precise relationship between the incorporated base and cytotoxicity has been controversial. Adequate data exist to establish that radiolabeled ara C is incorporated into both DNA and RNA of a variety of cell types (e.g., SILAGI, 1965; CREASEY et al., 1968a; CHU, 1971; ZAHN et al., 1972; NUTTER and RAPP, 1973), and that such incorporation also takes place in isolated enzyme systems (FURLONG and GRESHAM, 1971; GRAHAM and WHITMORE, 1970; MOMPARLER, 1972). However, GRAHAM and WHITMORE (1970) could find no correlation between lethality and incorporation of ara C into DNA, and as was discussed earlier, incorporated ara C apparently need not be removed during repair and plays no role in interfering with that process (DUNN and REGAN, 1979). On the other hand, the work of CHU and FISCHER (1968) and CHU (1971) showed that acute cell death could be correlated with analog incorporated into RNA, particularly the lower molecular weight fractions (2–16S). This acute cell death phenomenon is primarily associated with higher drug levels.

Other effects of ara C include inhibition of histone synthesis in L cells (SCHOCHETMAN and PERRY, 1972) and Chinese hamster cells (SMITH and CHU, 1972), not unexpected since the messenger RNA (7–9S) for histone synthesis is in the fraction with greatest ara C incorporation. Polyamine synthesis would also appear to be sensitive to ara C (RUSSELL, 1972).

Thus it is apparent that it is not yet possible to define the precise biochemical lesion most closely associated with cytotoxicity. Rather, several sites of action have been identified, and it may be that the cumulative effect of these actions, and the relative extent to which they occur in different cell types, are the determining feature.

C. Resistance to Ara C

The major mechanisms proposed to account for acquired resistance to ara C involve reduction in the activity of deoxycytidine kinase, or elevation of cytidine deaminase levels.

Reduced deoxycytidine kinase activity is by far the most frequently described feature of ara C-resistant cells, having been identified in resistant strains of L5178Y (CHU and FISCHER, 1965), L1210 (SCHRECKER and URSHEL, 1968) P815 (DRAHOVSKY and KREIS, 1970) and human leukemic cells (KESSEL et al., 1969; TATTERSALL et al., 1974). Thus it is clear that a wide range of cells may become resistant through this mechanism, but this is not true of all resistant strains, as CHANG et al. (1977) found in human cells.

An alternative hypothesis, first advanced by STEUART and BURKE (1971), invoked cytidine deaminase whose levels were found to be higher in human cells exhibiting innate and acquired resistance. They suggested that the ratio of the relative rates of phosphorylation to deamination was the critical factor that correlated with clinical response to drug. Other workers were unable to confirm a correlation between cytidine deaminase levels and remission induction (CHANG et al., 1977; SMYTH et al., 1976). Furthermore, the work of COLEMAN et al. (1975) using ficoll fractionation of lymphoid cells suggested that cytidine deaminase levels were higher in more mature cells, and that the activity of this enzyme may reflect the proportion of granulocytes rather than resistance to ara C.

In resistant lines of experimental tumors, other changes have been described. For example, an ara C-resistant mutant of L1210 described by BACH (1969) had reduced deoxycytidine kinase activity, but also showed interesting changes in the properties of its DNA polymerase such that ara CTP was a much poorer inhibitor, with a three- or fourfold change in K_i, than was true of the enzyme from the parent line; the affinity for dCTP was unchanged. There was also evidence of increased dependence on exogenous thymidine in this cell line. Finally, strains of L5178Y (MOMPARLER et al., 1968) and Chinese hamster cells (SMITH and CHU, 1972) have been described in which the pools of deoxycytidine nucleotides were elevated, possibly through enhanced nucleoside diphosphate reductase activity. This would presumably decrease the degree of inhibition of DNA polymerase exerted by ara CTP. Fundamentally, however, it is likely that change in the equilibrium level of the latter nucleotide is the final most critical factor (RUSTUM et al., 1978), and this reflects the balance between phosphorylation on the one hand, and the combined effects of deamination at the nucleoside and nucleotide levels and specific and nonspecific dephosphorylation on the other.

Efforts to Enhance the Efficacy of Ara C

Apart from the development of combination chemotherapy regimens with other antitumor agents, attempts have been made to enhance the efficacy of ara C by various pharmacological manipulations.

The short half-life of the drug has encouraged efforts to prolong cell exposure to it by methods other than the use of frequent infusions. As was discussed earlier, tetrahydrouridine is a potent inhibitor of cytidine deaminase, which was shown to markedly decrease ara C degradation in animals (MULLIGAN and MELLETT, 1968). Evidence that a similar process occurred in humans was provided by a Phase I trial of ara C in combination with tetrahydrouridine, in which myelosuppression was greatly enhanced, but no remissions were seen (WANG et al., 1979). Evaluation of the efficacy of the combination awaits completion of other studies. Cyclocytidine, which slowly releases ara C as it is metabolized, but which itself is not subject to deamination, has been disappointing in early clinical trials in acute leukemias and other childhood neoplasma (MIALE et al., 1979; FINKLESTEIN et al., 1979). Some very limited activity was also seen in solid tumors (BURGESS et al., 1977). Another slow release form that has entered clinical trial is behenoyl-ara C, a drug which showed no schedule dependency (AOSHIMA et al., 1977). In the clinic, behenoyl-ara C induced a remission in one of a series of 14 leukemic patients (YAMADA et al., 1980). Once again, further evaluation is in order.

Altered delivery of ara C has been examined from two viewpoints. In the first the aim was to tailor the magnitude of ara C doses in sinusoidal fashion to circadian rhythms. This has been done in mice bearing the L1210 tumor. It seems clear that appropriate scheduling, that is with the largest doses at 11.00 to 14.00 hours, and the minimal doses at 23.00 to 02.00 hours, leads to major reduction in toxicity (HAUS et al., 1972), such that it is possible to increase the total maximum tolerated dose by 50% to 100% (ROSE et al., 1978). However, whereas some investigators have obtained increased survival and cures of the mice using ara C alone (HAUS et al., 1972) or in combination with cyclophosphamide (SCHEVING et al., 1977), others have been unable to demonstrate such an effect (ROSE et al., 1978). In the second approach, which is one that has been applied increasingly to other antitumor agents also, liposome encapsulated ara C has been used experimentally. For example, such encapsulated drug administered into rat lungs was highly localized, and left the lungs very slowly with a clearance half-life of about 8 h (JULIANO and MCCULLOUGH, 1980). It would appear that liposomes containing 5 parts cholesterol to 4 of phosphatidylcholine and 1 part of phosphatidylglycerol were most effective in preventing leakage of encapsulated ara C (MAYHEW et al., 1979). Therapeutic trial of such complexes should be interesting.

Conclusions

In this review we have seen that ara C is a drug whose present clinical utility is rather limited, but which might have applications outside cancer chemotherapy as an antiviral and immunosuppressive drug. Its biochemistry is complex, and there is probably no one site of action that adequately explains the action of the drug. The findings may be summarized, however, as suggesting that damage to polynucleotides through interrupted synthesis, and incorpora-

tion into lower molecular weight RNA, may be critical. Resistance develops through several possible steps that lead to a less favorable kinetic situation for inhibition of DNA polymerase by ara CTP.

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Chloramphenicol

F.E. HAHN

Introduction

The most recent reviews on the mechanism of action of chloramphenicol are contained in the proceedings of a 1974 symposium (DREWS and HAHN, Edts., 1975) and in *Antibiotics* V-1 (PONGS, 1979). A computer search on chloramphenicol of the data banks of *Chemical Abstracts* and *Biological Abstracts,* covering the period from 1975 to late summer 1981, has yielded approximately 1000 titles, but little of this large literature is concerned with the mode or mechanism of action of chloramphenicol.

The antibiotic inhibits specifically protein biosynthesis in prokaryotic organisms such as bacteria and mitochondria. The past 20 years have seen a large volume of studies of the effects of chloramphenicol in cell-free bacterial ribosome systems. These have either employed synthetic polynucleotide messenger RNA's and are much less sensitive to chloramphenicol than in vivo protein biosynthesis (HAHN and GUND, 1975), or they have measured the formation of single peptide bonds, using puromycin as a catholic peptidyl acceptor and either an N-formylmethionyl-hexanucleotide fragment (MONRO and MARCKER, 1967), polylysyl-tRNA (COUTSOGEORGOPOULOS, 1967) or acetyl-phenylalanyl-tRNA (PESTKA, 1970) as peptidyl donors. Systems which transfer preformed peptides to puromycin are more sensitive to chloramphenicol than polyribonucleotide systems which start the reaction by mistakenly placing amino acyl-tRNA into the P (peptidyl)-site of the peptidyltransferase system under the influence of excessive concentrations of Mg^{2+}.

The activity of chloramphenicol on reactions, carried out by 50S prokaryotic ribosomal subunits, is not limited to peptidyl transfer. The antibiotic also inhibits transesterification such as the transfer of N-formylmethionine in the "fragment reaction" to the α-hydroxyl group of the phenyl-lactic acid amide of the puromycin base (FAHNESTOCK et al., 1970) or the synthesis of polyphenyllactic acid from phenyl-lactyl-$tRNA^{phe}$ (FAHNESTOCK and RICH, 1971). It may have been forgotten by the field that SMITH et al. (1949) discovered inhibitions of bacterial esterases by chloramphenicol.

Problems in the Mechanism of Action of Chloramphenicol

The fragment reaction with puromycin as peptidyl acceptor, a variety of F-met oligonucleotides as peptidyl donors, ethyl alcohol, and 50S ribosomal subunits (but without 30S subunits, messenger RNA's, or supernatant "factors") catalyzes the synthesis of single peptide bonds; this reaction is inhibited by chloramphenicol (MONRO et al., 1969). The experiment eliminates by design the cryptographic role of the whole intact ribosome in the sequential readout of codons in messenger RNA. The fact that intact ribosome systems with synthetic messengers are subject to only weak inhibition by chloramphenicol which is a direct function of the cytidylic acid content of copolymeric polynucleotide messengers (HAHN and GUND, 1975), as shown in Fig. 1, is perhaps explained by the observation of PONGS (1975) that the affinity of monoiodoamphenicol for whole intact 70S ribosomes is one order of magnitude greater than that of the affinity for the 50S subunit. The monoiodo affinity label binds inter alia to the 30S component protein S3. Should the 30S subunit contribute to the chloramphenicol binding site of the intact ribosome, this could suggest that this contribution influences the cryptographic readout of messenger RNA with a preference for decrypting cytidylic acid. Such influence on the cryptography of messenger RNA might be considered one mechanism of action of chloramphenicol which has not been very clearly expressed in the literature. PONGS (1979) has reviewed a small body of instances in which natural protein syntheses, encoded by bacteriophage messenger RNA's, are relatively insensitive to inhibition, and suggests that such insensitivity of protein biosynthesis to chloramphenicol is "related to the base composition of the mRNA template."

Efforts at pinpointing one single ribosomal protein as the site of action of chloramphenicol have not met with unambiguous success. A view of chloramphenicol as an antagonist of a unique ribosomal protein in the peptidyltransferase domain must be regarded with reservations because of the results of genetic analysis. Protein biosynthesis on 70S ribosomes of prokaryotic cells and by 80S ribosomes of eukaryotic cells operate with the same reactants, e.g., puromycin. Yet, only the 70S-based protein synthesis is susceptible to inhibition by chloramphenicol. This difference could conceivably reside in structural differences between peptidyltransferase isoenzymes or isoproteins. If this were the case, one-step mutations to high-level chloramphenicol resistance might occur which would be phenotypically expressed by an altered peptidyltransferase.

Genetic analysis of high-level chloramphenicol-resistant mutants of *Escherichia coli* K12 (CAVALLI and MACCACARO, 1952) showed, however, that this resistance was a polygenic cooperative phenomenon and that the individual resistance markers were located in different cistrons. One-step high-level resistance mutations for chloramphenicol have not been discovered.

Affinity labeling of 70S ribosomes with iodo-[^{14}C]-chloramphenicol (PONGS et al., 1975) has produced the distribution of the [^{14}C] label in Fig. 2. The relatively prominently labeled ribosomal proteins were L16, L6, L24, L26, and S3. Analogous affinity labeling with radioactive bromamphenicol (SONNENBERG, WILCHEK and ZAMIR, 1973) demonstrated the distribution of label shown

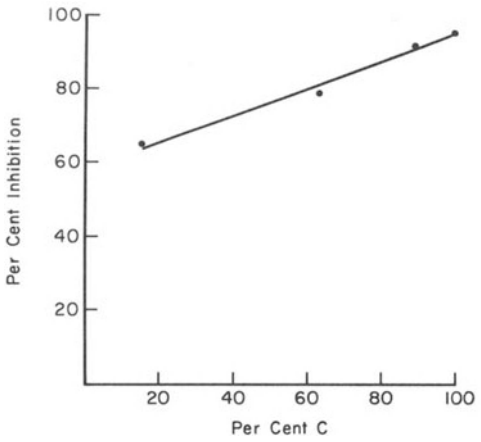


Fig. 1. Correlation between the inhibition by 3×10^{-5} M chloramphenicol of ribosome-poly-UC systems, polymerizing proline, and the cytidylic acid content of a series of poly-UC's

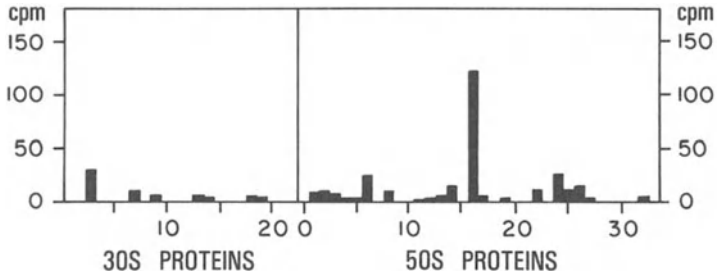


Fig. 2. Distribution of radioactivity in ribosomal proteins after incubation of 70S ribosomes with monoiodo-[^{14}C]-amphenicol

in Table 1. The preferentially labeled 50S proteins were L2, L27, L26, L17, L14, L10, L28, and L11. The authors did not study affinity labeling to intact 70S ribosomes so that data on the 30S proteins are not available. Except for a certain preference for L26, the two sets of data (Fig. 2 and Table 1) do not show a significant overlap among the affinity-labeled 50S proteins. The strong labeling of L16 in the work of PONGS et al. (1975) contrasts with the marked labeling of L2 and L27 in the study of SONNENBERG et al. (1973). While it cannot be excluded that affinity labeling with two differently halogenacetylated chloramphenicols may be responsible for the differences in the results of the two groups of investigators, it is difficult to decide which of the two labeling experiments more truly reflects the affinity of chloramphenicol for ribosomal proteins.

Tritiated chloramphenicol itself has been recently used by LEGOFFIC et al. (1980) in photoaffinity labeling of *E. coli* ribosomes in vitro. L11, S3, and S4 were specifically labeled. A detailed discussion of the results of the French group should await the publication of another report of work in which the yield of incorporation is significantly improved.

Table 1. Distribution of label in proteins of 50S ribosomal subunits treated with [^{14}C]-bromamphenicol

Ribosomal protein	Bromamphenicol	Ribosomal protein	Bromamphenicol
L1	0	L18	6
L2	480	L19	33
L3	0	L21	2
L4	0	L22	13
L5	0	L23	0
L6	40	L24	7
L7+L12	0	L25	0
L8+L9	10	L26	128
L10	67	L27	446
L11	50	L28	60
L13	11	L29	0
L14	71	L30	0
L15	38	L32	0
L16	11	L33	0
L17	81		

The total uptake of bromamphenicol per 50S ribosomal subunit corresponded to 0.5

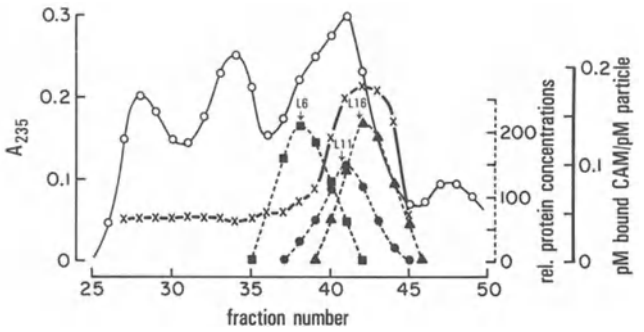


Fig. 3. Gel filtration profile from the basic fraction of the 50S derived split proteins SP 0.4–0.8 (o——o). The relative concentration curves of the three proteins are included (●---●). Each fraction was reconstituted with the nonbinding 0.8 c core. The reconstituted particles were tested for chloramphenicol binding (×——×)

In ribosomal degradation and reconstitution experiments, WERNER et al. (1975) separated a number of component proteins from 50S ribosomal subunits by exposure to 0.4 or 0.8 M LiCl. The 0.8 M LiCl particles regained chloramphenicol binding ability by the addition of the split protein L16 (but not by recombination with L6 or L11), as shown in Fig. 3. This result in conjunction with iodo-[^{14}C]-chloramphenicol affinity labeling (PONGS et al., 1975) has suggested a preferential function of L16 in chloramphenicol binding to 50S ribosomal subunits. It would be difficult to decide if this function is a direct consequence of the architecture of the peptidyltransferase domain or involves a less direct allosteric effect on the active site.

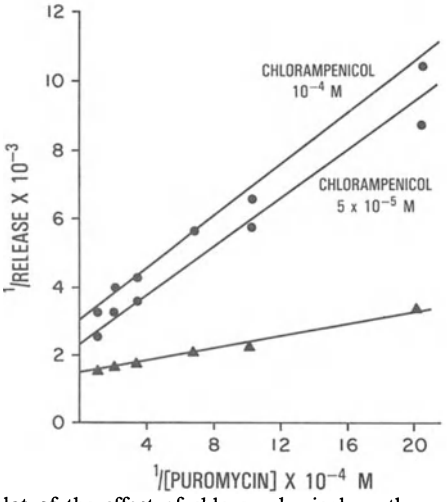


Fig. 4. Double-reciprocal plot of the effect of chloramphenicol on the puromycin-induced release of polylysine from tRNA bound to ribosomes

The inhibition of the formation of one single peptide bond between puromycin and growing peptide chains on the 50S subunit (TRAUT and MONRO, 1964) and the subsequent establishment of the fragment reaction in which N-formylmethionyl oligonucleotide fragments react with puromycin on the 50S subunit, the reaction being inhibited by chloramphenicol (MONRO and MARCKER, 1967), furnish proof that the antibiotic acts as an inhibitor of the formation of the single peptide bond. The as yet unresolved mechanistic problem in this action of chloramphenicol is centered on the question of whether the antibiotic is an antagonist of the amino acyl partner or the peptidyl partner in peptide bond synthesis (disc. HAHN and GUND, 1975). The inhibition of the puromycin reaction by chloramphenicol follows noncompetitive kinetics, as shown in Fig. 4 (GOLDBERG and MITSUGI, 1967). Other investigators (DAS et al., 1966; HAHN, 1968; HARRIS and SYMONS, 1973; CHENEY, 1974) have proposed that chloramphenicol acts as an antagonist of the peptidyl donor. This is consistent with observations that the drug inhibits peptide formation more strongly when the P-site is occupied by amino acyl amides, such as N-formyl methionine (MONRO and VÁZQUEZ, 1967) and acetylphenylalanine (PESTKA, 1970), or by actual peptides (COUTSOGEORGOPOULOS, 1967), than in those instances in which (such as in the poly-U system) amino acyl tRNA is forced into the P-site by excessive concentrations of Mg^{2+}. The alternatives of chloramphenicol acting as an antagonist of the peptidyl *acceptor* or of the peptidyl *donor* have been tested through an analysis of the reaction products of an *E. coli* ribosome-poly-A system which synthesizes oligolysines (JULIAN, 1965). Chloramphenicol does not inhibit the formation of the initial lysyl-lysine from two molecules of lysyl-tRNA, and, in fact, causes an *accumulation* of this dipeptide. The antibiotic, however, does inhibit the elongation of the dipeptide, which in uninhibited control systems leads to the preferential biosynthesis of hexa-, hepta-, and octapeptides.

Finally, the question must be asked if the A- and P-sites are structurally distinct ribosomal subentities or are merely kinetic terms. They must be structurally similar since the A-site must bind peptidyl-tRNA transiently after each elongation step and before translocation back to the P-site. It is not impossible that the attempts to assign the A- or P-sites as loci of the action of chloramphenicol point to a structural pseudoproblem. The real problem would be one of conformation induction of the peptidyltransferase domain brought about by the different substrates and/or inhibitors.

Antiphenicol, an Antagonist of Chloramphenicol

The first years following the disclosure of the structure of chloramphenicol saw a considerable effort at discovering antagonists or antimetabolites of the antibiotic as an approach to solving the then intractable problem of its mode of action. WOOLLEY (1950) proposed that chloramphenicol was an antagonist of phenylalanine or of some compound intermediary in structure between the two substances. TRUHAUT et al. (1951), and BERGMANN and SICHER (1952) thought of chloramphenicol in relation to tryptophan. MENTZER et al. (1950) recognized a similarity to phenylserine and MOLHO and MOLHO-LACROIX compared chloramphenicol to a dipeptide (1952). In no instance has the experimental testing of these antimetabolite hypotheses produced more than marginal results of diminished inhibitions of bacterial growth by very small quantities of chloramphenicol in terms of antagonism by a metabolite. Clearly, nothing like the relationship between sulfanilamide and p-aminobenzoic acid turned up to explain the mode of action of chloramphenicol.

In screening the fermentation products of approximately 1800 actinomycete isolates, strain 851 produced a compound, called I-851 or antiphenicol (IMAGAWA et al., 1979). The structure of antiphenicol is shown in Fig. 5.

Petri dishes, containing 10 μg/ml of chloramphenicol, 250 μg/ml thiamphenicol, 1500 μg/ml lincomycin, or 200 μg/ml erythromycin were inoculated with *E. coli* K12 IFO 3301. On these plates were placed paper disks soaked in 100 μg/ml antiphenicol. The zones of bacterial growth around the disks had diameters of 15, 13, 11, and 11 mm. Antiphenicol did not antagonize growth inhibitions by streptomycin, kanamycin, or tetracycline, i.e., by antibiotics whose sites of action are on the 30S ribosomal subunit.

Antiphenicol itself has weak antibacterial activity, the MICs for ten different bacterial strains varying between 25 and 50 μg/ml (IMAGAWA et al., 1979). From the preliminary work of the Japanese authors it appears that antiphenicol is not a specific antagonist of chloramphenicol but antagonizes the actions of inhibitors of protein synthesis whose site of action is on the 50S ribosomal subunit. It is reasonable to assume that antiphenicol reacts with, or binds to, the peptidyltransferase domain and denies access to the more active antibiotics. Detailed in vitro studies have not been published; they might yield interesting results concerning the relationship of antiphenicol and 50S ribosomes.

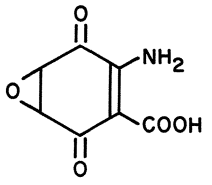


Fig. 5. Structure of antiphenicol

Antibiotics with a similar site of action on the larger ribosomal subunit but active solely against Gram-positive bacteria (mikamycins, thiopeptin) were not antagonized by antiphenicol in inhibition experiments with *Bacillus subtilis* (IMAGAWA et al., 1979).

Structure–Activity Relationships in the Chloramphenicol Series

Structure–activity relationships, established during the two decades, 1955–1975, and drawing on more than 500 synthetic derivatives of chloramphenicol, have been summarized and extensively discussed by HAHN and GUND (1975). They brought out the fact that certain moieties of the antibiotic molecule, especially the p-nitrophenyl ring system, can be varied with only partial loss of antibacterial activity, while others, especially the steric configuration, could not be changed without total loss of growth-inhibitory potency. Extensive and precise structure–activity relationship studies have not yielded derivatives which significantly surpass the antibacterial activity of the original antibiotic.

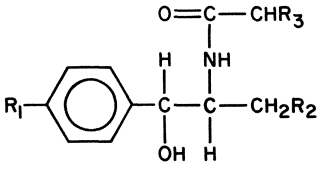
The intervening time has seen two additional structure–activity studies which shed some light on the essentiality of the p-nitro group and of the propanediol side chain. CORBETT and CHIPKO (1978) prepared analogs of chloramphenicol in which the nitro group was replaced by hydroxylamine, nitroso, hydroxamic acid, methyl hydroxamate, and O-acetylhydroxamate functional groups. These compounds were tested for antibacterial activity in *Staphylococcus aureus, Pseudomonas aeruginosa, E. coli,* and *B. subtilis.* Table 2 lists the MIC's in μg/ml of the five analogs as inhibitors of the four bacterial strains.

The lack of antibacterial activity of the hydroxamic acid analog has been interpreted as an effect of the hydrophilicity of hydroxamic acid. SHEMIAKIN et al. (1956) reported that the substitution in the para position of ionogenic and, hence, hydrophilic groups abolished antibiotic activity of chloramphenicol. In fact, conversion of the hydroxamic acid substituent to its methyl ester decreases the hyrophilicity and produced the reappearance of a modest antibacterial activity (CORBETT and CHIPKO, 1978). The authors express surprise that the nitroso derivative is less potent than chloramphenicol itself; they conclude that chemical reduction of the nitro group does not contribute to the antibacterial potency in the chloramphenicol series.

Table 2. Minimum inhibitory concentrations (MIC) for chloramphenicol and five reduced analogs against four bacterial strains

Para substitution	MIC (μg/ml)			
	S. aureus	*P. aeruginosa*	*E. coli.*	*B. subtilis*
$-NO_2$	0.007	0.003	0.001	0.001
$-NHOH$	0.062	0.062	0.125	0.062
$-NO$	0.062	0.062	0.062	0.062
$-NCOCH_3$ | OH	>2.5	>2.5	>2.5	>2.5
$-NCOCH_3$ | OCH_3	0.250	0.062	0.250	0.062
$-NCOCH_3$ | $OCOCH_3$	>0.250	>0.250	>0.250	>0.250

Table 3. Fluorinated derivatives of chloramphenicol and thiamphenicol



	R_1	R_2	R_3
Chloramphenicol	NO_2	OH	Cl_2
Thiamphenicol	SO_2CH_3	OH	Cl_2
Sch 24893	NO_2	F	Cl_2
Sch 25298	SO_2CH_3	F	Cl_2
Sch 25393	SO_2CH_3	F	F_2

Early studies on the structure–activity relationships in the chloramphenicol series of compounds concluded that the propanediol moiety of the antibiotic molecule was essential for antimicrobial activity (HAHN et al., 1956). Structural changes that affected the functional character of the hydroxyl group on carbon atom 3, or altered the length of the propane residue, abolished antibacterial action.

Clinically important resistance of bacteria to chloramphenicol is predominantly due to the formation of an R-plasmid gene product, chloramphenicol acetyltransferase, which catalyzes an acetyl-coenzyme A-dependent acetylation of both hydroxyl groups (SHAW, 1967). The assumed mechanism of these reactions is an initial acetylation of the hydroxyl on carbon 3, followed by spontane-

Table 4. Activities of fluorinated chloramphenicol and thiamphenicol against chloramphenicol-resistant bacteria (MIC's of chloramphenicol >200 μg/ml)

Organism	MIC (μg/ml)		
	SCH 24893	SCH 25298	SCH 25393
S. aureus	3.1	3.1	6.3
E. coli	6.3	3.1	3.1
K. pneumoniae	6.3	3.1	6.3
E. cloacae	12.5	12.5	12.5
C. freundii	25	12.5	12.5
S. marcescens	>200	>200	>200
S. typhimurium	6.3	3.1	3.1
S. sonnei	6.3	3.1	6.3
P. rettgeri	12.5	12.5	12.5
P. stuartii	6.3	6.3	3.1
A. anitratus	6.3	100	100
P. aeroginosa	200	100	200

ous migration of the acetyl group to the hydroxyl on carbon atom 1, and an enzymatic reacetylation of the 3-C hydroxyl group. NAGABUSHAN et al. (1980) reasoned on theoretical grounds that a substitution of the terminal OH by F should result in an active chloramphenicol derivative which lacks the point of attack by chloramphenicol acetyltransferase and, hence, should be growth inhibitory to bacteria which owe their resistance to chloramphenicol to the formation of this R-plasmid gene product. Table 3 lists the structures of chloramphenicol, thiamphenicol, and three of their fluorine-containing new derivatives (HAHN, 1980). SCHAFER et al. (1980), NEU et al. (1980), and NEU and FU (1980) have demonstrated susceptibilities of numerous chloramphenicol-resistant bacteria to the three fluorine derivatives. Table 4 (NEU and FU, 1980) lists the MIC's of the fluorine compounds against a series of bacteria, resistant to >200 μg/ml of chloramphenicol and thiamphenicol. All 16 strains of *Serratia* and most of the 10 strains of *Pseudomonas* were resistant to the three fluorine derivatives. These bacteria may express resistance through a permeability block mechanism (SCHAFER et al., 1980) rather than by enzymatic derivatization of chloramphenicol.

Chloramphenicol, Thiamphenicol and the Causation of Aplastic Anemia

Chloramphenicol-induced cases of aplastic anemia in man have been reported since 1950 and the first nationwide survey in the United States was published in 1954 (WELCH et al.). The assumed frequency of this toxic phenomenon varies widely, the most careful estimates being at 1/10,000 to 1/40,000.

It is probable that the idiopathic disposition to develop aplastic bone marrow after chloramphenicol is genetically determined (NAGAO and MAUER, 1969). As there does not exist an animal model for the study of aplastic anemia after chloramphenicol, experimental studies have remained somewhat circuitous, and the mechanism of pathogenesis of aplastic anemia from chloramphenicol remains uncertain.

Statistical evidence suggests that thiamphenicol in which $-SO_2CH_3$ has replaced the aromatic nitro group of chloramphenicol does not produce aplastic anemia. Between 1972 and 1979 approximately 50 million patients have been treated with thiamphenicol in Europe and Japan and no documented cases of fatal aplastic anemia have been reported.

In the aggregate, these negative observations support the hypothesis that the p-nitro group of chloramphenicol is the structural moiety of the antibiotic molecule, responsible for causing aplastic anemia in genetically predisposed individuals. A comparison of the effects of chloramphenicol and thiamphenicol on DNA biosynthesis in human lymphoblastoid cells has shown that at 10^{-3} M chloramphenicol inhibited DNA biosynthesis by 52%, while thiamphenicol produced only 8.2% inhibition (MANYAN et al., 1975). YUNIS et al. (1980) have suggested that the phenotypic nature of the predisposition to develop aplastic anemia after chloramphenicol is a metabolic conversion of the p-nitro group to reduction products such as $-N=O$ or $-NOH$. FREESE (1963) presented an early summary of the mutagenic effects of hydroxylamine which result from a chemical reaction with cytosine bases of DNA.

YUNIS et al. (1980) have compared the effects of chloramphenicol and its nitroso derivative on DNA synthesis and viability of human bone marrow cells. Chloramphenicol inhibited DNA synthesis reversibly at concentrations of $>3\times10^{-4}$ M and did not affect marrow cell viability. At the same concentration range, nitroso-chloramphenicol inhibited marrow cell DNA synthesis by 80%–90%; this inhibition was largely irreversible. The nitroso derivative at 3×10^{-4} M killed 90% of the bone marrow cells in 48 h. The authors suggest that nitroso-chloramphenicol reacts with DNA, a mechanism to which the carcinogenic property of nitroso compounds has also been ascribed.

Conclusions

Chloramphenicol acts as an inhibitor of the biosynthesis of individual peptide bonds on the 50S subunit of prokaryotic ribosomes. The unambiguous definition of the molecular site of action of the antibiotic awaits resolution of the peptidyltransferase domain. Molecular modifications of the chloramphenicol molecule have apparently overcome the risk of causing aplastic anemia as well as the plasmid-borne chloramphenicol resistance problem. Hence, one of the most important broad-spectrum antibiotics has been rescued for clinical use.

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Emetine, Cryptopleurine, Tylocrebrine and Other Functionally Related Alkaloids

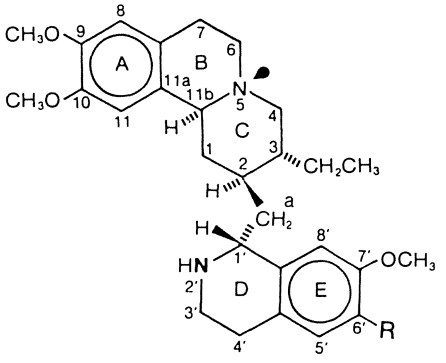
R.S. GUPTA

Introduction

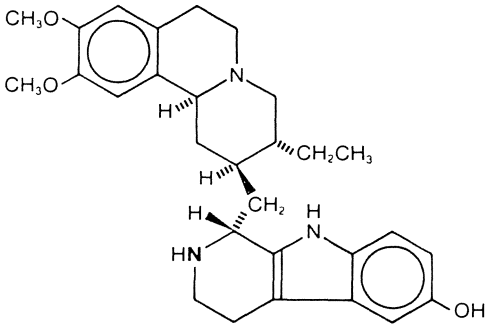
Emetine, cryptopleurine and tylocrebrine are plant alkaloids which belong to two different chemical families, namely the benzoisoquinolines (emetine) and the phenanthrene (tylocrebrine and cryptopleurine) alkaloids. Of these, emetine and related ipecac alkaloids, e.g., dehydroemetine, cephaeline, have long been used for medicinal purposes and have been extensively investigated. The biochemical and pharmacological properties of these compounds were earlier reviewed in this series by GROLLMAN and JARKOVSKY (1975). In contrast to the ipecac alkaloids, the pharmacological properties of various phenanthrene alkaloids, e.g., cryptopleurine, tylocrebrine, tylophorine, have not been systematically evaluated thus far. However, genetic and biochemical studies during the past few years, which are reviewed here, strongly indicate that the mechanism of action of tylocrebrine and cryptopleurine is very similar to that of emetine and related compounds (GUPTA and SIMINOVITCH, 1977b). These studies have led to the identification of common structural determinants shared by these two groups of alkaloids, which may be responsible for their biological activities (GUPTA et al., 1980).

Origin and Chemical Structures

Emetine (I) and a number of related compounds, namely cephaeline, emetamine, O-methylpsychotrine, psychotrine, were originally isolated from the roots of the South American plant *Cephaelis ipecacuanha*. Many additional compounds related in structure to emetine (*e.g.* alangicine, desmethylpsychotrine, dihydroprotoemetine, ankorine), and a number of indole alkaloids, which are also related to emetine in configuration and biological properties (e.g., tubulosine, isotubulosine, desmethyltubulosine, alangimarcine), have since then been isolated from the Indian plant *Alangium lamarckii* (see reviews by OPENSHAW, 1970; BROSSI et al., 1971; SHAMMA, 1972). The chemical structure of emetine and some of the other alkaloids is shown in Fig. 1. The structural formulae of other related compounds as well as the details of their isolation, chemical



I (−)-Emetine R = OCH_3
II (−)-Cephaeline R = OH



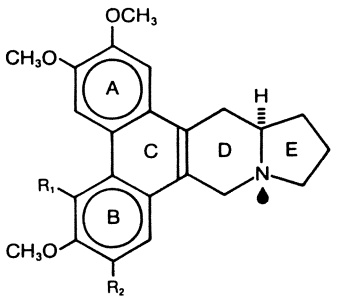
III Tubulosine

Fig. 1. Structural formulae of emetine, cephaeline, and tubulosine

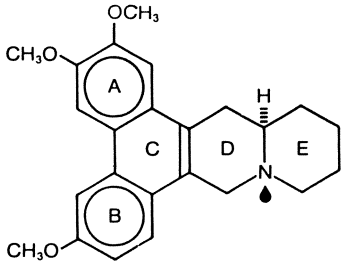
synthesis and establishment of structures could be found in the reviews cited above.

The phenanthroindolizidine alkaloids tylophorine (IV) (Fig. 2) and tylophorinine were first isolated from the Indian plant *Tylophora indica* (synonym: *Tylophora asthmatica*) in 1935 (see GOVINDACHARI, 1967). GELLERT et al. (1962) subsequently described the isolation of a third member of this group, tylocrebrine (V), from the related Australian plant *Tylophora crebriflora*. Four additional related alkaloids have since then been isolated from this latter plant (RAO et al. (1970). Another related alkaloid, antofine (VI), has been isolated from the plant *Antitoxicum funebre* (PLATONOVA et al., 1958). The chemical structures of tylophorine, tylocrebrine and tylophorinine have been established both by degradation and by synthesis by GOVINDACHARI and co-workers (see GOVINDACHARI, 1967).

The phenanthroquinolizidine alkaloid cryptopleurine (VII) was first isolated by DE LA LANDE (1948) from the bark of the Australian plant *Cryptocarya pleurosperma*. Subsequently its isolation from a number of related North American plants, e.g., *Boehmeria cylindrica, Boehmeria caudata, Cryptocarya laevigata* has been reported (FARNSWORTH et al., 1969; HOFFMAN et al., 1978). The chemical structure of cryptopleurine has been established by X-ray crystallography (FRIDRICHSON and MATHIESON, 1955) and several different routes for its chemical synthesis have been described (BRADSHER and BERGER, 1957; PATON et al., 1969; MARCHINI and BELLEAU, 1958). Many modified derivatives of cryptopleurine have also been synthesized (FOLDEAK, 1971). The absolute configurations of cryptopleurine (VII) and tylocrebrine (V) were recently proposed to be opposite (i.e., R and S, respectively) by GELLERT et al. (1978) based on the opposite signs of their respective Cotton effects (circular dichroism curves: $[\theta]_{233}+15{,}000$ for cryptopleurine, and $[\theta]_{255}-4370$ for tylocrebrine). However, our sample of tylocrebrine shows the same sign of the Cotton effect as (−)-cryptopleurine ($[\theta]_{232}+25{,}000$ for cryptopleurine, and $[\theta]_{232}+5300$ for tylocrebrine; measured with a Jouan Dichrographe II instrument in 95% ethanol $[C_I=0.63\times10^{-3}$;



IV Tylophorine $R_1=H$; $R_2=OCH_3$
V Tolycrebrine $R_1=OCH_3$; $R_2=H$
VI Antofine $R_1=R_2=H$



VII Cryptopleurine

Fig. 2. Structural formulae of tylocrebrine, cryptopleurine and related compounds

$C_{II}=0.2\times10^{-3}$]) suggesting that the absolute stereochemistry of both these compounds should be the same. Identical absolute configuration has also been found in antofine (VI) by its degradation to D-proline (WIEGREBE et al., 1971). Although the basis of the above differences in the signs of Cotton effect is not clear at present, based on our results we have assumed here that the absolute stereochemistry of tylocrebrine (V) and cryptopleurine is the same as that shown in Fig. 2.

Growth-inhibitory Effects of Emetine, Cryptopleurine and Tylocrebrine

The cytotoxic effect of emetine on amoeba and its inactivity against bacteria have been known since the early part of this century (ROGERS 1912; VEDDER 1912, 1914). In recent years, GROLLMAN and colleagues have reported that emetine inhibits protein synthesis in various eukaryotic organisms such as cultured animal cells (HeLa and rabbit reticulocytes), plants (gametophytes of *Anemia phylitidis*) and amoeba (*Entamoeba histolytica*) at very low concentrations (GROLLMAN, 1966; ENTNER and GROLLMAN, 1973). Emetine also inhibits protein synthesis in extracts of yeast cells, however intact yeast cells (*Saccharomyces cerevisiae* and *Saccharomyces pastorianus*) are resistant to emetine, due to their impermeability (GROLLMAN, 1966). In contrast to these eukaryotic organisms, protein synthesis in extracts of bacteria (e.g., *Escherichia coli*) and other prokaryotic cells is resistant to very high concentrations of emetine (GROLLMAN, 1966; GROLLMAN and JARKOVSKY, 1975). The effect of emetine on the growth and survival of cultured Chinese hamster ovary (CHO) cells has recently been examined by GUPTA and SIMINOVITCH (1976). In cultures of CHO cells which were treated with 2×10^{-7} M and 1×10^{-6} M emetine, cell growth was completely inhibited, however a large fraction of such cells remained viable in presence of the drug for the first few days. These results

indicate that the initial effect of emetine on cell growth is largely cytostatic, and can be reversed once the drug is removed (GUPTA and SIMINOVITCH, 1976).

In contrast to the impermeability of yeast cells to emetine, cryptopleurine and tylocrebrine caused 50% inhibition of the growth of *S. cerevisiae* at 2×10^{-6} M and 6×10^{-6} M, respectively (HASLAM et al., 1968). As in the case of emetine, protein synthesis in extracts of *E. coli* was resistant to high concentrations of cryptopleurine and tylocrebrine (HASLAM et al., 1968). GUPTA and SIMINOVITCH (1977b) have determined the cloning efficiencies of CHO cells in medium containing different concentrations of these drugs and, based on these studies, concentrations of cryptopleurine, tylocrebrine, and emetine which reduce the cloning efficiency of such cells to 10% level are 2×10^{-9} M, 7.5×10^{-9} M, and 3.5×10^{-8} M, respectively. The effects of low concentrations of tylocrebrine and cryptopleurine on the growth of CHO cells are also initially reversible (GUPTA, R.S., unpublished results).

Effects of Emetine, Tylocrebrine, Cryptopleurine and Related Compounds on Protein Synthesis

The primary effect of all the above compounds in various susceptible species is on protein synthesis, which is inhibited at very low concentrations (10^{-7} to 10^{-8} M) (GROLLMAN, 1966, 1967, 1968; DONALDSON et al., 1968; HUANG and GROLLMAN, 1972; GUPTA and SIMINOVITCH, 1976, 1977b). The effect of these compounds, e.g., emetine, tylocrebrine, on protein synthesis is observed almost immediately. DNA synthesis is also affected at the same time, but to a lesser extent and this effect appears to be indirect and related to the inhibition of protein synthesis (HUANG and GROLLMAN, 1972; GROLLMAN and JARKOVSKY, 1975; GUPTA and SIMINOVITCH, 1976, 1978b). In contrast to protein and DNA synthesis, RNA synthesis is only partially inhibited even at very high concentrations of these drugs. The inhibitory effect of emetine and tylocrebrine on protein synthesis was reported to be irreversible by GROLLMAN (1968), and HUANG and GROLLMAN (1972). However, in later studies we have observed that the effect of low concentrations of these inhibitors (e.g., 2×10^{-7} M emetine or 1×10^{-7} M tylocrebrine) on both cell viability and protein synthesis is reversible at least during the first few hours (GUPTA and SIMINOVITCH, 1976, and unpublished results). It has been reported that emetine is concentrated up to about 50-fold by HeLa cells and that the extent of this effect is dependent upon the concentration of emetine in the culture medium (GROLLMAN, 1968). In view of this observation, the irreversibility of the effect of these inhibitors in earlier studies could result from the fact that much higher concentrations of these drugs (1×10^{-6} M), which are difficult to remove completely by washing, were employed in such experiments.

When emetine or tylocrebrine is added to the culture of HeLa cells, an increase in polyribosome content and a concomitant decrease in the number of single ribosomes is observed, indicating that these compounds inhibit the

elongation step in protein synthesis (GROLLMAN, 1968; HUANG and GROLLMAN, 1972; OLEINICK, 1977). Upon addition of puromycin to such cells, about 90% of the nascent polypeptide chains which are attached to the polyribosomes are released, indicating that these compounds do not inhibit the peptide bond formation step which is required for the observed effect of puromycin. In contrast to emetine and tylocrebrine, other inhibitors of protein synthesis, such as cycloheximide and anisomycin, do not allow the release of nascent polypeptide chains by puromycin, indicating that these latter compounds also inhibit the peptide bond formation reaction (GROLLMAN and JARKOVSKY, 1975; OLEINICK, 1977).

The specific steps in protein synthesis which are inhibited by the above compounds have been further investigated using cell free systems derived from mammalian cells and yeast. GROLLMAN (1968), and HUANG and GROLLMAN (1972) have reported that in the presence of elongation factor 2 (EF2), GTP, and sodium fluoride, when the nascent peptides are located almost entirely at the peptidyl (P)-site on the ribosomes, their reaction with labeled aminoacyl tRNA (i.e., peptide bond formation) was not affected by emetine and tylocrebrine. On the other hand, when these inhibitors were added prior to the addition of EF2 and GTP, so that the nascent peptides were distributed equally among the aminoacyl and the P-sites, the incorporation of labeled amino acid into the released chains was reduced by about 50%, indicating that these compounds were inhibiting the ribosome translocation step. Similar results have been observed with cryptopleurine in yeast (BUCHER and SKOGERSON, 1976). CARRASCO et al. (1976), and JIMENEZ et al. (1977) have examined the effects of emetine, cryptopleurine, tubulosine, and cycloheximide on both the enzymic (requiring EF2) and nonenzymic (observed at high K^+ concentrations) translocation reactions in cell-free extracts of yeast. Results of their studies show that emetine, cryptopleurine, and tubulosine all inhibit only the EF2-dependent translocation reaction. In experiments in which polyribosomes were derived from a cryptopleurine resistant mutant, the translocation reaction was found to be resistant to these inhibitors. In contrast to these inhibitors, cycloheximide inhibited both the enzymic as well as the nonenzymic translocation reactions in both cryptopleurine-sensitive and -resistant cell extracts, indicating that its mechanism of action was different from that of the above inhibitors (JIMENEZ et al., 1977).

The inhibitory effect of emetine, cryptopleurine and related compounds on the translocation step also helps to explain the sensitivity to emetine of cell hybrids formed between emetine-resistant and -sensitive cells (GUPTA and SIMINOVITCH, 1977a, 1978a, c). It has been observed that when protein synthesis is carried out in cell-free extracts using either ribosomes from such hybrid cells or a 1:1 mixture of ribosomes from emetine-sensitive and -resistant cells, the emetine sensitivity of the mixture is determined by the relative ratio of ribosome to mRNA. When mRNA is present in limiting amounts, a condition which favors attachment of several ribosomes to each mRNA, protein synthesis in the mixture is sensitive to emetine. On the other hand, when mRNA is present in excess, both emetine-resistant as well as the sensitive components are expressed in the mixtures (GUPTA and SIMINOVITCH, 1978c). These results can

because in such a case, the presence of a single sensitive ribosome in a polyribosome (containing both sensitive and resistant ribosomes) would be sufficient to block the polyribosome function in presence of emetine (GUPTA and SIMINOVITCH, 1977a, 1978c).

The binding of the above inhibitors to ribosomes has been investigated in a number of studies. Using [^{3}H]-labeled emetine of high specific activity, GROLLMAN (1968) reported no significant binding of the drug to HeLa cells ribosomes. BUCHER and SKOGERSON (1976) and later DOLZ et al. (1980) have examined the binding of cryptopleurine to ribosomes and based on their studies again no unique ribosome binding site for cryptopleurine could be inferred. However, studies of GROLLMAN (1968) and ENTNER (1979) indicate that emetine binds tightly to some component of intact cells. In view of the specific effect of emetine, cryptopleurine and related compounds on ribosomes and protein synthesis, and the observed binding of emetine to intact cells, the apparent failure to detect a specific ribosome binding site for these drugs is very intriguing and needs further investigation. One of the possibilities that should be examined in this regard is that the binding of these drugs to ribosomes may occur only in the presence of one or more of the specific protein synthesis factors.

The above inhibitors at concentrations which completely block cytoplasmic protein synthesis have no significant effect on mitochondrial protein synthesis (HASLAM et al., 1968; PERLMAN and PENMAN, 1970; MAHLER et al., 1971; OJALA and ATTARDI 1972). However, at very high concentrations of these compounds, some inhibition of mitochondrial protein synthesis has been observed in a number of studies (HASLAM et al., 1968; LEITMAN, 1971; CHAKRABARTI et al., 1972; IBRAHIM et al., 1974). Studies by LEITMAN (1971) show however that the mitochondrial protein synthesis was also inhibited at high concentrations by two emetine derivatives, isoemetine and o-methylpsychotrine, which do not inhibit cytoplasmic protein synthesis. These results indicate that the effect of high concentrations of emetine and related compounds on mitochondrial protein synthesis is most probably unrelated to their effect on cytoplasmic protein synthesis.

Genetic Studies with Emetine, Cryptopleurine, and Tylocrebrine Resistant Mutants

The approach that has proved particularly useful in understanding the mechanism of action of these alkaloids, and in establishing the similarity of their modes of action, involves the use of cellular mutants resistant to these compounds. The nature of the function affected in such mutants as well as their cross-resistance to various other inhibitors of protein synthesis is then examined. Although mutants resistant to cryptopleurine and tylocrebrine were first isolated in yeast cells (SKOGERSON et al., 1973; GRANT et al., 1974), much of the work that has led to our current understanding that emetine, cryptopleurine and tylocrebrine all act at the same site and may possess common structural determi-

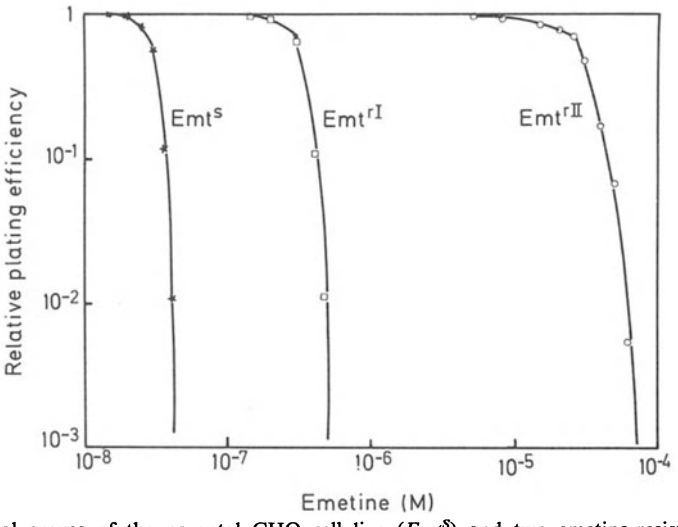


Fig. 3. Survival curves of the parental CHO cell line (*Emt^{S}*) and two emetine-resistant mutants [obtained after one (*Emt^{rI}*) and two (*Emt^{rII}*) step selections] in presence of different concentrations of emetine. (For details see GUPTA and SIMINOVITCH, 1978a)

nants responsible for their biological activity has been carried out with the emetine-resistant mutants of CHO cells. Hence the work on these latter mutants is reviewed below in somewhat greater detail.

In 1976 we reported that stable mutants which are resistant to up to 50 to 60 times higher concentrations of emetine can be readily selected in a single step (Emt^{rI} mutants) in CHO cells (GUPTA and SIMINOVITCH, 1976). Mutant cells exhibiting much higher levels of resistance to emetine were obtained from Emt^{rI} cells when a second-step selection in presence of higher concentrations of emetine was carried out (Fig. 3). In cell-free extracts from various Emt^{rI} and Emt^{rII} mutants, protein synthesis was inhibited at proportionately higher concentrations of emetine (Fig. 4), providing evidence that the genetic lesions in such mutants had affected some component of the protein synthesis machinery (GUPTA and SIMINOVITCH, 1976, 1977a, 1978a). Fractionation of extracts from the sensitive and resistant cells into supernatant (S-100) and ribosomal fractions, and examination of the effect of emetine on protein synthesis after mixing them into various combinations, showed that resistance to emetine was conferred by the mutant ribosomal fraction (Table 1). Further fractionation of ribosomes from the parental and mutant cells into 40S and 60S subunits and mixing them in different combinations with an S-100 fraction derived from sensitive cells established that the resistance of mutant cells to emetine was associated with the 40S ribosomal subunits (Table 1). Two-dimensional gel electrophoresis of ribosomal proteins from several Emt^{rI} and Emt^{rII} mutants has subsequently established that these mutant cells contain alteration in the protein S20 of the 40S ribosomal subunit, which is responsible for their Emt^{r} phenotype (BOERSMA et al., 1979a, b; REICHENBACHER and CASKEY, 1979).

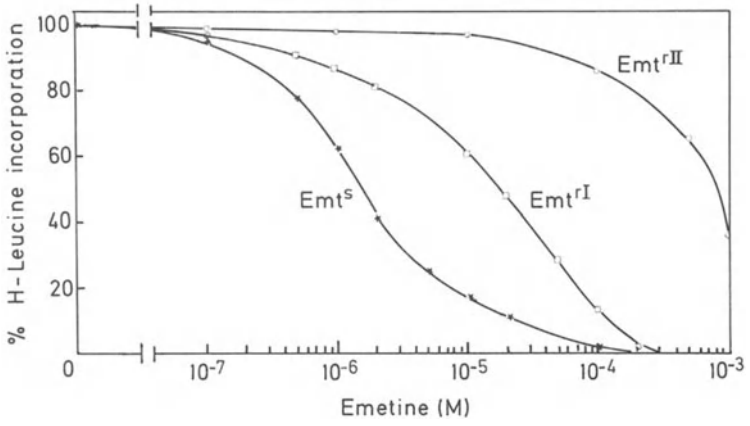


Fig. 4. Effect of increasing concentrations of emetine on [^{3}H]-leucine incorporation in the extracts of parental and two emetine-resistant mutant cell lines. (For details see GUPTA and SIMINOVITCH, 1978a)

Table 1. Localization of emetine resistance to the 40S ribosomal subunit[a]

Experiment	Source of *Polyribosome fraction*	Source of *Supernatant fraction (S-100)*	^{3}H-Leucine Incorporated (pmol/ml)		
			−Emetine	+Emetine 2×10^{-5} M	% Inhibition
I	Emt^S	Emt^S	6.1	2.5	59.0
	Emt^{rI}	Emt^{rI}	12.0	9.4	21.7
	Emt^{rI}	Emt^S	13.8	11.4	17.4
	Emt^S	Emt^{rI}	7.4	3.1	58.1
	Source of *60S Subunit*	Source of *40S Subunit*			
II	Emt^S	Emt^S	2.40	1.0	58.3
	Emt^{rI}	Emt^{rI}	0.92	0.88	4.3
	Emt^S	Emt^{rI}	1.08	1.0	7.4
	Emt^{rI}	Emt^S	2.24	1.12	50.0

[a] Protein synthesis was carried out in cell-free extracts after combining different fractions from emetine-sensitive and -resistant cells. In Exp. II S-100 fraction derived from Emt^S cells was utilized. (For details see GUPTA and SIMINOVITCH, 1977a.)

Our success in selecting and characterizing Emt^r mutants in CHO cells prompted us to select mutants resistant to other inhibitors of protein synthesis, including cryptopleurine and tylocrebrine. Mutants resistant to cryptopleurine and tylocrebrine had earlier been obtained in yeast by SKOGERSON et al. (1973), and the mutation in such cells was later shown to affect the 40S ribosomal subunit (GRANT et al., 1974). When mutants resistant to cryptopleurine (Cry^r) and tylocrebrine (Tyl^r) were selected from CHO cells, it was observed that their frequencies in both mutagen treated and untreated cultures were the same

Table 2. Cross–resistance patterns of mutant cell lines[a]

Types of mutants	Levels of resistance to various compounds (fold)						
	Emetine	Tylocrebrine	Cryptopleurine	Dehydroemetine	Tubulosine	Cephaeline	Cycloheximide
Emt^{rI}	10–50	2–6	2–5	4–20	2–20	5–30	1
Tyl^{rI}	10–50	2–6	2–5	4–20	2–20	5–30	1
Cry^{rI}	10–50	2–6	2–5	4–20	2–20	5–30	1
Emt^{rII}	200–1,500	40–350	30–250	100–400	300–1,500	400–1,500	1

[a] The levels of resistance of mutant cell lines are with respect to the parental sensitive cell line. The range shown here is for different mutant cell lines. rI and rII refer to mutants obtained after one and two selection steps, respectively. (For details see GUPTA and SIMINOVITCH, 1977b, 1978a; GUPTA et al., 1980.)

as that seen for the Emt^r mutants in the same cultures. Biochemical studies with the Cry^r and Tyl^r mutants indicated that like the Emt^r mutants the genetic lesions in these mutants also affected the protein synthesis machinery (GUPTA and SIMINOVITCH, 1977b). Cross-resistance studies with the above mutants showed that they all exhibited cross-resistance to the three selective drugs as well as to a number of other compounds, e.g., tubulosine, dehydroemetine, and cephaeline, which are related to emetine (Table 2). The most striking observation however was that the pattern of cross-resistance of all of the above mutants to these compounds was identical and was related to the degree of resistance of the mutant cells (GUPTA and SIMINOVITCH, 1977b). In the second-step mutants which are highly resistant to emetine (Emt^{rII} type), the level of cross-resistance to all of the above compounds increased in roughly the same proportion (GUPTA and SIMINOVITCH, 1978a). The cross-resistance of mutant cells to these compounds was seen in both whole cells as well as in cell-free extracts (Table 3), providing strong evidence that its basis was at the level of protein synthesis (GUPTA and SIMINOVITCH, 1977b, 1978a). Studies in yeast, which show that protein synthesis in cell-free extracts of Cry^r mutants exhibit cross-resistance to emetine and tubulosine, support these results (SANCHEZ et al., 1977).

In contrast to the effects with the above compounds, these mutants did not show any cross-resistance to various other inhibitors of eukaryotic protein synthesis such as cycloheximide, anisomycin, trichodermin, pactamycin, sparsomycin, harringtonine, bruceantin, diphtheria toxin, pederine, chalcomycin, pretazettine, etc. (Table 3; GUPTA and SIMINOVITCH, 1977b, 1978a; and GUPTA, R.S., unpublished results). In cases where mutants resistant to these other inhibitors have been selected in CHO cells, e.g., resistance to trichodermin, pactamycin, and diphtheria toxin, no cross-resistance to emetine, cryptopleurine, or tylocrebrine has been observed (GUPTA and SIMINOVITCH, 1978b, d, 1980). These results show that the observed cross-resistance of the mutants is highly specific and that the sites of action of these latter inhibitors are different from that of emetine, cryptopleurine, and tylocrebrine. The failure of Emt^r mutants to

Table 3. Sensitivity of wild-type CHO cells and an Emt^{rI} mutant cell line to various inhibitors of protein synthesis, in vivo and in vitro

Inhibitors	In vivo resistance[a] D_{10} values (M)			Resistance in extracts[b] I_{50} values (M)		
	Wild type	Emt^{rI} mutant	Degree of resistance	Wild type	Emt^{rI} mutant	Degree of resistance
Emetine	3.1×10^{-8}	2.0×10^{-6}	64.0	2.4×10^{-6}	4.5×10^{-5}	19.0
Tylocrebrine	7.5×10^{-9}	4.5×10^{-8}	6.0	4.0×10^{-7}	1.8×10^{-6}	4.5
Cryptopleurine	2.1×10^{-9}	9.0×10^{-9}	4.3	3.3×10^{-7}	6.6×10^{-7}	2.0
Tubulosine	1.4×10^{-7}	4.2×10^{-6}	30.0	4.5×10^{-7}	4.4×10^{-6}	10.0
Cephaeline	2.2×10^{-8}	6.0×10^{-7}	27.0	1.4×10^{-6}	2.0×10^{-5}	14.0
Dehydroemetine	4.1×10^{-8}	7.0×10^{-7}	17.0	1.3×10^{-6}	4.0×10^{-5}	13.3
Pactamycin	2.0×10^{-7}	1.9×10^{-7}	1.0	1.5×10^{-7}	1.4×10^{-7}	1.0
Cycloheximide	4.5×10^{-7}	4.5×10^{-7}	1.0	3.5×10^{-6}	3.8×10^{-6}	1.1
Trichodermin	3.5×10^{-7}	3.6×10^{-7}	1.0	9.0×10^{-7}	1.0×10^{-6}	1.0
Anisomycin	6.0×10^{-8}	6.0×10^{-8}	1.0	4.0×10^{-7}	4.0×10^{-7}	1.0
Sparsomycin	1.0×10^{-6}	1.0×10^{-6}	1.0	5.0×10^{-6}	5.0×10^{-6}	1.0
Harringtonine	1.0×10^{-6}	1.0×10^{-6}	1.0	–	–	–
Pederine	2.0×10^{-10}	2.0×10^{-10}	1.0	–	–	–
Bruceantin	7.0×10^{-8}	7.0×10^{-8}	1.0	–	–	–
Pretazettine	1.5×10^{-6}	1.5×10^{-6}	1.0	–	–	–

[a] The D_{10} values represent drug concentrations which reduce plating efficiency of the cell line to 10% of that observed in the absence of any drug.

[b] The I_{50} values indicate drug concentrations which inhibit protein synthesis in cell extracts by 50%.

(For details see GUPTA and SIMINOVITCH, 1977b.)

show any cross-resistance to cycloheximide (Table 3) is of particular interest, because GROLLMAN (1966, 1967) has suggested earlier that emetine and cycloheximide possessed common structural determinants responsible for their protein synthesis inhibitory activity. This inference is not supported by the cross-resistance pattern or by other biochemical studies which are reviewed here, and furthermore (as discussed in the next section) the structural features which appear to be essential for the biological activity of emetine and related alkaloids are clearly not present in cycloheximide.

Based on their biochemical properties and cross-resistance pattern toward various inhibitors of protein synthesis, the mutants resistant to emetine, tylocrebrine, and cryptopleurine could not be distinguished from each other and appeared identical. This inference is further strengthened by the results of genetic complementation studies (GUPTA and SIMINOVITCH, 1977b). It was shown earlier that in cell hybrids formed between resistant and sensitive CHO cells all of the above mutants behaved recessively, i.e., hybrid cells are sensitive to the drugs (GUPTA and SIMINOVITCH, 1977a, b). Resistance to cryptopleurine in yeast has also been shown to occur due to a recessive nuclear mutation, which maps on chromosome III (SKOGERSON et al., 1973; GRANT et al., 1974). Due to the recessive behavior of mutants it became possible to determine if they complemented each other in cell hybrids. If resistance to these compounds involved

mutations in different genes, then such mutants are expected to complement each other, and hybrid cells should be sensitive to the drugs. If, on the other hand, mutants are in the same gene, then they will not complement each other and hybrids formed between them will also be resistant to the drugs. Results of such studies showed that mutants resistant to emetine, tylocrebrine, and cryptopleurine do not complement each other, providing strong support to the notion that mutations in all of the above cases lie in the same gene and are most likely identical (GUPTA and SIMINOVITCH, 1977b).

To account for the identity of the Emt^r, Tyl^r, and Cry^r mutants as indicated by various genetic and biochemical studies, we suggested that all of the above compounds to which the mutant cells exhibit cross-resistance, e.g., emetine, cryptopleurine, tylocrebrine, cephaeline, tubulosine, and dehydroemetine, possessed common structural determinants responsible for their biological (i.e., protein synthesis inhibitory) activity, such that the mutants in every case are selected against the same common structural determinants and hence they behave identically (GUPTA and SIMINOVITCH, 1977b).

Structure–Activity Relationship Studies

In view of the clinical and pharmacological activities of emetine and related compounds, a large number of stereoisomers and derivatives of emetine have been synthesized (see YARDLEY et al., 1967; OPENSHAW et al., 1969; OPENSHAW, 1970; BROSSI et al., 1971; JONDORF et al., 1971). The structure–activity relationships between these compounds have been examined by a number of investigators in an attempt to identify the structural features which are essential for the biological activities of these compounds. The two requirements for the activity of emetine-like compounds which have been identified by previous studies include the (R) configuration at C-1 and the secondary nitrogen at the 2′-position (see review by GROLLMAN and JARKOVSKY, 1975). Based on this information and certain topochemical considerations GROLLMAN (1966, 1967; GROLLMAN and JARKOVSKY, 1975) suggested that the structural features which are essential for the biological activity of emetine and related compounds are also present in, and responsible for, the activity of the glutarimide antibiotic cycloheximide. However, this inference is clearly incorrect as several lines of evidence now show that emetine and cycloheximide do not act in the same manner (see earlier sections, and GUPTA and SIMINOVITCH, 1977b).

In studies of structure–activity relationships one major problem that is encountered is to make sure that different compounds whose activities are being compared act in the same manner and at exactly the same cellular site. For example, the biological activity of emetine and related compounds are determined with regard to either their amoebicidal (i.e., cytotoxic) or protein synthesis inhibitory activity (ENTNER and GROLLMAN, 1973; GROLLMAN and JARKOVSKY, 1975). However, one or both of these activities are also shown by a large number of other compounds, some of which also bear limited structural resemblance

to emetine (see GUPTA et al., 1980). Therefore, unless there is a way to establish that these other compounds also act in exactly the same manner as emetine, the structure–activity relationship between them could be very misleading, as was observed in the case of emetine and cycloheximide (GROLLMAN, 1966; GROLLMAN and JARKOVSKY, 1975). One possible way to establish that the two compounds act in the same way is by means of examining their cross-resistance to mutant cells which bear specific alterations at the target site. The rationale of this cross-resistance approach could be described as follows: "If any two given compounds owe their toxicity to the same structural determinants, then cellular mutants which have become resistant to one compound due to an alteration in its target site should at the same time also exhibit cross-resistance to the other compound. At the same time, the failure of such mutants to exhibit cross-resistance to a given drug provides strong evidence that the compound either lacks the appropriate structural determinants or that these are sterically blocked in the compound, so that its observed toxicity is due to other mechanisms" (GUPTA et al., 1980; GUPTA, 1981). Based on this premise we have examined the cross-resistance of Emt^r mutants (which bear specific alterations in the 40S ribosomal subunit) to a variety of compounds that show different degrees of structural similarities to the emetine and cryptopleurine groups of alkaloids (GUPTA et al., 1980). The cross-resistance in these studies was examined against two different Emt^r mutants (Emt^{r_I} and $Emt^{r_{II}}$) which show different degrees of resistance to emetine. Based on earlier studies it was expected that the $Emt^{r_{II}}$ mutants, which are more resistant to emetine, would exhibit a higher degree of cross-resistance to various compounds which may possess structural determinants in common with emetine.

The results of cross-resistance studies for some of the compounds which were examined are shown in Table 4. Of these compounds, Emt^r mutants exhibited cross-resistance to cryptopleurine, tylocrebrine, cephaeline, dehydroemetine, tubulosine, and to compounds NSC 134754 and NSC 134756. As expected, in every case $Emt^{r_{II}}$ mutants showed a higher level of resistance than the Emt^{r_I} mutant. Among the compounds which were active, cryptopleurine and tylocrebrine were much more active than emetine on a molar basis, whereas the compounds NSC 134754 and NSC 134756 possessed much less activity. The remainder of the compounds listed in Table 4, which included O-methylpsychotrine, 1,2-secoemetine and a number of other compounds, were inactive in the test system. The activity of various compounds in this test system was also consistent with amoebicidal and antileukemic activities (see Table 4). Since according to the rationale outlined above common structural determinants should be present among the active compounds, the chemical structures of such compounds were closely examined (GUPTA et al., 1980).

The structural similarities between the two basic types of active compounds (Table 4; Figs. 1, 2, and 6), i.e., the benzoisoquinoline (emetine, dehydroemetine, etc.) and the phenanthrene (e.g., cryptopleurine, tylocrebrine) alkaloids, are not immediately apparent. Whereas emetine (and its congeners) possess relative freedom of rotation around the connection between the two cyclic systems (2, a and a, 1′ bonds; see Fig. 1), structurally simpler phenanthrene-based compounds, which also show much greater activity than emetine, are more rigid

Table 4. Cross-resistance and relative activity of compounds related to emetine

Compounds	Structure	Relative degree of resistance for mutants lines			Activity[b] relative to emetine	Amoebicidal[c] Activity	Activity against mouse L1210 leukemia
		$Emt^S(D_{10}M)$[a]	$Emt^{rI}41$	$Emt^{rII}63$			
(−)-Cryptopleurine	VII	$1.0(2.1\times10^{-9})$	4.8	200.0	1,400	100	active
(−)-Tylocrebrine	V	$1.0(7.5\times10^{-9})$	6.0	330.0	400	100	active
(−)-Cephaeline	II	$1.0(2.2\times10^{-8})$	23.0	480.0	140	100	active
(−)-Emetine	I	$1.0(3.0\times10^{-8})$	33.0	1,500.0	100	100	active
(−)-2,3-Dehydroemetine	VIII	$1.0(4.0\times10^{-8})$	25.0	120.0	80	100	active
(−)-Tubulosine	III	$1.0(1.4\times10^{-7})$	17.0	450.0	22	100	active
(±)-NSC134754	IX	$1.0(1\times10^{-6})$	15.0	>50.0	3.3	[f]	inactive[d]
(±)-NSC134756	X	$1.0(1\times10^{-6})$	15.0	>50.0	3.3	[f]	inactive[d]
(+) O-methylpsychotrine	XI	$1.0(5\times10^{-5})$	1.0	1.0	[e]	<1	inactive
1,2-secoemetine	XIII	$1.0(2\times10^{-5})$	1.0	1.0	[e]	<1	[f]
(−)-compound XII	XII	$1.0(-1\times10^{-3})$	1.0	1.0	[e]	[f]	[f]
(+)-compound XIV	XIV	$1.0(5\times10^{-5})$	1.0	1.0	[e]	<1	[f]
(±)-compound XV	XV	$1.0(1\times10^{-4})$	1.0	1.0	[e]	<1	[f]

[a] The D_{10} value indicates the concentration of drug which reduces plating efficiency of parental CHO cell line to 10% of that observed in the absence of any drug. (For details see GUPTA et al., 1980.)

[b] The activity relative to emetine has been calculated from the ratio of the D_{10} value of Emt^s cells for emetine as compared to the drug X100.

[c] Data obtained from GROLLMAN (1966), ENTNER and GROLLMAN (1973), YARDLEY et al. (1967), and BUZAS et al. (1977).

[d] NSC134754 and NSC134756 were tested at the same concentrations as emetine, cephaeline, etc, which may account for their inactivity (JONDORF et al., 1971).

[e] Do not show emetine-type activity in the test system.

[f] Unknown.

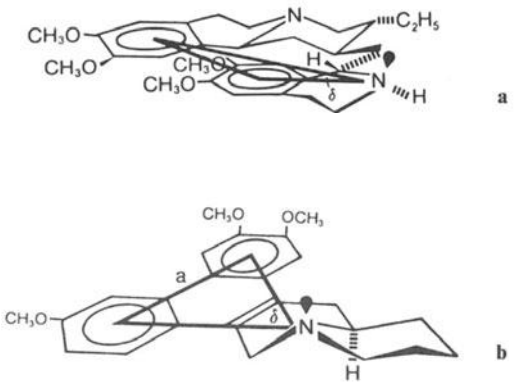
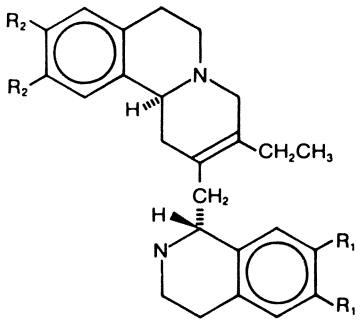


Fig. 5. Structural similarities between the emetine (**a**) and cryptopleurine (**b**) types of compounds. The *triangles* identify some of the common structural features which appear to be essential for the biological activity of these compounds

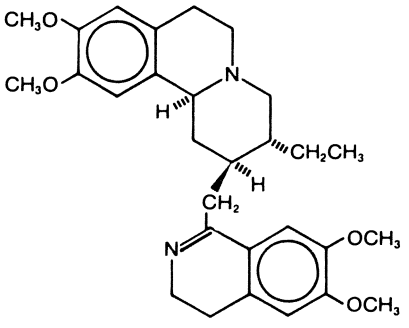
and nearly planar molecules (see Fig. 2). The planar character of the phenathrene moiety in cryptopleurine and tylocrebrine is determined by the rigid benzene rings and, in addition, the other important common structural features which are present in both these compounds include the nitrogen atom with its free pair of electrons and the methoxyl substituents in the benzene rings. The methoxyl groups, because of their electron-releasing nature, confer a slightly electronegative character on the relatively easily polarizable aromatic rings, thus underlining the dipolar character of this part of the molecule. Based on the above facts, the most likely active structures in cryptopleurine and tylocrebrine should comprise the two aromatic rings (in the same plane) rendered slightly electronegative by methoxyl (or hydroxyl) substitutents, and the nitrogen atom with its free pair of electrons located at a certain distance from the aromatic rings, forming a sort of triangle as depicted in Fig. 5b.

In the less rigid emetine and related compounds, rings A, B, C, and rings D, E, form two nearly planar systems which can assume various relative positions, i.e., they can either be perpendicular to each other or can assume various angular forms, or they may lie in the same plane while the distance between the aromatic rings could vary from the minimum determined by atomic space requirements to about 7–8 Å (see GUPTA et al., 1980). All emetine derivatives are expected to exist in various degrees in any of these forms, depending on a combination of external factors and structural features. The planar forms which can be assumed by emetine and its derivatives closely resemble the shape of phenanthrene-based molecules discussed above (Fig. 5). If a triangle is drawn with its points at the two aromatic rings and N-2′ in emetine (or through corresponding structural elements in cryptopleurine or tylocrebrine), the distance between N and side *a* remains the same in both types, although the angle δ is slightly larger in emetine than in cryptopleurine or tylocrebrine (GUPTA et al., 1980).

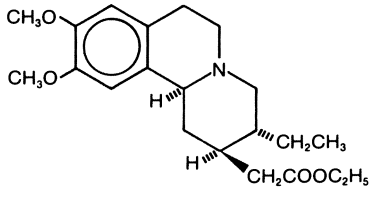
The importance of these structural features in the activity of the above compounds was tested by an examination of the relative activity of various



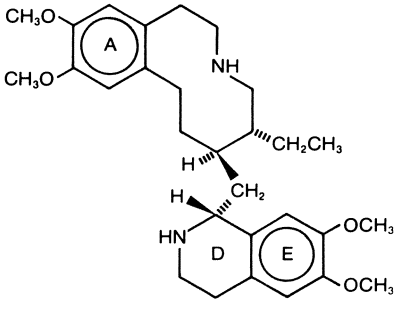
VIII (−)-2,3-Dehydroemetine $R_1=R_2=OCH_3$
IX NSC 134754 $R_1=H$; $R_2=OCH_3$
X NSC 134756 $R_1=OCH_3$; $R_2=H$



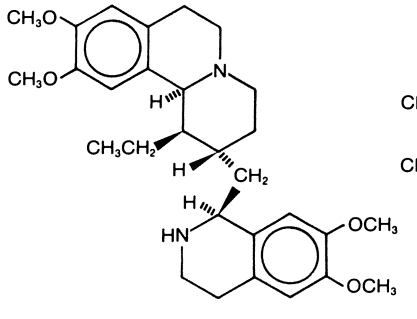
XI (+)-O-Methylpsychotrine



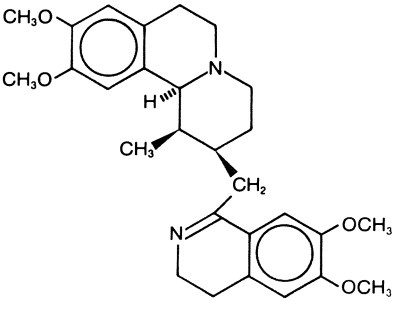
XII



XIII 1,2-Secoemetine



XIV



XV

Fig. 6. Structural formulae of some of the compounds related to emetine

compounds in which some of the structural features have been modified (Fig. 6) (GUPTA et al., 1981). Among the active compounds the more rigidly planar cryptopleurine and tylocrebrine, in which the position of the nitrogen atom with respect to the aromatic rings is also fixed, are expected to be more active than emetine and its congeners, which is what is observed (Table 4). The lower

activity of tylocrebrine (V) as compared to cryptopleurine (VII) is most likely due to the presence of the additional methoxyl group in tylocrebrine, which may force the two aromatic rings slightly out of the ideal plane. The 2,3-double bond in 2,3-dehydroemetine (VIII) restricts the freedom of assuming "angular" conformations while making ring C somewhat flatter, but the system can still become as planar as emetine itself, so the activity remains almost unchanged. Tubulosine (III), however, deviates slightly from planarity which is probably the reason for a slightly diminished activity. The effect of electron-releasing substituents in the aromatic rings can be followed in cephaeline (II) and in compounds IX and X. Cephaeline is slightly more active than emetine (I) or 2,3-dehydroemetine (VIII) because one OMe is replaced by OH, which is a better electron-releasing group. The complete removal of both OMe groups in either ring A (X) or ring E (IX) greatly reduces the activity of emetine, indicating the importance of methoxyl or hydroxyl groups. The importance of methoxyl groups in the activity of these compounds is also evident from the work of FOLDEAK (1971), who has synthesized a number of derivatives of cryptopleurine in which the methoxyl groups are replaced with hydrogen. Results of his studies show that the antifungal activity of cryptopleurine derivatives is progressively diminished as the methoxyl groups are replaced with hydrogen.

The necessity of the two aromatic rings (of phenanthrene type), as well as the distance a and the angle δ in the active site (see Fig. 5), were shown by examining the cross-resistance of several compounds which differed in these respects (GUPTA et al., 1980). This group of compounds, which included cularine, dicentrine, O-methylbulbocapnine and its dimer, glaucine, liriodenine, pontevedrine, corunnine, isocorydine, boldine, uvariopsine, allocryptopine, magnoflorine, (+)-thalicarpine, erybidine, laudanosine, hydrastine, ethaverine, octaverine, papaverine, tetrabenazine, pavine, argemonine, phaenthine, tetrahydropalmatine, capaurine, corydaline, were all inactive in the test system (GUPTA et al., 1980). Similarly, no cross-resistance was exhibited by compounds such as colchicine, brucine, reserpiline, calycanthine, rotenone, tropolone, or by various other inhibitors of protein synthesis as noted before. An additional indication that the benzoisoquinoline ring system in emetine by itself is not sufficient for protein synthesis inhibitory activity is provided by the inactivity of compound (XII), which has all the necessary features of the more complex half of the emetine molecule. That the planar geometry of the molecule of emetine as a whole plays a role in assuming the "active" shape is demonstrated by complete loss of the cross-resistance activity by 1,2-secoemetine (XIII) in which the B, C rings become a medium-sized ten-membered ring whose geometry totally differs from the parent bicyclic system (YARDLEY et al., 1967). The importance of both aromatic rings of emetine being in one plane is further demonstrated by compound (XIV), whose inactivity could be accounted for by the unnatural configuration of the 1′, a bond (as in isoemetine, which is also inactive) making it impossible for the molecule to become planar. Emetine and its congeners contain two nitrogen atoms in their molecules; however, only the isoquinoline ring nitrogen (i.e., 2′) of I–III and VIII–XI corresponds to

the sole nitrogen in IV–VII spacewise, and so this N atom should be the one which should be essential for the activity. The fact that this is so is indicated by the inactivity of the compounds O-methylpsychotrine (XI) and derivative XV (BUZAS et al., 1977), in which the tetrahedral nitrogen becomes planar and its electronic properties are changed accordingly (e.g., lower basicity; see also SCHUIJ et al., 1979). The importance of the 2′ nitrogen atom in the activity of emetine has also been stressed in earlier studies, where substitution at this position (e.g., N-methylemetine) results in complete loss of activity (see GROLLMAN and JARKOVSKY, 1975).

Based on the aforementioned structure–activity relationships studies, the structural requirements for the biological activity of emetine, cryptopleurine and related compounds can be summarized as follows: "The requirement for biological activity appears to be a planar molecule which contains two aromatic rings that are rendered electronegative by methoxyl or hydroxyl substituents, and a nucleophilic element such as nitrogen at a certain distance from the aromatic rings. The distance between the two aromatic rings, the angle between the nitrogen atom and the rings, and the planarity of the structure, all appear to be critical features in determining the biological activity" (GUPTA et al., 1980).

The cross-resistance studies described here also make it possible to draw some conclusions concerning the stereochemistry of tylocrebrine and cryptopleurine. As the cross-resistance phenomenon is extremely sensitive to the spatial relationships, and even small deviations from the required arrangements result in the loss of activity, we believe that the same degree of activity in very closely related compounds implies identical chiralities of the chiral centers of the compounds in question. Thus, the relative configuration of (−)-cryptopleurine is well established, while that of (−)-tylocrebrine is supported mainly by the structural analogy with (−)-cryptopleurine and thermodynamic considerations. The cross-resistance of these two compounds strongly suggests that not only their relative stereochemistry should be identical, but that their absolute chiralities should correspond to that of (−)-emetine and its derivatives as portrayed in Fig. 1.

Therapeutic Properties

The medicinal properties of ipecac, which contains emetine as its main active principle, has long been known to the natives of South America, and the drug has been used for the past three centuries in Europe for the treatment of dysentery (LLOYD, 1921; GROLLMAN and JARKOVSKY, 1975). The widespread use of emetine as a specific remedy for the treatment of amoebic dysentery and extraintestinal amoebiasis, began after 1912, when ROGERS (1912) demonstrated its amoebicidal effect on the human pathogen, *Entamoeba histolytica*. At about the same time, VEDDAR (1912, 1914) showed that dilution of emetine as low as 1:100,000 could kill the amoebae, but was inactive against bacteria. From

1912 until recently, when other less toxic drugs, e.g., metronidazole, which are effective against amoebiasis, were developed (POWELL et al., 1966), emetine and its derivative (±) 2,3 dehydroemetine (which is claimed to be superior to emetine because of its lesser degree of toxicity) were the drugs of choice in the treatment of parasitic infections, such as those caused by *Entameoba histolytica* (see ROLLO, 1975). Even at present, in cases where metronidazole treatment proves ineffective, emetine or dehydroemetine are widely used clinically (ROLLO, 1975). The amoebicidal action of emetine and related compounds seems to result from their effect on protein synthesis, as a good correlation between the amoebicidal, antiprotozoal, and protein-inhibitory activity of such compounds is observed (NEAL, 1970; ENTNER and GROLLMAN, 1973; GROLLMAN and JARKOVSKY, 1975). This view is further supported by the fact that other inhibitors of protein synthesis in eukaryotic cells, e.g., cycloheximide, puromycin, anisomycin, are also effective amoebicidal agents (ENTNER and GROLLMAN, 1973).

Emetine and related compounds have been extensively investigated also for their antitumor activity. The effect of emetine on tumor regression was first described by LEWISOHN in 1918. In the following year, VAN HOOSEN (1919) reported results of a study in which 35 of the 100 patients with advanced malignant diseases responded to emetine treatment. Since then a number of other reports describing the usefulness of emetine or dehydroemetine in the treatment of various forms of malignancies (e.g., chronic myelogenous leukemia, Hodgkin's disease, bronchogenic carcinoma) have appeared (ABD RABBO, 1966, 1969; WYBORN-MASON, 1966). However, in many recent reports only very limited success with emetine in the treatments of different types of cancers has been observed (PANETTIERE and COLTMAN, 1971; MASTRANGELO et al., 1973; SIDDIQUI et al., 1973; MOERTEL et al., 1974; KANE et al., 1975). In the experimental animal studies emetine has proved effective against rat Yoshida sarcoma (ISAKA, 1950), and mouse L1210 leukemia, P388 leukemia, B-16 melanoma, and the Ehrlich ascites carcinoma (JONDORF et al., 1971; JOHNSON and JONDORF, 1974). The effect of emetine in the mouse tumor screen was found to be schedule-dependent and the maximum response (72% increase in life-span) was observed in the mouse L1210 leukemia (JONDORF et al., 1971). JONDORF et al., (1971) have also examined the effectiveness of various derivatives of emetine in the mouse L1210 system and the results of their studies show a good correlation between the antitumor activity of these compounds and their protein synthesis inhibitory activity in other systems. It has been reported that emetine in combination with 5-azacytidine is much more effective against L1210 leukemia than either of these drugs alone (KLINE, 1974).

In addition to its amoebicidal and antitumor activities, the usefulness of emetine in the treatment of certain viral diseases, e.g., herpes zoster (VIDAL, 1952), ophthalmic herpes zoster (HANSICH et al., 1966), virus hepatitis (DEL PUERTO et al., 1968), and foot and mouth disease (KHALITOVA, 1972) has also been reported. In experimental viral infection studies in mice, emetine showed some activity against Columbia SK virus, but was ineffective against Coxsackie virus B1 and influenza A viruses (GRUNEBERG and PRINCE, 1966). Emetine, at low concentrations, has also been reported to be an effective antifungal agent (ABD-RABBO and YUSEF, 1966).

In comparison to emetine the pharmacological activities of the phenanthrene alkaloids cryptopleurine and tylocrebrine have only been investigated in a few experimental systems. The pharmacological activity of tylophora alkaloids was first investigated by CHOPRA and CHAKERBURTY (1935), who observed that tylophorine was toxic to *Paramecium caudatum* at a dilution of 1:50,000. In the mouse tumor screen, tylocrebrine has shown activity against mouse leukemia L1210, adenocarcinoma 755, and Walker 256 systems, but was inactive against the Dunning leukemia (GELLERT and RUDZATS, 1964; GOLDIN et al., 1966). GOLDIN et al., (1966) have also made mention of some clinical trials on tylocrebrine, which were inconclusive as they were terminated early because of the observed toxicity. FARNSWORTH et al., (1969) have reported that cryptopleurine shows a highly specific and extremely cytotoxic action against Eagle's 9KB carcinoma in cell culture, but was inactive against the sarcoma 180, adenocarcinoma 755, Lewis lung carcinoma, L-1210 leukemia, PS leukemia, and the Walker 256 sarcoma. However, no details of these experiments or the concentrations of cryptopleurine, which were employed in such studies, were provided. Cryptopleurine also exhibits antiviral activity against herpes virus, but was reported to be inactive against Coxsackie B5 and poliotype I viruses (KRMPOTIC et al., 1972).

Mammalian Toxicity and Tissue Distribution

The clinical usefulness of emetine in human subjects is somewhat limited due to the various side effects that are often observed (see reviews by KLATSKIN and FRIEDMAN, 1948; MANNO and MANNO, 1977). The primary sign of ipecac toxicity include; cardiotoxicity (symptoms: tachycardia, electrocardiographic irregularities and hypotension), gastrointestinal disturbances (symptoms: severe diarrhea, nausea, vomiting and abdominal cramping), neuromuscular effects (symptoms: muscle weakness, aching or stiffness, convulsion, mild tremor, edema), and other general symptoms such as fever, shock, and dehydration (see review by MANNO and MANNO, 1977). The mechanism of cardiotoxicity of emetine, which represents the most serious of its side effects, has been investigated in heart preparations (isolated from animals treated with acute and chronic doses of emetine) using biochemical, structural, and electrophysiological approaches. The literature on this subject has recently been reviewed by YANG and DUBICK (1980). Some of the various effects of emetine that have been reported in the isolated heart or heart extract are: decreased contractility and conduction velocity of heart atria, reduced rate of protein synthesis, etc. However, as reviewed by these authors, the relationships of various biochemical and physiological changes that are observed in heart to the cardiotoxic effect of emetine are not clear at present (YANG and DUBICK, 1980).

The LD 50 (i.p.) of emetine in rats has been estimated to be about 12.0 mg/Kg (RADOMSKI et al., 1952). The distribution and excretion of emetine in rats, mice, and dogs have been studied by GIMBLE et al., (1948). In rats and dogs, which received either single or multiple doses of emetine, concentration of the drug

in various tissues was found to be in the following order: liver, kidney, lung, brain, heart, skeletal muscle, and blood. PARMER and COTTRILL (1949), and later DAVIS et al. (1962) reported that soon after injection the heart levels of emetine were relatively high, but that after 6 h they had dropped to the lower levels seen over the next 6 days. SCHWARTZ and HERRERO (1965) have compared the tissue distribution and excretion of emetine and dehydroemetine in guinea pigs. Results of their studies showed that when the drugs were administered intraperitoneally dehydroemetine disappeared faster from the heart than the liver, whereas the opposite results were observed for emetine. The reduced cardiotoxicity of dehydroemetine compared to emetine may, therefore, be a result of its weaker binding to heart.

In contrast to emetine, the data on the mammalian toxicity of cryptopleurine and tylocrebrine are very scanty. DE LA LANDE (1948) had examined the toxicity of cryptopleurine toward guinea pigs, rats, mice, rabbits, cats, and dogs, and the LD50 of cryptopleurine for these animal species was reported to be in the range of 1.5 to 5 mg/kg. The toxic effects which were observed in these studies included marked diarrhea and inflammation of the stomach. Tylocrebrine has also been reported to be extremely toxic to various animal species. It is lethal to frogs at a dose of 0.4 mg/kg, but is less toxic toward mice and guinea pig (CHOPRA and CHAKERBURTY, 1935; GOVINDACHARI, 1967). Upon administration of either tylocrebrine or cryptopleurine to animals, an initial drop in blood pressure followed by immediate recovery to normal levels has been reported (DE LA LANDE, 1948; see GOVINDACHARI, 1966). Both tylocrebrine and cryptopleurine also exhibit powerful skin irritant and vesicant activities.

Summary and Prospects

Genetic and biochemical data reviewed here show that the benzoisoquinoline alkaloids, emetine, cephaeline, dehydroemetine, tubulosine, etc., and the phenanthrene alkaloids, cryptopleurine, tylocrebrine, etc., possess similar biological activities and appear to act in an identical manner. The primary effect of these alkaloids in eukaryotic cells and organisms is on cytoplasmic protein synthesis, which is inhibited at very low concentrations (10^{-7} to 10^{-9} M range). These compounds inhibit the enzymic translocation of peptidyl tRNA from the acceptor site to the donor site on ribosome and thereby block movement of mRNA along the ribosome. Mutants of mammalian cells resistant to these alkaloids are indistinguishable from each other by various genetic and biochemical criteria, but they do not exhibit any cross-resistance to other inhibitors of protein synthesis, such as cycloheximide, trichodermin, anisomycin, sparsomycin, bruceantin, etc., indicating that the mechanism of action of the latter inhibitors is different from that of the above compounds. The biochemical lesion in these mutants has been shown to affect the protein S20 of the 40S ribosomal subunit.

To account for the striking similarity in their modes of action, it has been suggested that the biological activity of these two groups of alkaloids is due

to common structural determinants. Based on the structure–activity relationship between these and a number of other related compounds, the requirement for biological activity for these compounds appears to be "a planar molecule with two aromatic rings rendered electronegative by methoxyl or hydroxyl substituents and a nucleophilic element, such as nitrogen, at a certain distance from the aromatic rings." Cross-resistance studies with the mutant cells provide a sensitive means for determining the presence of similar structural determinants in other compounds.

Emetine and related ipecac alkaloids, e.g., 2,3-dehydroemetine, have long been used for therapeutic purposes and have been extensively investigated. Although the toxic side effects somewhat diminish their clinical value, these compounds still constitute one of the important drugs in the treatment of invasive intestinal amoebiasis and amoebic liver abscesses. These compounds have also shown promise in some studies as antifungal, antiprotozoal, antiviral, and antitumor agents. In contrast to emetine and related compounds, various phenanthrene alkaloids, e.g., tylocrebrine, tylophorine, cryptopleurine, have not been properly evaluated for their clinical and pharmacological activities. However, in view of the fact that the biological activity of these compounds is very similar (or identical) to that of the ipecac alkaloids, and that these compounds may in fact be more active than the latter, further clinical evaluation of these compounds is warranted. Our current understanding of the structural features of these compounds, which appear important in their biological activity, should also prove very useful in the design and synthesis of new drugs, some of which may have higher therapeutic index than these compounds.

Acknowledgments. I am particularly grateful to Dr. L. SIMINOVITCH, in whose laboratory much of our work reviewed here was carried out for providing inspiration and encouragement, and to Dr. J.J. KREPINSKY, whose participation made some of the studies possible. I also thank Drs. L. BRANDA and D. MCCALLA for many helpful comments on the manuscript. The author is currently a scholar of the Medical Research Council of Canada.

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Kirromycin and Related Antibiotics

H. WOLF and E. FISCHER

Introduction

Most of the antibiotics reported to interfere with polypeptide chain elongation bind to ribosomes. There are only three kinds of antibiotics whose target is not the ribosome, but an elongation factor. The antibiotic fusidic acid inhibits the translocation reaction catalyzed by the elongation factor G (EF-G). The antibiotics kirromycin and pulvomycin inhibit the substrate supply by acting on the elongation factor Tu (EF-Tu); however, the mechanism of action is quite different. Unlike fusidic acid, kirromycin and pulvomycin selectively act upon the elongation factor from prokaryotes.

This article is intended to review the mechanism of action of kirromycin in comparison with that of pulvomycin.

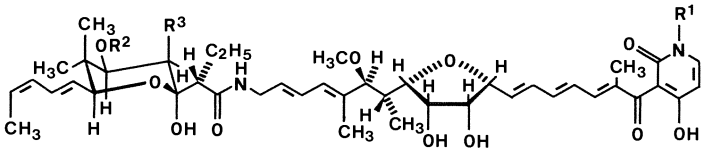
Chemical Structures

Recently, a group of antibiotics with novel structures was discovered (Fig. 1).

In 1972 the antibiotics kirromycin (WOLF and ZÄHNER, 1972), mocimycin (VOS, 1972), and aurodox (BERGER, 1972) were isolated from the culture fluids of *Streptomyces collinus, Streptomyces ramocissimus,* and *Streptomyces goldiniensis,* respectively. The complete structures of these antibiotics, including stereochemical details, have been elucidated. Kirromycin is identical with mocimycin, whereas aurodox is the pyridone N-methylated form (VOS and VERWIEL, 1973; MAEHR et al., 1973; MAEHR et al., 1980).

Closely related to aurodox are heneicomycin (ZIMMERMANN et al., 1979) and efrotomycin (WAX et al., 1976), produced by *Streptomyces filipinensis* and *Streptomyces lactamdurans;* heneicomycin is the 3-deshydroxy pyran modification, efrotomycin a disaccharide derivative of aurodox.

Dihydromocimycin (JONGSMA et al., 1977) and kirrothricin (THEIN, 1977) were isolated more recently from cultures of *Streptomyces ramocissimus* and *Streptomyces cinnamomeus*. Both antibiotics contain a 4-hydroxy-5,6-dihydro-2-pyridone. Kirrothricin differs further from dihydromocimycin in lacking the

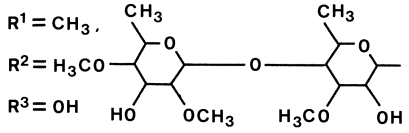


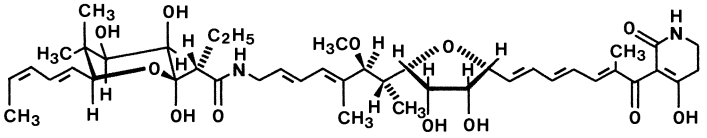
MOCIMYCIN, KIRROMYCIN: $R^1 = R^2 = H$, $R^3 = OH$

AURODOX, X-5108, GOLDINOMYCIN: $R^1 = CH_3$, $R^2 = H$, $R^3 = OH$

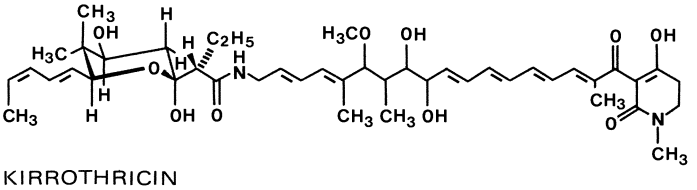
HENEICOMYCIN, A 21A: $R^1 = CH_3$, $R^2 = R^3 = H$

EFROTOMYCIN: $R^1 = CH_3$,

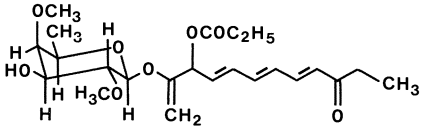




DIHYDROMOCIMYCIN



KIRROTHRICIN



LABILOMYCIN, PULVOMYCIN

Fig. 1. Structures of kirromycin and related antibiotics. (MAEHR et al., 1980; AKITA et al., 1964; ZEECK et al., 1981)

central tetrahydrofuran and the hydroxyl at the C-3 position of the pyranose (ZEECK et al., 1981).

Azdimycin is a fermentation product of *Streptomyces diastatochromogenes*. This antibiotic is also assumed to be a derivative of kirromycin, although its detailed structure is as yet unknown (NIMECK et al., 1975).

The antibiotic pulvomycin (ZIEF et al., 1957; ASSMANN and WOLF, 1979), identical with labilomycin (AKITA et al., 1963; SCHWARTZ et al., 1976), was isolated from cultures of *Streptoverticillium mobaraense* and *Streptomyces albosporeus*. Pulvomycin resembles the 5′-substituent of the central tetrahydrofuran of

kirromycin (WOLF et al., 1978). Based on this structural similarity and the common target site of pulvomycin and kirromycin, pulvomycin was placed in this group.

Antibacterial Spectrum and Toxicity

The antibiotics of the kirromycin group are active against a limited variety of bacteria, but are ineffective against fungi (WOLF, 1971; FROST et al., 1976). In general, they function as bactericidal agents at high concentrations. *Bacillus anthracis, Bacillus brevis, Bacillus cereus, Bacillus megaterium, Clostridium pasteureanum, Haemophilus influenzae, Moraxella bovis, Mycoplasma gallisepticum, Streptomyces viridochromogenes,* and other bacteria are highly sensitive (MIC <1 μg/ml).

The insensitivity of most bacteria to these compounds is considered to be due to inefficient uptake. If the resistant wild type of *Proteus mirabilis* is converted to the L-form with a defective or absent cell wall, this organism becomes highly sensitive (MIC 0.07 μg/ml) to kirromycin (WOLF and ZÄHNER, 1972). The impermeability of the cell wall, however, is not the only principle of natural resistance to kirromycin. Some bacteria are less sensitive by virtue of a nonresponding target site. For instance, the insensitivity of *Halobacterium cutirubrum* (KESSEL and KLINK, 1981), *Lactobacillus brevis* (WÖRNER and WOLF, 1981), and *Streptoverticillium mobaraense* (GLÖCKNER et al., 1981) toward kirromycin or pulvomycin is mediated by EF-Tu that naturally is less susceptible toward the action of the drugs.

The antibiotics are well tolerated in animals. LD_{50}'s of mocimycin, aurodox, and efrotomycin for mice are >4000 mg/kg given orally or >1000 mg/kg given intraperitoneally (VOS, 1972; BERGER et al., 1973; FROST et al., 1976). Pulvomycin is more toxic; the LD_{50} of pulvomycin for mice is >250 mg/kg given intraperitoneally (ZIEF et al., 1957). The addition of 1000 ppm mocimycin to the food of chickens or rats for 2 months did not reveal any adverse effect (VOS, 1972).

Antibiotics of the kirromycin group are suitable as food additives for farm animals because of their high nutritive effectiveness and low toxicity. In this respect, they can serve as chemotherapeutic agents to cure the dysentery caused by *Treponema* infections (BERGER, 1972; VOS, 1972; FOSTER and HARRIS, 1976).

Mechanism of Action of Kirromycin and Pulvomycin

Inhibition of Protein Synthesis

First evidence for kirromycin and pulvomycin being specific inhibitors of protein synthesis emerged from studies with whole cells of *Bacillus brevis* (WOLF

et al., 1972; ASSMANN and WOLF, 1979). Incorporation of radioactively labeled precursors into DNA, RNA, or protein showed that either antibiotic selectively inhibits protein synthesis. This finding was further supported by in vitro experiments (WOLF et al., 1972; ASSMANN and WOLF, 1979). In extracts of *Bacillus brevis* and *Escherichia coli,* poly(Phe) synthesis was 50% inhibited at 5×10^{-7} M kirromycin and 10^{-6} M pulvomycin, respectively. The high susceptibility of poly(Phe) synthesis and the insensitivity of amino acylation of tRNA to these antibiotics (WOLF et al., 1977; ASSMANN and WOLF 1979) suggested that kirromycin and pulvomycin act primarily on polypeptide chain elongation.

Inhibition of Polypeptide Chain Elongation

To locate the action of kirromycin and pulvomycin, the effect on partial reactions of the elongation cycle in the cell-free system of *E. coli* was investigated.

In the experiment shown in Fig. 2, ribosome·poly(U) complexes bearing poly(Phe)-tRNA in the ribosomal P-site were incubated with [^{14}C]Phe-tRNA in the presence and absence of EF-Tu and [γ-^{32}P]GTP. In the presence of EF-Tu, Phe-tRNA binding to the ribosome was not affected by kirromycin, while GTPase activity was stimulated and the peptidyl transfer reaction was inhibited. In the absence of EF-Tu, however, the peptidyl transfer reaction was not inhibited by the antibiotic. These results showed that kirromycin permits AA-tRNA binding to the ribosome with concomitant hydrolysis of GTP. In the presence of the antibiotic, enzymatically bound AA-tRNA could not function as a substrate for the peptidyl transfer reaction. Consequently, the inhibition

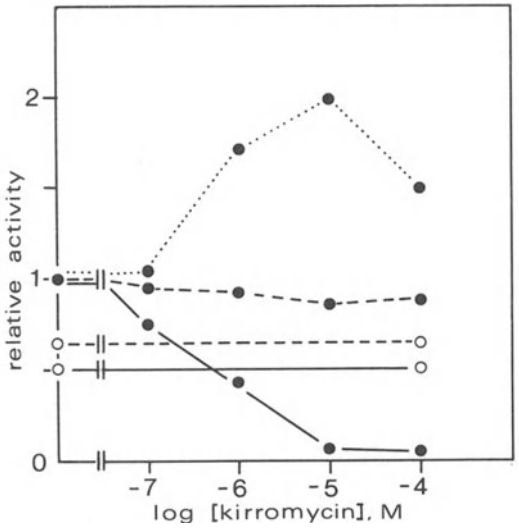


Fig. 2. Effect of kirromycin on (···) GTP hydrolysis, (– – –) Phe-tRNA binding to ribosomes, (——) peptidyl transfer reaction, (●) with or (○) without EF-Tu. (WOLF et al., 1974)

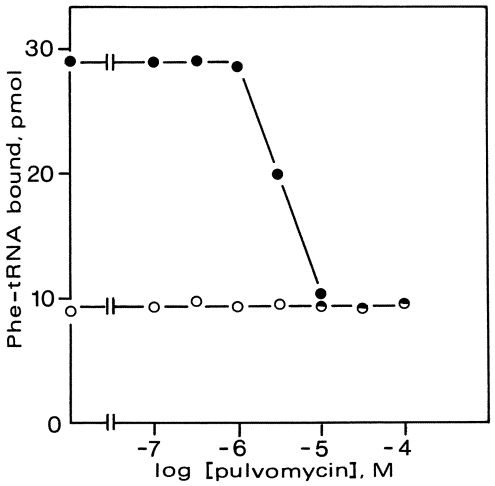


Fig. 3. Effect of pulvomycin on (●) enzymatic and (o) nonenzymatic binding of Phe-tRNA to ribosomes. (WOLF et al., 1978)

of peptide bond formation ought to be due to an incorrect positioning of the AA-tRNA on the ribosome. These results pointed out that EF-Tu, rather than the peptidyl transferase, was the target site of kirromycin (WOLF et al., 1974).

In the case of pulvomycin, the EF-Tu-catalyzed binding of AA-tRNA to ribosomes was identified as the target reaction of the elongation cycle (WOLF et al., 1978):

The antibiotic did not affect nonenzymatic binding of Phe-tRNA to the ribosome, whereas efficient EF-Tu-mediated binding was suppressed to the level of nonenzymatic binding (Fig. 3). If pulvomycin were to inhibit EF-Tu-catalyzed binding by blocking the A-site on the ribosome, Phe-tRNA binding ought to be prevented entirely. Since only nonenzymatic binding of Phe-tRNA to the ribosomal A-site and to the P-site could occur, these data suggested that pulvomycin inhibited the formation of the ternary complex between EF-Tu, GTP, and Phe-tRNA through interaction with EF-Tu.

The susceptibility of other partial reactions of the elongation cycle toward kirromycin and pulvomycin was also examined (WOLF et al., 1974; WOLF et al., 1978). Neither antibiotic did interfere at concentrations up to 10^{-5} M with the formation of AcPhe-puromycin generated through the peptidyl transferase. Similarly, two parameters for translocation activity were found to be unaffected by the antibiotics: augmentation of the AcPhe-puromycin synthesis in the presence of EF-G plus GTP, and the EF-G-dependent GTPase activity.

Change of EF-Tu Affinity for Guanine Nucleotides

Elongation factor Tu promotes the binding of AA-tRNA to ribosomes during polypeptide chain elongation in *E. coli* (for reviews, see MILLER and WEISSBACH, 1977; KAZIRO, 1978). The protein is a monomer composed of 393 amino

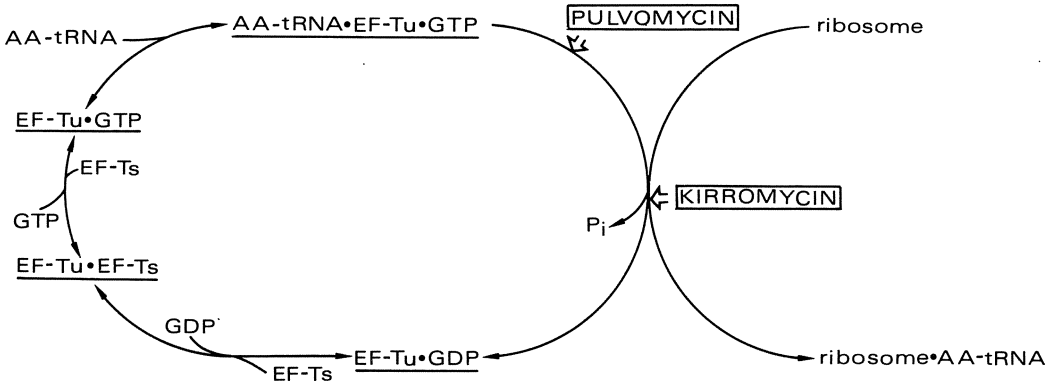


Fig. 4. The substrate supply cycle. (MILLER and WEISSBACH, 1977; KAZIRO, 1978)

acids, M_r 43,225 (ARAI et al., 1980). In this process EF-Tu·GTP interacts with AA-tRNA to form the ternary complex AA-tRNA·EF-Tu·GTP. In response to the appropriate codon, AA-tRNA is bound irreversibly to the ribosome in the form of the ternary complex, GTP is hydrolyzed, EF-Tu·GDP and Pi are released. The regeneration of EF-Tu·GTP from EF-Tu·GDP is catalyzed by elongation factor Ts (EF-Ts), through a transient EF-Tu·EF-Ts complex (Fig. 4).

Essentially, the attachment of kirromycin and pulvomycin to EF-Tu results in strong changes in the affinity of the factor for guanine nucleotides. Thus either antibiotic can promote a rapid exchange of EF-Tu-bound GDP toward unbound GTP (CHINALI et al., 1977; WOLF et al., 1978) and can stimulate as well the EF-Tu·GDP/GDP exchange at 0° C (BLUMENTHAL et al., 1977; our unpublished data). In both cases, the enhancement of the exchange reactions of guanine nucleotides with EF-Tu is more pronounced with pulvomycin than with kirromycin.

EF-Tu undergoes conformational transitions upon substitution of the bound nucleotide GDP by GTP and vice versa (MILLER and WEISSBACH, 1977; KAZIRO, 1978). GTP stabilizes a reactive conformation having high affinity for AA-tRNA and ribosomes; conversely, GDP induces a nonreactive conformation having low affinity for AA-tRNA and ribosomes. By several studies, kirromycin has been shown to interfere with this transition from one EF-Tu conformation to another.

In a kinetic study, it has been reported that the equilibrium constant of (kirromycin·EF-Tu)·GTP is about 100-fold less than that of EF-Tu·GTP, i.e., equal to that of EF-Tu·GDP (FASANO et al., 1978). These results suggested that kirromycin makes EF-Tu·GDP more like EF-Tu·GTP.

Conformational changes of EF-Tu can be detected by the method of controlled proteolytic cleavage. Therefore, the action of kirromycin on the rate of proteolytic conversion of EF-Tu (M_r 43,225) to the fragment A (M_r 36,000) by trypsin was studied (DOUGLASS and BLUMENTHAL, 1979). EF-Tu·GDP was cleaved slowly (50% at 2.9 min), whereas EF-Tu·GTP was cleaved rapidly (50% at 0.7 min). In the presence of kirromycin the cleavage rate of EF-Tu·GDP

(50% at 0.9 min) was indistinguishable from the cleavage rate observed with EF-Tu·GTP, suggesting an EF-Tu·GTP-like conformation in kirromycin·EF-Tu·GDP.

NMR studies indicated that kirromycin indeed stabilizes a conformation in EF-Tu·GDP, which is similar to that of EF-Tu·GTP. Conversion of EF-Tu·GDP to EF-Tu·GTP resulted in altered signals, which could be in part simulated by the addition of the antibiotic (RÖMER et al., 1981).

In ESR and water relaxation studies, the effect of aurodox on EF-Tu·MnGDP has been investigated. These studies revealed that upon binding of the antibiotic the metal-nucleotide binding site on the factor became more exposed (WILSON and COHN, 1977). This finding is in agreement with the observations described above.

Effect on EF-Tu Interaction with AA-tRNA

AA-tRNA interacts with EF-Tu·GTP to form the ternary complex AA-tRNA·EF-Tu·GTP. This complex passes through cellulose nitrate filters, while EF-Tu·GTP is retained. This filtration technique has been used to assay the effect of both antibiotics on the formation of the ternary complex. In the presence of kirromycin, EF-Tu·GTP was released from the filters by Phe-tRNA due to the formation of the ternary complex (WOLF et al., 1974). Virtually, the inversed effect was found with pulvomycin. All EF-Tu·GTP was retained on the filters because pulvomycin prevented the binding of Phe-tRNA to EF-Tu·GTP; the values obtained in the presence of Phe-tRNA coincided with those found for EF-Tu alone (WOLF et al., 1978).

The divergent effect of pulvomycin and kirromycin on EF-Tu affinity for Phe-tRNA was also found when the formation of the ternary complex was measured by gel filtration (CHINALI et al., 1977; WOLF et al., 1978). Chromatographic analysis of the reaction mixture containing pulvomycin revealed no detectable formation of Phe-tRNA·EF-Tu·GTP (Fig. 5). In the presence of the antibiotic, uncomplexed Phe-tRNA (partially deacylated during the gel filtration process) was eluted from the Sephadex G-100 column in the same way as in the absence of EF-Tu·GTP. By contrast, when Phe-tRNA was added to EF-Tu·GTP, kirromycin caused a significant increase in the Phe-tRNA·EF-Tu·GTP peak. EF-Tu·GTP protects the ester linkage between amino acids and tRNA against nonenzymatic hydrolysis. Therefore, the effect of both antibiotics on the formation of the ternary complex was also investigated by hydrolysis protection experiments. Addition of pulvomycin completely abolished the protective effect of EF-Tu·GTP on the nonenzymatic hydrolysis of Phe-tRNA (WOLF et al., 1978). Kirromycin is significantly less effective in the elimination of the hydrolysis protection effect. However, due to an altered conformation of the ternary complex in the presence of kirromycin, this antibiotic also renders the ester bond in Phe-tRNA·EF-Tu·GTP slightly more accessible to spontaneous hydrolysis (PINGOUD et al., 1978).

GTP stabilizes a conformation of EF-Tu having an AA-tRNA binding site which is not exposed in EF-Tu·GDP (MILLER and WEISSBACH, 1977; KAZIRO,

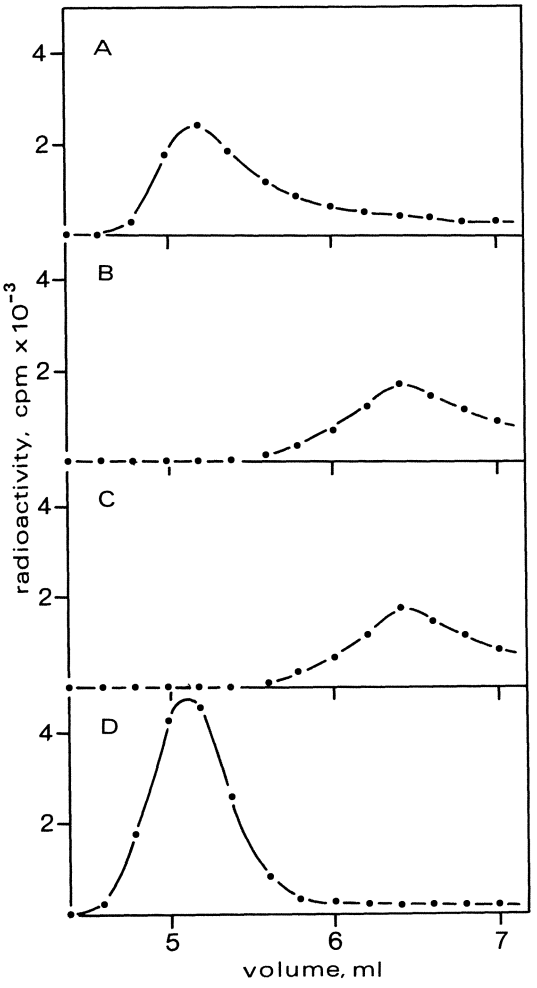


Fig. 5. Effect of kirromycin and pulvomycin on the interaction of Phe-tRNA with EF-Tu·GTP as measured by gel filtration. *A* Control; *B* Phe-tRNA alone; *C* pulvomycin; *D* kirromycin. (WOLF et al., 1978)

1978). As kirromycin induces an EF-Tu·GTP-like conformation in EF-Tu, the existence of unusual complexes such as AA-tRNA·EF-Tu·GDP·kirromycin or AA-tRNA·EF-Tu·kirromycin seemed possible. Indeed, a detectable interaction of EF-Tu·GDP with Phe-tRNA in the presence of kirromycin was demonstrated using gel filtration chromatography (CHINALI et al., 1977). The antibiotic-induced interaction of EF-Tu·GDP with AA-tRNA was also inferred from the stimulation of the AA-tRNA binding to ribosomes by EF-Tu·GDP (WOLF et al., 1974; WOLF et al., 1977). Moreover, using the latter method, evidence was obtained that kirromycin could substitute for the guanine nucleotide as a whole. Even the entirely nucleotide-free complex Phe-tRNA·EF-Tu·kirromy-

cin promoted the binding of Phe-tRNA to the ribosome (WOLF et al., 1974; WOLF et al., 1977).

The rate of proteolytic conversion of EF-Tu into fragment A by trypsin is modified by the ligands that bind to EF-Tu. Since EF-Tu is cleaved far more slowly in the ternary complex AA-tRNA·EF-Tu·GTP than in the binary complex EF-Tu·GTP, the rate of tryptic hydrolysis was used as a probe to study the effect of pulvomycin upon ternary complex formation. Pulvomycin did not alter the cleavage rate of EF-Tu within the ternary complex. The sensitivity of EF-Tu toward trypsin remained unchanged in the presence of the antibiotic, and hydrolysis occurred apparently more slowly than with pulvomycin·EF-Tu·GTP (FISCHER and WOLF, 1981). The protection of EF-Tu·GTP from hydrolysis by trypsin shows that, despite the disintegration of the ternary complex functions, AA-tRNA does interact with EF-Tu·GTP also in the presence of pulvomycin.

Interference with EF-Tu GTPase

The distortion of allosteric configurations of EF-Tu·guanine nucleotide complexes through the action of kirromycin stops protein synthesis finally by locking AA-tRNA·EF-Tu·GDP or AA-tRNA·EF-Tu to the ribosomal A-site. The accomplishment of kirromycin action is favored and may be accelerated by another principal effect of kirromycin upon EF-Tu, namely the uncoupling of the elongation factor borne GTPase activity (WOLF et al., 1974). Powered by the GTPase reaction EF-Tu switches back into the EF-Tu·GDP configuration and hereby looses the affinity for both AA-tRNA and ribosomes. By this mechanism the A-site is cleared from EF-Tu (MILLER and WEISSBACH, 1977; KAZIRO, 1978). Through the uncoupling of the GTPase reaction from the transition of the EF-Tu·GTP configuration into the one of EF-Tu·GDP kirromycin inhibits the release of EF-Tu from the ribosomal A-site (FASANO et al., 1978).

The stimulation in vitro of the GTPase reaction involved in the enzymatic binding of AA-tRNA to the ribosome requires the presence of major components of protein synthesis, ribosomes, AA-tRNA, and EF-Tu. The addition of mRNA stimulates the reaction (MILLER and WEISSBACH, 1977). When kirromycin is added to such an assay, the GTPase activity is markedly enhanced but soon levels off. The yield of the reaction approximates 1 mol GTP hydrolyzed per 1 mol EF-Tu and cannot be increased through prolonged incubation. This means that by the addition of the antibiotic the substrate supply cycle can function one round and then is blocked (WOLF et al., 1977). The kirromycin-dependent limitation of the substrate supply cycle to one round can easily be broken up by the omission of any integral component of the physiological GTPase reaction. Through the action of the antibiotic the absence of all physiological effectors can be compensated for. A comparable hydrolysis of GTP does occur even under such otherwise nonpermissive conditions (WOLF et al., 1974). From the data shown in Table 1 it can be derived that poly(U) (mRNA) does not stimulate the kirromycin-induced GTPase reaction, whereas tRNA, AA-tRNA and, most of all, ribosomes act as potent catalysts of the antibiotic-

Table 1. Interference of kirromycin and pulvomycin with the GTPase reaction catalyzed by EF-Tu. (WOLF et al., 1978)

	GTP hydrolyzed, pmol			
	Control	+kirromycin	+ kirromycin +pulvomycin	+pulvomycin
EF-Tu·EF-Ts	0.2	3.2	1.4	1.0
EF-Tu·EF-Ts+Phe-tRNA	0.2	14.3	6.2	0.2
EF-Tu·EF-Ts+ribosomes	1.8	44.7	14.6	1.9
EF-Tu·EF-Ts+ribosomes +Phe-tRNA	7.4	54.2	16.9	1.1
EF-Tu·EF-Ts+ribosomes +Phe-tRNA+poly(U)	21.0	27.8	10.7	1.2

dependent GTPase. In the absence of poly(U), kirromycin can catalyze the GDP/GTP exchange reaction. Consequently, the hydrolysis of GTP by EF-Tu becomes a cyclic reaction, the yields of which even exceed the level of the physiological GTPase reaction.

Because the presence of both EF-Tu and ribosomes is crucial for the induction of the GTPase reaction if no kirromycin is added to the assay, it had been impossible for a long time to decide whether the enzyme to catalyze the hydrolysis of GTP is an integral part of either the ribosome or the elongation factor. This question could not be answered unequivocally until it was found that kirromycin can compensate for ribosomes in the GTPase reaction and that this antibiotic is capable to induce the hydrolysis reaction of GTP with EF-Tu alone (WOLF et al., 1974). No such activity can be stimulated with the ribosome through any physiological effector or antibiotic in the absence of EF-Tu. The action of kirromycin thus has proven an indispensable means for the discovery that the GTPase center is an integral domain of the elongation factor, and that the catalysis of GTPase reaction is a primary function of EF-Tu (WOLF et al., 1974).

Pulvomycin on the other hand is an effective inhibitor of the GTPase reaction associated with the enzymatic binding of AA-tRNA to the ribosome. The inhibition of the GTPase reaction is another aspect of the mechanism of action of pulvomycin, by which this antibiotic is clearly distinguished from kirromycin (WOLF et al., 1978). Although pulvomycin stimulates the exchange reaction of GDP vs GTP, the catalysis of the guanine nucleotide exchange by itself is not sufficient for the induction of the GTPase reaction. As shown in Table 1, inhibition by pulvomycin is not limited to the GTPase reaction promoted by the natural ligands of EF-Tu, but also concerns the GTPase reactions induced by kirromycin. Both antibiotics are effectively counteracting each other. The mutual exclusion suggests that both drugs bind to a common site of the EF-Tu molecule (WOLF et al., 1978). With EF-Tu alone, pulvomycin stimulates a GTPase reaction, which is less pronounced, however, than the corresponding activity stimulated by kirromycin. The pulvomycin-dependent hydrolysis of GTP by EF-Tu is completely suppressed by the addition of AA-tRNA (Table 1).

Apparently, the interaction of AA-tRNA with EF-Tu·GTP suffices to eliminate the inactive GTPase reaction caused by pulvomycin. Despite this corrective effect of AA-tRNA upon EF-Tu·GTP, pulvomycin prevents a productive interaction of AA-tRNA and EF-Tu (WOLF et al., 1978). The ternary complex AA-tRNA·EF-Tu·GTP, which is formed in the presence of the antibiotic, does not promote the binding of AA-tRNA to the ribosome (Fig. 3).

Effect on EF-Tu Interaction with EF-Ts

When EF-Tu·GDP together with EF-Ts was chromatographed on Sephadex G-25, GDP was found to be replaced by EF-Ts in EF-Tu·GDP. By addition of kirromycin (2×10^{-4} M) to the reaction mixture, this replacement was inhibited. Thus, at high concentration kirromycin prevented the association of EF-Tu with EF-Ts to the EF-Tu·EF-Ts complex (BROWN and BLUMENTHAL, 1976).

Kirromycin cannot dissociate a preformed EF-Tu·EF-Ts complex. This conclusion is based on experiments on reconstitution of Qß replicase (BROWN and BLUMENTHAL, 1976). *E. coli* phage Qß replicase, an RNA-dependent RNA polymerase, is a tetramer composed of one phage-coded polypeptide and three host-coded polypeptides: EF-Tu, EF-Ts, and ribosomal protein S1. Qß replicase, denatured in 8 M urea, can be renatured by addition of EF-Tu or EF-Tu·EF-Ts. Kirromycin, which did not affect native replicase activity, inhibited the renaturation when EF-Tu was added, but did not affect renaturation when the EF-Tu·EF-Ts complex was added. It was suggested that EF-Tu and EF-Ts function as a complex in Qß replicase. The antibiotic inhibited the renaturation by preventing the complex formation, but (relevant in this context) did not separate a preformed complex.

This result contrasts with the finding that kirromycin can induce complete dissociation of EF-Tu·EF-Ts into EF-Tu and EF-Ts when analyzed by analytical polyacrylamide gel electrophoresis without detergent (CHINALI et al., 1977). The different experimental approaches, especially the exposure to an electric field, might be responsible for the opposite effect on EF-Tu·EF-Ts. By contrast, pulvomycin did not dissociate EF-Tu·EF-Ts on gel electrophoresis (our unpublished data).

Binding to EF-Tu

Parameters for the binding of kirromycin to EF-Tu were determined by CD titration (PINGOUD et al., 1978). In the CD spectrum of the antibiotic the negative Cotton effect at 320 nm increases in its amplitude upon the addition of EF-Tu. Using this effect, the binding constant and the number of binding sites were calculated from the titration curve to be $K_{ass} = (4 \pm 2) \times 10^6\ M^{-1}$ and $n = 1$ for the interaction between kirromycin and EF-Tu. The binding constant and the number of binding sites were the same for both EF-Tu·guanine nucleotide complexes, EF-Tu·GDP and EF-Tu-GTP.

The binding of kirromycin to AA-tRNA·EF-Tu·GTP cannot be investigated by the same technique because of the interference of CD signals from tRNA with those from the antibiotic. Therefore, the binding constant was derived indirectly. The association constants for the interaction between kirromycin and EF-Tu·GTP were compared to those for ternary complex formation – measured by hydrolysis protection experiments – in the presence and in the absence of the antibiotic. By this method, binding parameters for the interaction between kirromycin and the ternary complex were evaluated to be $K_{ass} \approx 2 \times 10^6\ M^{-1}$ and $n = 1$ (PINGOUD et al., 1978).

The affinity of kirromycin for EF-Tu was also estimated by additional methods: In these experiments the antibiotic concentration inducing half-maximal effect upon association and dissociation of EF-Tu·nucleotide (FASANO et al., 1978), upon trypsin cleavage, and upon nucleotide exchange (DOUGLASS and BLUMENTHAL, 1979), was used as a probe to quantify the interaction of the antibiotic with EF-Tu. In contrast to the data obtained by CD titration, the affinity of kirromycin determined by these techniques was approximately 20- to 40-fold higher for EF-Tu·GTP than for EF-Tu·GDP.

Structural Basis for Action on EF-Tu

The 5′-substituent of the tetrahydrofuran ring of kirromycin might be responsible for the action of kirromycin on EF-Tu (WOLF et al., 1978). This suggestion is based on the following observations. The structure of the 5′-substituent resembles the structure of pulvomycin. Both antibiotics probably bind to a common site on EF-Tu (WOLF et al., 1978). Moreover, hydrolysis of the amide bond located in the 5′-substituent produces two fragments which are completely inactive on EF-Tu (WOLF et al., 1977).

Recent work (CHINALI, 1981) provides experimental evidence for this suggestion. A fragment, which results from periodate oxidation of kirromycin and virtually corresponds to the entire 5′-substituent, promotes all effects of the intact antibiotic on EF-Tu reactions, although at elevated concentrations (50% inhibition of poly(Phe) synthesis at 10^{-4} M). It has been suggested that kirromycin and pulvomycin induce different conformational changes at the same EF-Tu site as a consequence of differences between the structures of pulvomycin and that of the 5′-substituent.

Kirromycin, aurodox, efrotomycin, and kirrothricin appear to share a common mode of action. These compounds have been examined but were found indistinguishable from one another in the poly(Phe) synthesis, EF-Tu·GDP/GTP exchange reaction, and formation of the ternary complex Phe-tRNA·EF-Tu·GTP measured by gel filtration chromatography or hydrolysis protection experiments (WOLF et al., 1977; our unpublished data). Thus, substitutions of the hydroxyl at C-2 position of the sugar by hydrogen (kirrothricin), or at C-3 position of the sugar by a disaccharide (efrotomycin), seem to be irrelevant to the action of the 5′-substituent upon EF-Tu.

Resistant Mutants with Altered EF-Tu

The response of EF-Tu to kirromycin can be eliminated through the alteration of the primary structure of the enzyme. Mutations that cause the genes of EF-Tu to code for kirromycin resistance of the enzyme have been reported for *E. coli* (two different mutants: FISCHER, 1976; VAN DE KLUNDERT et al., 1977) and *Bacillus subtilis* (several mutants: SMITH and PARESS, 1978). No such resistant mutant has yet been described for pulvomycin. The decreased sensitivity of EF-Tu prepared from the producing strain *Streptoverticillium mobaraense* (GLÖCKNER et al., 1981), indicates, however, that modifications of the EF-Tu structure to diminish the interaction of pulvomycin with EF-Tu may not be lethal.

The selection of kirromycin-resistant mutants of *E. coli* harboring alterations in the target site of kirromycin is particularly hampered by inherent properties of this strain:

1. *E. coli* is not sensitive toward kirromycin because of the impermeability of its outer membrane (WOLF and ZÄHNER, 1972). In consequence, *E. coli* cells have to be permeabilized by a treatment with EDTA, or else kirromycin-sensitive mutants of *E. coli* must be used for the selection procedure. Upon application of either method, the selection of mutants with modified EF-Tu is obscured by mutants that have become insensitive to the permeabilizing agent, or have regained the impermeability for kirromycin by modifications of the cell wall (FISCHER, 1976).

2. Two distinct structural genes are coding for EF-Tu, *tuf* A and *tuf* B, located at 72 and 88 min of the recombination map, respectively (JASKUNAS et al., 1975). A mutation of only one gene of EF-Tu consequently yields a mixed population composed of resistant and sensitive EF-Tu. Additional mutations in the second gene of EF-Tu are required to obtain a homogeneous population of resistant EF-Tu only (FISCHER et al., 1977).

3. Kirromycin resistance mediated by EF-Tu is a recessive property (FISCHER, 1976; FISCHER et al., 1977). In mutant strains containing mixed populations of sensitive and resistant EF-Tu the kirromycin resistance is not expressed in the phenotype, due to the excess amount of EF-Tu over ribosomes. The molar ratio of EF-Tu:ribosomes depends upon the growth conditions and varies between 8:1 and 14:1 (FURANO, 1975). Because of the particular mode of action of kirromycin, all the A-sites of the ribosomes are blocked by immobilized EF-Tu if sensitive EF-Tu is present in at least a 1:1 molar ratio to ribosomes. The acceptor function of ribosomes being crucial for the turnover of the substrate supply, protein synthesis can no longer be promoted despite the presence of kirromycin-resistant EF-Tu (FISCHER et al., 1977).

Both mutants of *E. coli* described to display kirromycin resistance in vivo and in vitro carry additional mutations in the second gene of EF-Tu. In the kirromycin-resistant mutant *E. coli* D2216, a modified *tuf* B gene (*tuf* B1) codes for the kirromycin-resistant EF-Tu (FISCHER, 1976; FISCHER et al., 1977). The mutant has been selected from the kirromycin-sensitive strain *E. coli* D22

(NORMARK et al., 1969) with a modified cell envelope (*env* A), after induced mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine as a mutagen. The *tuf* A gene in this strain is silenced and does not code for an EF-Tu-like gene product. The EF-Tu population of D2216 consists of homogeneous kirromycin-resistant EF-Tu only. In the poly(U)-directed poly(Phe) synthesis catalyzed by resistant EF-Tu a 50% inhibition requires 10^2- to 10^3-fold the amount of kirromycin, depending upon the incubation temperature (FISCHER et al., 1977).

There is no cross-resistance of EF-Tu from D2216 to pulvomycin. The in vitro protein synthesis catalyzed by kirromycin-resistant EF-Tu is even more susceptible to pulvomycin than a corresponding assay which is catalyzed by wild-type EF-Tu (WOLF et al., 1978).

A proportional decrease in sensitivity toward kirromycin is found with the induction of the GTPase reaction (FISCHER et al., 1977). The purified elongation factor shows low autocatalysis of the GTP hydrolysis in the absence of natural effectors or even kirromycin. The level of GTP hydrolysis in the presence of effectors is higher than with wild-type EF-Tu. In the absence of kirromycin, the affinity of resistant EF-Tu for GTP is increased (FASANO and PARMEGGIANI, 1981). These alterations of the properties of the enzyme must result from minute alterations in the enzyme structure, because in SDS-polyacrylamidegel-electrophoresis (FISCHER, 1976), or in isoelectric focusing (IVELL et al., 1981), the EF-Tu from D2216 is indistinguishable from kirromycin-sensitive EF-Tu. In fingerprints of the tryptic hydrolysate of denatured resistant EF-Tu, one peptide bearing a modification could be identified by an altered mobility in chromatography (FISCHER et al., 1977). The alteration in the amino acid composition causing the modification of the hydrophobicity coefficient of the peptide has not been analyzed.

The other mutant, *E. coli* LBE2012, isolated by means of an EF-Tu-mediated resistance toward mocimycin contains a heterogeneous population of EF-Tu comprising *tuf* A as well as *tuf* B gene products (VAN DE KLUNDERT et al., 1977). In this mutant, the *tuf* A gene codes for the kirromycin-resistant EF-Tu, whereas the *tuf* B gene product can still sustain protein synthesis, but is kirromycin-sensitive (VAN DER MEIDE et al., 1980). That kirromycin resistance is expressed in the phenotype of this mutant is a consequence of a mutation in the *tuf* B locus causing a peculiar modification of the EF-Tu response toward kirromycin action. The modified *tuf* B gene product can form a ternary complex AA-tRNA·EF-Tu·GTP, which promotes the substrate supply reaction in the absence of kirromycin. Unlike the ternary complex containing wild-type EF-Tu, which is locked to the A-site of the ribosome, the ternary complex with EF-Tu coded by the modified *tuf* B gene fails to associate with the ribosomes in the presence of the antibiotic (DUISTERWINKEL et al., 1981a; VAN DER MEIDE et al., 1981). This explains both the phenotypic expression of kirromycin-resistant EF-Tu despite the presence of otherwise dominating sensitive EF-Tu, and the inhibition of protein synthesis independent of the immobilization of EF-Tu on the ribosome.

The mutant LBE2012 bearing these modifications has been isolated from *E. coli* KMBL1001 (VAN DE KLUNDERT et al., 1977). It was selected on mocimycin-containing plates after mutagenesis with ethylmethane sulfonate as a

mutagen and permeabilization of the mutagenized strain with EDTA. The mocimycin-resistant EF-Tu is 70-fold less sensitive to the antibiotic in poly(U)-directed poly(Phe) synthesis. The affinity to mocimycin is low, and compared to the wild-type EF-Tu, less than 10% of [^{14}C]-labeled mocimycin are bound to the resistant EF-Tu. The mocimycin-resistant elongation factor carries a threonine instead of an alanine residue at position 375. This single amino acid substitution is assumed to be sufficient for the reduction of mocimycin sensitivity of EF-Tu (DUISTERWINKEL et al., 1981 b).

With *Bacillus subtilis,* resistance to kirromycin based on a modified elongation factor becomes a very frequent mutation, which occurs spontaneously at a frequency of 10^{-7} (SMITH and PARESS, 1978). The elongation factor Tu prepared from two resistant mutants is less sensitive in the poly(U)-directed poly(Phe) synthesis than EF-Tu prepared from wild-type *Bacillus subtilis.* In general kirromycin-sensitive and kirromycin-resistant EF-Tu from this organism are less sensitive toward the antibiotic than EF-Tu from *E. coli* (SMITH and PARESS, 1978). The specifity of kirromycin action, together with the high frequency of elongation factor mediated resistance makes kirromycin a versatile tool to investigate the genetics of EF-Tu in *Bacillus subtilis.* By means of kirromycin resistance, it was demonstrated that there is only a single gene in *Bacillus subtilis* coding for EF-Tu, which maps in the cluster of genes for ribosomal proteins and elongation factor G inside the *str* A – *spc* region of the *Bacillus subtilis* chromosome (SMITH and PARESS, 1978).

Effect on Eukaryotic Cells

Pulvomycin is known to be cytotoxic for malignant cells in tissue culture and for cells of the ascitic form of Ehrlich carcinoma (ECA) in mice (ISHIZUKA et al., 1964; SCHWARTZ et al., 1976). Incorporation of radioactive precursors into macromolecules of ECA and HeLa cells showed that pulvomycin and kirromycin inhibited RNA and, to a lesser degree, DNA synthesis, while protein synthesis continued. Similarly, for both cell types 50% inhibition of uridine incorporation into acid-insoluble material was observed at 3×10^{-6} M pulvomycin and 10^{-5} M kirromycin, respectively (SCHMID et al., 1978). In bacteria these antibiotics do not interfere with RNA and DNA synthesis but inhibit protein synthesis by acting on elongation factor Tu. Therefore, the action of kirromycin and pulvomycin on eukaryotic cells is different from the action on prokaryotic cells.

Since the toxicity of pulvomycin and especially kirromycin is very low, transformed cells differ in sensitivity from normal cells. The cytotoxic effect of either drug on transformed eukaryotic cells is related to the inhibition of the RNA synthesis. Preliminary results suggest an interference of both antibiotics with the salvage pathway (SCHMID, 1980), a reaction which is essential for the supply of rapid-growing cells with nucleotides (LUCAS, 1967).

Summary

Pulvomycin and the kirromycins are inhibitors of bacterial origin that act upon the elongation factor Tu (EF-Tu) from prokaryotes. The antibiotics induce short circuits or stop the substrate supply reaction cycle of protein synthesis.

Kirromycin causes the spontaneous exchange of guanine nucleotides complexed with EF-Tu and distorts the structural transitions of EF-Tu necessary for the cyclic interaction with AA-tRNA and ribosomes. In the GTPase reaction associated with the binding of every AA-tRNA to the ribosomal A-site, kirromycin can substitute for the physiological effectors ribosomes and AA-tRNA. The hydrolysis of GTP can even be induced with EF-Tu alone. In this antibiotic-induced GTPase reaction of the elongation factor, ribosomes and AA-tRNA remain active as catalysts. As a consequence of these molecular actions of kirromycin, a nonfunctional ternary complex AA-tRNA·EF-Tu·GDP is formed, which equally shows affinity for the ribosome. Similarly, the orderly ternary complex AA-tRNA·EF-Tu·GTP does not leave the ribosome after hydrolysis of GTP, because the GTPase reaction becomes uncoupled from the transition of the EF-Tu·GTP configuration into the one of EF-Tu·GDP. However, only this switchover in the structure of EF-Tu – which is inhibited by kirromycin – could eliminate the affinity for both AA-tRNA and the ribosome, and thereby initiate a new round of the substrate supply reaction cycle. The ongoing distortion of EF-Tu-dependent partial reactions by kirromycin finally leads to the breakdown of protein synthesis through the obstruction of the AA-tRNA binding site on the ribosomes.

The inhibition of protein synthesis by pulvomycin does not require the participation of ribosomes because this antibiotic inhibits a preceding reaction in substrate supply. In the presence of pulvomycin the enzymatic binding of AA-tRNA to the ribosome is stopped by rendering the ternary complex AA-tRNA·EF-Tu·GTP nonfunctional. Like kirromycin, this antibiotic interferes with the exchange reaction of guanine nucleotides. The binding of GTP to EF-Tu is stimulated and the EF-Tu·GTP complex is stabilized. The EF-Tu·GTP complex formed in the presence of pulvomycin, however, does not interact with AA-tRNA to yield a functional ternary complex AA-tRNA·EF-Tu·GTP. Especially the interaction of EF-Tu with the 3′-OH Terminus of AA-tRNA which is charged with the amino acid is susceptible to pulvomycin. Through the action of the antibiotic the protection of AA-tRNA against hydrolysis is totally eliminated and AA-tRNA is prone to hydrolysis as in the absence of EF-Tu·GTP. Nevertheless, some interaction of AA-tRNA with EF-Tu·GTP does occur even in the presence of pulvomycin. This is shown by the conservation of the insensitivity of EF-Tu toward tryptic hydrolysis, as well as through the suppression of the EF-Tu-dependent GTPase reaction by AA-tRNA. In the absence of physiological ligands a GTPase reaction of EF-Tu is stimulated by pulvomycin. This hydrolysis of GTP is less pronounced than that induced by kirromycin and is inhibited by the addition of AA-tRNA, ribosomes, poly(U), or by combinations of these ligands. Even the kirromycin-dependent GTPase reaction is suppressed by pulvomycin. From these data it is clear that pulvomycin action

is different from kirromycin action: Pulvomycin blocks the substrate supply by eliminating the efficient enzymatic binding of AA-tRNA to the ribosome through the creation of a nonfunctional ternary complex.

By their mode of action and their low toxicity pulvomycin and kirromycin are particularly suited for use in chemotherapy. The inhibitory action is very specific and solely directed toward well-known constituents of the prokaryotic protein synthesis machinery. Because of their precise mode of action these drugs have been recognized as precious tools to dissect the molecular mechanism of the enzyme EF-Tu, the functions of which are essential for the protein synthesis of bacteria.

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Anti-β-Lactamase Agents

J.R. KNOWLES

There are several ways in which bacterial populations can become resistant to the antibiotic action of β-lactams. First, the enzymes of cell-wall biosynthesis that are the targets of the β-lactam antibiotic may become less susceptible to acylation and inactivation. Secondly, changes in outer membrane permeability (for Gram-negative bacteria) may limit the access of the β-lactam antibiotic to the target enzymes. Thirdly, alterations in the activities of other enzymes that are responsible for the lysis of cells whose cell-wall synthesizing apparatus has been blocked by β-lactams may lead to bacterial stasis rather than to bacterial death. Finally, the appearance of either a chromosomal or a plasmid-encoded β-lactamase may cause the hydrolytic destruction of the β-lactam antibiotic before it can reach its target. Of all these types of resistance, it appears that production of a β-lactamase is the most common defense mechanism of bacteria, and the elimination of this problem is necessary to ensure the continuing clinical utility of β-lactams as antibacterial agents. This challenge is particularly important, since the plasmid- and transposon-mediated spread of the β-lactamase gene both within and between bacterial species is a facile process. The promiscuity of bacteria in terms of the sharing of the genetic information for elaboration of a β-lactamase is depressingly high.

There are two obvious approaches to solving the problem posed by the β-lactamase. The first is to devise β-lactams that are resistant to hydrolysis by the β-lactamase, yet retain their specificity for the target enzymes and hence their antibiotic potency. This route was the first to be followed, interest being spurred by the availability of 6-aminopenicillanic acid (which can be *N*-acylated with a variety of acid derivatives, leading to a range of penams of different enzyme specificities) in the late 1950's (BATCHELOR et al., 1959; SHEEHAN and HENERY-LOGAN, 1959; DOYLE and NAYLER, 1964; NAYLER, 1973). The quest for penam (and cephem) derivatives that were resistant to β-lactamase-catalyzed hydrolysis was quite successful, though antibiotic potency was often lost along with β-lactamase sensitivity. This led to a number of attempts to use relatively inert β-lactam derivatives in synergy with β-lactam antibiotics that were only effective against non-β-lactamase-producing strains. The hope was that the inert β-lactam derivative would bind competitively to the β-lactamase, thereby protecting the sensitive antibiotic from hydrolytic destruction by the enzyme (GREENWOOD and O'GRADY, 1975). This exemplifies the second approach to the β-lactamase problem: to use a hydrolytically sensitive but otherwise powerful antibiotic in synergy with a compound that will inhibit or inactivate the β-lacta-

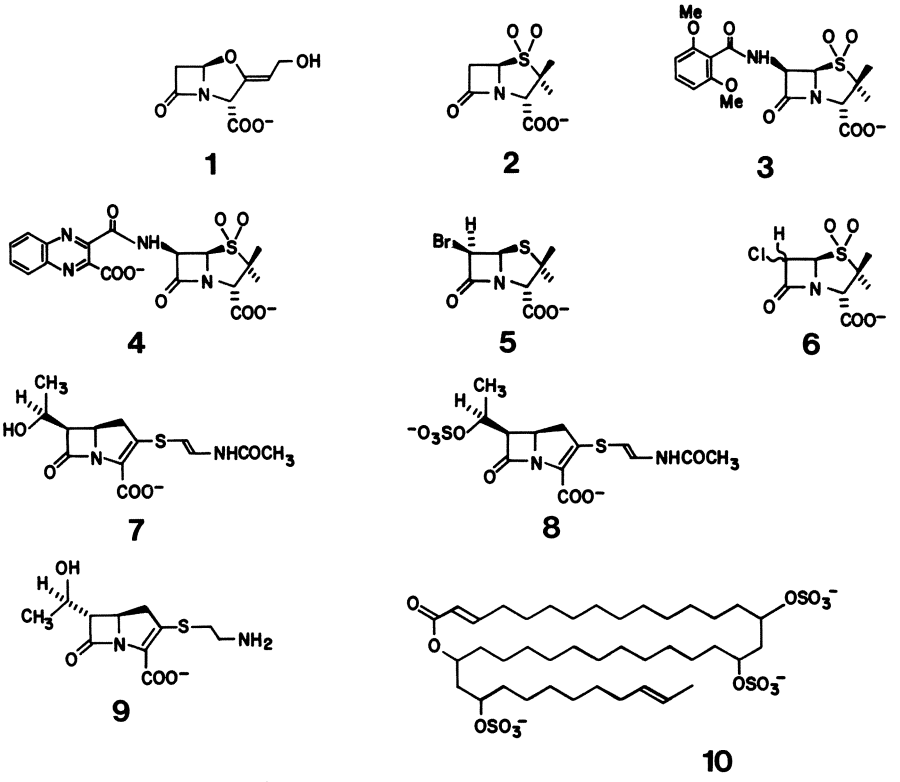

Fig. 1. Structures of β-lactamase inhibitors

mase (COLE, 1980). The approach has the merit of extending the therapeutic usefulness of the existing armory of excellent but β-lactamase-sensitive antibiotics. These efforts to inhibit the β-lactamase competitively were not especially successful in vivo, and the breakthrough only came in 1976 with the discovery in extracts of *Streptomyces clavuligerus* of an *inactivator* of β-lactamase, clavulanic acid (1 of Fig. 1) (BROWN et al., 1976). This material proved to be a very effective synergist. The LD_{50} values for a 1:1 (w/w) mixture of clavulanic acid with the hydrolytically sensitive antibiotic ampicillin, are, for β-lactamase-producing strains of *E. coli,* lower than the LD_{50} values for ampicillin alone by about a thousand fold.

The discovery of clavulanic acid led quickly to reports of a number of naturally occurring and semisynthetic β-lactamase inactivators, including a number of penicillanic acid sulfones (2, 3, 4 of Fig. 1) (ENGLISH et al., 1978; FISHER and KNOWLES, 1980), 6-halopenams (5, 6 of Fig. 1) (PRATT and LOOSEMORE, 1978; KNOTT-HUNZIKER et al., 1979), and carbapenems (7, 8, 9 of Fig. 1) (KAHAN et al., 1976; BROWN et al., 1977; MAEDA et al., 1977; OKAMURA et al., 1980; FUKAGAWA et al., 1980). It is the purpose of this chapter to describe what is known about the mechanism of action of these compounds at the molecular level.

A. The Mechanism of Action of the β-Lactamase

Since it is apparent that compounds 1 to 9 are "mechanism-based" or "suicide" inhibitors (BLOCH, 1969; ABELES and MAYCOCK, 1976; WALSH, 1977) of the β-lactamase, it is necessary first to review what is known about the mechanism of action of this enzyme, so that the mode of action of the inhibitors can be put in the appropriate mechanistic context.

The most basic question that one may ask about the mechanism of an amidohydrolase (for that is all a β-lactamase is), is whether a nucleophilic residue of the enzyme attacks the β-lactam's carbonyl group to form an acyl-enzyme intermediate that then hydrolyzes, or whether the enzyme acts as a general base and catalyzes the direct attack of a water molecule on the β-lactam. Amidohydrolases such as α-chymotrypsin, elastase and papain (e.g., KRAUT, 1977) clearly follow pathways involving acyl-enzyme intermediates, while for enzymes such as carboxypeptidase A and pepsin (e.g., FRUTON, 1970) the search for acyl-enzymes has been much less successful, and these enzymes may normally follow the general base path. From recent studies on the β-lactamase, it has become clear that, despite the intrinsic lability of the β-lactam ring, the enzyme-catalyzed reaction proceeds via an acyl-enzyme intermediate. Using the relatively poor substrate cefoxitin and the plasmid-encoded enzyme from *E. coli,* the following findings point unambiguously to the acyl-enzyme path (FISHER et al., 1980a): (1) When cefoxitin and β-lactamase are mixed, a rapid kinetic "burst" of cefoxitin consumption is seen, the size of which is 86% of that expected on the basis of the amount of enzyme used. This burst is followed by the slower steady-state reaction. (2) When an incubation of cefoxitin and enzyme that has reached the steady state is diluted into a solution of a very good substrate such as benzylpenicillin, the reappearance of enzyme activity (as monitored by the accelerating rate of benzylpenicillin hydrolysis) is identical with the steady-state rate of cefoxitin hydrolysis. (3) By quenching an incubation of [^{14}C]-cefoxitin and the enzyme from the steady state into cold denaturant, a cefoxitinoyl-enzyme can be isolated. (4) The cefoxitinoyl group is attached either to Ser_{45} or Thr_{46} (the former is more likely: vide infra). (5) When the hydrolysis of cefoxitin is followed by Fourier transform infrared spectroscopy, a new absorption band appears and (when all the cefoxitin has been consumed) disappears, at rates consistent with its deriving from the ester linkage of the acyl-enzyme intermediate. The new band is at 1753 cm^{-1}, at a frequency expected for the α-methoxy alkyl ester bond of a cefoxitinoyl-enzyme. All these data provide clear evidence for an acyl-enzyme pathway in which the relatively rapid acylation of the enzyme by cefoxitin is followed by a steady-state reaction, the rate of which is limited by the slower deacylation reaction (FISHER et al., 1980a). In addition, it has been shown that the *B. cereus* β-lactamase I is inactivated by methicillin and cloxacillin by stoichiometric acylation of the enzyme (KIENER et al., 1980), and that cloxacillin acylates a unique serine residue of the inducible chromosomal β-lactamase from *Ps. aeruginosa* (KNOTT-HUNZIKER et al., 1981). Finally, the acyl-enzyme intermediate from the interaction between *N*-dansyl-6-aminopenicillanic acid (an excellent substrate) and the β-lactamase

I from *B. cereus* has been isolated by quenching the hydrolytic reaction from the steady state (CARTWRIGHT and FINK, 1982). These studies show that the acyl-enzyme pathway is followed not merely by poor substrates of one class of β-lactamase, but is likely to be the common mechanism adopted by all the β-lactamases.

B. β-Lactamase Inhibitors

I. Penicillanic Acid Sulfone, 2

Of all the inhibitors of β-lactamase listed earlier (1 through 9), penicillanic acid sulfone, 2, is the simplest, and the material whose interaction with the enzyme is probably best understood. This compound will therefore be discussed first, since its mechanistic behavior provides the framework for discussion of the more complex interactions of clavulanic acid (1).

Penicillanic acid sulfone, 2, was first described by the Pfizer group in 1978 (ENGLISH et al., 1978). The compound shows only weak antibiotic activity against most bacterial species, but acts in synergy with β-lactam antibiotics against many resistant strains that produce a β-lactamase (WISE et al., 1980). Mechanistically, compound 2 interacts in three different ways with the enzyme (FISHER et al., 1980b; BRENNER and KNOWLES, 1981; KEMAL and KNOWLES, 1981). First, it is a substrate in the sense that the enzyme catalyzes the opening of the β-lactam ring. This cleavage can be followed by monitoring the changes in the ultraviolet (a new chromophore appears at 235 nm), the infrared (the β-lactam carbonyl absorption at 1770 cm^{-1} disappears), or the proton NMR. In each case, the same steady-state kinetic parameters (k_{cat}, 2.0 s^{-1}; K_m, 0.8 μM) are obtained. [For comparison, the steady-state parameters for the very good substrate benzylpenicillin are k_{cat}, 2000 s^{-1}; K_m, 20 μM. The sulfone 2 is therefore (in terms of k_{cat}/K_m) only about 10^2-fold less reactive as a substrate.] The reaction product from this enzyme-catalyzed hydrolytic reaction is not penicilloic acid sulfone, but malonsemialdehyde and the sulfinate of penicillamine. That is, the action of the enzyme results in the cleavage of *three* bonds in 2, and the fragmentation of the molecule into two parts. On the basis that the β-lactamase reaction proceeds via an acyl-enzyme intermediate (see the previous section), the reaction products can readily be rationalized (see Fig. 2). In the first step of the reaction, the hydroxyl group of Ser_{45} attacks the β-lactam carbonyl to yield the tetrahedral intermediate, A. This species then collapses, either synchronously (to C) or in a stepwise fashion (via B), to yield an acylated enzyme C in which *both* rings of 2 have been opened. The species C only differs from the normal penicilloyl-enzyme intermediate in that the oxidation of the thiazolidine sulfur to the sulfone makes the cleavage to the imine (step B → C) a very facile process (WOODWARD et al., 1949). The acyl-enzyme C then deacylates and the imine hydrolyzes, to regenerate the native enzyme and the two known products.

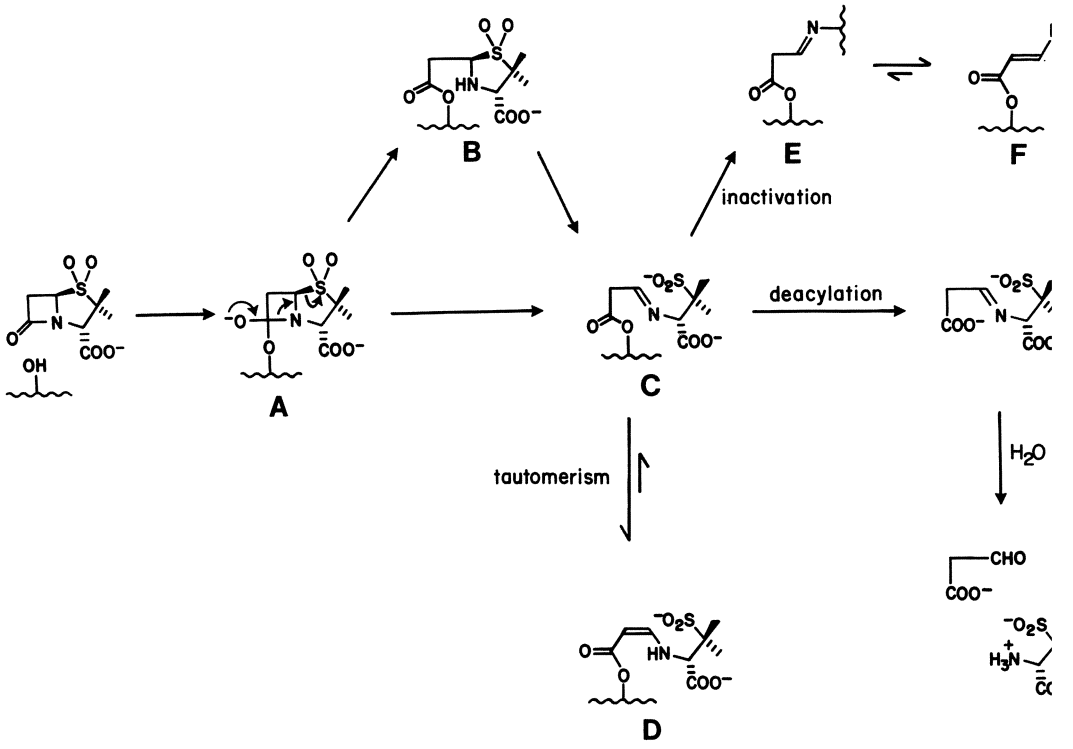

Fig. 2. The interaction of penicillanic acid sulfone with the β-lactamase

The second reaction of penicillanic acid sulfone is as a transient inhibitor of the enzyme. When a portion of an incubation of enzyme and sulfone is diluted into a solution of benzylpenicillin, the rate of benzylpenicillin hydrolysis accelerates during the first minute or so, with a rate constant, k_{react}, of 0.05 s^{-1}. This reactivation process cannot be due to the deacylation of the acyl-enzyme, since deacylation is at least 20 times faster (i.e., k_{cat} is 2.0 s^{-1}), and the reaction pathway must therefore be branched, one branch leading to a transiently inhibited form of the enzyme that decomposes (regenerating active enzyme) at 0.05 s^{-1}. The existence of a branched pathway is confirmed by the observation of a large "burst" of product when sulfone is added to enzyme. At pH 8, about ten molecules of sulfone are consumed as the enzyme accumulates into the transiently inhibited form, and there is a large increase in absorption (of ε 20,000) at about 290 nm (KEMAL and KNOWLES, 1981). These data are all consistent with the transiently inhibited form of the enzyme being the enamine D (Fig. 2), which is the more stable tautomer of the imine C. As a β-aminoacrylate ester, D is expected to absorb strongly at around 290 nm.

In addition to the processes of hydrolysis and of transient inhibition, there is a third fate of the acyl-enzyme C. This last reaction leads to irreversible inactivation of the enzyme, and is considerably slower than either the hydrolytic reaction or the transient inhibition process (k_{inact} is 2.8×10^{-4} s^{-1}, at saturating

levels of sulfone). From the ratio of k_{cat} to k_{inact}, and from experiments using different molar ratios of sulfone to enzyme, it is clear that about 7000 molecules of sulfone are hydrolyzed before the enzyme is irreversibly inactivated. The inactivated enzyme has a strong absorption at about 280 nm (ε 16,000), which is similar though not identical to the absorption spectrum of the transiently inhibited enzyme, D. When radiolabeled sulfone (tritiated in one of the methyl groups at C-2) is used to inactivate the enzyme, the purified inactive protein contains less than 5% of the amount of radiolabel expected on the basis of a stoichiometric inactivation reaction (D. BRENNER, unpublished work). These results may be explained by postulating that the third fate of the acyl-enzyme C is transimination by a lysine residue at the active site. In this way the existence of the new chromophore in the inactive enzyme and the absence of enzyme-bound radiolabel from the methyl-labeled sulfone are accounted for. The imine C would be attacked by a protein amino group giving the transimination product E (Fig. 2), and the penicillamine part of the original sulfone would be lost. The imine E would then rapidly tautomerize to give the more stable, chromophoric, enamine (F, Fig. 2), in which two active site groups (Ser_{45} and a Lys) have been cross-linked.

The chemistry presented in Fig. 2 is consistent with all that we know about the interaction of penicillanic acid sulfone with the β-lactamase (FISHER et al., 1980b; LABIA et al., 1980; BRENNER and KNOWLES, 1981; KEMAL and KNOWLES, 1981). While parts of this scheme are still only postulate, the credibility of these proposals is much enhanced by the results obtained using 6,6-dideutero-penicillanic acid sulfone. When the β-lactamase is incubated with this isotopically labeled analog, the unusual consequence is that both the hydrolytic reaction (the sulfone acting as a substrate) and the irreversible inactivation reaction are *accelerated* about threefold (BRENNER and KNOWLES, 1981). Since the isotope effect is much too large to be ascribed to a secondary isotope effect, we must devise a mechanistic pathway that uses a primary isotope effect to produce the observed accelerations. This is not as paradoxical as it appears, and is indeed demanded by the formulations presented in Fig. 2. If, for an enzyme-catalyzed reaction, an enzyme-substrate reaction intermediate partitions — *in an isotopically sensitive step* — into a transiently inhibited complex, then all of the other fates of that intermediate will show an acceleration equal to the (normal) isotope effect for the isotopically sensitive step. [This statement is only true, of course, if the isotope that is removed in the conversion of C to D is then lost (either directly or via an enzymic base) to the medium. Only in this way is the equilibrium between C and D perturbed by the isotopic substitution.] The acyl-enzyme from the dideutero sulfone partitions less often to the corresponding transiently inhibited complex, and more of the enzyme is available for the other two processes, hydrolysis and inactivation (each of which is insensitive to isotopic substitution at C-6). [The tautomerism of E to F is presumably a fast step following the slow C → E reaction, so the inactivation reaction (C → F) is isotopically insensitive.] These relationships are illustrated in Fig. 3: the tautomerism step (C → D) is slower, so less enzyme accumulates as D in the steady state, resulting in an acceleration of the other two fates of C, deacylation and inactivation. These results with the deuterium-labeled

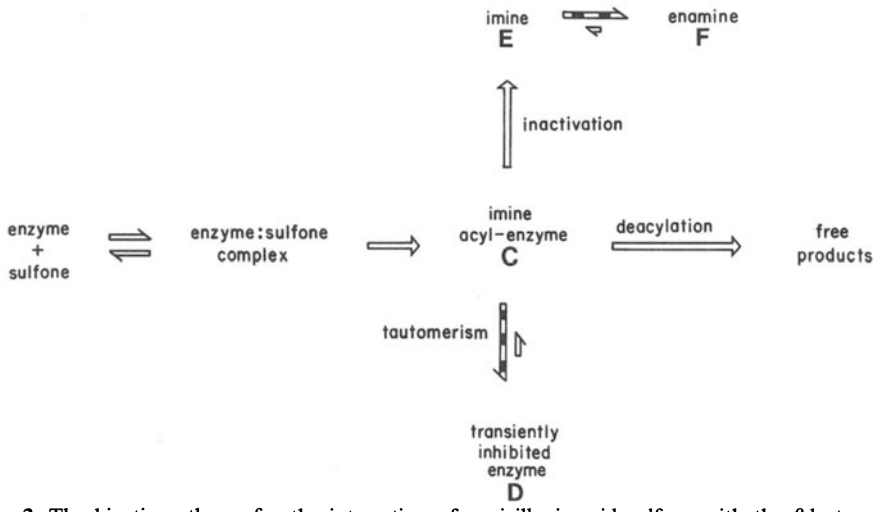

Fig. 3. The kinetic pathway for the interaction of penicillanic acid sulfone with the β-lactamase. The *open arrows* represent steps that are unaffected by isotopic substitution at C-6. The *dashed arrows* represent steps that are threefold slower for the 6,6-dideutero sulfone

sulfone (BRENNER and KNOWLES, 1981) provide strong support for the scheme of Fig. 2, both in terms of the kinetic events, and in terms of the chemical changes from which these events derive. We may now, with the scheme of Fig. 2 as a backdrop, analyze the kinetic, spectroscopic, and chemical behavior of other sulfones (e.g., 3 and 4) and clavulanic acid (1) with the plasmic-encoded β-lactamase.

II. Other Penam Sulfones (e.g., 3 and 4)

On the basis of the interactions illustrated in Fig. 2, it is the oxidation of the thiazolidine sulfur to the sulfone that ensures the fragmentation of the 5-membered ring and the production of the imine C (WOODWARD et al., 1949). This imine may deacylate and regenerate active enzyme, or it may suffer tautomerism or transimination and inhibit or inactivate the enzyme. Any factor that slows the hydrolysis of the acyl-enzyme relative to the other two reactions should increase the efficacy of the compound as an inhibitor. We therefore reasoned (FISHER and KNOWLES, 1980) that while the sulfones of *good* substrates of the β-lactamase would rapidly deacylate, the sulfones of *poor* substrates could lead to acyl-enzymes (analogous to C) whose lifetime would be longer and allow more chance for the inhibitory processes of tautomerism and transimination. These expectations were fully realized from studies on penam sulfones substituted at C-6. Such substituents are known to change the rate of β-lactam hydrolysis. As shown in Table 1, when penicillin V, which is an excellent substrate for the β-lactamase, is oxidized to the sulfone, the product is not an inactivator of the enzyme. Presumably the acyl-enzyme (which from product analysis is

Table 1. Kinetic characteristics of the interaction of penams and penam sulfones with β-lactamase

Penam	k_{cat} of penam (s^{-1})	k_{cat} of sulfone[a] (s^{-1})	$t^1/_2$ of inactivation by sulfone (min)	Number of hydrolytic events before inactivation by sulfone
Penicillin V	1,000	~1,500	–	>250,000[b]
Penicillanic acid	40	~2	44	~7,000
Methicillin	7	~80	1.5	10,000
Quinacillin	2	~10	<1	400

[a] These values are approximate. [b] Benzylpenicillin sulfone behaves analogously.

the imine analogous to C) deacylates so rapidly that no partitioning into the tautomerism (to D) or transimination (to F) routes is seen. Yet when the sulfones of the *poor* substrates methicillin and quinacillin (3 and 4) are used, the rate of enzyme inactivation is increased dramatically (FISHER and KNOWLES, 1980) (see Table 1). Here, we presume, a much slower deacylation rate allows the acyl-enzyme time to partition into the pathways that result in enzyme inhibition and inactivation. As with penicillanic acid sulfone, the inactivated enzymes have a strong new absorption (of ε 18,000) at around 290 nm, and it has been shown that a single serine residue, Ser_{45}, is labeled by quinacillin sulfone (FISHER et al., 1981).

It is evident, therefore, that the mechanistic analysis that led to the formulation of the scheme of Fig. 2 can rationally be used to design more effective β-lactamase inactivators. It must be said, however, that neither methicillin sulfone nor quinacillin sulfone is an effective synergist with ampicillin in vivo, either for normal resistant *E. coli* or for resistant *coli* mutants that have an especially permeable outer membrane (C. EASTON, unpublished experiments). These findings only stress, of course, the obvious fact that accessibility and pharmacokinetic problems must also be solved before one arrives at a β-lactamase inactivator of therapeutic potential.

III. Clavulanic Acid, 1

The interaction of clavulanic acid with the plasmid-encoded β-lactamase from *E. coli* is the most complex of all the inactivators so far investigated. The main features of the behavior of this molecule are, however, nicely accommodated by the pathways developed in the scheme of Fig. 2 for penicillanic acid sulfone. Clavulanic acid acts in three ways: it is a substrate for the β-lactamase, it acts as a transient inhibitor of the enzyme, and it leads to irreversible inactivation of the enzyme (FISHER et al., 1978; CHARNAS et al., 1978). From studies on clavulanate itself and by analogy with the more detailed work on penicillanic acid sulfone, it is evident that the first-formed tetrahedral intermediate (G, Fig. 4: compare A, Fig. 2) collapses with the opening of both rings, to give the acyl-enzyme (H, Fig. 4: compare C, Fig. 2). The A → C conversion

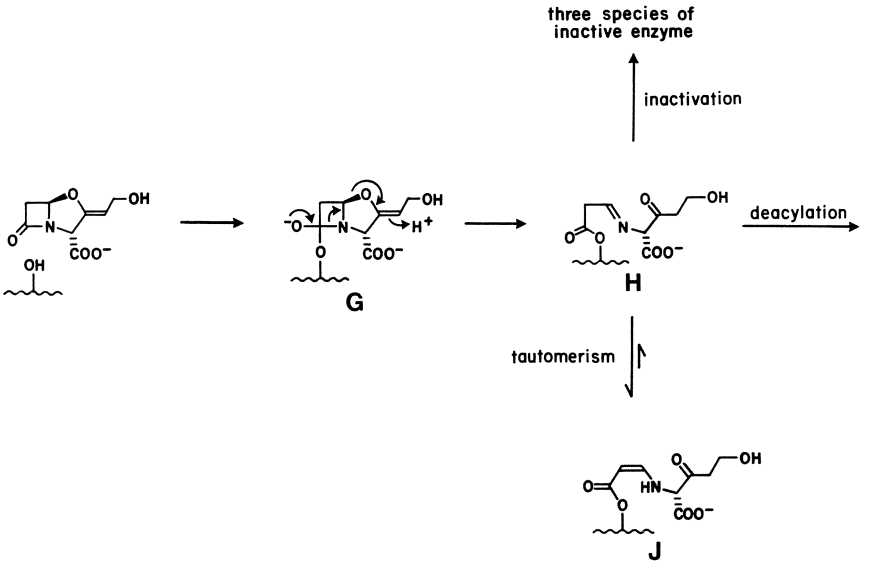

Fig. 4. The interaction of clavulanic acid with the β-lactamase

is facile because the sulfinate is a reasonably good leaving group; the G → H conversion is facile because of the ketonization (in H) of the enol ether (in G). The acyl-enzyme H may tautomerize to the enamine J, which is the chromophoric transiently inhibited enzyme. Deacylation of H leads, presumably, to malonsemialdehyde and the δ-hydroxy, β-keto, α-amino acid, or products derived from them. The suggested chemistry in Fig. 4 is consistent with the kinetic interactions between clavulanic acid and other plasmid-encoded β-lactamases from *E. coli* reported by LABIA and PEDUZZI (1978). The main difference between the behavior of penicillanic acid sulfone and clavulanic acid (aside from differences in the rates of the various processes) is that clavulanic acid leads to *three* forms of inactivated enzyme that can be discerned by isoelectric focussing (CHARNAS et al., 1978). While the structure of these species has not yet been completely defined, studies with both extensively labeled ([2,3,5,6,7,8,10-$^{14}C_7$]) and specifically-labeled ([5,6,7-$^{14}C_3$]) material (CHARNAS and KNOWLES, 1981 a) have shown that one of the three inactive enzyme species retains only the carbon atoms of the original β-lactam ring, while the other two inactive species retain the whole clavulanate skeleton. Finally, the close similarity between 9-deoxy-clavulanic acid and clavulanic acid in terms of their kinetic, spectroscopic, protein-chemical (CHARNAS and KNOWLES, 1981) and pharmacological properties (HOWARTH et al., 1977), shows that the allylic alcohol group is irrelevant to the action of clavulanate as a β-lactamase inactivator.

The interaction of clavulanic acid with β-lactamases from different sources is not identical to that seen with the *E. coli* enzyme. Thus the β-lactamase I from *B. cereus* appears not to hydrolyze clavulanate effectively, but the enzyme is both competitively inhibited (K_i is 35 μM) and irreversibly inactivated by clavulanic acid (DURKIN and VISWANATHA, 1978). Benzylpenicillin protects the

enzyme from inactivation. The fact that the enzyme is inactivated by clavulanate more rapidly in the presence of the substrate ampicillin may reflect the importance of multiple conformational states of the enzyme, which have been proposed by CITRI and his group to account for the kinetic behavior of the β-lactamase when the enzyme is taken from an incubation with one substrate and presented with a different one (CITRI et al., 1976). With the β-lactamase from *Staphylococcus aureus* it has been shown that fully inactive enzyme can be prepared by treatment with only a small molar excess of clavulanate (CARTWRIGHT and COULSON, 1979). Substrates protect the enzyme from inactivation, and there are spectral changes around 290 nm that are consistent with the generation of β-aminoacrylate species in the reaction. Unlike the enzymes from other sources, incubation (especially at low pH) leads to the recovery of all the catalytic activity with the concomitant loss of the new chromophore (CARTWRIGHT and COULSON, 1979; READING and HEPBURN, 1979). While the studies on the enzymes from other sources are less extensive than those on the plasmid-encoded enzyme from *E. coli,* it seems probable that the observed differences may merely reflect relatively small changes in the partitioning behavior of the reaction intermediates shown in Fig. 4 or of species derived from them.

The multiply branched pathways followed by penicillanic acid sulfone (Fig. 2) and by clavulanic acid (Fig. 4) make it difficult to assess a priori which compound is the more effective inhibitor in vivo. Even if both compounds have free and equal access to the cell's β-lactamase, it would be naive to conclude, because the sulfone suffers about 7000 hydrolytic turnovers before enzyme inactivation and clavulanic acid suffers only 115, that clavulanate is the more effective compound. This is because both transient inhibition *and* irreversible inactivation may preoccupy the β-lactamase and so prevent the destruction of the β-lactam antibiotic. The effectiveness of a β-lactamase inhibitor is a complex function of the kinetics of occupancy of the β-lactamase by the inhibitor, the kinetics of β-lactamase-catalyzed hydrolysis of the antibiotic, the kinetics of the antibiotic's attack on its target enzymes, and the relative and absolute local concentrations of inhibitor and antibiotic. The above notwithstanding, it does appear that, for clavulanate at least, β-lactamase inactivation (as distinct from transient inhibition) accompanies clavulanate-mediated synergy in vivo (C. EASTON, unpublished experiments).

IV. 6-β-Bromopenicillanic Acid, 5

It was shown independently by PRATT and LOOSEMORE (1978) and by WALEY and co-workers (KNOTT-HUNZIKER et al., 1979) that whereas 6-α-bromopenicillanic acid is without effect on β-lactamase, incubation at high pH allows epimerization at C-6 to produce a small equilibrium percentage (about 10%) of the 6-β-bromo compound, which is a powerful inactivator of the enzyme. Mechanistic work from the laboratories both of PRATT (COHEN and PRATT, 1980; LOOSEMORE et al., 1980) and of WALEY (KNOTT-HUNZIKER et al., 1980; ORLEK et al., 1980) have elucidated the mode of action of this inactivator. With the *B. cereus*

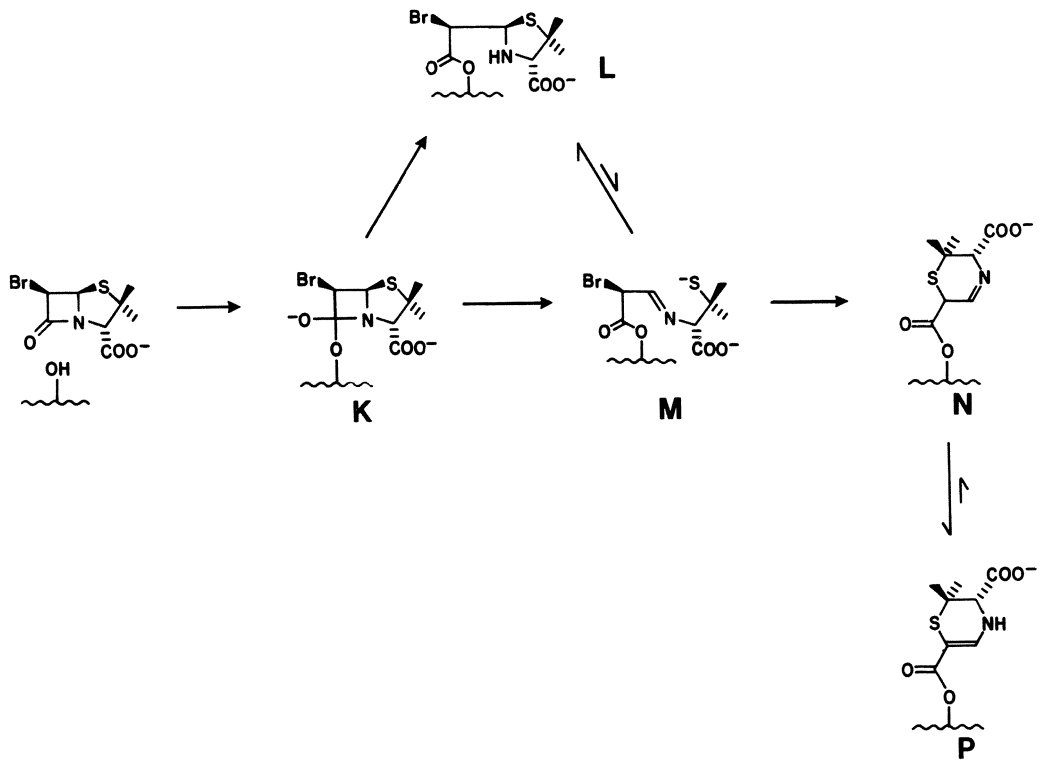

Fig. 5. The interaction of 6-β-bromopenicillanic acid with the β-lactamase

β-lactamase I, there is a rapid reaction ($10^6\ M^{-1}\ s^{-1}$) that leads to inactive enzyme that contains a single dihydrothiazine group (λ_{max} 326 nm in the native enzyme, λ_{max} 314 nm in the denatured protein) attached to the hydroxyl group of Ser_{44} by an ester linkage. The enzyme is inactivated by less than 1.5 molar equivalents of reagent, showing that there is, in this case, little or no hydrolytic turnover by the β-lactamase before inactivation. Use of the 6-β-bromo penam free from its 6-α epimer confirms these findings. The β-lactamases from *Bacillus spp.* are very sensitive to inactivation, those from *Staphylococcus* and *E. coli* are less so, and the metallo-β-lactamase II from *B. cereus* is unaffected. The inactivation pathway is illustrated in Fig. 5. The chemical feasibility of the transformation shown has been demonstrated using model compounds.

In comparison with the behavior of the sulfone 2 and of clavulanic acid 1, the breakdown of the first-formed tetrahedral intermediate (K, Fig. 4) to yield the imine M, is a slight surprise. The bonds between C-5 and the heteroatom in 1 and 2 are weak, due to the possibility of ketonization in 1 (the conversion of G → H) and the existence of a good leaving group in 2 (the conversion of A → C). Yet in the K → M conversion a thiolate is the leaving group, and if this fragmentation were always easy one would expect that all penams would be β-lactamase inactivators, which they patently are not. The

answer to this problem must lie *either* in a greater instability of the bond between C-5 and sulfur in K because of the bulk of the β-bromo substituent, *or* in the fact that a small proportion of M in equilibrium with L can rapidly be trapped as the dihydrothiazine N by the displacement of bromide ion from C-6 by the thiolate. For whatever reason, K is converted smoothly to P, which is the stable acylated enzyme. Model studies have shown that dihydrothiazines such as P are resistant to attack by nucleophiles like ammonia or hydroxylamine, and by analogy are expected to be stable to a hydrolytic deacylation reaction.

6-β-Iodopenicillanic acid has recently been reported (MOORE and BRAMMER, 1981) to be a potent inhibitor of β-lactamases, and to act in synergy with ampicillin both in vitro and in vivo against a number of β-lactamase-producing bacteria. A number of other 6-halo-penams have been synthesized (VON DAEHNE, 1980), though their relative potencies as enzyme inactivators have not yet been reported.

V. 6-Chloropenicillanic Acid Sulfone, 6

CARTWRIGHT and COULSON (1979) synthesized the 6-chloropenam sulfone, 6, and showed it to be both a substrate and an inactivator of the *Staphylococcal* β-lactamase. About 100 hydrolytic turnovers occur before enzyme inactivation, and it has been shown that either Ser or Thr (presumably the former, by analogy with the substrate and inhibitor labeling studies already discussed) is covalently labeled (CARTWRIGHT and COULSON, 1980). It is not clear whether this chlorosulfone more resembles the bromopenam 5 (after a rapid uncatalyzed epimerization at C-6 to yield the 6-β-chloro material), or the penam sulfone 2, in mechanistic terms.

VI. Carbapenems

The discovery of a number of examples of a new skeletal class of β-lactams, the carbapenems (KAHAN et al., 1976; BROWN et al., 1977; MAEDA et al., 1977), has been followed by reports of a variety of such compounds from *Streptomyces spp.* (see, e.g., BASKER et al., 1980; NAKAYAMA et al., 1980). Some of these materials are powerful broad-spectrum antibiotics, and some are effective β-lactamase inhibitors. The carbapenem nuclei may have the "normal" configuration (*R*) at C-5, a carbon substituent at C-6 that may be *R* (*cis* stereochemistry across C-5 and C-6) or *S* (*trans* stereochemistry across C-5 and C-6), a variety of C-6 side chains (e.g., 8-*S*-hydroxyethyl, 8-*S*-sulfatoethyl, 8-*R*-hydroxyethyl, 8-hydroxyisopropyl, 8-sulfatoisopropyl, alkyl, alkenyl) and, usually, a 2-acylamidoethenylthio (or 2-acylamidoethanylthio) substituent at C-2.

It appears that compounds with *trans*-β-lactam protons and 8 *R* stereochemistry (e.g., 9), and those with *cis*-β-lactam protons and 8 *S* stereochemistry (e.g., 7, 8) are the most potent antibiotics, though the unsulfated *cis* compounds

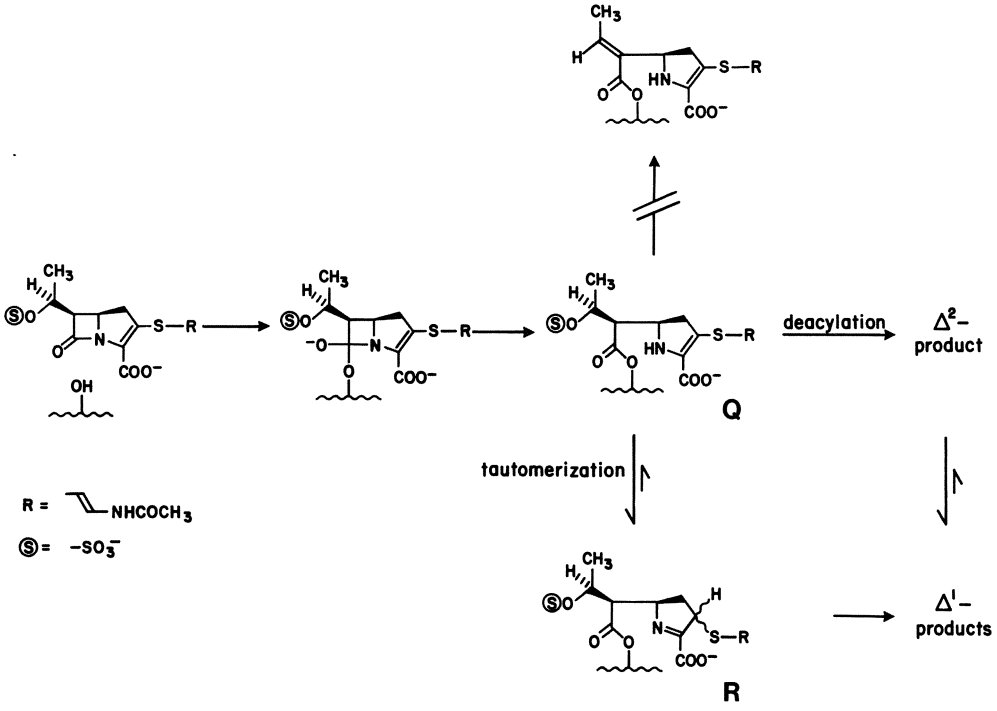

Fig. 6. The interaction of olivanic acids (e.g., 8) with the β-lactamase

(e.g., 7) are rather susceptible to hydrolysis by the plasmid-encoded β-lactamase from *E. coli* (BASKER et al., 1980). Thienamycin, 9, and its more stable *N*-acylated derivatives are powerful antibacterial agents, and are rather resistant to the β-lactamase. Sulfation of the C-8 hydroxyl group of the olivanates (as in 8) does not reduce antibacterial potency (and may enhance it), and gives the molecules β-lactamase-inhibitory properties. The carpetimycins (both hydroxy and sulfato) are also β-lactamase inhibitors. In in vivo experiments, many members of the olivanic acid family (loosely defined as carbapenems that are elaborated by *S. olivaceus*) are effective synergists with β-lactamase-sensitive β-lactam antibiotics.

Since it was known that sulfato compounds such as 8 are good inhibitors of β-lactamase, whereas the unsulfated analogs (e.g., 7) are not, it was suggested (FISHER et al., 1980) that the inhibition could be due to the formation of an α,β-unsaturated acyl enzyme by β-elimination between C-6 and C-8 and loss of sulfate (see Fig. 6). This proposal has, however, recently been shown (CHARNAS and KNOWLES, 1981b) to be unfounded, since free sulfate ion is not released during the interaction of 8 with the enzyme. Indeed, inhibited enzyme was shown to retain one mol of sulfate ester per mol of protein. The sulfato group appears to exert its effect by a binding interaction with the enzyme that slows the deacylation reaction and allows the acyl-enzyme to be diverted into a transiently inhibited form. Studies of a number of carbapenem derivatives

including 7, 8, and 9 have led to the formulation shown in Fig. 6 (EASTON and KNOWLES, 1982). While 7 behaves simply as a substrate for the enzyme, when the reaction of 8 is followed by the disappearance of the chromophore at 304 nm, a biphasic reaction is observed. A kinetic "burst" that is larger than stoichiometric with enzyme is seen, during which time the catalytic activity of the enzyme falls to a low level. This represents the accumulation of the transiently inhibited enzyme, R. From the spectroscopic changes and from the isolation of the products of the reaction (these are the two Δ^1-pyrrolines, epimeric at C-3), it is clear that the acyl-enzyme Q is the Δ^2-pyrroline form, and the transiently inhibited enzyme is the more stable Δ^1-pyrroline, R (Fig. 6) (EASTON and KNOWLES, 1982). At long times, when all the carbapenem is exhausted, all the catalytic activity of the enzyme returns: there is no irreversible inactivation of the β-lactamase. The fact that olivanic acid derivatives inhibit the β-lactamase but do not irreversibly inactivate it emphasizes the view that irreversible inactivation of the enzyme is not a sine qua non for the effective protection of a β-lactam antibiotic in vivo. Provided that the β-lactamase is blocked by a tightly binding and slowly reacting substrate, the enzyme will be preoccupied long enough for the antibiotic to reach its target, and in vivo synergy will have been achieved.

VII. Izumenolide (10) and Other Sulfate Esters

The SQUIBB group reported in 1980 the isolation and properties of a β-lactamase inhibitor produced by *Micromonospora* that, unlike the materials already discussed (1 through 9), does not contain a β-lactam ring (LIU et al., 1980). This material, izumenolide (10), is a macrolide containing three sulfate esters, and is a potent inhibitor of β-lactamases from Gram-negative bacteria. In tenfold molar excess over enzyme, 10 inactivates the RTEM β-lactamase irreversibly, though its effect as a synergist with ampicillin in vivo is limited, due to permeability problems (BUSH et al., 1980a). The mechanism of action of izumenolide has not been delineated, though hydrolytic opening of the macrocycle and reduction of the double bonds reduces the inhibitory potency somewhat. It appears that the apparent specificity of 10 for β-lactamases may be related to the constellation of anionic sulfates. This view is strenghtened by the fact that sodium dodecyl sulfate and panosialin [as well as other alkylbenzene sulfates (YAGINUMA et al., 1980)] also inhibit the β-lactamase (BUSH et al., 1980b).

VIII. Sulfenimines

It has been reported that some sulfenimine derivatives of 6-aminopenicillanic acid and of 7-aminocephalosporanic acid are very powerful inhibitors of a cephalosporinase (GORDON et al., 1980). No mechanistic work has yet appeared concerning these materials.

C. Overview

From all the activity in the area of β-lactamase inhibition and inactivation over the past five years or so, can any general lessons be learned? Aside from a very few examples of chemical reagents that will inactivate β-lactamase more or less specifically [e.g., the penam isocyanates of OGAWARA and UMEZAWA (1973, 1974), or phenylpropynal (SCHENKEIN and PRATT, 1970)], all the effective inactivators of β-lactamase appear to belong to the class of "mechanism-based" reagents. Each compound contains the structural elements required for recognition as a potential substrate by the enzyme, and is handled as such either as far as the formation of a tetrahedral intermediate at the β-lactam carbonyl (e.g., A, G, or K), or as far as the formation of the acyl-enzyme itself. The β-lactamase inactivators discussed in this chapter *either* have features that divert the breakdown of the tetrahedral intermediate from its normal course (see Fig. 7), *or* form acyl-enzymes that readily react to give more stable species (e.g., C→D, H→J, L→P, or Q→R) which deacylate only slowly. Figure 7 illustrates a frequent strategy, where the availability of an electron sink allows the tetrahedral intermediate to collapse (in one or two steps) with the cleavage of both the 4- *and* the 5-membered rings of the penam. The path followed by the carbapenems is, of course, analogous to that observed with cephalospor-

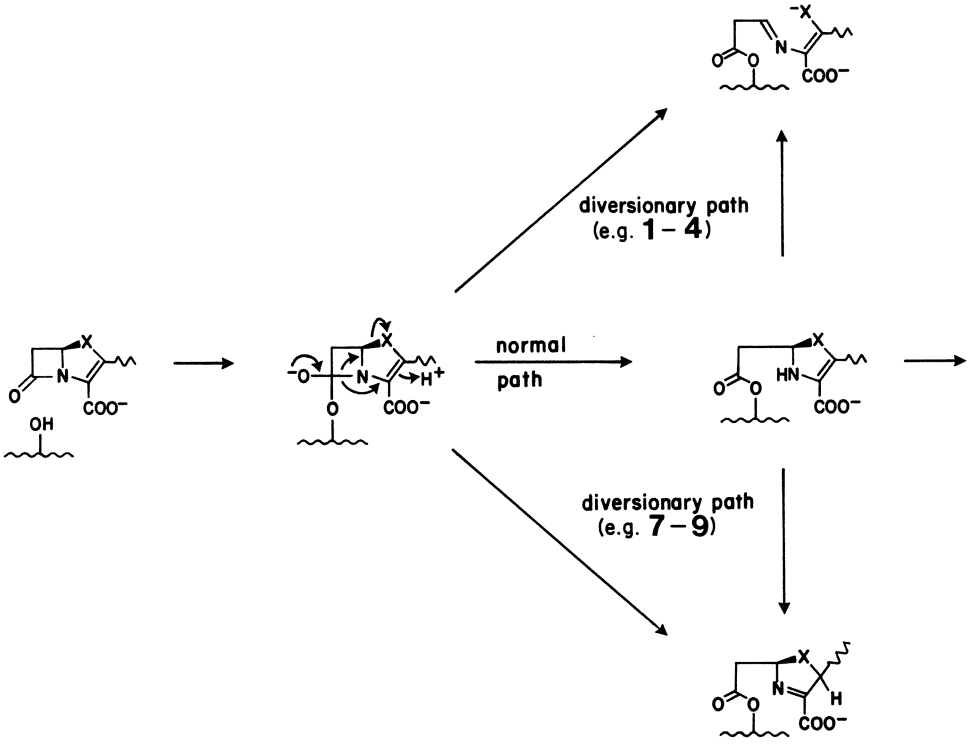

Fig. 7. Diversionary pathways that may lead to inhibition or inactivation of the β-lactamase

ins that have good leaving groups at the C-3′ position. Here, however, β-lactamase inhibition is not observed. The reasons for these differences remain obscure, and we shall presumably require a high resolution crystal structure of one or more β-lactamases before rational explanations can be put forward. It is clear, though, that the recognition requirements of a β-lactamase are quite strict: peptide analogs are not hydrolyzed, and monocyclic β-lactams are cleaved considerably more slowly than their more strained penam and cephem counterparts (PRATT et al., 1980).

The mechanistic and protein-chemical studies summarized in this chapter point to the existence of a strong similarity between the D-Ala-D-Ala carboxypeptidases that participate in bacterial cell wall biosynthesis, and the β-lactamases. Both groups of enzymes are acylated by β-lactam antibiotics on a unique serine residue, the peptide sequences around which are somewhat homologous (YOCUM et al., 1979). It is tempting to suggest that these carboxypeptidases and the β-lactamases are all serine amidohydrolases that proceed via acyl-enzyme intermediates, and that the two types of enzymes only differ from each other in terms of substrate specificity and the relative rates of their acylation and deacylation steps. Such similarities should give new momentum to the search for effective β-lactam antibiotics that are inert to (or can simultaneously inhibit) the β-lactamases.

Acknowledgments. The author gratefully acknowledges all the intellectual and practical contributions of J.G. BELASCO, S.M. BRADLEY, D.G. BRENNER, R.L. CHARNAS, C. EASTON, J. FISHER, C. KEMAL and S. KHOSLA; and representatives of BEECHAMS, BOOTS, GLAXO, ELI LILY, MERCK, PFIZER, SANRAKU-OCEAN and SQUIBB, who provided many of the materials essential for our studies in this area.

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Mefloquine

A.D. WOLFE

Introduction

Mefloquine (Fig. 1) is a quinolinemethanol blood schizontocide which is highly effective against drug-resistant as well as drug-sensitive *P. falciparum* and *P. vivax,* has a high therapeutic index, and has been accepted for clinical use. The mode and mechanism of mefloquine action are unknown, but available information suggests that cell membranes, internal as well as external, may be selective targets. A general review of mefloquine has recently been written by SWEENEY (1981), and the development of mefloquine has been recounted by SCHMIDT et al. (1978a), and by CANFIELD and ROZMAN (1974), all of whom have been instrumental in this process.

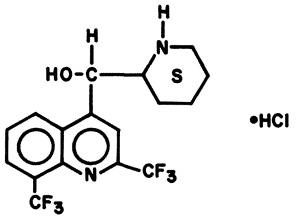

Fig. 1. The structure of mefloquine (WR 142,490), DL-*erythro*-α-2-Piperidyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol hydrochloride

History

Mefloquine is among a small group of clinically promising antimalarial agents which have resulted from the United States Army Research Program on Malaria. In excess of 200,000 compounds related to growth-suppressive agents of many chemical classes have been encompassed by this vast synthesis and development program which received impetus during the 1960's by the evolution of chloroquine-resistant strains of *Plasmodium falciparum.* Elucidation of the structure of the naturally occurring quinolinemethanol, quinine (COHEN and KING, 1938), and the suppression of its supply during the Second World

War, led to the synthesis and to the evaluation of 177 compounds structurally related to quinine. Of these, 156 exhibited suppressive activity against the blood forms of avian malarial species, and eight of these were further evaluated in human volunteers for efficacy against *P. vivax,* and for human tolerance. Of these eight compounds, one, SN-10,275, possessed three times the activity of quinine, and a second possessed activity equal to that of quinine. By 1944, however, clinical emphasis was placed upon use of quinacrine (atebrin, mepacrine), and sulfonamides, and experimental emphasis on the development of the 4-aminoquinolines. Cessation of the War renewed worldwide access to quinine, and was followed by introduction of chloroquine. Further development of arylmethanol blood schizontocides essentially ceased, although there remained in storage approximately 100 quinolinemethanols.

Restoration of the Drug Development Program after two decades entailed screening, against *P. berghei,* in mice, of approximately 300 quinolinemethanols, including those which remained from the initial group. SN-10,275, redesignated WR 7,930, and SN-15,068, redesignated WR 30,090, exhibited high activity, but WR 30,090 proved adventitious in that it failed to induce phototoxicity, a side effect common to arylmethanols (ROTHE and JACOBUS, 1968; ISON and DAVIS, 1969). The qualitities of WR 30,090 led to its testing and evaluation by human volunteers infected with either *P. falciparum* or *P. vivax* (MARTIN et al., 1973; CLYDE et al., 1973), and inclusion among a group of 12 quinolinemethanols for pilot studies in *Aotus* monkeys infected with drug-resistant, as well as with drug-sensitive strains of *P. falciparum* and *P. vivax*. Five compounds exhibited activity equal to or greater than chloroquine, and among these was WR 142,490, which proved five times as effective as chloroquine against a pyrimethamine-resistant strain of *P. falciparum*. WR 142,490 was submitted for further evaluation, and ultimately accepted for clinical use after convincing demonstration of its efficacy and safety (SCHMIDT et al., 1978a). This compound was subsequently named mefloquine.

Chemistry

Mefloquine was first synthesized (OHNMACHT et al., 1971) as the free base, with a molecular weight of 378, and is converted to the hydrochloride or methanesulfonate forms for advanced testing and clinical use. Mefloquine hydrochloride is soluble to the extent of a 1% aqueous solution at 60° C, but is highly lipid-soluble, and possesses a partition coefficient of 1300 in a 2-phase extraction system consisting of N-butanol/Sorenson buffer, pH 7.4 (MU et al., 1975). Mefloquine hydrochloride has a pK of 8.6. Mefloquine contains two asymmetric centers, the carbinol carbon and the amine carbon, and therefore occurs in two racemic forms, the erythro, which was the original synthesis product (OHNMACHT et al., 1971), and the threo racemate (WR 177,602). All four optical isomers have been separated, and their absolute configurations determined (CARROLL and BLACKWELL, 1974). [^{14}C]mefloquine has been synthesized

(YANKO and DEEBEL, 1980), and has proven of unusual value. Patents related to mefloquine include: German patents: 2,806,909 (GRETHE and MITT), 2,820,991 (MERKLI), and 2,940,443 (HICKMAN, OESER, and MOEBIUS).

Procedures used to quantitate mefloquine in blood and urine include (1) ion pair, or conventional solvent extraction, followed by trimethylsilylation and gas liquid chromatography with electron capture or flame ionization detection, with the capability to determine mefloquine derivative concentrations as low as 10 ng/ml blood (NAKAGAWA et al., 1979), (2) high pressure liquid chromatography, with a detection limit of 0.05 μg/ml blood or plasma, and 0.25 μg/ml urine (GRINDEL et al., 1977), and (3) extraction with ether via the trichloroacetate ion pair, followed by alkylation, and plastic ion-selective electrode analysis (MENDENHALL et al., 1979). The latter procedure detects derivatives at concentrations as low as 0.004 ng/ml. Detection by means of thin-layer chromatography has also been found useful (SCHWARTZ, 1980).

Activity Spectrum

Mefloquine has a broad action spectrum, and has not only been successfully tested against schizonts of drug-resistant as well as drug-sensitive rodent, primate, and human schizonts, but upon *Leishmania major* and *L. maxicana amazonensis* (PETERS et al., 1980), and upon selected species of bacteria (WOLFE, 1979). Prophylactic and radical therapeutic use of mefloquine does not appear promising, since the drug failed to prevent recurrence of sporozoite-induced *P. vivax* in human volunteers (CLYDE et al., 1976), or development of *P. cynomolgi* blood infections from tissue schizonts in rhesus monkeys (SCHMIDT et al., 1978a). However, mefloquine and chloroquine are almost equally effective when used in conjunction with primaquine in radical curative therapy (SCHMIDT et al., 1978a).

Screening studies on mefloquine revealed it to be 8 times as active as chloroquine, and 64 times as active as quinine against *P. berghei* infections in mice (OHNMACHT et al., 1971; STRUBE, 1975). A dose of 10 mg/kg yielded a 50% increase in mean survival time, and a dose of 40 mg/kg was curative (CARROLL and BLACKWELL, 1974). Mefloquine was effective, at low dosage, against a variety of drug-resistant *P. falciparum,* including the chloroquine-resistant Vietnam Oak Knoll, and Monteray strains, the pyrimethamine-resistant Uganda Palo Alto and Malayan Camp CH/Q strains, the chloroquine-amodiaquine-antifolate resistant Vietnam (Marks) strain, the pyrimethamine-chloroquine-quinine resistant Vietnam Smith strain, and the multidrug resistant Cambodian (Buchanan) strain (RIECKMAN et al., 1974; TRENHOLME et al., 1975; SCHMIDT et al., 1978a). The drug-sensitive Chesson strain, and the pyrimethamine-resistant Vietnam Palo Alto strain of *P. vivax* were equally sensitive to mefloquine (SCHMIDT et al., 1978a). Use of a semiautomated microdilution technique (DESJARDINS, 1979a) permitted quantitative assessment of the antimalarial activity of clinical and candidate antimalarials against several *P. falciparum* strains.

Drug concentrations, in ng/ml, which resulted in 50% suppression of [^{3}H]hypoxanthine incorporation by the Uganda I strain were mefloquine, 6.7, quinine, 26.1, and chloroquine, 9.5, whereas similar suppressive concentrations required by the Smith strain were mefloquine 7.8, quinine, 109, and chloroquine, 182.

Resistance to mefloquine has been primarily limited to experimental induction in mice infected with *P. berghei* subjected to serial passage under constant drug pressure (PETERS et al., 1977b; KAZIM et al., 1979; MERKLI and RICHLE, 1980; MERKLI et al., 1980). In one series of experiments, the initial maximum parasite-tolerated mefloquine dose was 15 mg/kg, but after 45 serial passages this dose had risen to 550 mg/kg, the highest dose tolerated by the mouse host (MERKLI and RICHLE, 1980). Combinations of mefloquine with antifolates retarded evolution of resistance and reduced its potency (PETERS et al., 1977b; MERKLI and RICHLE, 1980; MERKLI et al., 1980). Resistant parasites exhibit a preference for polychromatophilic red cells, and appear deficient in pigment (PETERS et al., 1977b). These characteristics are similar to those of the *P. berghei* RC line, which is highly resistant to chloroquine, and which may actually be partially resistant to mefloquine, since the 90% effective mefloquine dose is 3.8 times that of the chloroquine-sensitive *P. berghei* N line (14 mg/kg vs 3.8 mg/kg) (PETERS et al., 1977a). Induced *P. berghei* resistance to mefloquine was phenotypic, however, and disappeared upon drug withdrawal. A decrease in the susceptibility of *P. falciparum* in culture has been reported (BROCKELMAN et al., 1981) and a single human case of *P. falciparum* resistance to mefloquine has been observed in Thailand (Research Reports, 1981). Induction of resistance to quinine has been accomplished through serial passage of the Panama II strain of *P. falciparum* in monkeys administered sublethal quinine doses (GLEW, 1978), and it suggests the potential for the evolution of *P. falciparum* mefloquine resistance (PETERS, 1977b; CANFIELD, 1980).

Therapeutic Use, Pharmacokinetics, and Toxicity

Mefloquine is normally administered as the hydrochloride. The drug has been given to humans orally, in tablet or capsule form, or in aqueous suspension, but attempts to give the drug intravenously resulted in severe venous irritation (TRENHOLME et al., 1975; DESJARDINS et al., 1979). In addition, animals have received mefloquine intraperitoneally and subcutaneously. Mefloquine has been found effective when given as a single, total dose or, equivalently, in partial doses, and has a therapeutic index of 12 (SCHMIDT et al., 1978a). A single, human, oral dose of 400 mg mefloquine was sufficient to clear blood of patent falciparum malaria, but recrudescence occurred. A 1 g dose cured blood infections with the exception of the Cambodian (Buchanan) strain, which required a dose of 1.5 g (TRENHOLME et al., 1975). Comparison of the efficacy of a single, 1.5 g dose of mefloquine with Fansidar showed mefloquine to cure all 37 patients suffering from falciparum malaria, while the antifolate combination cured 34 of 38 patients (DOBERSTYN et al., 1979). Erythrocytic *P. vivax* infections were

cleared by doses of 400 mg or greater, but blood infections reappeared, the more rapidly the lower the dose. Experimental, sporozoite-induced *P. vivax* infections were suppressed by single doses of 250 mg mefloquine, but blood infections appeared upon termination of drug administration. Radical cure required combination therapy (TRENHOLME et al., 1975; CLYDE et al., 1976; SCHMIDT et al., 1978a).

The pharmacokinetics of mefloquine administered to human volunteers has been studied by DESJARDINS et al., 1979b, and in man and dog by SCHWARTZ et al., 1980. Additional studies on mefloquine distribution and excretion have been conducted in rodents (MU et al., 1975; ROZMAN et al., 1978). Aqueous suspensions of mefloquine are much more completely absorbed than mefloquine administered by tablet. Administration by tablet results in slow and incomplete absorption. Mefloquine exhibits a high affinity for lung, liver, and lymphoid tissue. The half-life of a single, 1 g human oral dose, by 250 mg tablets, was 13.89 days, although with considerable variation; this aids in understanding the long duration of mefloquine protection. In addition, the volume of distribution of mefloquine is large. Bile excretion and gastric secretion have been detected in rats, and suggest the occurrence of enterohepatic circulation (MU et al., 1975). Three mefloquine metabolites have been discovered (MU et al., 1975; JAUCH et al., 1980), and the occurrence of a fourth inferred. One metabolite, 2,8-trifluoromethyl-quinoline-4-carboxylic acid, was tested in rodents, and found to be as toxic as orally administered mefloquine, but without antimalarial activity (JAUCH et al., 1980). A large proportion of [^{14}C] derived from mefloquine is excreted in feces, and secondarily in urine. MU et al. (1975) found the radioisotope to be in the form of mefloquine derivatives, whereas others (ROZMAN et al., 1978; JAUCH et al., 1980) detected fecal excretion of large quantities of mefloquine itself.

A 1 g oral dose was nontoxic. Human volunteers, given a single, 1.5 g dose experienced minor gastric disturbances, while a more heterogeneous patient group, in Thailand, suffered from abdominal pain, dizziness, nausea, vomiting, and weakness. Side effects were not intense, however (DOBERSTYN et al., 1979). Investigations of toxic effects in animals revealed dogs to be more sensitive to mefloquine than monkeys. Toxicity depended upon total dosage, and the duration, the route, and the frequency of administration (CRAWFORD and LEE, 1975; KORTE et al., 1978; KORTE et al., 1979). Single doses of 141 mg/kg were lethal to dogs; toxicity at lower dosages include hemolysis, reticulocytosis, erythrocytopenia, hepatic damage, and organ necrosis and atrophy. Intravenous administration produced transient cardiovascular changes and local irritation. Extended low dose administration produced gastrointestinal disturbance, weight loss, and lymphoid tissue depression. A concentration of one μg/ml partially suppressed mitogen-induced lymphocyte responses, while a concentration of 4 μg/ml suppressed both human and mouse lymphocyte responses (THONG et al., 1979). At the latter concentration cell viability was reduced by 50% or more. High (100 mg/kg) mefloquine doses administered daily from the 6th through the 15th day of gestation produced skeletal and soft tissue anomalies in rat fetuses, and occasional cleft palates in mouse fetuses (MINOR et al., 1976). Mefloquine, in rats, was not carcinogenic at concentrations of 30 mg/kg or lower, and fluctuation tests showed mefloquine to be nonmutagenic (SCHUPACH, 1979).

Structure–Activity Relationships

The threoracemate of mefloquine, WR 177,602, is slightly more potent, and more toxic than mefloquine itself (SCHMIDT et al., 1978a; KORTE et al., 1978). In addition, many structural variants of mefloquine have been synthesized and tested for antimalarial activity. The presence of the trifluoromethyl substituent, as opposed to hydrogen, in the 8 position of the quinoline ring, is necessary for high antimalarial activity, and a trifluoromethyl group in the 2-position maintains this activity (a minimum effective dose of 10 mg/kg in mice). A phenyl substituent in position 2 of the 8-trifluoro- compound yields an active but phototoxic structure. Introduction of a methoxy group in position 6 of mefloquine does not alter activity but also produces phototoxicity. Variation in the placement of the bond between the carbinol and the piperidine ring yields only one active isomer in the 3-position, whereas the 4-piperidine structure is inactive (STRUBE 1975; LOEW and SAHAKIAN, 1977). The key to the pharmacological action of these compounds is thought to be a pharmacophoric ring in which the carbinol oxygen is hydrogen bonded to the piperidine nitrogen, either to help stabilize an active conformation, or to match a 3 Å distance required by a putative bioreceptor (CHENG, 1971; CHIEN and CHENG, 1973; CHIEN and CHENG, 1976; LOEW and SAHAKIAN, 1977).

Evaluation of 12 quinolinemethanols against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* revealed five compounds to exhibit schizontocidal activity equal to or greater than that of chloroquine (SCHMIDT et al., 1978a and b). These five compounds, in order of activity, were WR 226,253, WR 177,602, WR 142,490 (mefloquine), WR 184,806, and WR 7,930. When compared against the multidrug-resistant Smith strain, the four most active compounds possessed 90% curative doses of 12.5/21/28/90 (mg total base/kg body wt). WR 226,253, the most active drug, differs from mefloquine only in possession of chlorine substituents in positions 6 and 8, while retaining the 2-trifluoromethyl group, and the piperidine ring; WR 177,602 is the threoracemate of mefloquine. WR 184,806, in turn, retains the 2,8-trifluoromethyl substituents on the quinoline ring, but the piperidine ring has been replaced with an ethyl tertiarybutylamino substituent. Thus variation in the quinoline and methanol substituents has yielded active and promising compounds related to mefloquine.

Mode of Action

Information from cytological, physiological, and binding studies is compatible with the hypothesis that mefloquine is deleterious to membranes, although the mechanism by which mefloquine acts is not presently discernible.

Cytology

Administration of mefloquine to monkeys infected with *P. falciparum,* or *P. vivax,* produces progressive dissolution of intraerythrocytic parasite forms. Changes which occurred within 24 h of drug administration included swelling

of the blue portion of the ring forms, vacuole enlargement, and "rarefaction" of chromatin. The ring substance collapsed, the chromatin coalesced, and, ultimately, the internal structure of the intraerythrocytic parasite disappeared (SCHMIDT et al., 1978a). The cytological influence of mefloquine has been likened to that of the related arylmethanols, quinine and WR 122,455 (PETERS and PORTER, 1976; PORTER and PETERS 1976; PETERS et al., 1977a). In *P. berghei,* the most apparent morphological changes occur in the membrane-bound pigment granules (WARHURST et al., 1972; PETERS et al., 1977a). The number of granules per trophozoite is reduced. Vesicles ultimately lose almost all particulate material. Mefloquine, like quinine, inhibits or reverses chloroquine-induced pigment clumping, although mefloquine is 100 times as effective as quinine. Neither chloroquine-induced pigment clumping, nor its reversal by arylmethanols, is understood. In *P. berghei,* the action of mefloquine was additive with that of pyrimethamine, primaquine, and sulphaphenazole, suggesting that the action of these drugs was different and nonpotentiating. Similarly, the property of mefloquine to suppress chloroquine-, amodiaquine-, and pyrimethamine-resistant *P. falciparum* (TRENHOLME, 1975; SCHMIDT et al., 1978a; DESJARDINS et al., 1979a) suggests differences in modes of action between mefloquine and these drugs. The property of mefloquine to act equally strongly against quinine-sensitive and quinine-resistant strains (DESJARDINS et al., 1979a) is somewhat troublesome in view of their structural relation. However, mefloquine has been observed to be 64–100 times as potent as quinine (STRUBE 1975; PETERS et al., 1977a).

Mefloquine lyses both nonparasitized and rodent red cells parasitized with *P. berghei,* as a function of drug and cell concentration, and, potentially, of duration of cell exposure to drug (EMERY et al., 1982). In addition, suspensions of saponin-released plasmodia undergo rapid turbidimetric decreases, indicative of lysis, upon exposure to mefloquine (EMERY et al., 1982). Hemin caused similar turbidimetric decreases deemed lytic (ORJIH et al., 1981).

Mefloquine is also bactericidal, and causes exponential loss of viability in cultures of *Escherichia coli* (BROWN et al., 1979a). Loss of viability is accompanied by decreases in turbidity which suggests the lytic properties of mefloquine. In addition, spheroplasts of *E. coli,* obtained by EDTA-lysozyme treatment, and stabilized by a concentration of 2.0 M sucrose, were lysed by mefloquine, and polymyxin B as well. It is noteworthy that *E. coli,* in addition to *P. berghei,* may be phenotypically adapted to mefloquine by continuous exposure of cultures to bactericidal concentrations of mefloquine (BROWN et al., 1979b). Spheroplasts obtained from such cultures appear less sensitive to mefloquine than spheroplasts derived from susceptible cultures (BROWN et al., 1979b).

Binding Studies: Cellular

Binding of mefloquine to cells, and to cell components, has been measured through use of [^{14}C]mefloquine. Approximately one-half the mefloquine which

associates with uninfected erythrocytes is membrane bound (MU et al., 1975). Membranes of human erythrocytes bind 49% of the cell-associated drug; membranes of mouse erythrocytes bind 66%. Mouse erythrocytes infected with *P. berghei,* whether chloroquine-sensitive or chloroquine-resistant, bind approximately twice as much mefloquine as do parasite-free cells (FITCH et al., 1979). Membrane binding of mefloquine is energy and temperature independent, and occurs by two processes, one of which is saturable. The apparent dissociation constant (K_d) for the saturable process is 2.5×10^{-6} M. Chloroquine, as well as radioisotope-free mefloquine, competitively inhibits binding of isotopically labeled mefloquine, but K_i mef $= 2.5 \times 10^{-6}$ M, while the K_i chl $= 1.7 \times 10^{-3}$ M. Thus, chloroquine has a much lower affinity than mefloquine for at least one class of binding sites. Mefloquine, conversely, competitively inhibits cellular binding of [^{14}C]chloroquine. The respective K_is for this process were 5×10^{-7} M_{chl}, and 2×10^{-6} M_{mef}, (FITCH, 1972). Direct measurement revealed cellular binding of chloroquine to occur with a $K_d = 10^{-8}$ M (FITCH, 1969). Thus, there appears to be a second cellular site which possesses a high affinity for mefloquine, but an even greater affinity for chloroquine.

Kinetic data from this laboratory revealed mefloquine to bind to both parasite-free, and *P. berghei* parasitized rat red cells, by a very rapid initial process, followed by a subsequent, slower process, suggestive of (1) reduction in cellular permeability controls, (2) cooperative binding, or (3) relatively slow accessibility to a second binding site. Parasitized cells bound more mefloquine by each process (WOLFE, unpublished observation).

Binding Studies: Subcellular

Stepwise degradation of mouse erythrocytes, both parasitized, and parasite-free, has contributed to identification of one group of high affinity mefloquine receptors (CHEVLI and FITCH, 1981). [^{14}C]mefloquine bound with a $K_d = 3 \times 10^{-7}$ M to erythrocyte membranes, membrane ghosts, membranes stripped of protein by detergent, or by treatment with a protease from *Streptomyces griseus.* However, membrane exposure to phospholipase C eliminated mefloquine binding. Lipids extracted from normal mouse erythrocytes, or erythrocytes parasitized with either chloroquine-sensitive or chloroquine-resistant *P. berghei* exhibited equal affinity for mefloquine, and bound 80 nmol mef/mg lipid. Parasitized cells contained almost twice the quantity of recoverable lipid, however, consistent with the ratio of mefloquine which bound to parasitized, in contrast to nonparasitized cells. Analysis of mefloquine binding to purified phosphatides revealed phosphatidylserine and phosphatidylinositol to bind 300–400 nmol mef/mg, while phosphatidylcholine and phosphatidylethanolamine bound 100 nmol mef/mg. All four phospholipids had a $K_d = 3 \times 10^{-7}$ M, however (CHEVLI and FITCH, 1981), suggesting that these membrane components constituted the cellular, high affinity mefloquine receptors. Since plasmodia contain appreciable quantities of phosphatidylinositol, not only may parasitized erythro-

cytes bind more mefloquine as a result of their greater lipid content, but also as a result of the type of phospholipid present in the respective cells. It is of interest that membrane phospholipids have also been implicated in the mode of action of polymyxin B, although the mechanisms by which this antibiotic acts remain to be elucidated (FEINGOLD et al., 1974; STORM et al., 1977).

Mefloquine binds to the inner and outer membranes of *E. coli* (BROWN et al., 1980). Binding was of sufficiently high affinity as to be demonstrable through sucrose gradient fractionation of the *E. coli* cell envelope.

Mefloquine, however, binds to cell components other than phospholipids. Thus molecular complexes of quinolinemethanols with iron porphyrin components have been studied, and mefloquine binding to plasmodial hemozoin, and its component, ferriprotoporphyrin IX, demonstrated (JEARNPIPATKUL and PANIJPAN, 1980). Ferriprotoporphyrin IX has been proposed to be the high affinity chloroquine receptor in plasmodia (CHOU et al., 1980). The respective affinities of chloroquine and mefloquine for ferriprotoporphyrin IX were $K_{d\,chl} = 3.5 \times 10^{-9}$ M, and $K_{d\,mef} = 3 \times 10^{-7}$ M. Thus studies in vitro have adduced results which parallel cellular studies, and demonstrate a class of sites which possess a greater affinity for chloroquine than mefloquine, but permit competitive inhibition by mefloquine. Determination of the affinity of mefloquine for still further binding sites has been limited, but the observation that mefloquine possesses equal affinities for phospholipids and ferriprotoporphyrin IX suggests interpretive caution. It should be noted that mefloquine binds with high but undetermined affinity to albumin, a_2, and B globulin (MU et al., 1975).

Mefloquine, in contrast to quinine (ESTENSEN et al., 1969; ALLISON and HAHN, 1977), and chloroquine (ALLISON et al., 1965), does not intercalate into DNA, or interact strongly or specifically with varied forms of this polymer (DAVIDSON et al., 1975; DAVIDSON et al., 1977). These conclusions were reached after extensive biophysical and biochemical study failed to detect changes in the properties of either DNA or mefloquine normally associated with intercalation, or other specific modes of ligand binding. The interaction of both sonicated calf thymus DNA, and closed circular superhelical colicin E_1 plasmid DNA with mefloquine, its 8-fluoro and 2-carboxamide congeners, and with quinine, chloroquine, quinacrine, and putrescine was compared by viscosimetric titration, thermal denaturation, changes in absolute and difference spectra, and inhibition of RNA polymerase. For example, the comparative viscosity of Col E_1 DNA was maximally increased approximately one and a half times by quinacrine and chloroquine, while mefloquine and its 8-fluoro congener failed to alter viscosity. The T_m of sonicated calf thymus DNA, under experimental conditions, was 62.0 C, 63.0 C in the presence of a concentration of 10^{-4} M mefloquine, 61.5 C in the presence of the 8-fluoro congener, and 88 C and 75 C, respectively, in the presence of concentrations of 10^{-5} M quinacrine and chloroquine. The presence of DNA minimally influenced the spectrum of mefloquine, and mefloquine did not inhibit RNA polymerase. Theoretical analysis, utilizing CPK space-filling models, supported the experimental findings, and suggested that the bulky, trifluoromethyl group in the 2-position could not be rotated into the plane of the quinoline ring, with the result that intercalative stacking was structurally improbable.

Physiological and Enzymatic Studies

Mefloquine inhibits incorporation of [^{3}H]adenosine, [^{3}H]deoxyadenosine, [^{3}H]hypoxanthine, and [^{14}C]phenylalanine into erythrocytes parasitized with either *P. berghei* or *P. falciparum,* and into *Escherichia coli* (EMERY et al., 1982; DESJARDINS et al., 1979a; BROWN et al., 1979a). The influence of arylmethanols, and mefloquine in particular, on macromolecular precursor incorporation was dependent upon the drug structure, the drug concentration, the cell concentration, as determined by hematocrit, and the cell-drug exposure time (EMERY et al., 1982). For example, a concentration of 8×10^{-5} M mefloquine caused 67% inhibition of [^{3}H]adenosine incorporation into a *P. berghei* parasitized rat red cell suspension at 5% hematocrit, and only 19% inhibition into a similar suspension at 10% hematocrit. Precursor entry into low molecular weight pools, measured by parasitized red cell uptake of 1 min pulses of [^{3}H]adenosine, was reduced by approximately 20–30%, during a period in which incorporation of [^{3}H]adenosine had been reduced by more than 75%. After a 1 h incubation mefloquine had reduced pulse uptake by 52%. Mefloquine did not appear to selectively inhibit the synthesis of RNA, DNA, or protein, in *P. berghei* parasitized rat red cells, or in *E. coli* (Emery et al., 1982; BROWN et al., 1979a).

Enzyme Inhibition

Mefloquine inhibited the plasmodial enzyme, ornithine decarboxylase, whose activity is found in lysates of mouse erythrocytes parasitized with *P. chabaudi* (KONIGK et al., 1981). Conversely, mefloquine stimulated ATP synthesis by both the Na^+/K^+ and Ca^{2+}/Mg^{2+} ATPases in both intact and membrane ghost preparations of *P. chabaudi* parasitized mouse erythrocytes (KONIGK et al., 1981). The membrane acetylcholinesterase of these erythrocytes was only partially suppressed by a concentration of 10^{-3} M mefloquine.

The inner, or cytoplasmic, membrane of *E. coli* contains the enzyme NADH oxidase. Mefloquine, the membrane-active antibiotic polymyxin B, and antimycin A, which is considered to inhibit the membrane-bound b cytochromes, all suppressed the activity of this enzyme in isolated *E. coli* membranes (BROWN et al., 1980). NADH oxidase has also been detected on the outer membrane of *P. lophurae* (LANGRETH, 1977).

Conclusion

Intraerythrocytic plasmodia, species of leishmania, and bacteria, are susceptible to mefloquine. The drug possesses a high affinity for cell membranes, their component phospholipids, and specific plasma proteins. Data from a variety of studies suggest that the binding of mefloquine to cell membranes is deleterious, if not ultimately lethal. Detailed study of drug–membrane interaction is

required to determine the relation between this binding and the mode of action of mefloquine, and to elucidate the operative mechanisms.

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Metronidazole

D.I. EDWARDS

Introduction

Metronidazole was the first nitroimidazole drug to be marketed for the systemic treatment of trichomonal vaginitis and has been available since 1960. During that time its use has increased to include other protozoal and bacterial infections with no serious problems of resistance being encountered.

The drug (see Fig. 1 for structure) is 1-β-hydroxyethyl-2-methyl-5-nitroimidazole and is a white, crystalline, nonhygroscopic compound with a molecular weight of 171.15 and melting point of 160° C. It is soluble in water to about 1% and in ether, chloroform, and ethyl alcohol to about 0.5%. The pH of a saturated solution at 20° is 6.6. Two recent reviews as to the clinical applications of metronidazole have been published recently (FINEGOLD, 1977; PHILLIPS and COLLIER 1979) so this review will mainly deal with the mechanism of action and selective toxicity of the drug.

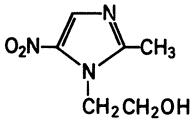

Fig. 1. Structure of metronidazole (1-2′-hydroxyethyl-2-methyl-5-nitroimidazole)

Clinical Uses

Metronidazole is the current drug of choice for the treatment of several protozoal diseases including trichomoniasis (COSAR and JULOU, 1959) caused by *Trichomonas vaginalis* and intestinal infections caused by *Giardia lamblia* (POWELL et al., 1966), all forms of *Entamoeba histolytica* infections (KHAMBHATTA, 1971; ADAMS, 1977), and *Balantidium* (GARCIA-LEVERDE and de BONILLA, 1975). The drug appears useful against *Leishmania mexicana* but not against *L. brasiliensis* (BELTRAN et al., 1967; LAPIERRE et al., 1975).

Since 1975 metronidazole has been found to be exceptionally useful against bacterial infections caused by nonsporing anaerobes, particularly *Bacteroides*. The first indication came from an observation that a patient with Vincent's

disease (an acute ulcerative gingivitis) was rapidly cured whilst receiving metronidazole therapy for trichomoniasis (SHINN, 1962). The condition is caused by a variety of anaerobic bacteria, and subsequent controlled trials proved the drug remarkably effective (WADE et al., 1966). The recognition that *Bacteroides* is the major cause of postoperative sepsis, particularly from the gut, has led to the widespread use of metronidazole in successfully controlling such conditions, and these have been reviewed recently (FINEGOLD, 1977; PHILLIPS and COLLIER, 1979).

Metronidazole has also been investigated as a potential radiosensitizer of hypoxic tumours in man. As a tumour grows it tends to outstrip its blood supply causing hypoxic regions to develop. Such cells are resistant to radiation but their resistance is reduced in the presence of certain drugs known as radiosensitizers. Metronidazole is potentially useful in this condition since it selectively sensitizes hypoxic cells only and has an additional cytotoxic effect. As will be described later, the mechanism of this effect appears to be identical to that which occurs in microorganisms.

Effect of Metronidazole on Microorganisms

The spectrum of activity of metronidazole is unique in that it encompasses both Gram-positive and Gram-negative bacteria, protozoa, even a few nematodes and hypoxic tumour cells. However, the spectrum is limited to those organisms which are anaerobic or at least are capable of anaerobic metabolism. The exception appears to be the inhibition of *Gardnerella vaginalis* (*Hemophilus vaginalis*) which is killed by a metabolite of metronidazole (BALSDON and JACKSON, 1981).

Anaerobes differ from aerobes in their biochemistry of respiration in several important respects. Redox mechanisms in anaerobes occur at a much lower potential and one of the products of respiration is usually H_2 gas since O_2 as a final electron acceptor is absent. The evolution of H_2 is the result of electron transport mechanisms and is characteristic of both anaerobic protozoa and bacteria. It was of particular interest therefore that metronidazole was found to inhibit specifically H_2 output from *T. vaginalis* (EDWARDS and MATHISON, 1970a and b; EDWARDS et al., 1973) and in bacteria (EDWARDS et al., 1973). These studies established that the mechanism of the H_2 output in *T. vaginalis* was a modified pyruvate dehydrogenase reaction, the clostridial-type pyruvate phosphoroclastic reaction (Fig. 2).

Metronidazole does not affect the synthesis of acetyl phosphate nor CO_2 production (inhibition of the latter occurs much later and is a consequence of cell viability loss) and thus the site of inhibition must occur in the cycle involving H_2 generation.

The inhibition point was shown to be the reduced electron transfer protein ferredoxin in bacteria or its equivalent in protozoa which has a redox potential of -470 mV, far lower than that attainable in aerobic cells. It was also demonstrated that the drug was not detectable in cell-free *T. vaginalis* or *Clostridia*, indicating reduction of the nitro group (EDWARDS et al., 1973). This observation

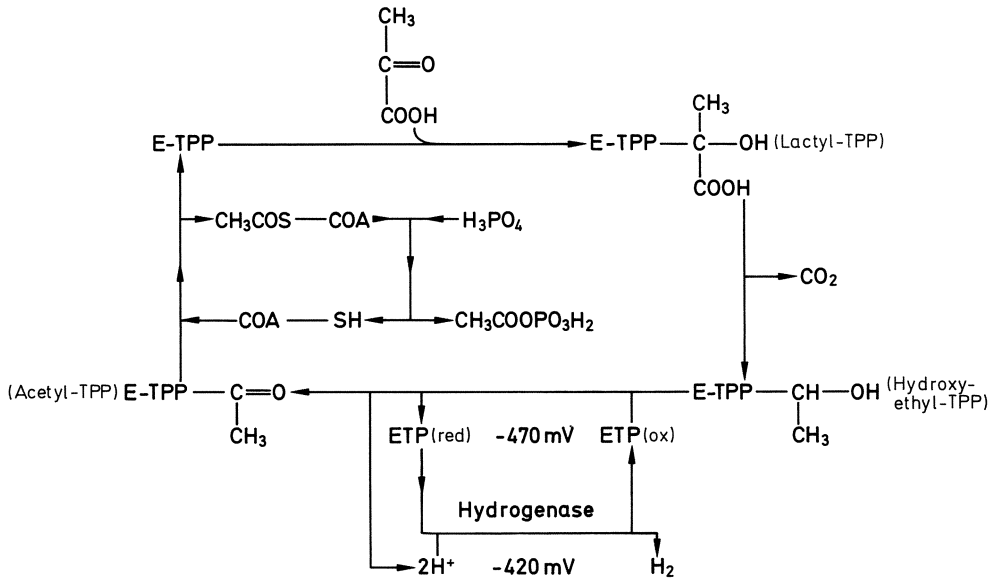

Fig. 2. The pyruvate phosphoroclastic reaction (from EDWARDS et al., 1973). E-TPP is a membrane-bound enzyme-thiamine pyrophosphate complex and ETP is a low redox electron transfer protein known to be ferredoxin in Clostridia and, as yet, uncharacterized in protozoa. The redox potentials in millivolts are given for ferredoxin and the hydrogenase reaction

and one showing that the drug inhibited electron transport to various dyes (EDWARDS and MATHISON, 1970b) demonstrated that metronidazole acted as an electron sink in the phosphoroclastic reaction accepting electrons from reduced ferredoxin via the nitro group and becoming reduced in the process.

Such a mechanism of action explains the selective toxicity of metronidazole towards anaerobes in that only they possess redox mechanisms of sufficiently low potential that can couple with the drug. However, it was elegantly demonstrated that once all the drug was reduced in *Clostridia,* the phosphoroclastic reaction recovered (O'BRIEN and MORRIS, 1972).

A similar effect demonstrated in protozoa (LINDMARK and MULLER, 1974) thus showed that inhibition of H_2 evolution was transient and interrupted electron flow in the phosphoroclastic reaction was not the biochemical lesion responsible for death of susceptible cells. It was tacitly assumed, therefore, that the compound responsible for cell death was a reduction product of the drug, and which exerted its effect at some other site in the cell.

Mechanism of Action of Metronidazole

I. Effect of Metronidazole on DNA

Evidence that metronidazole inhibited the uptake of [^{14}C]-labelled thymidine in *T. vaginalis* suggested that DNA was the site of action of the drug (INGS

et al., 1974). At the same time, during radiosensitization studies it was shown that radiation induced binding of the drug to DNA, but only under hypoxic or anoxic conditions (WILLSON et al., 1974). Later studies not only confirmed this work but showed that the drug degraded existing DNA in *Clostridium bifermentans* (PLANT and EDWARDS, 1976). Once the target was identified, studies in vitro of the drug–target interaction could be made. The first of these used metronidazole reduced by sodium dithionite in the presence of DNA (EDWARDS, 1977) which was subsequently analyzed by melting profiles and sucrose gradient sedimentation.

These studies demonstrated that the drug decreased the temperature of the mid-point of the helix-coil transition (the T_m value) of DNA, increased the melting range, i.e. the temperature over which the helix-coil transition occurs, but had no effect on the cooling profiles. This indicates that metronidazole destabilizes DNA by a non-intercalative and non-cross-linking mechanism, but by strand-breaking. This effect was subsequently corroborated by sucrose gradient sedimentation experiments which demonstrated both single and double strand breakage. Significantly, these effects only occurred under anaerobic conditions and do not occur if DNA is added after drug reduction, indicating that the reduced drug responsible for DNA damage is a short-lived compound.

Sodium dithionite, although a powerful reducing agent necessary for drug reduction, causes DNA strand breaks (CONE et al., 1976) so quantitative measurement of strand breakage is ambiguous. Indeed, some workers have been unable to detect DNA damage using viscosity measurements with dithionite-reduced drug (LA RUSSO et al., 1977). These problems may be overcome by using an electrolytic reduction technique in which the drug is reduced at constant potential. Such a technique developed in our laboratory (EDWARDS et al., 1978; KNIGHT et al., 1978) enables unambiguous measurements of DNA damage to be made, provided the applied potential does not exceed -1.1 V because otherwise degradative interactions occur between the electrodes and DNA (BRABEC and PALECEK, 1976; NURNBERG and VALENTA, 1978).

DNA in this in vitro system does not show damage in air, or if metronidazole is unreduced. The system has been used to investigate DNA damage caused by nitroimidazoles, nitrofurans, and nitrobenzenoid compounds (EDWARDS et al., 1978, 1980; KNIGHT et al., 1978, 1979; ROWLEY et al., 1979, 1980; SKOLIMOWSKI et al., 1981).

Detailed studies using a variety of techniques to measure DNA damage show that metronidazole causes a marked decrease in the viscosity of DNA by strand breakage and/or strand separation, but not helix "bending" or intercalation which tends to increase viscosity (REINERT, 1972). Since the viscosity decrease falls below that for the helix-coil transition or conversion from double to single stranded forms, the major effect is not merely strand separation (EDWARDS et al., 1978). Such an interpretation is supported by results obtained from an analysis of thermal hyperchromicity and renaturation profiles of DNA (EDWARDS et al., 1978; KNIGHT et al., 1979), which show that metronidazole decreases the thermal hyperchromicity and inhibits the renaturation of random-coil DNA to the double-helix form. If thermal renaturation is controlled, the Rowley equation (ROWLEY et al., 1979) enables a direct calculation to be made

of the amount of intact helix in any DNA preparation. Using this type of measurement helix loss can be as high as 70%–80%, indicating extensive single-strand nicking.

Further corroboration of single- and double-strand breakage as a major effect of reduced metronidazole comes from studies using techniques of alkaline and neutral sucrose gradient sedimentation, hydroxyapatite chromatography and agarose gel electrophoresis (ROWLEY et al., 1979; KNIGHT et al., 1979).

However, the extent to which the drug causes DNA damage depends on the source of DNA used, indicating that the induced damage may not be random.

II. Nature of DNA Damage

Damage to DNA induced by a variety of 2- and 5-nitroimidazoles including metronidazole varies with the base composition of the DNA (EDWARDS et al., 1982a and b; EDWARDS, 1980; KNOX et al., 1980; ROWLEY et al., 1980). In general, irrespective of which method is used to measure DNA damage, a nitroimidazole will damage DNA of a high A+T content far more than that of a low A+T content. Damage is maximal with the artificial polymer poly (d[A+T]) and absent with poly (d[G−C]). This suggests that DNA damage results from an attack by the reduced active species upon either A or T residues in DNA.

III. The Target of Nitroimidazole Action

Recent evidence indicates that the 2-nitroimidazole misonidazole specifically releases thymidine phosphates from DNA (KNOX et al., 1980; KNOX et al., 1981) and this effect appears to be common to both 2- and 5-nitroimidazoles (KNOX, KNIGHT, and EDWARDS, unpublished). At drug-nucleotide ratios far lower than that obtainable clinically DNA damage is sufficiently extensive that degradation products are released and may be separated by dialysis and chromatography, and subsequently characterized. Using such methods KNOX and colleagues have shown that the only significant products of nitroimidazole-induced damage are thymine-derived (KNOX et al., 1980, 1981). Misonidazole for example liberates about 5% of the total thymine from *E. coli* DNA as a mixture of thymine (3%), d-thymidine (18%), d-thymidine-3-phosphate (32%), d-thymidine-5-phosphate (22%), and d-thymidine-3,5-diphosphate (15%). The value for d-thymidine-3-phosphate is probably artificially high and that for the di-phosphate low because the latter tends to hydrolyse to yield the 3-phosphate.

These effects also occur in calf thymus DNA, apurinic acid and RNA (where uridine is released), but is absent in apyrimidinic acid and poly (d[GC]). Significantly, these results parallel the damage measured by other techniques, indicating that nitroimidazole-induced strand breakage results in thymidine (and its derivatives) release. These effects have been observed in a variety of nitroimidazoles (EDWARDS et al., 1982a). In general, those nitroimidazoles of low electron

Table 1. Relationship between DNA base composition and the susceptibility of microorganisms to metronidazole. MIC is the minimum inhibitory concentration

Organism	MIC ($\mu g/cm^3$)	% A+T
Entamoeba histolytica	0.5–5.0	73–78
Clostridium bifermentans	0.1–2.0	73
Trichomonas vaginalis	0.5–5.0	71
Fusobacterium fusiformis	0.1	68.5
Veillonella parvula	1.6	63.5
Bacteroides ovatus	0.5	60
B. fragilis	1.0	58
B. distasonis	2.0	56
Bifidobacterium bifidum	3–16	42
Rhodopseudomonas acidophila	25	35
Actinomyces spp	32	30

affinity release more thymidine than those of high electron affinity. The thymidine release and strand breakage effects also occur with single-stranded DNA indicating that the topological requirements of a double helix are absent. Further, since thymidine release is constant irrespective of the base composition, there appears to be no specificity for any particular base sequence (KNOX et al., 1981). The fact that nitroimidazoles also release uridine derivatives from RNA indicate that the specificity does not involve the 5-methyl group of the thymine ring nor the 2-position of the ribose sugar.

The nuclease-like action of these nitroimidazoles is superficially similar to the bleomycins, which also specifically release thymidine residues from DNA. However, bleomycin does not release uridine from RNA (CARTER et al., 1978; HECHT, 1979), the mechanism of each must therefore differ.

The evidence thus far indicates that the major target of metronidazole after reduction is the phosphodiester bonds around thymine residues in DNA. It is logical to predict, therefore, that those bacteria or protozoa which possess DNAs rich in T-residues would be more susceptible to the action of metronidazole than those organisms of a low A+T content. This is generally true (EDWARDS, 1981; EDWARDS et al., 1981) as may be seen from Table 1. It is clear that organisms with DNAs of high A+T content are more susceptible to metronidazole, at least as demonstrated by MIC determination, and it is germane that those organisms against which metronidazole is the drug of choice and consequently clinically most effective are those of particularly high A+T content. The base composition of an organism is therefore important in determining the relative susceptibility of an organism to nitroimidazole drugs.

IV. Reduction and the Nature of the Active Species

It is tacitly assumed in the above discussion that the agent responsible for DNA damage is a reduction product with a short (less than one second) half-life.

Table 2. Reduction products of metronidazole reduced by xanthine oxidase and electrolytically. The yields are not additive due to different fragmentation patterns of the imidazole ring

Reduction product	Reduction method (%)	
	Xanthine oxidase	Electrolytic
Ethanolamine	15.3	10.0
Acetamide	14.1	28.3
Glycine	8	5.0
Acetate	4.8	3.8
N-glycolethanolamine	2.7	
N-acetylethanolamine	2.4	0
N-2-hydroxyethyloxamic acid	2.2	
Oxamate		14.0
Nitrite		16.1
CO_2		0.35
N-acetylglycine		0.5

The identification and characterization of the cytotoxic agent is by no means an easy task. Pulse radiolysis and rapid mixing experiments have shown that the cytotoxic effect of metronidazole and other nitroimidazoles develops after the radiosensitization effect (ca. 100 ms), but may be prevented by aminothiols which act within 500–850 ms. The cytotoxic effect therefore is generated and brought about within about 500 ms by a reduction product of the original drug. Classic chemistry indicates a possible 6 electron reduction of metronidazole (and other nitroimidazoles) thus.

$$R-NO_2 \xrightarrow{1E} R-\dot{N}O_2^- \xrightarrow{1E} R-NO \xrightarrow{2E} R-NHOH \xrightarrow{2E} R-NH_2$$

(nitro-radical anion) (nitroso) (hydroxylamine) (amino)

The reduction scheme, based on nitrobenzene reduction, does not obtain with most imidazoles since any reduction product is not amenable to identification, because they tend to be extremely labile at room temperature and in air. This difficulty is compounded by the fact that reduction products have no UV or visible absorption spectrum making detection, and therefore separation, difficult. If metronidazole is reduced with xanthine oxidase, seven reduction products are formed (CHRYSTAL et al., 1980) but these account for less than half the original drug. A somewhat different picture is obtained if the drug is reduced electrolytically. The major products listed in Table 2 have no antimicrobial activity and are consequently not the active species responsible for cytotoxicity. One approach to the study of reduction products is coulometry (coulombetry), a technique in which the number of electrons involved in the reduction process may be measured. Generally, *p*-nitrobenzenoids such as chloramphenicol show a 6-electron reduction indicating the ultimate formation of the corresponding amine (SKOLIMOWSKI et al., 1981). The 2-nitroimidazoles such as misonidazole and benznidazole give a 4-electron reduction indicating the formation of a hydroxylamine derivative (EDWARDS et al., 1982a). Metronida-

zole however, in common with other 5-nitroimidazoles show a non-integral value, commonly between 3 and 4 and which tends to increase in the presence of DNA (EDWARDS et al., 1982a). This may suggest that a reduced derivative below the 4-electron stage disproportionates and this increases in the presence of DNA (EDWARDS et al., 1982a and b).

Another product of 5-nitroimidazole reduction is the nitrite ion formed by the disproportionation of the 1-electron radical anion.

$$R-NO_2 \xrightarrow{1E} R-N\dot{O}_2^- \longrightarrow \dot{R}+NO_2^-$$

Metronidazole, electrolytically reduced, provides 16% nitrite which increases to 20.7% in the presence of calf thymus DNA (EDWARDS et al., 1982a). This effect, which is more marked with metronidazole than any other nitroimidazole, not only explains the non-integral electron requirements (2-nitroimidazoles do not yield nitrite), but strongly suggests that the active cytotoxic agent is the 1-electron radical anion. Since nitrite is not cytotoxic to DNA, it should follow that its production as a result of disproportionation of the nitro radical anion ($R-N\dot{O}_2^-$) should parallel that of DNA damage (cytotoxicity) which is related to DNA base composition, notably A+T content (ROWLEY et al., 1980; KNOX et al., 1981). This is precisely observed experimentally; nitrite production from reduced metronidazole being related to the A+T content of DNA (EDWARDS et al., 1982b). This evidence that the cytotoxic agent is the nitro radical anion explains the short half-life of the agent but not its specificity for strand breakage of phosphodiester bonds resulting in thymidine release from DNA.

It has been known for many years that radiation-induced damage to DNA is caused by attack of the hydroxy radical. Interestingly, thymine residues in DNA seem most susceptible to hydroxy radical attack and this appears to be due to electron transfer from sugar radicals to electron-affinic compounds, d-ribose being rather more sensitive than ribose in this respect (MICHAELS et al., 1976). Such an effect would logically lead to strand breakage with such agents as the nitroimidazoles, as has been demonstrated with radiation (HOHMAN et al., 1976), and electrolytically, as mentioned previously. Further evidence comes from studies using electron spin resonance of free radicals formed in irradiated drug-DNA complexes (WASHINO et al., 1979). This also shows an increase of radical formation with misonidazole (a 2-nitroimidazole), which was most efficient with dTMP residues and which increased the incidence of sugar damage, which would naturally lead to strand breakage. In summary, both the hydroxy radical and the nitro radical appear to exert a specificity towards thymine residues, but the electronic basis for such an action remains to be elucidated.

Metronidazole and Aminothiols

Aminothiols, such as glutathione, cysteine and cysteamine, are known to inhibit both the radiosensitizing and anaerobic cytotoxicity of nitroimidazoles

including metronidazole (HALL et al., 1977). Such compounds are generally regarded as being radical scavengers and radioprotectors, and many theories have been put forward to explain their mechanism of action including protection against radical-induced DNA damage (JELLUM and ELDJARN, 1965; RIX-MONTEL et al., 1978). A more widely accepted theory involves the SH group in the repair caused by radical damage (WILLSON et al., 1974) as shown in the equation depicted below.

$$\dot{X} + R-SH \rightarrow XH + R\dot{S}$$

Here, $\dot{X}$ is damaged DNA and the aminothiol R−SH repairs the damage by H-atom transfer, forming a sulphydryl radical which may undergo further reactions. In the case of the inhibition of the cytotoxic effect of metronidazole and other nitroimidazoles the inhibition mechanism may be due to a reaction between the nitro radical anion $R-N\dot{O}_2^-$ and the thiol, thus

$$R-N\dot{O}_2^- + R^1-SH \rightarrow R-NO + R^1\dot{S} + OH^-$$

This mechanism, however, does not explain the nature of protection as observed by electrolytically reduced drugs in the presence of cystamine or cysteamine (EDWARDS, 1981; EDWARDS et al., unpublished) since this appears to vary with the redox potential of the drug, suggesting a relationship between the redox potential of the drug and that of the aminothiol. Although the precise mechanism of aminothiol protection is unclear, it has an important bearing on considering the relative cytotoxicity of the drug.

Mutagenicity and Carcinogenicity

I. Mutagenicity

A major target of metronidazole is DNA and it may logically be predicted that the drug may show mutagenic and possibly carcinogenic toxicity related to its mechanism of action in susceptible bacteria and anaerobic cells. There is no doubt that metronidazole is mutagenic to bacteria, but whether it has any relevance to its use in human medicine is questionable. The basic method used for mutagenicity tests is that developed by AMES and co-workers (AMES et al., 1973) in which mutant histidine-requiring strains of *Salmonella typhimurium* are exposed to the chemical under test and the number of colonies of the bacterium which revert to the wild type are scored. Metronidazole, when tested in this fashion give a positive Ames test, i.e. is mutagenic (WANG et al., 1975; VOOGD et al., 1974; ROSENKRANZ and SPECK, 1975; LINDMARK and MULLER, 1976; CHIN et al., 1978; ROSENKRANZ et al., 1976) as are many other nitroimidazoles and nitroheterocyclic drugs. In addition, metronidazole has been found after therapeutic dosing to be mutagenic in the urine of mice using the Ames test (LEGATOR et al., 1975; SPECK et al., 1976).

However, in those Ames tests which lacked a liver microsomal fraction mutation only occurred under anaerobic conditions, but occurs seemingly under

both aerobic and anaerobic conditions in the presence of the microsomal fraction. It is well established that metronidazole and other nitroimidazoles are only capable of exerting antimicrobial effects as a result of reduction, a conversion which is only possible under anaerobic conditions generally. It is apparently not generally realized that the microsomal preparation used in the Ames test has a very high respiratory quotient and oxygen demand. This results in the preparation quickly being made anaerobic in a seemingly aerobic environment. LINDMARK and MULLER (1976) have unequivocally shown that mutagenesis of metronidazole in the Ames test only occurs under anaerobic conditions leading to drug reduction. It follows that the conclusions drawn from mutagenicity studies of nitroimidazoles using the Ames test and related to potential human toxicity are not only highly questionable but most probably irrelevant. Nitroimidazoles are not alone in this respect (EDWARDS, 1980).

II. Carcinogenicity

Reports of carcinogenicity of metronidazole are conflicting. The first of these indicated that metronidazole caused lung tumours at high dose levels in mice and also lympho-reticular neoplasms in female mice only (RUSTIA and SHUBIK, 1972). The same authors found hepatomas and mammary tumours in rats also at high dose levels (RUSTIA and SHUBIK, 1979). Other studies have shown lung tumours in male mice only or in both sexes (RUST, 1977), while in the rat (female) one study was negative (COHEN et al., 1973) while the other was non-significant (RUST, 1977). In hamsters, two studies have shown negative carcinogenicity of metronidazole (ROE, 1977a and b, 1979). As ROE (1979) points out, increased incidences of lung tumours have been seen in three experiments in mice, but two in hamsters and two in mice were negative. The typical type of tumour which develops in these studies is also typical of those which arise by overfeeding leading to the increased incidence of mammary tumours (ROE and TUCKER, 1974; TUCKER, 1979).

In other studies metronidazole has proved negative in the dominant lethal test in rats (BOST, 1977), the micronucleus test in mice and human cells in vitro (HARTLEY-ASP, 1979a), in vitro studies of unscheduled DNA synthesis in human lymphocytes and fibroblasts (BOST, 1977; LAMBERT et al., 1979), and in chromosome aberration frequency tests and mitotic indices in human lymphocytes (HARTLEY-ASP, 1979b). Probably the most significant report in this respect is that concerning a 10-year study showing that metronidazole did not increase the risk of cancer in women (BEARD et al., 1979).

Metronidazole Resistance

Metronidazole has been in clinical use for over 20 years and during that time its use has increased. Surprisingly, very little resistance has been encountered with the drug either with protozoa or bacteria. Treatment of trichomonal

vaginitis occasionally results in clinical failure, but the protozoan is usually fully sensitive to the drug in vitro (KANE et al., 1961; SQUIRES and MCFADZEAN 1962; NICOL et al., 1966). In many cases the flora of the vagina includes many bacteria capable of "inactivating" the drug. In the case of anaerobes these are killed by the drug, but aerobes may "absorb" the drug without loss of viability (RALPH and CLARK, 1978; EDWARDS et al., 1979) thus reducing the level of the drug below that required therapeutically. However, under enhanced anaerobiosis metronidazole appears to exert a killing effect on *E. coli* which is normally relatively refractory to the drug (INGHAM et al., 1979, 1980). The relevance of this observation to the in vivo situation is, at present, a moot point because the redox levels and oxygen tension of the vagina, for example, are unknown. True resistance does exist in *Trichomonas vaginalis*. Recent reports have demonstrated that the organism obtained clinically is resistant to levels of drug attainable clinically (MULLER et al., 1980). The mechanism of resistance of these strains does not involve an altered pyruvate dehydrogenase complex as might be expected, but involves an increased tolerance to oxygen (MULLER et al., 1980). Metronidazole-resistant *T. vaginalis* have also been found in Vienna (THURNER and MEINGASSNER, 1978) and in Scandinavia (FORSGREN and FORSSMAN, 1979), and were not only clinically resistant but also in vivo in mice. The important point raised by this work is that these organisms are sensitive under anaerobic conditions but resistant under partially aerobic systems, as has been demonstrated for *T. foetus* (MEINGASSNER et al., 1978), indicating that anaerobic sensitivity tests for metronidazole may not be a reliable indicator of its efficacy if performed in isolation (MULLER et al., 1980; MEINGASSNER and THURNER, 1979). However, this does raise the point that if tolerance to oxygen can lead to metronidazole resistance, what are the prevailing O_2 tensions in the normal and infected vagina, and its redox potential? At present the answer is unknown.

The biochemical mechanisms of resistance of *Bacteroides fragilis* to metronidazole are better understood. Resistance of this organism to the drug was first reported in 1977 but no in vitro levels of resistance were determined (CHARDON et al., 1977). Later, however, a strain was isolated with a minimum inhibitory concentration of 32 $\mu g/cm^3$, and metronidazole uptake was markedly less than in a susceptible strain (INGHAM et al., 1978; WILLIS et al., 1978; TALLY et al., 1979). Studies indicated that the decreased uptake was not a consequence of altered cell permeability, but of an altered reduction mechanism. In strains of *B. fragilis,* made resistant to metronidazole by alkylating mutagens, it has been shown that the levels of the pyruvate dehydrogenase complex, the mechanism responsible for reduction of the nitro group of the drug, are markedly decreased not only in the artificial mutant strain but also in a strain clinically resistant (BRITZ and WILKINSON, 1979). As has been mentioned previously, the rate of entry of metronidazole into a cell depends upon the rate of intracellular reduction which generates a concentration gradient favouring uptake.

Decreased reduction rates would thus lower the rate of entry of the drug into the cell. This mechanism also results in altered end products of glucose metabolism and increased lactate levels in resistant cells (BRITZ and WILKINSON, 1979; BRITZ, 1982).

Similar results have been obtained with the 2-nitroimidazole misonidazole in resistant *B. fragilis,* which showed depressed levels of pyruvate dehydrogenase, but in *E. coli* a permeability mechanism appears to confer resistance to the drug (ROWLEY and EDWARDS, 1982), indicating at least two resistance mechanisms operating in bacteria and a third in protozoa. At the time of writing, however, even though the use of metronidazole is increasing, the occasional clinical failure is often resolved by increasing the drug dose and resistance is not a serious problem.

Acknowledgments. Work from this laboratory is supported by the Cancer Research Campaign and the Medical Research Council, whose support is gratefully acknowledged. I also wish to thank my colleagues in the Research Unit, namely, RICHARD C. KNIGHT, RICHARD J. KNOX, and IRENA M. SKOLIMOWSKI, whose work is described herein.

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Neothramycin

N. TANAKA

Neothramycin has been isolated from *Streptomyces* MC916–C4 in the laboratory of Dr. HAMAO UMEZAWA (TAKEUCHI et al., 1976). It is a potent antitumor antibiotic of the pyrrolo(1,4)benzodiazepine group, which anthramycin (cf. a review by KOHN, 1975), sibiromycin (see a review by GAUSE, 1975), tomaymycin (ARIMA et al., 1972) and mazethramycin (KUNIMOTO et al., 1980) belong to. Neothramycin[1] contains two stereoisomers A and B in nearly equal amounts. The structural elucidation and total synthesis have been reported by MIYAMOTO et al. (1977). Both antibiotics are hydrated within 60 min after being dissolved in water, forming the 10-hydro-11-hydroxyl-derivatives (Fig. 1). The hydroxyl groups at C-3 and C-11 epimerize in aqueous solution.

Neothramycin exhibits a significant activity against transplantable animal tumors, such as Ehrlich carcinoma, leukemia P388 and L1210, sarcoma 180 and mammary adenocarcinoma (CCMT) of mice, and hepatoma AH130 and Walker tumor 256 of rats, although the antimicrobial activity is weak (TAKEUCHI et al., 1976; HISAMATSU et al., 1980). Neothramycin is less toxic than the other pyrrolo(1,4)benzodiazepines. At present, clinical investigations of the antitumor effect on human neoplasms are in progress in Japan.

DNA appears to be the chemoreceptor of neothramycin. The reaction with DNA is highly dependent upon the proper conformation of DNA double helix. The drug may bind covalently to 2-amino group of guanine base of DNA through C-11 and/or C-3 of the molecule, and exist within the narrow groove of the DNA duplex. The rate of adduct formation is slow. DNA possesses

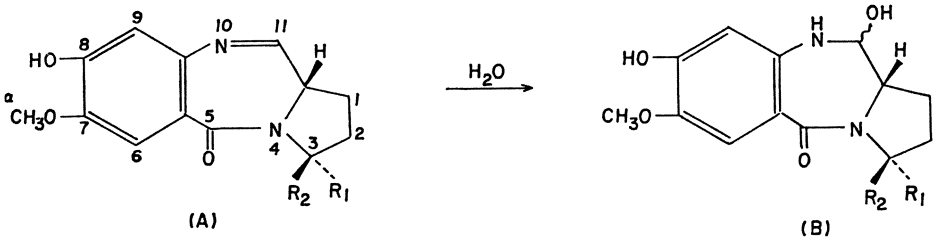


Fig. 1. Structures of neothramycins (**A**) and their hydrated forms in water (**B**). Neothramycin A: R_1=OH, R_2=H; neothramycin B: R_1=H, R_2=OH. [a] C: This carbon of [^{14}C]neothramycin was labeled with radioisotope (MIYAMOTO et al., 1977)

[1] "Neothramycin" is used for the mixture of neothramycins A and B in this article.

one binding site per ca. three base pairs with an association constant of $4.7 \times 10^3\ M^{-1}$ (MARUYAMA et al., 1981a, b). Neothramycin preferentially inhibits RNA over DNA synthesis (MARUYAMA et al., 1978, 1979).

A few review articles on the mechanism of action of the pyrrolo(1,4)benzodiazepine group antibiotics appeared lately: KOHN (1975), GAUSE (1975), and HURLEY (1977).

Growth Inhibition in Culture

Neothramycins A and B exhibit weak growth-inhibitory activities against some Gram-positive and -negative bacteria, and fungi (Table 1). Neothramycin A displays slightly higher antimicrobial activity than neothramycin B (TAKEUCHI et al., 1976).

Neothramycin prevents growth of mouse lymphoblastoma L5178Y and HeLa cells in culture at concentrations of 0.5 and 1.0 μg/ml, and shows a lethal effect at 5.0 μg/ml. The partial inhibition is observed in the range of 0.2 to 0.5 μg/ml (Fig. 2) (MARUYAMA et al., 1978). Neothramycins A and B also inhibit multiplications of Yoshida rat sarcoma cells and C3H cells transformed by SV40 in culture (TAKEUCHI et al., 1976).

Table 1. The antimicrobial spectra of neothramycins

Test organisms	Minimum inhibitory concentrations (μg/ml)	
	Neothramycin A	Neothramycin B
Staphylococcus aureus SMITH	50	100
Staphylococcus aureus FDA 209P	>100	>100
Bacillus subtilis PCI 219	100	>100
Klebsiella pneumoniae PCI 602	50	100
Escherichia coli NIHJ	100	100
Escherichia coli K-12	100	100
Escherichia coli W677	50	100
Escherichia coli JR66/W677	100	>100
Pseudomonas aeruginosa No. 12	>100	>100
Aeromonas salmonecida ATCC14174	25	50
Vibrio anguillarum NCBM6	50	100
Xanthomonas citri	>100	>100
Xanthomonas oryzae	50	100
Saccharomyces cerevisiae	50	>100
Candida albicans 3147	>100	>100
Aspergillus niger	100	>100
Piricularia oryzae	50	>100

Bacteria were incubated on nutrient agar plates at 37 °C for 17 h and fungi on nutrient agar plates containing 1% glucose at 27 °C for 40 h (TAKEUCHI et al., 1976).

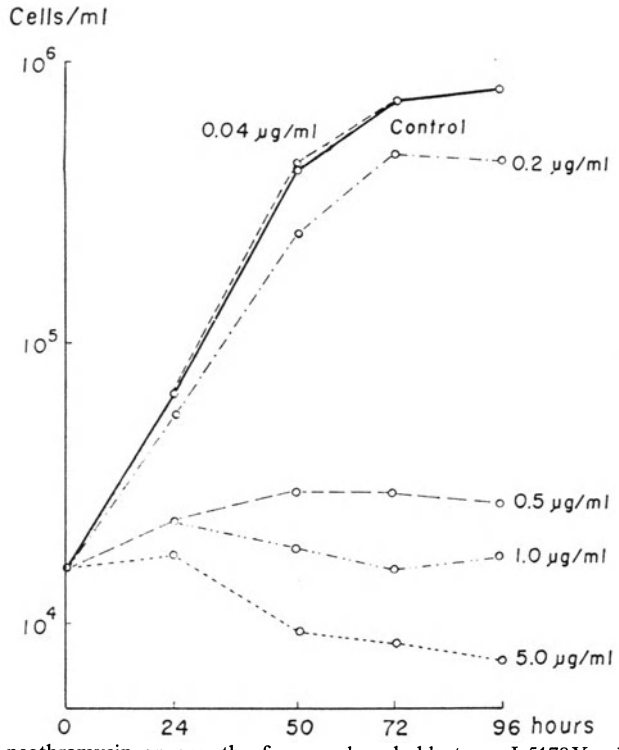


Fig. 2. Effects of neothramycin on growth of mouse lymphoblastoma L5178Y cells (MARUYAMA et al., 1978)

Toxicity

The acute LD_{50} of neothramycin A or B has been described by TAKEUCHI et al. (1976) to be in the range of 20–30 mg/kg by intravenous or intraperitoneal injection into mice. The LD_{50} of neothramycin has been also reported by HISAMATSU et al. (1980) to be 17.5 mg/kg (male) and 22.0 mg/kg (female) upon intravenous administration to mice, observed for 14 days.

Effects on Experimental Tumors

TAKEUCHI et al. (1976) have found that neothramycins A and B display significant activities against Ehrlich carcinoma and leukemia L1210 in mice, and markedly prolong the survival period of tumor-bearing animals. HISAMATSU et al. (1980) have described, in detail, the effects of neothramycin on transplant-

Table 2. Effects of neothramycin on leukemia P388

Dose (mg/kg/day)	Administration	Schedule	ILS (%)
20	ip	day 1	−74.5
10			32.7
5			45.5
2.5			20.0
12	ip	days 1, 5, 10	−15.6
8			56.6
4			37.7
2			35.8
8	ip	days 1–5	−30.8
4			53.8
2			40.4
1			34.6
6	ip	days 1–10	−21.2
4			73.1
2			50.0
1			41.3
80	po	days 1–3	−45.1
60			9.8
40			58.0
20			16.0
10			3.0

Leukemia P388 cells (10^6) were intraperitoneally inoculated into CDF_1 mice, and the administration of neothramycin started 24 h after the tumor inoculation (HISAMATSU et al., 1980).

able animal tumors: leukemia P388 and L1210, sarcoma 180, Ehrlich carcinoma, mammary adenocarcinoma (CCMT) of mice, and AH130 and Walker tumor 256 of rats.

Neothramycin is active against P388 leukemia in the ip-ip system (Table 2). The optimal effective dose in the four ip injection schedules, 5 mg/kg (a single administration on day 1), 8 mg/kg/day (injections for 3 days on days 1, 5, and 10), 4 mg/kg/day (injections for 5 days from day 1 to 5), and 4 mg/kg/day (injections for 10 days from day 1 to 10) have produced 46%, 57%, 54%, and 73% ILS (increase of life span), respectively. The effects of neothramycin depend on the administration schedule, and successively divided doses show higher activity than a single dose. Oral administration for 3 consecutive days has shown 58% ILS with an optimal dose of 40 mg/kg/day, and the activity is comparable to that with ip injection.

ILS of tumor-bearing animals, produced by optimal ip dosage of neothramycin, have been 39% with leukemia L1210, more than 148% with sarcoma 180, 39% with Ehrlich carcinoma, 75% with adenocarcinoma CCMT, and more

than 136% with AH130. Complete growth inhibition of sc Walker tumor 256 has been observed by ip injection of neothramycin: 2–4 mg/kg/day for 8 days.

Effects on Macromolecular Syntheses in Culture

Neothramycin produces a preferential inhibition of RNA over DNA synthesis in the intact cells of mouse lymphoblastoma L5178Y. Approximately 50% inhibition of uridine uptake is observed at a drug concentration of 1.4 μg/ml, and that of thymidine incorporation at 12.0 μg/ml. Protein synthesis is not significantly affected (Fig. 3) (MARUYAMA et al., 1978).

Contrary to the effect in the mammalian cells, thymidine uptake into DNA is more markedly blocked than uridine incorporation into RNA by neothramycin in the intact cells of *E. coli*. On the other hand, the antibiotic causes a significant degradation of DNA (Fig. 4), and the apparent inhibition of thymidine uptake seems to be due to the DNA cleavage, but not to the inhibition of net DNA synthesis (MARUYAMA et al., 1978). The effects of neothramycin on RNA and DNA polymerase reactions, described below, support the assumption that the drug prevents RNA synthesis more profoundly than DNA synthesis in the bacterial cells as in the mammalian cells. However, the molecular mechanism of DNA breakage in bacteria remains to be determined.

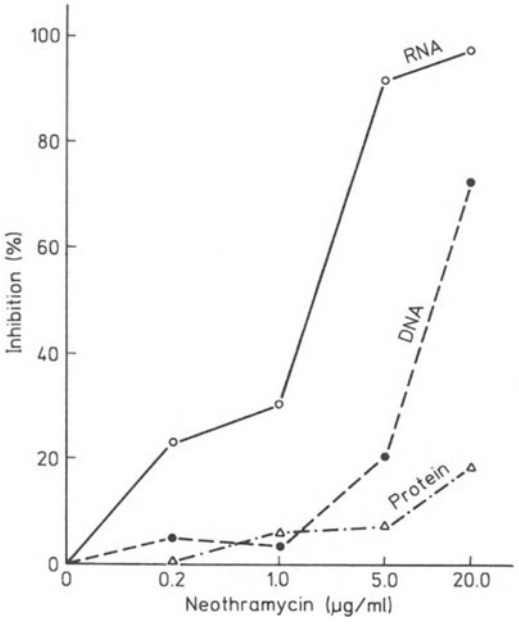


Fig. 3. Effects of neothramycin on macromolecular syntheses in the intact cells of mouse lymphoblastoma L5178Y cells (MARUYAMA et al., 1978)

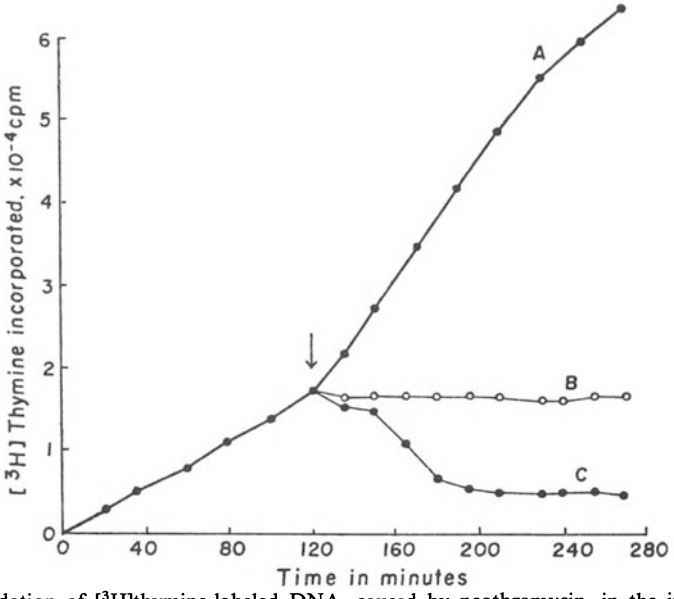


Fig. 4. Degradation of [^{3}H]thymine-labeled DNA, caused by neothramycin, in the intact cells of *E. coli*. *Arrow* shows the time, at which neothramycin (39.2 μg/ml) was added to the culture. *A* Control without addition of unlabeled excess thymine; *B* Control with addition of unlabeled excess thymine; *C* The same as *B* in the presence of neothramycin (MARUYAMA et al., 1978)

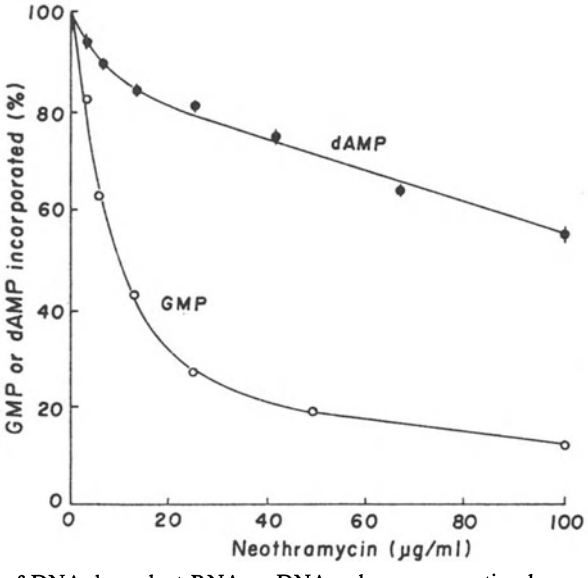


Fig. 5. Inhibition of DNA-dependent RNA or DNA polymerase reaction by neothramycin (MARUYAMA et al., 1978)

Effects on DNA and RNA Polymerase Reactions

ATP-dependent DNA synthesis with toluene-treated cells of *E. coli* polA$^-$ is not significantly affected by neothramycin, while RNA synthesis with the same system is markedly blocked by the antibiotic (MARUYAMA et al., 1978).

Neothramycin prevents DNA-dependent RNA and DNA polymerase reactions, using isolated *E. coli* enzymes and calf thymus DNA as a template. RNA polymerase reaction is more profoundly inhibited than DNA polymerase I: i.e., approximately 50% inhibition of RNA polymerase reaction is observed at an antibiotic concentration of 11 μg/ml, and that of DNA polymerase I at 100 μg/ml (Fig. 5).

The inhibition of DNA polymerase I is reversed by increasing concentrations of the template but not by that of the enzyme, suggesting the direct interaction of the antibiotic with DNA. The degree of inhibition of the polymerase reactions depends upon the period of preincubation of template DNA and neothramycin; increasing gradually until the preincubation reaches 60 min. The results suggest that the interaction of DNA and the antibiotic may be a slow reaction (MARUYAMA et al., 1978).

Evidence for Binding of Neothramycin to DNA

As described above, the biochemical investigations suggest that DNA is the chemoreceptor of neothramycin. The interaction of the antibiotic with DNA has been further demonstrated by ultraviolet, circular dichroism and fluorescence spectroscopy, and a [^{14}C]neothramycin binding method. The ultraviolet and circular dichroism spectra of neothramycin exhibit bathochromic shifts and hypochromic changes upon reaction with native calf thymus DNA, indicating a binding of the antibiotic to DNA (MARUYAMA et al., 1979).

The fluorescence emission maximum of neothramycin A or B is observed at 420 nm (excitation at 316 nm). The fluorescence quantum yields have been calculated, using quinine as a standard: neothramycin (A:B=5.5:4.5) 0.046, A 0.031, and B 0.061 (Table 3). The fluorescence intensity of neothramycins

Table 3. Fluorescence quantum yields of neothramycins and tomaymycin (MARUYAMA et al., 1979)

Antibiotic	Quantum yield (φ)	Emission λ_{max} (nm)
Neothramycin [A&B (5.5:4.5)]	0.046	420
Neothramycin A	0.031	420
Neothramycin B	0.061	420
Tomaymycin	0.045	416

is enhanced by the reaction with DNA (Table 4). The binding of [^{14}C]neothramycin to DNA has been also demonstrated (Table 5) (MARUYAMA et al., 1979, 1981 a).

Mode of Reaction with DNA

Since the fluorescence intensity of neothramycin is enhanced by the reaction with DNA, the mode of reaction has been studied by fluorescence spectroscopy (MARUYAMA et al., 1979, 1981 a).

As presented in Fig. 6, the reaction rate of neothramycin A or B with DNA is slower than that of tomaymycin, indicating that neothramycin shows the slowest reaction rate with DNA in the pyrrolo(1,4)benzodiazepine group of antibiotics, which react with DNA more slowly than mitomycins, actinomycin, and adriamycin (cf. a review by HURLEY, 1977). The reaction rate of neothramycin is dependent upon both drug and DNA concentrations. The same tendency of reaction rate has been also demonstrated by ultraviolet absorption spectroscopy and [^{14}C]neothramycin binding method.

The binding rate of neothramycin to DNA is highly dependent upon pH of the reaction buffer, and markedly promoted by hydrogen ion (H^+) in a range of pH 5–7 (Fig. 7).

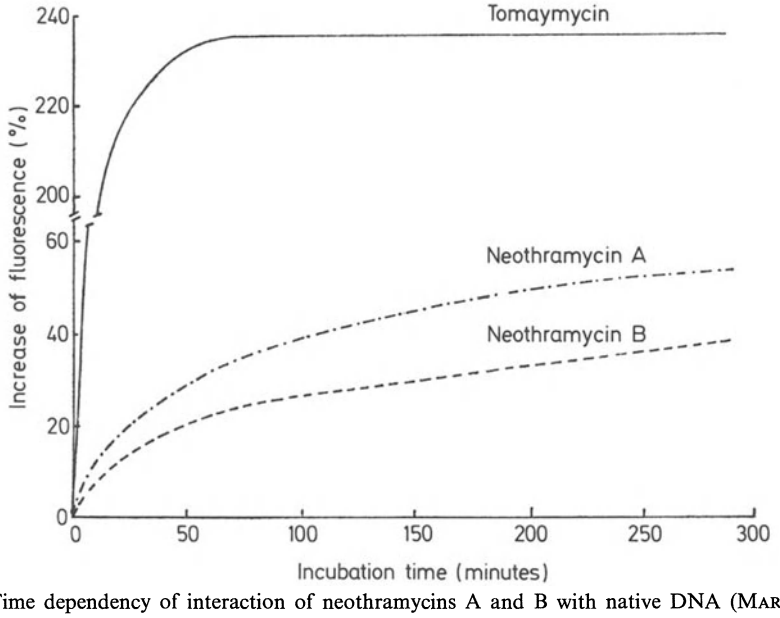


Fig. 6. Time dependency of interaction of neothramycins A and B with native DNA (MARUYAMA et al., 1979)

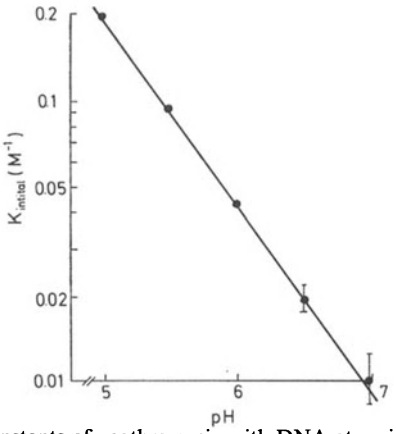


Fig. 7. Initial reaction rate constants of neothramycin with DNA at various pH's (MARUYAMA et al., 1981a)

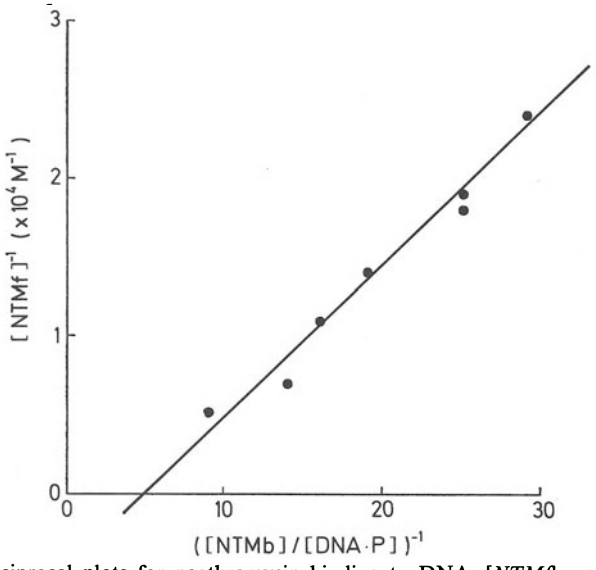


Fig. 8. Double reciprocal plots for neothramycin binding to DNA. [*NTMf*] represents the molar concentration of free neothramycin, [*NTMb*] that of bound neothramycin, and [*DNA·P*] that of DNA·P (MARUYAMA et al., 1981a)

The amount of neothramycin bound to DNA has been calculated from the fluorescence intensity increase (ΔF) over a range of concentrations of neothramycin (1–100 μM) and DNA nucleotide (100–1700 μM). The double reciprocal binding plot of the data is illustrated in Fig. 8. There appears to exist a linear relationship between the molar concentration of bound neothramycin per nucleotide or DNA·P and the molar concentration of free neothramycin. Native calf thymus DNA seems to possess one binding site for the antibiotic

per ca. three base pairs with homogenous affinity: an association constant is ca. $4.7 \times 10^3\ M^{-1}$.

DNA Reactive Sites on the Neothramycin Molecule

The interaction of neothramycin derivatives with DNA has been studied by fluorospectroscopy in comparison with that of neothramycins. The results are summarized in Table 4. Fluorescence intensity of 10,11-dihydroneothramycins A and B, and their 3-O-butyl derivatives, which do not form the 11-hydroxyl compounds in water, is not stimulated by DNA, indicating that the C-11 of the antibiotics may react with DNA. 3-O-Alkyl derivatives of neothramycin B appear to react with DNA but display less interaction, indicating that C-3 of the antibiotic may not react with DNA. On the contrary, 3-O-alkyl derivatives of neothramycin A do not interact with DNA, suggesting that neothramycin A may bind to DNA through C-3, or the 3-O-alkyl groups may cause steric hindrance for the binding to DNA in the case of neothramycin A derivatives (MARUYAMA et al., 1979). The steric hindrance due to 3-O-alkylation of neothramycin A may be explained by the space-filling molecular model of CPK (COREY, PAULING, and KOLTUN) for the drug-DNA adduct proposed by HURLEY et al. (1980) and PETRUSEK et al. (1981), in which hydrogen bonding between the 3-hydroxy proton and 0-1′ of the deoxyribose phosphate backbone of DNA is possible for neothramycin A, but not for neothramycin B.

The result, described in this section, suggests that neothramycin binds to DNA through C-11, as well as through C-3 in the case of neothramycin A.

Table 4. Fluorescence enhancement of neothramycins and their derivatives by the presence of native calf thymus DNA. Excitation at 316 nm and emission at 420 nm

Antibiotics	$\Delta F^a\%$
Neothramycin (A and B mixture)	30.0
Neothramycin A	37.0
Neothramycin B	24.5
3-O-Methylneothramycin A	0.0
3-O-Methylneothramycin B	13.1
3-O-Butylneothramycin A	0.0
3-O-Butylneothramycin B	24.0
10,11-Dihydroneothramycin (A and B)	0.0
3-O-Butyl-10,11-dihydroneothramycin A	0.0
3-O-Butyl-10,11-dihydroneothramycin B	0.0
Tomaymycin	235.0

[a] Increase of fluorescence intensity (MARUYAMA et al., 1979)

The assumption is also supported by the studies on a neothramycin-2′-deoxyguanosine adduct, described below.

Requirement of Guanine Base and Double Helical Conformation for Neothramycin Binding to DNA

The interactions of neothramycin with nucleic acids and synthetic deoxyribonucleotides have been studied by fluorospectrometry and [^{14}C]neothramycin binding method (MARUYAMA et al., 1979, 1981a).

The binding of [^{14}C]neothramycin to polydeoxynucleotides has been measured by radioactivity remaining on DNA, after five times rapid extraction of the free antibiotic with three-fold volumes of water-saturated *n*-butanol. The results are summarized in Table 5. By the method employed, the drug seems to bind to native DNA of calf thymus or *E. coli* approximately five times more than the heat-denatured DNA. Neothramycin interacts with poly[dG–dC] copolymer more effectively than poly[dA–dT] copolymer and poly[dG]-poly[dC] homopolymer, suggesting that the specific base and conformation of DNA are required for the antibiotic binding. Poly[dG–dC] shows a lesser magnitude of reaction with neothramycin than does native DNA. Slight binding of the drug to heat-denatured DNA, poly[dA–dT], or poly[dG]-poly[dC] may be due to nonspecific binding or to incomplete extraction of the free antibiotic by the solvent.

Heating the neothramycin-DNA complex results in releasing a part of the drug from native DNA. However, the amount of neothramycin, remaining on the DNA, is much larger than the amount released from the adduct, suggesting

Table 5. Binding of [^{14}C]neothramycin to polynucleotides

Polynucleotide		Neothramycin bound nmol/100 nmol nucleotide
Calf thymus DNA		
	native	1.50±0.13[a]
	heat-denatured	0.27±0.13
E. coli DNA		
	native	1.31±0.12
	heat-denatured	0.32±0.13
Poly[dG·dC]		0.80±0.13
Poly[dG]·poly[dC]		0.22±0.13
Poly[dA·dT]		0.32±0.13
Calf thymus DNA[b]		1.04±0.13

[a] Mean±S.D.; n=3.

[b] DNA-neothramycin complex was warmed at 100 °C for 5 min, cooled rapidly to room temperature, and then free antibiotic was removed (MARUYAMA et al., 1979).

Table 6. Fluorescence intensity of neothramycin in the presence of various nucleotides

Nucleotide		(F − Ff)/Ff (%)
Calf thymus DNA	native	28.0
	heat-denatured	0.0
E. coli tRNA		0.7
poly[dG − dC]		9.6
poly[dI − dC]		0.0
poly[dA − dT]		1.5
poly[dG]		2.6
dATP		0.0
dGTP		0.0
TTP		0.0

The increase of neothramycin fluorescence by various nucleotides (F − Ff) was compared with neothramycin fluorescence without nucleotides (Ff) (MARUYAMA et al., 1981 a).

that most of the antibiotic bound to native double-stranded DNA retains its binding to single-stranded DNA even after denaturation of DNA.

The results, studied by fluorospectrometry, are presented in Table 6. Again, neothramycin binds to native DNA, but not significantly to heat-denatured DNA or tRNA (*E. coli*), suggesting that the double helical structure of native DNA is essential for the binding. The requirement of the double helix suggests the intercalation between the base pairs as a possible mechanism of the drug binding. Enhancement of fluorescence intensity has been also reported for the interaction of DNA with ethidium bromide, a well-known intercalator (cf. a review by WARING, 1975), as in the case of neothramycin (OLMSTED and KEARNS, 1977). It appears to support an intercalation model for the neothramycin binding. However, the intercalation of neothramycin between the base pairs is not generally accepted (cf. a review by HURLEY, 1977; PETRUSEK et al., 1981).

The interaction with poly[dG − dC] is more intensive than those with poly[dI − dC] or poly[dA − dT], suggesting that the guanine base participates in the drug-DNA binding. The nonreactivity of poly[dI − dC], which differs from poly[dG − dC] in that it lacks a 2-amino group, suggests that neothramycin binds to the 2-amino group of DNA guanine base. Since most 2-amino groups of guanine are exposed to the narrow groove of DNA, neothramycin may be bound within the minor groove of the double helix.

On the other hand, poly [dG] shows less affinity for the antibiotic than poly[dG − dC], and no significant binding is observed with dATP, dCTP, dGTP, or TTP. The results again suggest the importance of the structural conformation of nucleotides.

In conclusion, neothramycin binds through C-11 or C-3 to the 2-amino group of DNA guanine base by an amine linkage, which is stabilized by secondary hydrogen bonding.

A Neothramycin-2′-Deoxyguanosine Adduct

Since the neothramycin-DNA adduct is unstable to enzymic denaturation and the nucleoside adduct cannot be isolated by deoxyribonuclease digestion, MARUYAMA et al. (1981 b) have investigated adduct formation of the antibiotic with deoxynucleosides as a model for the drug-DNA binding.

Neothramycin reacts with 2′-deoxyguanosine in aqueous (optimum 30%) dimethyl sulfoxide, producing two reaction products, which are stained with both fast blue B (for neothramycin) and diphenylamine aniline (for deoxynucleoside) reagents on thin-layer chromatograms (TLC). The reaction is promoted by acid. In the reaction with 2′-deoxyadenosine, lesser amounts of two reaction products are detected on TLC. However, 2′-deoxycytidine, 2′-deoxyinosine, and thymidine do not react with neothramycin in 30% aqueous dimethyl sulfoxide.

Approximately 6 mg of the major adduct has been obtained as a colorless powder from 1 g of neothramycin and 1 g of 2′-deoxyguanosine, but the yield is less than 1%. The poor yield seems to be due to the low association constant of the reaction.

The chemical structure of the major reaction product of neothramycin and 2′-deoxyguanosine has been determined by UV absorption spectra, ^{1}H-NMR (nuclear magnetic resonance), and mass spectrometry to be a one-to-one adduct of both substances, as described below. A covalent bond is formed between C-3 of the antibiotic and 2-NH_2 of 2′-deoxyguanosine by dehydration.

The UV absorption spectra reveal that the adduct consists of neothramycin and 2′-deoxyguanosine in a molar ratio of 1:1, because the spectrum of the adduct can be dissected into those of the antibiotic and 2′-deoxyguanosine (Fig. 9).

The field-desorption mass spectrum of the adduct shows M^+ (m/z 511), which coincides with a calculated value for the one-to-one adduct of neothramycin and 2′-deoxyguanosine. Another molecular ion at m/z 534 corresponds to $(M+Na)^+$.

The ^{1}H-NMR spectrum of the adduct has been compared with that of 3-O-butylneothramycin A in the region of 4–8 ppm (Fig. 10). A doublet signal (δ 7.57, J = 7.0 Hz), which can be assigned to 2-amino of the 2′-deoxyguanosine moiety, is observed in a low field. A doublet signal (3-H) at δ 5.56 in the ^{1}H-NMR of 3-O-butylneothramycin A changes to a multiplet signal at δ 5.94 in that of the adduct, while a doublet signal of the 11-H (δ 7.82, J = 4.2 Hz), does not shift.

The results suggest that neothramycin covalently binds through C-3, but not through C-11, to the 2-amino group of 2′-deoxyguanosine by dehydration. The stereochemistry at C-3 in the neothramycin moiety of the adduct remains to be determined.

The fluorescence intensity of neothramycin is markedly increased by the presence of native DNA, but not significantly altered by 2′-deoxyguanosine. The results appear to be in accord with the assumption that the antibiotic binds through C-3 to 2′-deoxyguanosine but mainly through C-11 to double-stranded DNA.

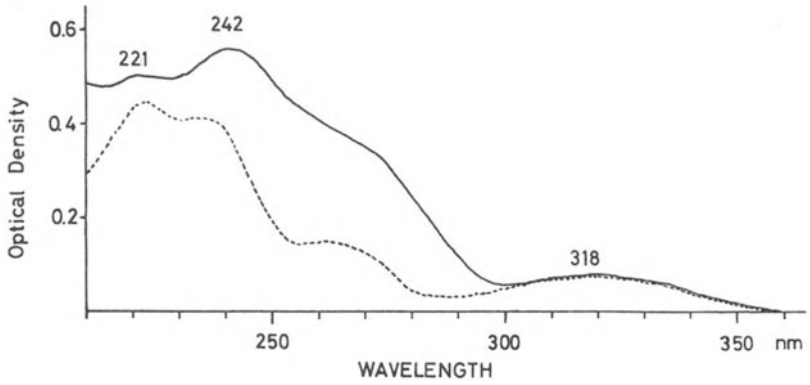


Fig. 9. UV absorption spectra of neothramycin (----) and the antibiotic-2′-deoxyguanosine adduct (——) (MARUYAMA et al., 1981 b)

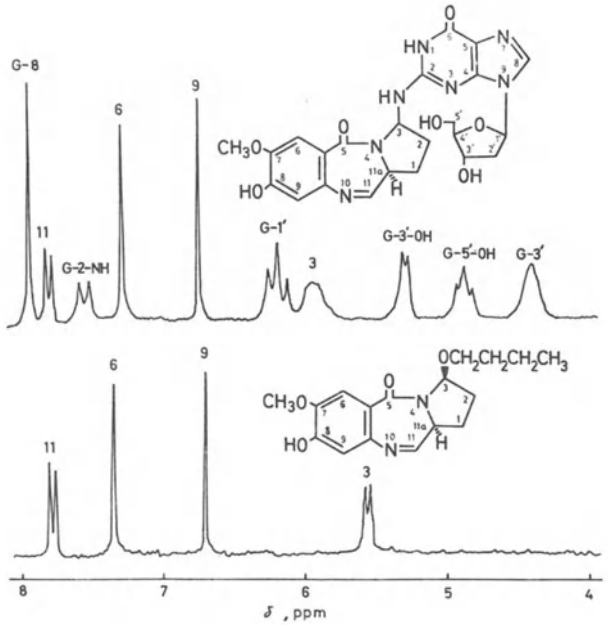


Fig. 10. PMR spectra of 3-O-butylneothramycin A and the adduct of neothramycin and 2′-deoxyguanosine (MARUYAMA et al., 1981 b)

The neothramycin-2′-deoxyguanosine reaction is similar to the antibiotic-DNA interaction in the following characteristics: (1) the same base specificity, (2) reversible reactions, (3) slow binding rates, (4) low yields, and (5) acid catalysis. It suggests that neothramycin covalently binds to the 2-amino group of guanine moiety of DNA to produce aminal linkage in a similar manner to that of the drug-2′-deoxyguanosine adduct formation.

CPK Space-filling Models of the Neothramycin-DNA Adduct

HURLEY et al. (1979, 1980) and PETRUSEK et al. (1981) have proposed space-filling molecular models of CPK (COREY, PAULING and KOLTUN) for the pyrrolo-(1,4)benzodiazepine antibiotic-DNA adducts, in which the antibiotic binds to the 2-amino group of guanine base and lies hidden within the narrow groove.

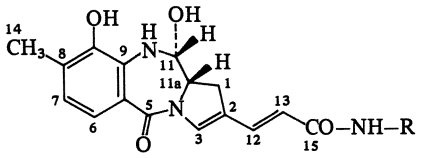
The 40–50° right-handed twist of the neothramycin molecule fits very snugly along the contour of the narrow groove without any detectable distortion of DNA in form B. According to the CPK models, hydrogen bonding between the 3-hydroxy proton and O-1′ of the deoxyribose of DNA is possible with neothramycin A, but not with neothramycin B. This explains the stronger binding of neothramycin A relative to neothramycin B. Furthermore, steric hindrance due to alkylation of neothramycin A at C-3 prevents only these derivatives of this isomer of the antibiotic from binding to DNA (MARUYAMA et al., 1979).

Discussion

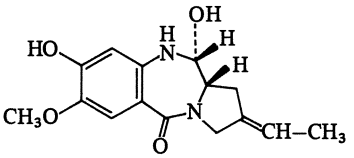
The reaction of pyrrolo(1,4)benzodiazepine antibiotics with native DNA is unusual in that the binding rate is slow in comparison with other DNA-binding agents, such as mitomycin C, adriamycin, and actinomycin D (cf. a review by HURLEY, 1977). Moreover, the reaction rate of neothramycin with DNA is slower than those of other pyrrolo(1,4)benzodiazepines: tomaymycin, anthramycin, and sibiromycin. The side chain at C-2 of anthramycin, tomaymycin, and sibiromycin, and the aminosugar of sibiromycin may enhance the binding rate to DNA. Both are lacking in the molecule of neothramycin (Figs. 1 and 11). The binding rate of pyrrolo(1,4)benzodiazepines to DNA appears to parallel the toxicity. Neothramycin is less toxic than the other pyrrolo(1,4)benzodiazepines. However, the detailed relationship between the binding rate and toxicity has been not well elucidated (TAKEUCHI et al., 1976; MARUYAMA et al., 1979, 1981a; and see a review by HURLEY, 1977).

Neothramycin binds to the 2-amino group of guanine base of DNA through C-11 and/or C-3. The base specificity is the same with the other pyrrolo(1,4)benzodiazepines. Since neothramycin is unique in structure possessing a hydroxyl group at C-3, only neothramycin, among pyrrolo(1,4) benzodiazepines, displays potentiality of binding to DNA both through C-11 and through C-3.

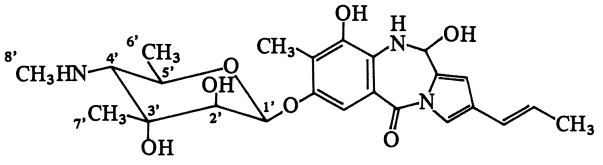
In mammalian cells, neothramycin preferentially prevents RNA synthesis over DNA synthesis, whereas anthramycin shows slightly more inhibition of RNA than DNA synthesis, and sibiromycin blocks both syntheses to the same degree. On the other hand, in bacterial cells, all the pyrrolo(1,4)benzodiazepines inhibit DNA synthesis more markedly than RNA synthesis. In the case of neothramycin, the apparent inhibition of DNA synthesis may be due to the degradation of DNA. However, the mechanism of DNA breaks induced by



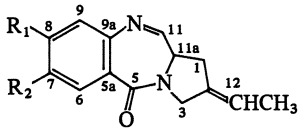
Anthramycin: R=H
Mazethramycin: R=CH_3



Tomaymycin



Sibiromycin



Prothracarcin: R_1=R_2=H
Pretomaymycin: R_1=OH, R_2=OCH_3

Fig. 11. Structures of pyrrolo(1,4)benzodiazepine antibiotics

neothramycin in the bacterial cells remains to be determined (MARUYAMA et al., 1978; NISHIOKA et al., 1972; and cf. reviews by KOHN, 1975, and by GAUSE, 1975). The significance of the inhibition pattern of RNA and DNA syntheses by various pyrrolo(1,4)benzodiazepine antibiotics remains open to discussion.

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Pyrazofurin

E. CADMAN

Pyrazofurin (PF) (3,β-D-ribofuranosyl, 4-hydroxyprazole-5-carboxamide) is a C-nucleoside antibiotic, one in which the ribose joins the base-ring carbon instead of a base-ring nitrogen (Fig. 1). PF was isolated from fermentation filtrate of *Strepomyces candidus* (GERZON et al., 1969; WILLIAMS et al., 1969; GUTOWSKI et al., 1973; WENKERT et al., 1973). This compound was initially found to have inhibitory activity against the vaccinia, herpes simplex, rhino and measles viruses in vitro and the vaccinia virus in vivo (STREIGHTOFF et al., 1969; WILLIAMS et al., 1969). More recently the antiviral spectrum has been extended to include the polio, Coxsackie, Sindbis and vesicular stomatitis viruses (DESCAMPS and DECLERCQ, 1977). A review of the antiviral activity of PF has been published by HAHN (1979) and therefore this aspect of PF will not be detailed. PF has also been shown to inhibit the Friend leukemia virus (DELONG et al., 1971), the Gross leukemia virus, and Rauscher MLV-induced splenomegaly in mice (SHANNON, 1977). Because of these effects of PF on oncologic viruses, SWEENEY et al. (1973) began investigations in humans to determine if this agent were an effective antitumor agent.

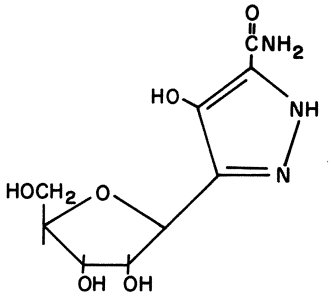

Fig. 1. Pyrazofurin (3,β-D-ribofuranosyl,4-hydroxyprazole-5-carboxamide)

Biochemistry

PF is converted intracellularly to the mono-, di-, and triphosphorylated nucleotide derivative (DIX et al., 1979). The initial phosphorylation occurs by

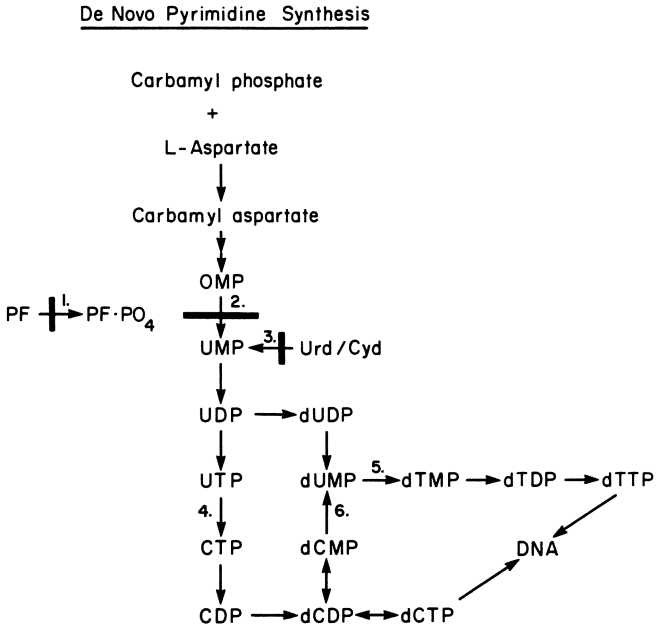

Fig. 2. The pyrimidine synthetic pathway demonstrating the inhibitory site of pyrazofurin (PF). *1* adenosine kinase; *2* orotidylate decarboxylase; *3* uridine/cytidine kinase; *4* cytidine triphosphate synthetase; *5* thymidylate synthetase; *6* deoxycytidylate deaminase. The *verticle bars* by enzyme *1* and *3* represent the cell wall. The *horizontal bar* represents a block of enzyme 2 by PF-monophosphate

adenosine kinase (EC 2.7.1.20) (DIX et al., 1979) (Fig. 2). Therefore it was not unexpected that cells which lack adenosine kinase were resistent to PF (DIX et al., 1979; SUTTLE et al., 1981). The monophosphate form of PF competitively inhibits orotidylate decarboxylase (EC 4.1.1.23) with a K_i of 5 nM. Under identical conditions orotidylate has a K_m of 7 μM for orotidylate decarboxylase. PF is a considerably more potent inhibitor of this enzyme in the de novo pyrimidine synthetic pathway than the inhibition resulting from 6-azauridine, which has a K_i of 700–800 nM (HANDSCHUMACHER, 1960).

The consequence of this profound block in de novo pyrimidine synthesis results in reduction of cytidine triphosphate (CTP) and uridine triphosphate (UTP) levels to below 20% of control levels by 6 h in L5178Y cells (CADMAN et al., 1978; WENKERT et al., 1973). By 24 h the adenosine triphosphate (ATP) and guanosine triphosphate are also reduced to these low levels (CADMAN and BENZ, 1980).

Cytotoxicity from PF can be prevented when exogenous uridine is provided in the cell culture medium, indicating that the lethal effect of PF is a result of the nucleotide depletion (CADMAN et al., 1978; PLAGEMANN and BEHRENS, 1976; CADMAN and BENZ, 1980; OLAH et al., 1980). Uridine is incorporated at an increased rate into PF-treated cells, indicating the attempt by the salvage pathways to preserve cell viability (CADMAN et al., 1978; PLAGEMANN and BEHRENS, 1976; CADMAN and BENZ, 1980). Cytidine will also prevent the nucleo-

tide pool depletion and cytotoxicity (CADMAN and BENZ, 1980). However, if tetrahydrouridine, which is nontoxic to the cells studied (L1210), is added with the cytidine, cell viability is not perserved. This indicates that cytidine, which can be deaminated to uridine by cytidine deaminase (EC 3.5.4.5), the enzyme inhibited by tetrahydrouridine, must at least in part be converted to uridine or uridine nucleotides to reverse the PF effects (CADMAN and BENZ, 1980).

PF is unusual in that it is initially phosphorylated by an enzyme specific for a purine (adenosine kinase) and the product, PF monophosphate, inhibits de novo pyrimidine synthesis. This active derivative of PF resembles structurally 5-amino-imidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate (AICAR) which is an intermediate nucleotide in the de novo purine synthetic pathway. Because of these chemical similarities, PF monophosphate was examined as an inhibitor of AICAR formyl transferase (EC 2.1.2.3). This enzyme of rat liver supernatants was inhibited 46%, 69%, and 89% at PF monophosphate concentrations of 0.2, 0.4, and 1 mM, respectively (WORZALLA and SWEENEY, 1980). The K_i was 30 μM. The significance of this secondary inhibitory site of PF is unknown. The fact that total reversal of PF toxicity in cell cultures can be prevented by the addition of uridine or cytidine to the cell culture medium would indicate that at least in these cells the antipurine effect is not toxic.

Effect on Cell Cycle

PF is more toxic in logarithmic growth cell cultures (OLAH et al., 1980). The rat hepatoma 3924A cells in plateau-phase growth were unaffected by PF while the logarithmic-phase growth cells were inhibited by 50% with 0.4 μM PF. This finding is consistent with the known action of PF on the de novo pyrimidine synthesis, in that the majority of cells during logarithmic-phase growth would be utilizing this pathway. Cells in plateau-phase growth would not require extensive de novo pyrimidine synthesis and therefore would be less sensitive to PF. This differential sensitivity to PF occurred in spite of the observation that in the logarithmic-phase cells the target enzyme, orotidylate decarboxylase activity, was increased 3.3 times over that of plateau-phase cells.

In synchronized 3924A cultures, the cells which were found to be sensitive to PF were those in early G_1 and early and mid-S phases of the cell cycle (OLAH et al., 1980). A 1 h exposure to 10 and 25 μM PF resulted in equal toxicity to early G_1 and early S-phase cells. When the concentration was increased to 300 μM, the killing of early and mid-S phase cells dominated, although early G_1-phase cells were still sensitive to PF. In synchronized NIL8 cells, however, cell kill was almost exclusively seen in cells during G_2 phase with 1 μM PF for 1 h. At higher concentrations of PF maximum cell killing occurred in G_2, although there was no toxicity observed in S-phase cells also (HILL and WHELAN, 1980) with these cells. In contrast to the hepatoma 3924A, NIL8 cells in mitosis and early G_1 were minimally affected. These effects in NIL8 cells were present irrespective of the synchronization method employed.

When the cell cycle progression of NIL8 cells in asynchronous growth were examined by Flow cytometry following PF treatment, the findings were consistent with those observations made with synchronous cultures. Following 2 μM and 4 μM PF there was a buildup of cells at the S-phase G_1-phase boundary. This suggests that cell killing was predominately occurring in G_2- and S-phase cells (HILL and WHELAN, 1980), however, concentrations of PF appeared to primarily affect G_2-phase cells.

Combining Pyrazofurin with Other Drugs

It was logical to assume that since cells which were sensitive to PF could be spared the toxicity of PF by uridine and/or cytidine, then analogs of these nucleosides could have an enhanced accumulation within the PF-treated cells. Analogs which required uridine-cytidine kinase (EC 2.7.1.48) for phosphorylation would be particularly interesting compounds to combine with PF because of the rapid reduction of UTP and CTP, which act as feedback inhibitors of this enzyme. 5-azacytidine rapidly accumulated into L5178Y and human leukemia cells exposed to PF. The result was an enhanced killing of cells, which was greater than additive (CADMAN et al., 1978). 3-deazauridine, also a uridine analog, also accumulated more quickly within PF-treated cells. The result is greater toxicity (CADMAN and GRANT, unpublished). Other drugs which have been studied in combination with PF are represented in Table 1. In each case where synergistic effects were observed a reasonable explanation exists based on the reduction in nucleotide pools which occurs following PF. 3-dea-azuridine, which inhibits CTP synthetase (EC 6.3.4.2), has less UTP to contend with at the active site of this enzyme. Cytosine arabinoside is more active because of the ultimate reduction in the deoxycytidine nucleotide levels. 5-fluoropyrimidines and methotrexate would have a more prolonged inhibitory effect on thymi-

Table 1. Drugs combined with pyrazofurin

Drug	Effect	Cell	References
5-Aza-cytidine	+	L1210/human leukemia	CADMAN et al., 1978
5-Aza-cytidine	+	L5178Y	HILL and WHELAN, 1980
3-Deazauridine	+	L1210/human leukemia	CADMAN and GRANT
Cytosine arabinoside	+	L5178Y	CADMAN et al., 1978; HILL and WHELAN, 1980
5-Fluorouracil	+	L5178Y	HILL and WHELAN, 1980
5-Fluordeoxyuridine	+	L5178Y	CADMAN et al., 1978
Methotrexate	+	L5178Y	HILL and WHELAN, 1980
DDMP	+	L5178Y	HILL and WHELAN, 1980
Vincristine	0	L5178Y	HILL and WHELAN, 1980
Hydroxyurea	0	L5178Y	HILL and WHELAN, 1980
Adriamycin	0	L5178Y	HILL and WHELAN, 1980
Persantin	−	Novikoff	PLAGEMANN and BEHRENS, 1976

+ = synergistic growth inhibition, 0 = additive effect, − = antagonistic effect.

dylate synthetase (EC 2.1.1.45) because of a presumed inability of PF-treated cells to synthesize maximum quantities of dUMP, the deoxynucleotide which normally increases prior to the resumption of thymidylate synthesis.

Persantin [2,6-bis(diethanolamino)-4,8-diperidinopyrimido (5,4-D)pyrimidine] is a drug which inhibits facilitated and simple diffusion of nucleosides and most likely results in less intracellular PF accumulation. Although this was not determined (PLAGEMANN and BEHRENS, 1976), it is a likely explanation for the reduction of PF cytotoxicity. Likewise, persantin also reduced the ability of uridine to rescue cells from the lethal effects of PF.

Effects on Tumors

The use of PF has been evaluated in several tumor systems and are represented in Table 2. Similar effects on nucleotide pools and an enhancement of [^{3}H] uridine incorporation into nucleic acid relative to controls have been documented in vivo with implanted tumors (BROCKMAN et al., 1977). The effects of PF on in vivo tumors is presented in Table 3.

Table 2. Effect of pyrazofurin on tumor cells in culture

Tumor (in vitro)	% Inhibition	μM	References
L1210	100	5	WORZALLA and SWEENEY, 1980
L5178Y	50	0.05	CADMAN et al., 1978
W-256	50	1	CADMAN et al., 1978
Morris rat hepatoma 3924A	50	0.04	SUTTLE et al., 1981; OLAH et al., 1980
Novikoff	100	0.1	PLAGEMANN and BEHRENS, 1976
HeLa	100	>0.1	PLAGEMANN and BEHRENS, 1976
L-cells	100	>0.1	PLAGEMANN and BEHRENS, 1976

Pharmacology of Pyrazofurin

In rats given 10 mg/kg of PF po or im, the peak levels occurred at 6 to 8 h. Fifty percent of the concentration persisted beyond 24 and 36 h; by the 5th day there were undetectable levels of PF (SWEENEY et al., 1973). Daily administration of PF to rats at a dose of 10 mg/kg ip is lethal. When the drug is given every 2nd or 3rd day no animal died (SWEENEY et al., 1973). Because of the prolonged plasma concentrations of PF, the in vivo studies in Table 3 generally used PF every 3 days. The maximum tolerated PF dose in mice was 5 mg/kg ip for 5 consecutive days (SWEENEY et al., 1973).

In humans the effect of PF was also quite prolonged. Patients have been given 3 to 5 mg/kg as an intravenous push once weekly without toxicity. Higher doses, however, resulted in mucositis. At the conservative PF dose, daily 24-h urine collections showed enhanced excretion of orotate which peaked on the 2nd and 3rd days. Control levels were not achieved for 7 to 12 days (GUTOWSKI et al., 1975). When [carboxyl-^{14}C] orotate is given, the rate at which the [^{14}C]O_2

Table 3. Effect of pyrazofurin on tumors in rodents

Tumor (in vivo)	% Inhibition	Route	Dose
L1210 lymphocytic leukemia	0	ip	2–10 mg/kg
P1534 lymphocytic leukemia	0	ip	Every 3rd day
P388 lymphocytic leukemia	0	ip	Every 3rd day
ARR lymphocytic leukemia	0	ip	Every 3rd day
C1498 myelogenous leukemia	0	ip	Every 3rd day
Ehrlich ascites	0	ip	Every 3rd day
Sarcoma 180 ascites	0	ip	Every 3rd day
Ridgeway osteogenic sarcoma	0	ip	10 mg/kg/3d
S91 melanoma	0	ip	10 mg/kg/3d
W-256 carcinosarcoma	75–100	ip, po	Every 3rd day
Gardner lymphosarcoma	50–74	ip	10 mg/kg/3d
X5563 plasma cell myeloma	50–74	ip	10 mg/kg/3d
Mammary carcinoma 755	50–74	ip	10 mg/kg/3d
DMBA-induced mammary carcinoma	↓tumor size	ip	10 mg/kg/3d
C_3H mammary carcinoma	30–49	ip, po	10 mg/kg/3d
Mecca lymphosarcoma	30–49	ip, po	10 mg/kg/3d
Taper liver tumor	30–49	ip, po	10 mg/kg/3d
Mammary carcinoma 115	30–49	ip, po	10 mg/kg/3d

Reference: SWEENEY et al., 1973.

is released into exhaled breath from the decarboxylation of the [carboxyl-^{14}C] OMP, which is converted intracellularly from the labeled orotate, is an indication of orotidylate decarboxylase inhibition induced by PF.

This method has been used to determine the activity of orotidylate decarboxylase in humans previously as a measure of the inhibition resulting of 6-azauridine (CARDOSO et al., 1961). Following an intravenous injection of 200 mg/m^2 PF the appearance of [^{14}C]O_2 in the expired air was less than 1% of the pretreatment values. By the 8th day the amount of [^{14}C]O_2 exhaled was only approximately 75% of pretreatment values. In addition, during the 24 h after PF treatment there was a maximum excretion in the urine of orotate and orotidine. These pyrimidine precursors, which are normally not present in urine, remained detectable even on the 7th day post PF treatment (CADMAN et al., 1978). These findings indicate that PF can have a prolonged effect on total orotidylate decarboxylase activity in humans. This observation does not suggest, however, that this drug would necessarily have similar effects in tumors within humans. Because of the continuous and dynamic state of cellular metabolism and catabolism there are continued amounts of nucleosides in the plasma which can be excluded or added from culture medium of in vitro experiments. Therefore, tumor cells in patients could conceivably be rescued continually from the effects of PF.

Human Studies

PF was considered initially to have potential as an antineoplastic agent, primarily because of the tight binding and profound inhibition of orotidylate

decarboxylase. Probably for the reasons just outlined, the use of PF has not proven to be an effective drug for the treatment of cancer. The potential of PF may be in its ability to modulate the intracellular nucleotide metabolism which might allow certain other drugs to be more effective.

PF has been documented to enter human leukemic cells, and an occasional patient has benefitted transiently from PF treatment (CADMAN et al., 1978). However, there have been no remissions of disease which would indicate continued use of this agent alone (GRALLA et al., 1978; SALEM et al., 1977; VOGLER and TRULOCK, 1978; OHNUMA and HOLLAND, 1977; CREAGAN et al., 1977; BUDMAN et al., 1977; GRALLA et al., 1978). The limiting toxicity has been mucositis. Some patients have had a mild reduction in platelet and white blood cells counts and hemoglobin levels.

Table 4. Summary of clinical trials of pyrazofurin

Tumor	No patients	No responding	References
Colon/Rectum	47	0	SALEM et al., 1977; OHNUMA and HOLLAND, 1977; CREAGAN et al., 1977; CADMAN et al., 1978
Lung	40	0	SALEM et al., 1977; OHNUMA and HOLLAND, 1977; GRALLA et al., 1978
Sarcomas	34	0	SALEM et al., 1977; GRALLA et al., 1978; OHNUMA and HOLLAND, 1977
Melanoma	27	0	SALEM et al., 1977; BUDMAN et al., 1977
Acute myelogenous leukemia	27	0	VOGLER and TRULOCK, 1978; SALEM et al., 1977; OHNUMA and HOLLAND, 1977; CADMAN et al., 1978
Kidney	12	0	SALEM et al., 1977; OHNUMA and HOLLAND, 1977; CADMAN et al., 1978
Breast	8	1	SALEM et al., 1977; OHNUMA and HOLLAND, 1977; CADMAN et al., 1978
Myeloma	4	0	SALEM et al., 1977
Salivary	3	0	SALEM et al., 1977
Lymphoma	3	0	SALEM et al., 1977; OHNUMA and HOLLAND, 1977
Others	21	0	SALEM et al., 1977; OHNUMA and HOLLAND, 1977; CADMAN et al., 1978
Totals	226	1	

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Ribavirin

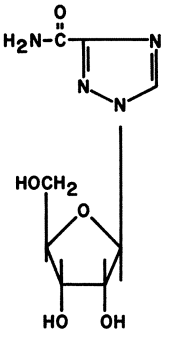
A Review of Efficacy, Toxicity and Mechanisms of Antiviral Activity

P.G. CANONICO

I. Introduction

The antibiotic pyrazomycin (3-β-D-ribofuranosyl-4-hydroxypyrazole-5-carboxamide) was isolated from *Streptomyces candidus* (WILLIAMS et al., 1969) and shown to have significant antiviral activity in cell culture against a large number of viruses including rhinovirus, measles, herpes simplex, and vaccinia virus (STREETER et al., 1973). Previously, NISHIMURA et al. (1964) isolated the antibiotic, showdomycin, from the bacterium *Streptomyces showdoensis*. Both showdomycin and pyrazomycin are nucleosides composed of a five-membered moiety attached to a β-D-ribofuranose (HARRIS and ROBINS, 1980). Elucidation of their structure prompted the synthesis and testing of a number of imidazole and triazole ribonucleosides in a search for compounds with broad-spectrum antiviral activity. In principle, the search, directed by scientists at ICN Pharmaceuticals, concentrated on synthesis of compounds with the potential to affect enzymatic processes common to all viruses such as viral-induced nucleic acid and protein synthesis. Ribavirin, 1-β-ribofuranosyl-1,2,4-triazole-3-carboxamide, was synthesized by WITKOWSKI et al. (1972) as part of this program to search for compounds with broad-spectrum antiviral activity (Fig. 1).

Early in vitro testing against a broad spectrum of viruses showed that ribavirin was markedly active against both DNA and RNA viruses (HARRIS and ROBINS, 1980). Significant activity against phage virus in prokaryotic cells



MOLECULAR FORMULA: $C_8H_{12}O_5H_5$

MOLECULAR WEIGHT: 244.2

Fig. 1. Structure of ribavirin: 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole)

(MENZEL and STEUZ, 1978) and against viral infection of eukaryotic systems in cell culture, plants, animals and in man have been reported (HARRIS and ROBINS, 1980).

II. Antiviral Activity in Experimental Systems

A. Antiviral Effects in Cell Culture

Early work on the inhibitory activity of ribavirin, recently reviewed by HAHN (1979), revealed that the range of ribavirin's antiviral activity is indeed wide (Tables 1 and 2). Unlike other antivirals, development of ribavirin-resistant virus strains has not been demonstrated. In most resting cell lines in which antiviral evaluations are performed the antiviral activity can usually be separated from any cytotoxic dose which ranges 200–1000 μg of ribavirin per ml. The minimum virus inhibitory concentrations, on the other hand, range as low as 0.001 μg/ml. Such observations suggest that in the case of the more sensitive viruses there is a considerable selectivity of antiviral effect.

In part, this selectivity is dependent on the cell line which is used to assay for antiviral activity (Table 3). Cell lines such as Vero are particularly refractory and require ribavirin concentrations which are 10–100 times greater than required by more sensitive cell lines (SIDWELL, 1980). The sensitivity of Lassa virus replication to ribavirin in Vero cells is influenced also by drug concentration and viral multiplicity of infection (MOI) (JAHRLING et al., 1980). In Vero cells infected at a high MOI (1.3), a dose of 50 μg/ml of ribavirin has a negligible

Table 1. DNA viruses reported to be inhibited in vitro by ribavirin[a]

Virus	References
Adeno 3	SIDWELL et al. (1972); HUFFMAN et al. (1973)
Adeno 19	SCHEFFLER et al. (1975)
Herpes 1	SIDWELL et al. (1972); HUFFMAN et al. (1973); DESCAMPS and DE CLERCQ (1978)
Herpes 2	SIDWELL et al. (1973); HUFFMAN et al. (1973); DESCAMPS and DE CLERCQ (1978)
Turkey herpes	C. EIDSON, cited by SIDWELL et al. (1974)
Mareks disease	EIDSON et al. (1974)
Human cytomegalo	SIDWELL et al. (1974)
Murine cytomegalo	SIDWELL et al. (1972); HUFFMAN et al. (1973); DOWLING et al. (1976)
Feline rhinotracheitis	POVEY (1978)
Infectious bovine rhinotracheitis	HUFFMAN et al. (1973)
Vaccinia	SIDWELL et al. (1972); HUFFMAN et al. (1973); KATZ et al. (1976); DESCAMPS and DE CLERCQ (1978)
Myxoma	SIDWELL et al. (1972); HUFFMAN et al. (1973)

[a] From SIDWELL et al. (1979).

Table 2. RNA viruses reported to be inhibited in vitro by ribavirin[a]

Virus	References
Influenza A and/or B	SIDWELL et al. (1972); HUFFMAN et al. (1973); TOGO (1973); SUGANUMA and ISHIDA (1973); OXFORD (1975); DURR and LINDH (1975); APPLEYARD and MABER (1975); TISDALE and BAUER (1975, 1977)
Parainfluenza 1 and 3	SIDWELL et al. (1972); HUFFMAN et al. (1973); SIDWELL et al. (1975b)
Parainfluenza 2	POVEY (1978)
Measles	HUFFMAN et al. (1973); STREETER et al. (1973); DESCAMPS and DE CLERCQ (1977)
Subacute sclerosing panencephalitis	HUFFMAN et al. (1973)
Rhino 1A, 2, 8, 13, 56	SIDWELL et al. (1972); HUFFMAN et al. (1973)
Type 22A Corona	G. WERNER, cited by SIDWELL et al. (1974)
Type A21 Coxsackie	G. WERNER, R. WENHAM, C. ENGLE, cited by SIDWELL et al. (1974)
Type B Coxsackie	G. WERNER, C. ENGLE, cited by SIDWELL et al. (1974); DESCAMPS and DE CLERCQ (1977)
Type 1 and 2 Polio	HUFFMAN et al. (1973); N. ISHIDA and C. ENGLE, cited by SIDWELL et al. (1974); DESCAMPS and DE CLERCQ (1977)
Sindbis	G. WERNER, cited by SIDWELL et al. (1974); DESCAMPS and DE CLERCQ (1977)
Vesicular stomatitis	SIDWELL et al. (1972); HUFFMAN et al. (1973); N. ISHIDA, cited by SIDWELL et al. (1974)
Semliki Forest	SIDWELL et al. (1972); HUFFMAN et al. (1973)
Newcastle disease	HUFFMAN et al. (1973); G. WERNER, cited by SIDWELL et al. (1974)
Moloney sarcoma	M. CHIRIGOS, cited by SIDWELL et al. (1974)
Gross AKR leukemia	SHANNON (1977)
Rota	SCHOUB and PROZESKY (1977)
Chandipura	ODELOLA (1977)
Chikungunya	ODELOLA (1977)
Lassa fever	JAHRLING et al. (1980)
Rift Valley fever	STEPHEN et al. (1980)
Machupo	STEPHEN et al. (1980)
Sandfly fever	STEPHEN et al. (1980)

[a] Adapted from SIDWELL et al. (1979).

effect on viral replication. However, significant inhibition is observed at this drug concentration when lower input MOI are used (Table 4). To demonstrate an inhibitory activity of ribavirin at lower concentrations in Vero cells, very low input multiplicities of Lassa virus are required.

In contrast, the antiviral activity of ribavirin is more pronounced when tested in rhesus alveolar macrophages. In these cells, ribavirin at 10 μg/ml completely inhibits viral replication even when high MOI are used. Cell cultures treated with ribavirin show a decreased yield of infectious virus and contain less viral antigen (as detected by immunofluorescence). The reduced virus yield suggests that ribavirin inhibits replication of Lassa virus at an early stage, before the accumulation of viral antigens within cells (JAHRLING et al., 1980).

Differences in cell line sensitivity cannot be accounted for by differences in transport of ribavirin into the cells. The extent of ribavirin uptake by sensitive and insensitive cell lines has been measured and found to be quite similar among

Table 3. In vitro inhibition of Rift Valley fever virus replication by ribavirin in different cell types[a]

Cell type	μg/ml giving 2 log inhibition[b]		Toxicity[c]
	Day 1	Day 2	
Vero	50[d]	48	>150
Lewis rat thymus	27	23	>150
Wistar-Furth rat thymus	25	–	>150
Maxx rat thymus	14	18	>150
LLC-MK_2	10	8	>150
Fetal rhesus lung	9[d]	2	> 50
MRC-5	8	6	>150
Guinea pig kidney	5[d]	2	>150
Guinea pig kidney-SV40	2	1	>150
Chick embryo fibroblast	1	3	>150

[a] Data of C.J. PETERS (unpublished).
[b] Concentration resulting in 2 log reduction in viral titer compared to controls.
[c] Minimum concentration of drug causing cytopathic effect.
[d] No evidence of replication at higher concentrations of drug, but only 1.4–1.8 log decrease compared to control rat.

Table 4. Effect of ribavirin on replication of Lassa virus in cultured cells tested 4 days after inoculation[a]

Cells $\log_{10}$ MOI[b]	Infectivity ($\log_{10}$ pfu/ml)[c]					% positive by direct fluorescent antibody assay after ribavirin (μg/ml)			
	Residual inoculum at time 0	Ribavirin (μg/ml)							
		0	1	10	50	0	1	10	50
Vero									
+1.3	2.3	5.3	5.2	5.4	4.8	100[d]	100[d]	100[d]	100[d]
−1.3	0.3	6.2	6.3	6.4	3.9	100[d]	100[d]	100[d]	5
−3.3	<	7.0	6.8	5.8	<	100[d]	100[d]	100	0
−5.3	<	6.0	6.2	3.1	<	100	60	5	0
−6.3	<	5.6	<	<	<	75	0	0	0
Rhesus alveolar macrophages									
+1.3	2.6	5.2	5.2	1.7	1.5	100	80	0	0
−1.3	0.6	5.0	4.7	<	<	40	20	0	0
−3.3	<	3.6	2.8	<	<	1	0	0	0
−5.3	<	3.5	<	<	<	1	0	0	0

[a] Data of JAHRLING et al. (1980).
[b] MOI (multiplicity of infection) = $\log_{10}$ Vero pfu − ($\log_{10}$ cell number/well).
[c] Geometric mean titer for three determinations; < = 0.3 $\log_{10}$ pfu/ml.
[d] Significant CPE, with most cells detached from monolayer.

the various cell lines (CANONICO, unpublished observation). These studies also show that 90–95% of intracellular ribavirin is in the cytoplasmic fraction while the remaining 5–10% becomes sequestered in lysosomes.

B. Antiviral Effects In Experimental Animal Systems

1. Nonprimates

Ribavirin has proven to be efficacious in the treatment of animals infected with DNA or RNA viruses. The large volume of literature which exists on the subject is summarized in Tables 5 and 6.

A number of in vivo evaluations of ribavirin have been performed with influenza (orthomyxo-) and parainfluenza (paramyxo-) viruses in many animal systems (ALLEN, 1980). The therapeutic indices in these studies range from 1 to 16. Variable results also are found for certain DNA viruses, such as herpes. Depending on the model, therapeutic indices ranging 0–>125 are reported (ALLEN, 1980). These variations emphasize the importance of optimizing treatment dosages, routes and schedules. In addition, there may be species differences in drug metabolism and viral pathogenesis which affect the severity of illness (ALLEN, 1980). The stress of physical manipulation also may offset benefits of the drug. The stresses of infection, the physical manipulation of treatment, and drug toxicity in laboratory animals may act synergistically to reduce drug efficacy (ALLEN, 1980).

Table 5. DNA virus infection significantly inhibited in vivo by ribavirin[a]

Virus	Animal host	Route of virus inoculation[b]	References
Herpes 1	Rabbit	c.s. (eye)	SIDWELL et al. (1972, 1973); SHIOTA et al. (1977); SIDWELL et al. (1972, 1973)
	Hamster	c.s. (eye)	SIDWELL et al. (1972, 1973)
	Mouse	s.c. (tail)	SIDWELL (1977); SIDWELL et al. (1972)
	Mouse	s.c. (back)	G. MAYER, cited by SIDWELL et al. (1974)
	Mouse	i.n.	DE CLERCQ et al. (1976)
Herpes 2	Mouse	i.d. (genital)	ALLEN et al. (1977)
Mareks disease	Chicken	s.c.	EIDSON et al. (1974)
Equine abortion	Hamster	i.p.	SIDWELL (1977)
Vaccinia	Rabbit	c.s. (eye)	SIDWELL et al. (1972, 1973)
	Mouse	i.v. (tail)	DE CLERCQ et al. (1976)
	Mouse	s.c. (tail)	SIDWELL (1977); SIDWELL et al. (1972)
	Rabbit	s.c. (back)	SIDWELL (1977)
Fibroma	Rabbit	i.d.	G. WERNER, cited by SIDWELL et al. (1974)

[a] From SIDWELL et al. (1979).

[b] c.s.: corneal scarification; i.d.: intradermal; i.n.: intranasal; i.p.: intraperitoneal; i.v.: intravenous; s.c.: subcutaneous.

Table 6. RNA virus infections significantly inhibited in vivo by ribavirin[a]

Virus	Animal host	Route of virus inoculation[b]	References
Influenza A	Mouse	i.n.	SIDWELL et al. (1972); KHARE et al. (1973); DURR et al. (1975); TISDALE and BAUER (1975); JACOBI et al. (1977); STEPHEN et al. (1977); ARENSMAN et al. (1977); others cited by SIDWELL et al. (1974)
Influenza A	Hamster	i.n.	RENIS (1977)
Influenza	Ferret	i.n.	SCHOFIELD et al. (1975); POTTER et al. (1976)
Influenza A	Monkey	i.n.	STEPHEN et al. (1977)
Parainfluenza 1	Mouse	i.n.	SIDWELL et al. (1972, 1975b); LARSSON et al. (1978)
Parainfluenza 3	Hamster	i.n.	SIDWELL et al. (1975b)
Murine hepatitis	Mouse	i.p.	G. WERNER, cited by SIDWELL (1977); SIDWELL et al. (1974)
Rift Valley fever	Mouse	i.p.	G.A. EDDY et al. (1981)
Foot and mouth disease	Mouse	s.c.	G. WERNER, cited by SIDWELL et al. (1974)
West Nile	Mouse	i.p.	ODELOLA (1977)
Friend leukemia	Mouse	i.p.	R. GORDES, cited by SIDWELL et al. (1974); SIDWELL et al. (1975a)
Moloney leukemia	Mouse	i.p.	G. WERNER, cited by SIDWELL et al. (1974)
Rauscher leukemia	Mouse	i.p.	SHANNON (1977); G. WERNER, cited by SIDWELL et al. (1974)
Gross (AKR) leukemia	Mouse	Spontaneous	BEKESI et al. (1976)
Lassa fever	Monkey	s.c.	JAHRLING et al. (1980)
Machupo	Monkey	s.c.	STEPHEN et al. (1980)

[a] Adapted from SIDWELL et al. (1979).
[b] i.n.: intranasal; s.c.: subcutaneous; i.p.: intraperitoneal.

The inability of ribavirin to adequately concentrate in the central nervous system (CNS) makes the drug ineffective against viruses causing primary encephalitis (rhabdo-), Western, or Venezuelan equine encephalitis (alpha-), Japanese B encephalitis (flavi-), and intracerebrally initiated infections of mice, regardless of the viral nucleic acid type (ALLEN, 1980). These results have led to the search for ribavirin derivatives which may be more likely to penetrate the blood brain barrier and combat viral encephalitic diseases.

2. Nonhuman Primates

The most impressive in vivo demonstration of ribavirin's therapeutic effects has been the treatment of monkeys infected with Lassa fever or Machupo virus.

JAHRLING et al. (1980) reported that 60% of the monkeys infected with about 10^6 palque-forming units (pfu) of Lassa virus die between 10 and 14 days after inoculation (Fig. 2). In contrast to untreated controls, all monkeys treated with

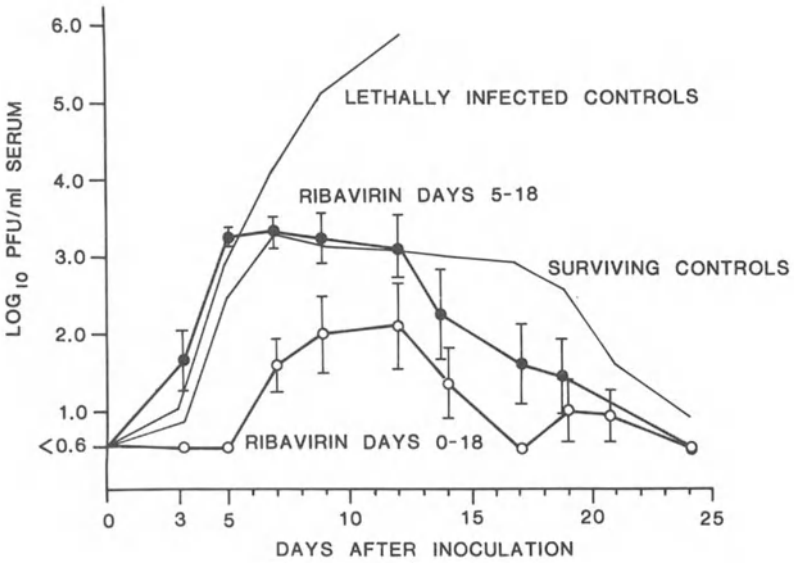


Fig. 2. Effect of ribavirin on Lassa fever viremia, given in $\log_{10}$ pfu/ml of serum. Groups of four monkeys received ribavirin initially on day 0 (*open circles*) on day 5 (*solid circles*) after inoculation with Lassa virus. *Points* are geometric mean titers ±SE (JAHRLING et al., 1980)

ribavirin survive. In monkeys treated with ribavirin (50 mg/kg loading dose, followed by 10 mg/kg, three times a day), from day 0 through day 18, the onset of detectable viremia is delayed until day 7, and peak viremia titers are significantly lower than those of surviving control monkeys. All monkeys survive, even when ribavirin therapy is delayed until day 5. Viremia titers remain relatively low and never exceed 10^4 pfu/ml. Clinical illness is mild and brief in monkeys that receive ribavirin initially on day 0. Some monkeys exhibit no clinical signs at all, while others become only slightly depressed, and develop a minimal facial rash during the 2nd week. Monkeys receiving ribavirin first on day 5 experience a moderately severe disease course. However, all monkeys treated therapeutically with ribavirin by day 5 eventually recover, with no evident sequelae.

The therapeutic potential of ribavirin was evaluated in the treatment of Machupo infection of rhesus monkeys (STEPHEN et al., 1980). Sham-treated virus control monkeys reached peak viremia by days 12–14 and began to die. Treatment of individual monkeys was initiated at the time of onset of fever and was continued every 8 h for 10 days. Quite remarkably, viremia responses of treated monkeys were lower by day 7 compared to sham-treated control monkeys and virtually undetectable by day 10 (Fig. 3). Regardless of the time of initial treatment or regimen of therapy, ribavirin (or its triacetate) prevented death during the acute phase of illness. The late neurological syndrome, seen in 20% of infected, untreated control monkeys, however, is not prevented. It is presumed that the late neurological phase of the disease results from the inability of ribavirin to concentrate in the CNS.

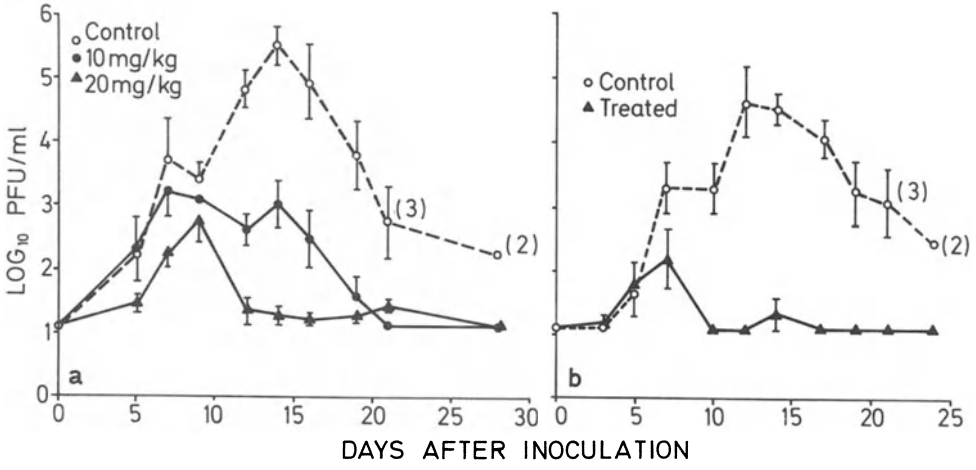


Fig. 3. Effect of ribavirin triacetate on viremia of Machupo virus infected rhesus monkeys. Treatment was given twice daily on days 0–17 (STEPHEN et al., 1980)

3. Man

Results from a number of clinical studies evaluating the efficacy of ribavirin against influenza A and involving some 300 patients have been generally disappointing. In four studies designed to evaluate the drug as a prophylactic agent, ribavirin was not found to prevent the development of influenza A (FERNANDEZ, 1980). In another study, the antiviral efficacy of ribavirin was evaluated in young adult males during an outbreak of infection with influenza virus type A/Brazil/11/78 (HlNl) (SMITH et al., 1980). Analysis of symptoms and signs of influenza illnesses indicated that ribavirin at a dose of 1000 mg/day for 5 days beginning 1 or 2 days after onset of influenza had no detectable effect on the clinical course of the disease.

More recently, KNIGHT et al. (1981) studied the efficacy of ribavirin in an outbreak of illness among college students caused by influenza A/England/330/80 (HlNl). In contrast to the previous study by SMITH et al. (1980), only patients who presented with influenza-like symptoms of less than 24-h duration were included in the study. Subjects were treated by inhalation of ribavirin by small-particle aerosol through a face mask; they retained an average estimated 1.15 g of drug in 23 h of treatment given during a 3-day period.

By the second day, temperatures declined in treated patients to a mean of 36.9° C compared to 37.4° C for controls, a difference which is regarded to be highly significant. When the duration of fever was assessed by measuring the hours to sustained reduction of temperatures below 37.4° C, it was found that treated patients had fever for a mean of 22.8 h versus 38.1 h for control subjects. The systemic illness scores in treated and control patients showed that at admission both groups of patients had nearly equal degrees of severe systemic illness. However, within 24 h, treated subjects had improved ($p = 0.004$) and the trend continued on day 2. Control patients had also shown great improvement by this time as well. Titers of influenza virus were measured on

nasal wash specimens at admission and twice daily thereafter. There was a highly significant and rapid reduction in mean virus titer of treated patients by 18 h after initiation of treatment. In a study involving therapeutic administration of ribavirin, male subjects were given influenza virus type A/Victoria/3/75 (H3N2) and received a ribavirin regimen of 1000 mg/day for 5 days. Drug therapy was reported to have effectively ameliorated clinical manifestations indicative of moderate to severe illness (MAGNUSSEN et al., 1977). While there are inconsistencies, it appears that under some circumstances ribavirin may have therapeutic activity in the treatment of influenza. Therapeutic effectiveness is most frequently reported when treatment is started early in the course of influenza.

As a prophylactic agent, ribavirin was marginally effective in preventing influenza B illness in humans challenged with influenza B virus and treated with ribavirin, 800 mg/day for 2 days prior to viral challenge and for 8 days after challenge. Ribavirin has been evaluated in eight clinical trials involving 226 patients in the treatment of acute and chronic viral hepatitis. Using the standard criteria of SGPT, SGOT and total bilirubin level reduction, drug treatment is reported to produce a faster return to normal levels when compared to a placebo (FERNANDEZ, 1980). Response to therapy in two studies with chronic type B hepatitis was inconclusive. Positive results have been reported with the use of ribavirin in the treatment of herpetic gingivostomatitis (ESPER-DIB et al., 1977), herpes genitalis, and measles (FERNANDEZ, 1980).

In summary, the therapeutic efficacy of ribavirin in the treatment of a number of viral illnesses in experimental animal models is clearly well established. On the other hand, the available clinical data on the drug's effectiveness in human illness are less impressive. Hundreds of patients have been treated with ribavirin in uncontrolled trials for various viral infections. The inconsistencies and borderline efficacy reported from some of these studies conducted by various laboratories throughout the world have been of concern. Ribavirin clearly merits full evaluation in properly controlled clinical studies. A currently ongoing clinical trial on the use of ribavirin in Lassa fever infections (MCCORMICK et al., 1980) and a proposed multicentered trial on the treatment of influenza by inhalation of small particle aerosol of ribavirin may yet assure the future of this drug as a clinically useful antiviral agent.

III. Toxicity and Safety Evaluations

A. Man

Important information on the safety of ribavirin was contributed by studies evaluating the drug for the treatment of advanced cancer and other malignancies (cited by FERNANDEZ, 1980). These data indicate that adult patients tolerate the drug well at dosages approaching 3300 mg/day for 7 days. At very high doses, 3900 mg/day to 12,600 mg/day, a decline in blood hemoglobin levels may be seen by days 7–13 of treatment. The decline in hemoglobin is rapidly

reversible upon discontinuance of the drug. When transfusions are administered to correct the anemia, cancer patients were able to maintain their posttransfusion hemoblobin levels. No effect on bone marrow or other alterations were seen in any of the patients studied.

The cancer studies were among the first human trials conducted with ribavirin. All subsequent studies have used lower doses of the drug, ranging from 400 to 800 mg/day for 10 days or 1000 mg/day for 5 days. Approximately 1000 human subjects have participated in these studies. Review of available clinical data indicates that ribavirin can be administered orally in dosage regimens of 600 mg/day for 28 successive days without significant adverse effects. At dosages greater than 600 mg/day, treatment for extended periods of time is associated with reduction in hemoglobin, hematocrit, and red blood cell count.

In their double-blind influenza study, SMITH et al. (1980) reported that the primary adverse effect of ribavirin was a transient elevation in serum indirect-reacting bilirubin. Abnormal values were detected in approximately 25% of the treated subjects. In the absence of abnormal values for serum SGOT, LDH- or alkaline phosphatase which would indicate hepatic cell toxicity, these authors suggested that ribavirin inhibits uptake and processing of serum bilirubin. The only hematologic abnormality associated with ribavirin treatment was a late (21-day) increase in reticulocyte counts. Although this suggests that ribavirin decreased synthesis and/or increased destruction of erythrocytes during ribavirin treatment, it was not possible to detect reductions in the erythrocyte counts, hematocrit or hemoglobin related to ribavirin therapy. These authors suggested that the clinical importance of the late rise in reticulocytes was probably not important and that it was not of sufficient magnitude to suggest that hemolysis is important in the pathogenesis of the elevated bilirubin values.

B. Laboratory Animals

Acute oral and intraperitoneal median lethal doses (LD_{50}) of ribavirin in mice, guinea pigs, and rats are given in Table 7. Compound-related clinical observations in acutely treated animals often include depression, rough coat, ataxia and soft feces. In general, no necropsy findings are noted in surviving, acutely treated animals. Ribavirin-related findings in nonsurvivors are limited to the presence of dark red material/fluid in the gastrointestinal tract suggesting hemorrhage.

Subacute toxicity evaluations conducted in rats given ribavirin orally for 28 days at levels of 30, 60, and 120 mg/kg resulted in no compound-related findings with regard to mortality or clinical observations. Food consumption and growth rates are generally lower in the mid- and high-dose groups. Hematologic data on days 14 and/or 29 of treatment show a treatment-related decrease in circulating red blood cell mass. This primarily consists of decreased values for mean hematocrit, hemoglobin, and erythrocyte counts in the high-dose males and mid- and high-dose females. Although considered different from controls, affected hematologic values remain within normal limits. Clinical chemistry findings are considered incidental, and evaluation of organ weight data fails to show any direct treatment-related effects on absolute and relative organ

Table 7. Acute LD_{50} of ribavirin in rats, mice, and guinea pigs[a]

Animal	Sex	Ribavirin LD_{50} (mg/kg) ±SEM	
		Oral	i.p.
Rat	Male	4,116±749	1,758± 99
	Female	5,827±391	1,554± 99
	Combined	5,006±463	1,655± 72
Mouse	Male	>10,000	1,268±120
Guinea pig	Male	2,313±278	823± 60

[a] Studies performed by Hazleton Laboratories America, Inc., in 1980 under Contract No. DAMD 17-80-C-0161.

weights. Ribavirin-related histomorphologic alterations are seen in the thymus of the high-dose rats. Lymphoid depletion characterized by decreased numbers of small lymphocytes in the cortex occurs in 75% of the rats. There are no other compound-related findings.

C. Clinical Studies in Monkeys

1. Clinical Findings

A number of studies evaluating ribavirin's effects on the hemogram of rhesus monkeys have been completed. These studies which use levels of ribavirin ex-

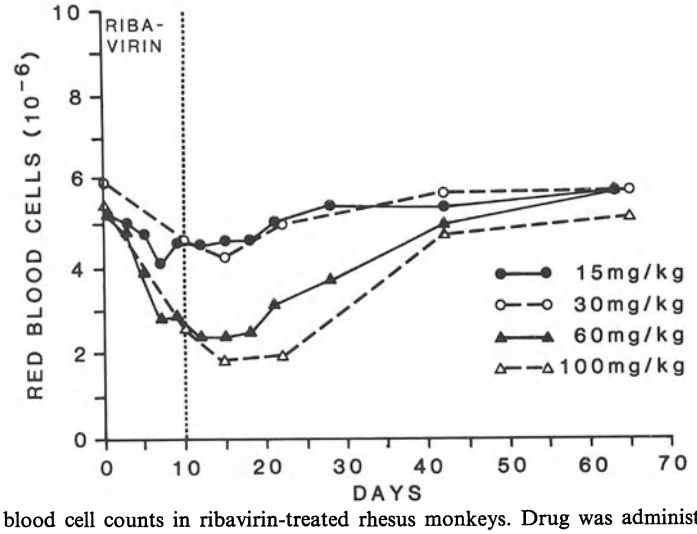


Fig. 4. Red blood cell counts in ribavirin-treated rhesus monkeys. Drug was administered IM for 10 days

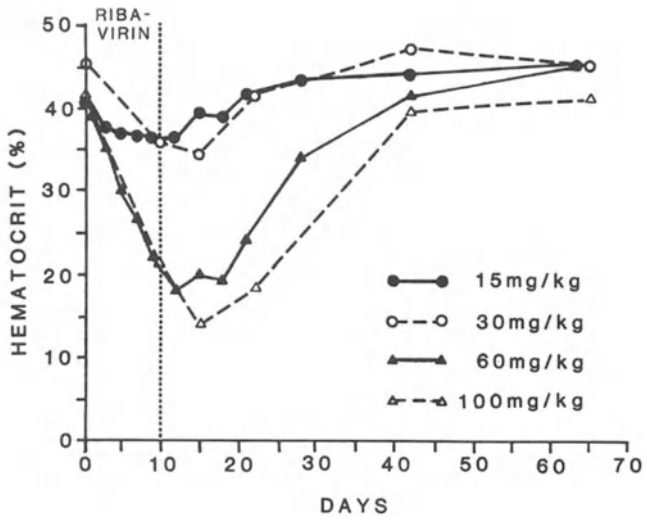


Fig. 5. Hematocrit values of ribavirin-treated rhesus monkeys. Drug was administered IM for 10 days

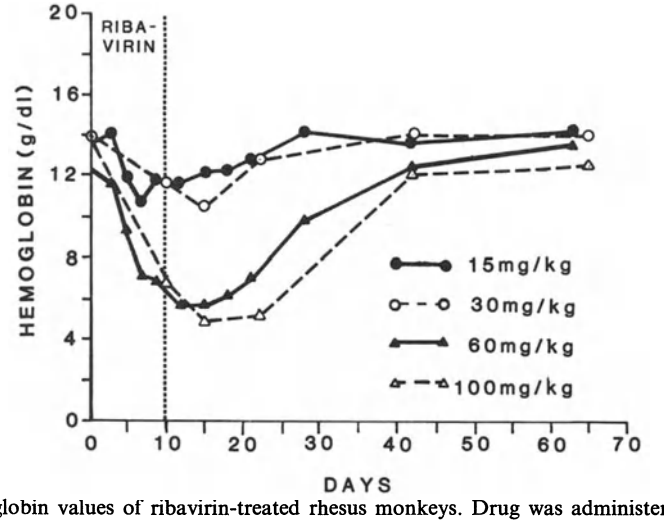


Fig. 6. Hemoglobin values of ribavirin-treated rhesus monkeys. Drug was administered IM for 10 days

ceeding the recommended dosage for man (10–15 mg/kg/day) show a reversible dose-related effect of ribavirin on red blood cell mass (Figs. 4–6). A mild anemia, characterized as normochromic and normocytic, is generally observed with dosages of 30–60 mg/day for 10 days. A severe anemia develops when dosages exceeding 60 mg are used. Despite the development of anemia, reticulocytosis does not occur until after the drug is withdrawn in those animals receiving doses of ribavirin in excess of 15 mg/kg/day (Fig. 7). Regardless of the dosage,

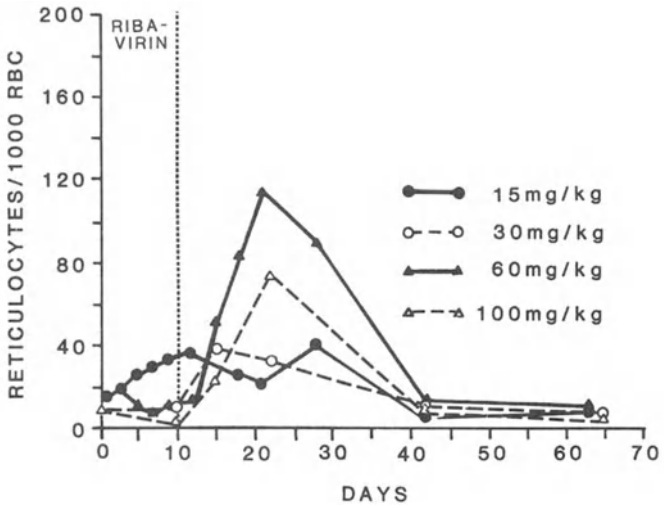


Fig. 7. Reticulocyte counts of ribavirin-treated rhesus monkeys. Drug was administered IM for 10 days

red blood cell mass values generally return to normal limits within 3–5 weeks after discontinuance of ribavirin.

2. Red Blood Cell Effects

The demonstration by ZIMMERMAN and DEEPROSE (1978) that red blood cells sequester large amounts of ribavirin raises the possibility that this may accelerate the destruction of red cells. In studies to be published, SPEARS and CANONICO compared in vitro the kinetics of ribavirin uptake by rat, monkey, and human red blood cells as well as their subsequent susceptibility to osmotic fragility.

These studies show that there is an initial rapid influx of ribavirin into red cells during the first 3 min of incubation (Fig. 8). Thereafter, the accumulation of drug is nearly linear with respect to time and directly related to the extracellular drug concentration. The accumulation of ribavirin in monkey cells is most impressive. After 150 min of incubation in the presence of 400 μM ribavirin, the concentration of drug in monkey red cells exceeds 900 μM. This is in contrast to values of 500 and 400 μM for human and rat cells. When drug-laden cells are reincubated in fresh drug-free media, rat cells release nearly 80% of their ribavirin content while monkey cells retain 80–90% of the drug. Tests to determine the susceptibility of ribavirin-treated red cells to lysis using a graded series of hypotonic salt solutions indicate that the sequestration of ribavirin by all three species of red cells does not lead to increased fragility (Fig. 9). Nonetheless, it is of interest that the disposition to develop anemia in these species appears to correlate with the rate of uptake and sequestration of ribavirin by their red cells. One possibility which is yet to be examined is that the uptake of ribavirin accelerates phagocytic removal from the circula-

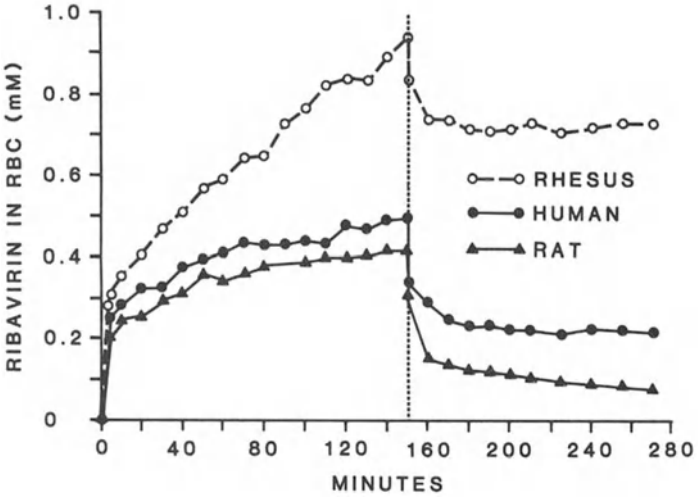


Fig. 8. Uptake of [^{14}C]-ribavirin in vitro by rhesus monkey, human and rat red blood cells. Uptake was followed for 150 min by incubation of red cells in balanced salt solution containing 100 μg/ml of radiolabeled ribavirin. Release of drug from red cells was followed after the initial uptake period (*vertical dashed line*) by washing red cell, then reincubating in drug-free media

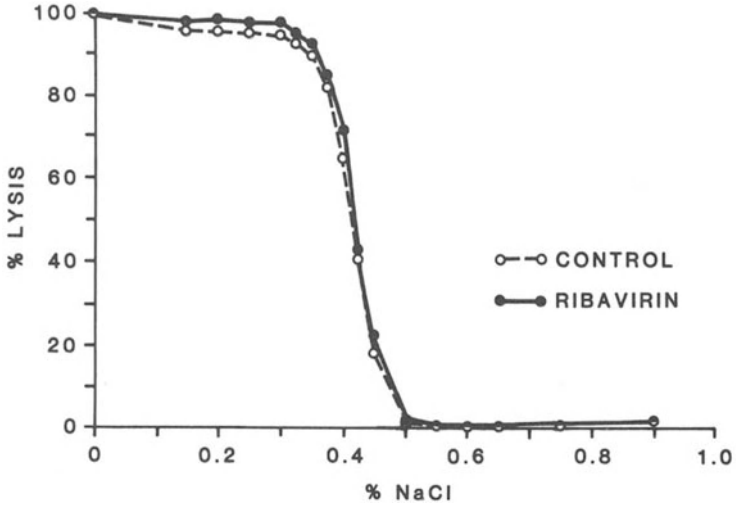


Fig. 9. Osmotic fragility of monkey red cells following ribavirin loading. Red cells were incubated in vitro for 150 min in the presence of 1000 μg/ml of ribavirin. Aliquots of control and treated cells were then mixed with a graded series of NaCl solutions and the degree of lysis determined

tion by elements of the reticuloendothelial system. In part, this may be related to the extent which red cells of different species sequester ribavirin.

The half-life of radiolabeled red cells was measured in ribavirin-treated monkeys to assess whether the drug-induced anemia is due to decreased production of erythrocytes, increased destruction or a combination of both factors.

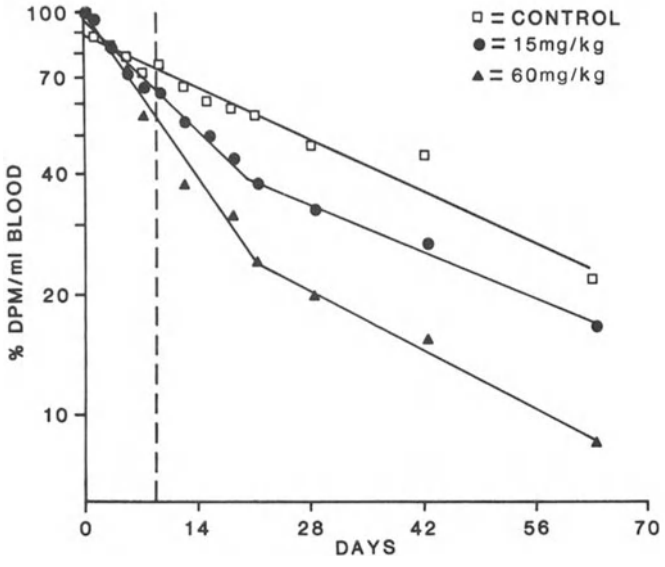


Fig. 10. Red cell survival in ribavirin-treated rhesus monkeys. Red cells labeled in vitro with [^{3}H]di-isopropyl fluorophosphate (DFP) were reinjected into each donor. Monkeys were given ribavirin IM for 10 days. The slope of each *curve* indicates the rate of removal of labeled red cells from the circulation

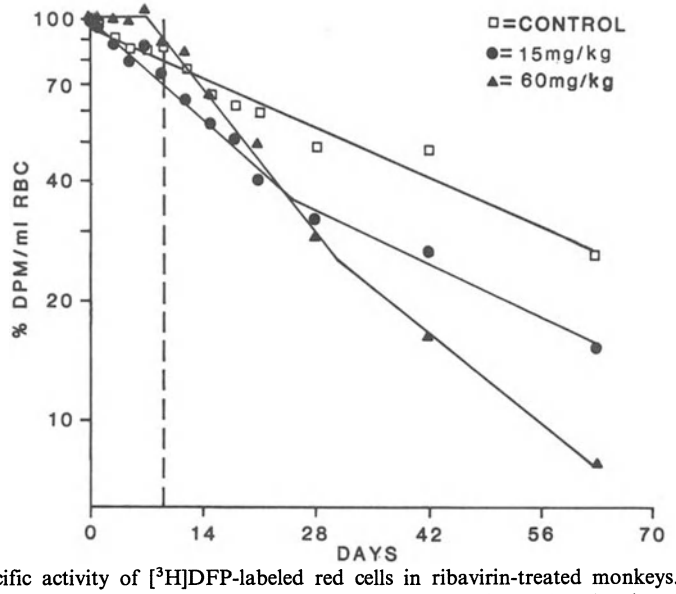


Fig. 11. Specific activity of [^{3}H]DFP-labeled red cells in ribavirin-treated monkeys. Data shown in Fig. 10 are expressed as radioactivity per number of circulating cells. The slope of each *curve* indicates the rate at which newly formed unlabeled red cells are released from the bone marrow, diluting the concentration of labeled circulating red cells

In monkeys treated with either 15 or 60 mg/kg for 10 days, a dose-related decrease in erythrocyte survival is observed from days 0–28 (Fig. 10). Thereafter, red cell half-lives are comparable to control values. These data show that ribavirin at a dose as low as 15 mg/kg decreases the half-life of monkey red cells. The effects, however, are fully reversible upon discontinuation of the drug. At a dose of 60 mg/kg, the specific activity of label in circulating red cells remains constant during the treatment period, suggesting that the release of red cells from the bone marrow is inhibited (Fig. 11). Upon termination of treatment, erythrocytes are again released from the marrow, as indicated by a reduction in the specific activity of radiolabeled cells (Fig. 11) and accompanying reticulocytosis (Fig. 7).

In the low-dose group, there is no evidence of any inhibition of red cell release from the bone marrow. It appears that the anemia associated with prolonged administration of ribavirin is due, in part, to a decrease in the half-life of circulating red cells as well as an inhibition of erythrocyte release from the bone marrow at higher dosages. Both effects appear fully reversible when treatment is withdrawn.

3. Bone Marrow Effects

Suppression of hematopoiesis by ribavirin is a serious clinical concern and prompted studies to characterize more fully the drug's effects on marrow elements. Serial examination of the bone marrow from monkeys treated with ribavirin for 10 days reveals a significant increase in myeloid to erythroid (ME) ratio in the high-dose group (100 mg/kg/day) (Fig. 12). The dose-related erythroid hypoplasia reaches a maximum on day 10, followed by hyperplasia on day 22, and a return to control values by day 42. Myeloid precursors are not affected. Differential counts of erythroid precursors show that the erythroid hypoplasia is due to a significant decrease in late erythroid forms and in particular the polychromophilic normoblast (Fig. 13). Early erythroid forms are unchanged or increased. Cytoplasmic vacuolization in erythroid precursors is seen on day 10.

In summary, the administration of ribavirin to rhesus monkeys at dosages exceeding 15 mg/kg for 5 or more days results in a dose-related hematologic toxicity which is characterized by a normochromic, normocytic anemia. The decline in late erythroid forms, with no effect on early forms in concert with the rapid appearance of reticulocytes immediately following withdrawal of the drug, imply an impairment in the maturation of the erythroid series rather than a direct toxic effect on erythroid stem cells. It is unclear why ribavirin should halt the maturation process of the erythrocytic series. One possibility is that the enzyme complement of late red cell precursors may be sufficiently differentiated as to more nearly resemble that of mature erythrocytes, at least with respect of their ability to transport, phosphorylate, and sequester large quantities of ribavirin. It is known that large intracellular concentrations of ribavirin have significant consequences on both the ribonucleotide and deoxyribonucleotide pools (LOWE et al., 1977; ZIMMERMAN and DEEPROSE, 1978). The

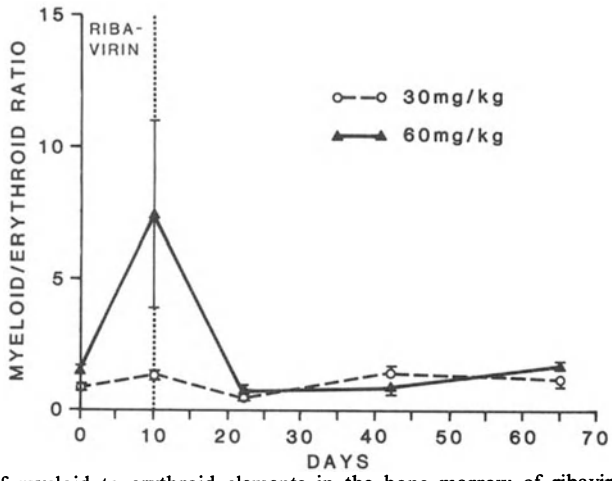


Fig. 12. Ratio of myeloid to erythroid elements in the bone marrow of ribavirin-treated rhesus monkeys

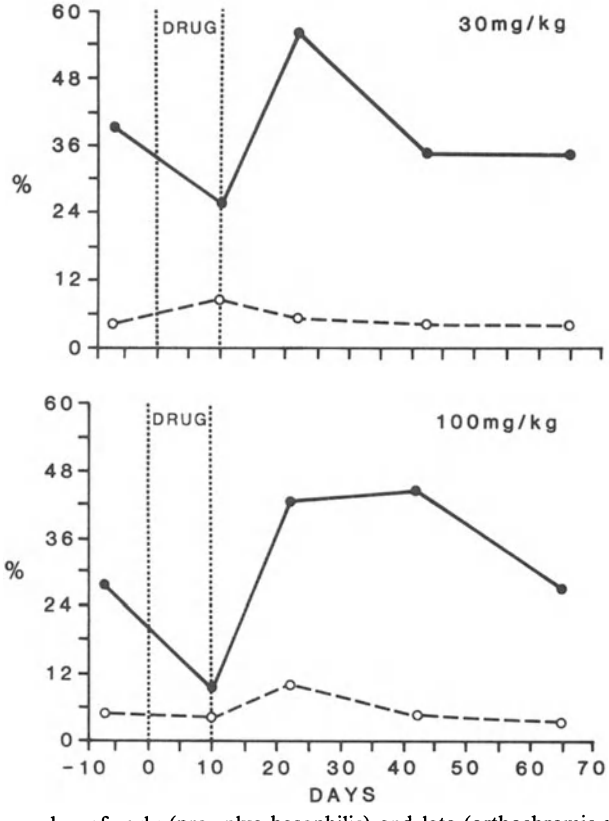


Fig. 13. Relative number of early (pro- plus basophilic) and late (orthochromic plus polychromic) erythroid elements in the marrow of ribavirin-treated rhesus monkeys. Values are expressed as the percentage of total cellular elements. *Closed circles* indicate late erythroid elements; *open circles* indicate early forms

Table 8. Changes in nucleotide pools in L5178Y cells treated with 100 μM ribavirin[a]

Nucleotide	pmol/10^6 cells[b]	Change over control, %[c]
GTP	142± 8	− 52
ATP	1819±178	+ 17
UTP	574± 53	+ 44
CTP	214± 37	+ 24
dGTP	–	− 70
dATP	–	− 20
dTTP	–	+250
dCTP	–	− 22
RTP[d]	103± 29	–

[a] From SMITH (1980).
[b] Values for ribonucleotides were taken from ZIMMERMAN and DEEPROSE (1978).
[c] Changes for deoxynucleotides were estimated from the published results of LOWE et al. (1977).
[d] Ribavirin triphosphate.

enormous perturbations which occur in nucleotide pool sizes (Table 8) may have significant effects on the regulation of metabolic events in such rapidly differentiating and metabolizing cells as the late forms of the erythropoietic series. Such potential difficulties may be prevented in other cells by their inherent capacity to metabolize ribavirin and prevent or at least limit the time where intracellular ribavirin attains concentrations which interfere with regulation of host metabolic events.

D. Dogs

1. Clinical Findings

When ribavirin is administered to beagles at dosage levels of 15, 30, and 60 mg/kg/day for 4 weeks, dose-related toxicity is observed at all levels. In studies performed at Hazelton Laboratories, Vienna, Virginia (Contract No. DAMD 17-80-C-0161), findings could be characterized as ranging from minimal/slight at the low dose to marked/severe at the high dose. Five high-dose dogs died or were sacrificed moribund. Clinical signs, such as soft feces/diarrhea and mucoid, sanguineous and tarry feces, in low-, mid- and high-dose animals indicate grossly an effect on the intestinal tract. Inappetence, signs of physical deterioration and cachexic effects are seen in mid- and high-dose animals. Hematologic data show evidence of a possible blood dyscrasia in the two highest dose groups and leukopenia in the 60 mg/kg/day group.

2. Pathology

Histomorphologic alterations are most widespread and severe in dogs given 60 mg/kg/day for 28 days, with primary involvement of the gastrointestinal

tract, thymus, spleen and bone marrow. Spleen sections show the presence of a brown granular pigment, which is also present in the Kupffer cells of the liver. There is an increased cytoplasmic vacuolation of cells in the fundic region of the stomach along with an apparent increase in mucous secretion. Drug-related enteritis occurs in all three sections of the small intestine and is characterized by inflammatory infiltrate, necrosis, and regeneration of crypt epithelium. Rib marrow reveals the occurrence of hypoplasia; there is a moderate lymphoid depletion in the thymus and tonsil. Changes in all these tissues are less severe in the mid-dose group. In dogs of the low-dose group, compound-induced changes are limited to the duodenum, jejunum, and ileum and are minimal to slight in severity.

IV. Mechanisms of Action

Although to the casual observer, ribavirin appears more as a pyrimidine analog than anything else, single crystal X-ray diffraction analysis has shown that it very closely resembles guanosine in structure. The geometry about the carboximide function renders ribavirin strikingly similar to guanosine. This structural similarity has been a leading principle guiding studies on the mode of action of ribavirin, namely, that it would probably involve biochemical pathways either leading to or involving guanosine nucleotides (SMITH, 1980).

The reversal of ribavirin's antiviral effect by guanosine when added to Vero cells infected with measles virus (Table 9) strengthens this concept (STREETER et al., 1973). In addition to guanosine, xanthosine effectively reverses the antiviral activity, but inosine is somewhat less effective. Orotidine, adenosine, deoxy-

Table 9. Effect of nucleotides and nucleosides on the antimeasles virus activity of ribavirin in Vero cell culture[a]

Ribavirin	Total pfu for ribavirin at different concentrations, μg/ml			
	500	100	20	0
Alone	0	3	176	213
+Guanosine	29	168	196	202
+Xanthosine	53	211	215	215
+Inosine	0	125	166	177
+Orotidine	0	16	191	210

Adenosine, deoxyadenosine, cytidine, uridine, thymidine, AICAR, AICAR-5′-P = no reversal.

[a] Adapted from SMITH (1980).

[b] 200 μg/ml of each nucleotide or nucleoside were incorporated in the agar overlay with each concentration of ribavirin. The agar overlay was added onto the cultures after 1–1.5 h of virus adsorption.

adenosine and aminoimidazole-4-carboxamide ribonucleotide, on the other hand, are without effect. Guanosine also abolishes the cytostatic effect of ribavirin on noninfected mouse lymphoma (L5178Y) (MÜLLER et al., 1977) and reverses the inhibition of hemagglutinin production by influenza A virus in MDCK cells (BROWNE, 1979). Thus, both chemically and biologically there is evidence that ribavirin simulates guanosine, so that its principal mode of action might be expected to involve guanosine nucleotides.

Intracellular ribavirin is phosphorylated; results obtained with human red blood cells and murine lymphoma L5178Y cells (ZIMMERMAN and DEEPROSE, 1978) show that ribavirin principally exists within the cell as the 5′-triphosphate form. Like other nucleotide pools, the ratio of the tri- to di- to monophosphate should be close to 25:5:1. The data of ZIMMERMAN and DEEPROSE (1978) show that more than 50% of the ribavirin taken up by human red blood cells is converted to the triphosphate and that the cellular concentration of ribavirin-5′-triphosphate equals that of adenosine triphosphate.

The mechanisms of ribavirin phosphorylation have also been examined. STREETER et al. (1974) demonstrated that rat liver extracts possess a kinase capable of phosphorylating ribavirin to its 5′-monophosphate. Studies by WILLIS et al. (1978) have led to the conclusion that it is adenosine kinase which converts ribavirin to its 5′-monophosphate and from that point to the triphosphate apparently is easily handled by the cell.

Inosine monophosphate (IMP) dehydrogenase is involved in the final steps of the de novo synthesis of guanosine monophosphate and normally is regulated in vivo by guanosine monophosphate. The earlier work of STREETER et al. (1973) showed that ribavirin monophosphate is a potent competitive inhibitor of IMP dehydrogenase. The Ki for ribavirin monophosphate is 2.2×10^{-7} M, whereas that for guanosine monophosphate is 2.2×10^{-4} M. Thus, ribavirin monophosphate is 50–100 times more potent than GMP as an inhibitor of IMP dehydrogenase. The ability of some other derivatives of ribavirin monophosphate to inhibit IMP dehydrogenase is shown in Fig. 14 as the ratio of the amount of inhibitor required to yield 50% inhibition to the substrate concentration. It is interesting that the analog containing the 1,4,5-triazole is as potent an IMP dehydrogenase inhibitor as ribavirin monophosphate, but is totally inactive as an antiviral drug (HARRIS and ROBBINS, 1980). In addition, the thiocarboxamide and carboxamidine derivatives are not very active IMP dehydrogenase inhibitors; however, the latter is an excellent antiviral, while the former is only mildly so (SMITH 1980).

While it is possible that the antiviral activity of ribavirin may be ascribed to its inhibition of IMP dehydrogenase and consequent effect on the guanosine nucleotide pools, it would appear more likely that the antiviral activity is closely related to another specific event(s). According to SMITH (1980), if the IMP dehydrogenase mechanism alone was operative, then ribavirin should show equal antiviral and cytotoxic effects; however, as was shown by SIDWELL (1980), there are a number of viruses against which ribavirin is inactive (notably polio, Semliki Forest, Coxsackie, and pseudorabies).

Since the principal intracellular form of ribavirin is the triphosphate, it is reasonable to assume that this may be the form in which it exerts its antiviral

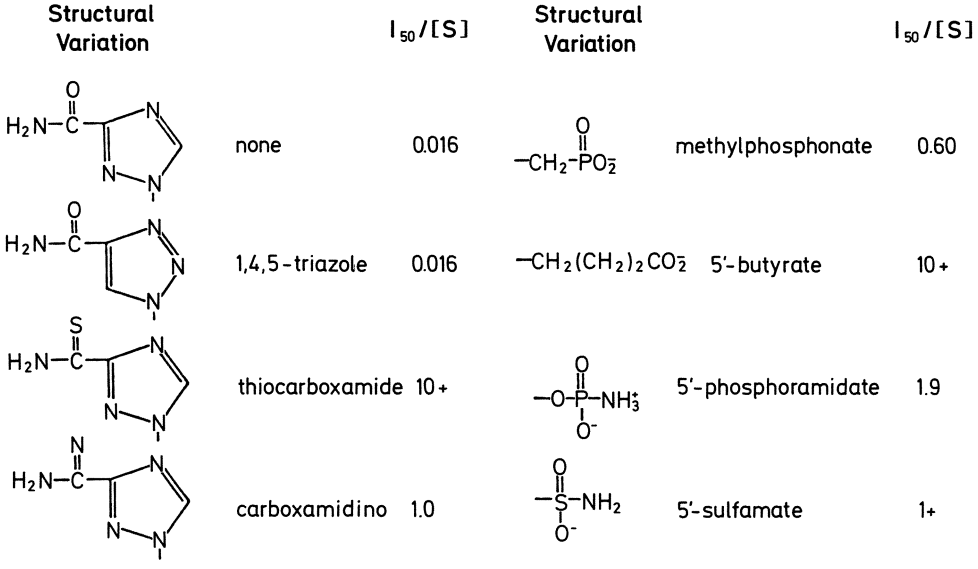


Fig. 14. IMP dehydrogenase inhibition by analogs of ribavirin-5-phosphate. All compounds are compared with ribavirin-5-monophosphate using the ratio of inhibitor concentration causing 50% inhibition to the substrate (IMP) concentration. (Data of STREETER, cited by SMITH, 1980)

activity. At least two observations relating to the effects of ribavirin triphosphate (RTP) are considered relevant in this regard. ERIKKSON et al. (1977) have shown that influenza A polymerase is inhibited by RTP. The inhibition is competitive with respect to ATP and GTP. The guanine nucleotide activation of this polymerase is particularly sensitive to the triazole nucleoside triphosphate. Calculations show that the Ki/Km ratio for RTP is about 1.0 for GTP and about 0.1 for ATP, indicating a powerful inhibition (SMITH, 1980). Ribavirin triphosphate, however, is not an inhibitor of a wide variety of animal cell RNA polymerases and appears to be selective for the influenza A RNA polymerase (SMITH et al., 1980).

Ribavirin has been found to exert an interesting effect on uridine incorporation into RNA of cells in culture (Fig. 15). When ribavirin is added to BHK-21 cells at low concentrations, the incorporation of uridine into acid-precipitable material is greatly inhibited. Remarkably, the inhibition is reversed when higher concentrations of ribavirin are used. This effect explains, in part, why ribavirin has been reported to inhibit RNA synthesis in some studies, but not in others. Ribavirin also appears to inhibit the uptake of uridine into the free soluble intracellular pool (Fig. 15). This effect would result in the synthesis of RNA with a lower specific activity of labeled uridine. Thus, the apparent inhibition of RNA synthesis may be an artifact due to a decrease in the specific activity of the labeled cellular uridine pool. In fact, ribavirin fails to alter the quantity and distribution of mRNA species in control and Venezuelan equine encephalomyelitis (VEE) virus-infected BHK-21 cells. In spite of similar levels of mRNA synthesis, protein synthesis in ribavirin-treated control, as well as in virus-infected, BHK-21 cells is inhibited by 40%–50% (Fig. 16). This inhibition occurs

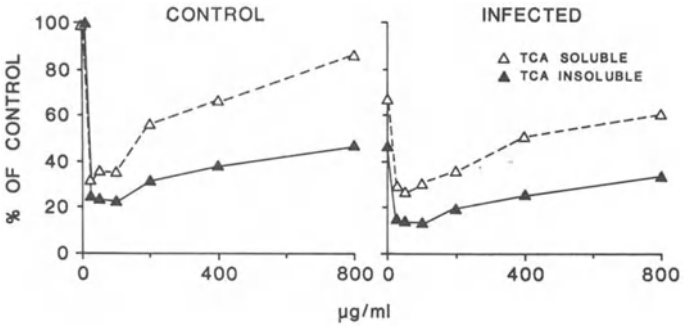


Fig. 15. Effect of ribavirin on uptake and incorporation of [^{3}H]uridine in control and infected BHK-21 cells. Results are expressed as a percentage of the uptake or incorporation of [^{3}H]uridine found for uninfected, untreated (control) cells

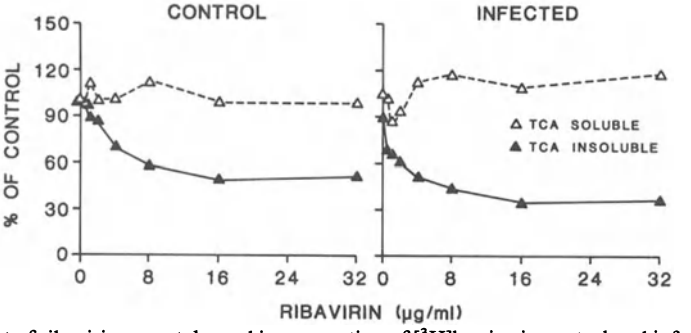


Fig. 16. Effect of ribavirin on uptake and incorporation of [^{3}H]leucine in control and infected BHK-21 cells. Results are expressed as a percentage of the uptake or incorporation of [^{3}H]leucine found for uninfected, untreated (controls) cells

in the presence of nearly constant levels in the soluble cellular pool of labeled leucine. The demonstration that mRNA from ribavirin-treated cells is translated in an in vitro system (Fig. 17) with only 30% efficiency when compared to control mRNA indicates that ribavirin treatment results in the production of altered mRNA species.

One explanation for these results is that ribavirin affects the formation of the 5′ "cap" common to many mRNA. This concept, first proposed by SMITH (1980), is based on the fact that mRNA of a large number of viruses and eukaryotic cells undergoes a posttranscriptional modification. This results in a 7-methylguanosine residue linked from its 5′-position via a triphosphate bridge to a 2′-0′-methylribonucleotide of the 5′-terminal. The 5′-terminal 7-methylguanosine cap in mRNA is required for efficient translation (MUTHUKRISHNAN et al., 1978). SMITH and collaborators (1980) studied the effect of ribavirin and its phosphorylated derivatives on in vitro directed RNA synthesis by vaccinia RNA polymerase. They demonstrated that RTP is a potent inhibitor of the 5′-terminal guanylation of synthesized uncapped mRNA (GOSWAMI et al., 1979). The inhibition is competitive and the Ki for RPT is 32 µM, while that for GTP is 22 µM. Thus, the Ki/Km ratio of 1.45 indicates a powerful inhibitory effect of RTP on the guanylation reaction. CANONICO et al. (1980) chromatographed mononucleotides resulting from the digestion of mRNA obtained from VEE-infected

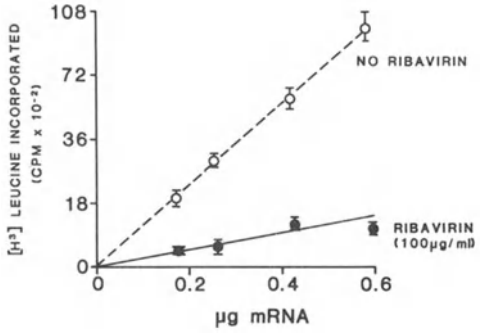


Fig. 17. In vitro translation of mRNA in a reticulocyte system. Incorporation of [^{3}H]leucine into trichloroacetic acid-insoluble products was measured using oligo-(dT) cellulose binding RNA from virus-infected cells treated for 5 h with or without ribavirin (CANONICO et al., 1980)

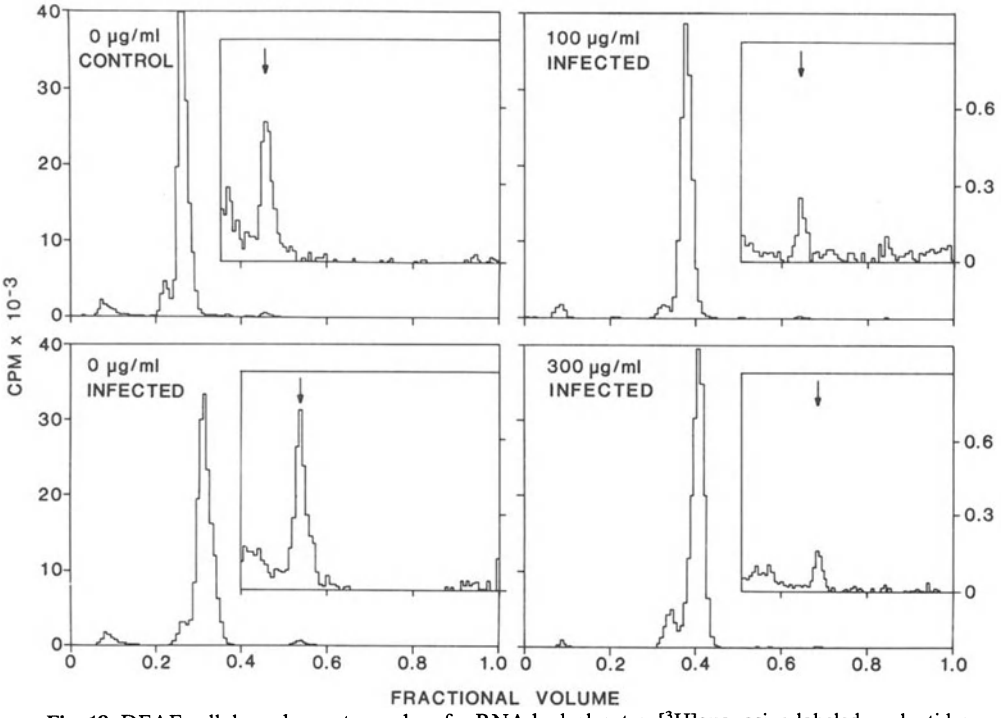


Fig. 18. DEAE-cellulose chromatography of mRNA hydrolysates. [^{3}H]guanosine-labeled nucleotides released by RNAase T_2 digestion of oligo-(dT) cellulose binding RNA from control and virus infected BHK-21 cells treated with or without ribavirin. The *insects* in each panel represent a 1000-fold expansion of the ordinate of the corresponding chromatographs. The elution peak of an mRNA "cap" standard (CANONICO et al., 1980)

and control cells treated with ribavirin. Results showed that the radioactive peak corresponding to the elution profile of a cap standard is reduced three- to tenfold in mRNA preparations from cells treated with ribavirin (Fig. 18). Inhibition of the normal modification of the 5′ cap would lead to the accumulation of mRNA that are inert or impaired in protein synthesis.

It is apparent that ribavirin exerts a myriad of effects on cellular metabolism; it is conceivable that its antiviral effects may be, in fact, expressed through an interaction of multiple mechanisms. Apparently its mode of action is multipronged, but generally involves perturbation of nucleotide pools and inhibition of 5′ cap formation.

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Rubradirin

F. REUSSER

I. Introduction

The antibiotic rubradirin impairs ribosomal functions in bacteria by selectively inhibiting protein chain initiation dependent on initiation factors. The compound was purified as an amorphous powder, which is an acid. Rubradirin possesses pH indicator properties. It is red in acidic or neutral solutions and green in basic solutions or in salt form. It is an extremely potent inhibitor of Gram-positive bacteria in vitro and effective in the treatment of experimental bacterial infections in mice.

The chemical structure of rubradirin is very complex. It consists of four distinctive moieties: a quinone, dihydroxydipicolinic acid, a coumarin and a nitrosugar (HOEKSEMA et al., 1979). Three of these structural entities are related to entities found in members of quite diverse antibiotic families with different modes of action: The quinone chromophore is related to the ansamycins (rifamycin, etc.). Most ansamycins inhibit bacterial RNA polymerase (RINEHART et al., 1974). A coumarin moiety is also present in novobiocin and related antibiotics. These drugs inhibit DNA replication in bacteria (STAUDENBAUER, 1975; GELLERT et al., 1976; REUSSER, 1977). Lastly, the nitrosugar is related to evernitrose occurring in some of the everninomicins (GANGULY et al., 1968, 1977). The mode of action of these later compounds is not known. The aglycone of rubradirin acts differently from its parent compound. Rubradirin aglycone predominantly inhibits bacterial RNA polymerase and retains the ability to inhibit ribosomal functions to some extent. Rubradirin itself inhibits ribosomal functions exclusively and does not affect RNA polymerase.

II. Discovery, Fermentation, Purification, and Chemistry

Rubradirin is produced by *Streptomyces achromogenes* var. *rubradiris* (UC 2630). The organism elaborates rubradirin in a medium containing Soludri (Brown Forman Co., Louisville, Kentucky), 20 g/l; corn steep liquor, 15 g/l; starch, 10 g/l; $NaNO_3$, 2 g/l. The pH of the medium was adjusted to 7.2 before sterilization. Usually titers peaked in shake flasks after 2–3 days of incubation

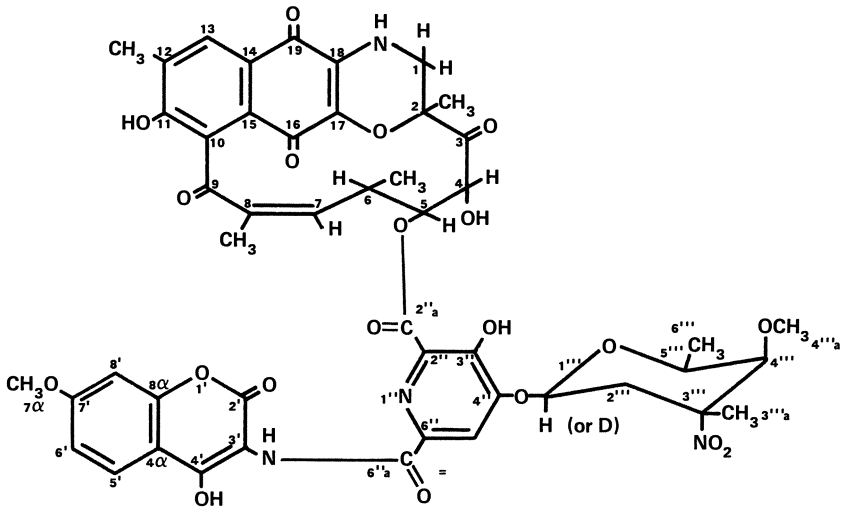

Fig. 1. The proposed structure for rubradirin

at 28° C. The antibiotic titer was measured by a disk plate agar diffusion assay using *Sarcina lutea* as the assay organism (BHUYAN et al., 1965).

Rubradirin was isolated by first removing the mycelium from the broth by filtration. The clear broth was then adjusted to pH 3 with conc H_2SO_4. The broth was extracted with methylene chloride, and the extract was concentrated under vacuum. Then the antibiotic was transferred to water at pH 8.7 and back into methylene chloride at pH 7.4. The methylene chloride solution was again concentrated under reduced pressure and 5 volumes of hexane were added. This precipitates the antibiotic as a dark red substance. The precipitate was recovered by filtration and freed from remnant solvent in vacuo. Then the antibiotic was isolated in pure form by countercurrent distribution in a hexane-acetone-water system (5:5:1).

Rubradirin recovered by the above procedures is a red amorphous powder. It is a pH indicator; acidic or neutral solutions are red, and basic solutions are green. Rubradirin has the empirical formula $C_{48}H_{46}N_4O_{20}$ and a molecular weight of 998. The visible and ultraviolet spectrum in 1 m*M* HCl in 85% methanol shows maxima at 510, 312, 270, and 222 nm. In 1 m*M* NaOH in 85% methanol maxima occur at 420 and 300 nm with a shoulder at 350 nm. The optical rotation is $[\alpha]_D^{25} = +920$ (c = 0.025, dilute acetone) (MEYER, 1965). Rubradirin is insoluble in water but its salts are water-soluble. It is soluble in most organic solvents and moderately soluble but unstable in alcohols.

The chemical structure for rubradirin is shown in Fig. 1 (HOEKSEMA et al., 1978a, 1979; MIZSAK et al., 1979). The antibiotic consists of four distinct moieties, a quinone, dihydroxydipicolinic acid, a coumarin, and a nitrosugar. The quinone chromophore (rubransarol) is related to the ansamycins. This moiety is linked through an ester bond to dihydroxydipicolinic acid. The hydroxydipicolinic acid in turn is glycosylated by a nitropyranose named rubranitrose. Rubranitrose is stereoisomeric with evernitrose found in the unrelated antibiotics ever-

ninomicins B and C. The fourth moiety is a substituted amide of coumarin linked to the dihydroxydipicolinic acid moiety via its amide. A coumarin moiety is also present in the novobiocin family of antibiotics. Rubradirin thus contains structural entities present in three other very diverse antibiotic families, such as the ansamycins, novobiocins, and everninomicins.

III. Biological Properties

Rubradirin primarily inhibits Gram-positive bacteria. The in vitro spectrum as determined in an agar diffusion test in brain heart infusion medium is shown in Table 1. Rubradirin is extremely active against *Staphylococci;* for that genus, its minimal inhibitory concentrations range from 0.003 to 0.1 μg/ml (C. LEWIS and G.E. ZURENKO, personal communication).

Rubradirin is nontoxic in animals and effective against experimental bacterial infections in mice. The CD_{50}'s (50% cure) in mice infected with a strain of *S. aureus* were 4.6 mg/kg of body weight orally and 2.3 mg/kg subcutaneously. With a *Streptococcus pyogenes* strain, the CD_{50}'s were 49 mg/kg orally and 11 mg/kg subcutaneously. The acute toxicity exceeded 5 g/kg when given orally and 240 mg/kg when given intraperitoneally.

Table 1. In vitro antimicrobial spectrum of rubradirin

Organism		Minimum inhibitory concentration (μg/ml) Rubradirin
S. aureus	UC 76	0.003
S. aureus	UC 570	0.012
S. aureus	UC 746	0.1
S. pyogenes	UC 152	0.20
St. faecalis	UC 694	0.78
E. coli	UC 45	>100
P. vulgaris	UC 93	>100
K. pneumoniae	UC 58	>100
S. schottmuelleri	UC 126	>100
Ps. aeruginosa	UC 95	>100
D. pneumoniae	UC 41	0.10

IV. Mode of Action

Rubradirin specifically impairs bacterial ribosomal functions (REUSSER, 1973a, b).

A. Effect on Synthetic Messenger-RNA-directed Amino Acid Incorporation Systems

Rubradirin substantially inhibited the incorporation of [^{14}C]phenylalanine, [^{14}C]proline, or [^{14}C]lysine directed by synthetic polyribonucleotides (Table 2). Reasonably strict dose–response relationships were obtained with all of these systems. A rubradirin concentration of 0.023 m*M* caused 50% inhibition of the poly(U)-directed phenylalanine incorporation system. The corresponding values were 0.056 m*M* for the poly(C)-proline and 0.035 m*M* for the poly(A)-lysine incorporation systems. Thus, polyphenylalanine synthesis was somewhat more susceptible to rubradirin inhibition than polylysine or polyproline synthesis.

Table 2. Effect of rubradirin on polyribonucleotide-directed amino acid incorporation

System	Sample	Cpm/Sample	% Inhibition
Poly (U)-phenylalanine incorp.	Control	2600	0
	0.05 m*M* rubradirin	1060	59
	0.02 m*M*	1270	51
	0.01 m*M*	1580	39
	0.005 m*M*	1670	36
Poly (C)-proline incorp.	Control	3150	0
	0.05 m*M* rubradirin	1620	48
	0.02 m*M*	2200	30
	0.01 m*M*	2290	27
	0.005 m*M*	2660	15
Poly (A)-lysine incorp.	Control	3030	0
	0.05 m*M* rubradirin	1440	52
	0.02 m*M*	1630	46
	0.01 m*M*	1870	38
	0.005 m*M*	2020	33

Reaction mixtures contained in a total volume of 0.25 ml: Tris-HCl buffer, pH 7.8, 25 µmol; magnesium acetate, 3.5 µmol; KCl, 15 µmol; phosphoenolpyruvate, K salt, 18.75 µmol; pyruvate kinase (Calbiochem), 10 µg; [^{12}C]amino acid mixture of 19 amino acids, 0.05-µmol each; [^{14}C]phenylalanine, [^{14}C]proline, or [^{14}C]lysine, 0.25 µmol containing 1.75 µCi; polyribonucleotide, 15 µg; S-30 enzyme, 430 µg of protein. The samples were incubated at 37° C for 15 min. Incorporation in the absence of messenger amounted to 1–3% of the controls.

B. Effect on Phage f_2 RNA-directed Amino Acid Incorporation Systems

In the presence of a relatively high Mg^{2+} concentration (14 m*M*) in the assay system, where Mg^{2+} alone initiated phage f_2 RNA-directed amino acid incorporation, rubradirin did not inhibit peptide biosynthesis.

In the presence of low Mg^{2+} concentrations (2 m*M*), the addition of leucovorin (the calcium salt of 5-formyltetrahydrofolic acid, Lederle) significantly stimulated phage RNA-directed amino acid incorporation. This indicated that leucovorin served as a formyl donor for the formation of formylmethionyl-

tRNA in the test system. Under these conditions peptide biosynthesis was inhibited substantially in the presence of rubradirin. A drug concentration of 0.05 m*M* inhibited the system 44%; a concentration of 0.025 m*M* inhibited the system 25%. Thus, in the presence of a high Mg^{2+} concentration, natural mRNA-mediated amino acid incorporation remained essentially insensitive to rubradirin. On the other hand, in the presence of low Mg^{2+} concentrations and leucovorin, rubradirin substantially inhibited peptide biosynthesis.

C. Effect on Polyribonucleotide (mRNA) Attachment to Ribosomes

Attachment of [^{3}H]poly(C) was measured by the Millipore filtration technique (REUSSER, 1973a). Ribosomal [^{3}H]poly(U) binding was measured by separation of the ribosome-poly(U) complex on sucrose density gradients because of the high nonspecific absorption of poly(U) to the filters in the Millipore filter assay. The results of these binding studies indicated that rubradirin did not change the binding capacity of ribosomes toward either poly(C) or poly(U).

D. Effect on tRNA Charging Activity

Rubradirin concentrations ranging from 0.01 to 0.1 m*M* did not affect the formation of prolyl-tRNA or phenylalanyl-tRNA.

E. Attachment of Aminoacyl-tRNA to Salt-washed 70S Ribosomes

Attachment of aminoacyl-tRNA was tested in two systems, one that produces phenylalanyl-tRNA-poly(U)-ribosome complexes and another that produces prolyl-tRNA-poly(C)-ribosome complexes. Rubradirin inhibited these reactions moderately. Phenylalanyl-tRNA as well as prolyl-tRNA binding were inhibited by 14% in the presence of 0.05 m*M* rubradirin. Higher antibiotic concentrations did not significantly increase inhibition in either system.

F. Effect on Formation of the 70S Initiation Complex

In low Mg^{2+} concentrations (4 m*M*), A-U-G mediated binding of fMet-$tRNA_f$ to 70S ribosomes depends on the presence of initiation factors and GTP. This initiation is usually referred to as enzymatic initiation. Under these conditions rubradirin inhibited the attachment of fMet-$tRNA_f$ to 70S ribosomes 87% (Table 3). In the presence of high Mg^{2+} concentrations (15 m*M*), the initiation complex will form without the initiation factors (nonenzymatic initiation). This type of initiation remained insensitive to rubradirin inhibition (Table 3).

These results demonstrated that rubradirin selectively inhibits initiation-factor-dependent initiation only and not Mg^{2+}-induced nonenzymatic initiation.

Table 3. Effect of rubradirin on the initiation-factor-induced and Mg^{2+}-induced formation of the 70S initiation complex

Sample		fMet-tRNA$_f$ bound (cpm/Sample)	% Inhibition
4 m*M* Mg^{2+}	Control	770	0
	Rubradirin, 0.05 m*M*	100	87
15 m*M* Mg^{2+}	Control	995	0
	Rubradirin, 0.05 m*M*	990	0

The reaction mixtures contained in 0.15 ml: 50 m*M* Tris-HCl (pH 7.8), 100 m*M* NH_4Cl, magnesium acetate as indicated, 4 m*M* mercaptoethanol, 2 m*M* GTP, 0.04 OD_{260} unit of A-U-G, 4.5 units of 70S ribosomes, 0.44 unit of [^{3}H]fMet-tRNA$_f$ containing ~140,000 cmp, and 25 µg of initiation factors (only present in the samples containing 4 m*M* Mg^{2+}). Binding was measured after incubation of the reaction mixtures for 10 min at 37° C.

G. Effect on Stability of 70S Initiation Complex

Rubradirin's inhibition of the factor-dependent 70S initiation complex could have been due to interference with either the formation of the complex per se or the rapid breakdown of the complex as it formed. To resolve this question the 70S initiation complex was formed in the absence of rubradirin, and the stability of the complex was assessed after addition of rubradirin.

If the initiation complex was formed in the presence of 15 m*M* Mg^{2+} and no initiation factors, the addition of rubradirin did not appreciably affect its stability (Fig. 2). If the initiation complex was formed under enzymatic initiating conditions, addition of rubradirin rapidly dissociated this complex (Fig. 2). Adjustment of the Mg^{2+} concentration to 15 m*M* immediately reassociated the rubradirin-dissociated initiation complex. Mg^{2+} therefore reversed rubradirin's dissociative action.

These studies showed that rubradirin could prevent the formation of the 70S initiation complex or dissociate the 70S complex if these complexes were formed or preformed with the active participation of initiation factors. Furthermore, since an increase of the Mg^{2+} concentration resulted in a rapid reassociation of the rubradirin-dissociated enzymatic initiation complex into a rubradirin stable form, fMet-tRNA$_f$ was displaced unmodified from the 70S ribosomes without concomitant deacylation.

H. Effect of fMet-tRNA$_f$ Binding to 50S Ribosomal Subunits in the Presence of Methanol (Alcohol Reaction)

In the presence of methanol, fMet-tRNA$_f$ binds to the 50S ribosomal subunit without the participation of the 30S subunit and initiation factors. This reaction is commonly referred to as the alcohol reaction. Rubradirin did not interfere with the alcohol reaction. This suggested that the antibiotic interfered with functional steps occurring at the level of the 30S rather than the 50S subunit.

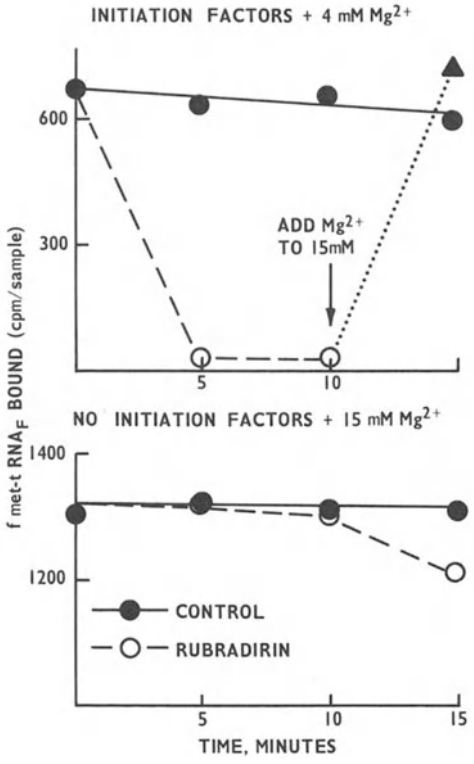

Fig. 2. Effect of rubradirin on the stability of the 70S initiation complex. Basic reaction mixtures were as described under Table 2. Top = 4 m*M* Mg^{2+} + initiation factors: bottom = 15 m*M* Mg^{2+}, no initiation factors. The mixtures containing 4 m*M* Mg^{2+} were preincubated for 15 min, those containing 15 m*M* Mg^{2+} were preincubated for 20 min; at this point rubradirin (0.05 m*M*) was added

I. Effect on Puromycin Reaction

Rubradirin did not interfere with the puromycin reaction and thereby does not affect peptide bond formation. These experiments were carried out in systems containing either N-acetylphenylalanyl-tRNA or fmet-$tRNA_f$ prebound to 70S ribosomes in the presence of high Mg^{2+} concentrations (15 m*M*).

J. Effect on the Formation of the 30S Initiation Complex

The foregoing results demonstrated that rubradirin inhibited the formation of the initiation-factor-dependent 70S initiation complex and caused rapid dissociation of this complex first formed in the absence of the antibiotic. This prompted us to investigate the effect of rubradirin on the formation of the initiation-factor-dependent 30S initiation complex. At a concentration of 0.05 m*M*, rubradirin completely abolished the enzymatic binding of fMet-

Table 4. Effect of rubradirin on the formation of the 30S initiation complex

Sample	fMet-tRNA$_f$ bound (cpm/Sample)	% Inhibition
Control	696	0
Rubradirin, 0.05 m*M*	48	93
Control	448	0
Rubradirin, 0.05 m*M*	2	~100
Streptomycin, 0.05 m*M*	144	63

The basic reaction mixtures are described in the legend of Table 3. They contained 4 m*M* Mg^{2+} and initiation factors. 30S ribosomal subunits = 4.2 units/sample.

$tRNA_f$ to 30S ribosomal subunits (Table 4). Since the formation of the 30S initiation complex precedes the formation of the 70S complex, rubradirin affected the formation or stability of the 70S complex by actually interacting with the 30S subunit and processes occurring at that unit.

K. Effect on the Stability of the 30S Initiation Complex

The initiation factor dependent 30S initiation complex was formed in the absence of rubradirin. Subsequent addition of rubradirin induced rapid dissociation of this complex (Fig. 3). An increase of the Mg^{2+} concentration to 15 m*M* resulted in an immediate reassociation of the 30S complex into a form now stable to the dissociative activity of rubradirin. The stiuation proved thus quite analogous to the one observed with the 70S complex.

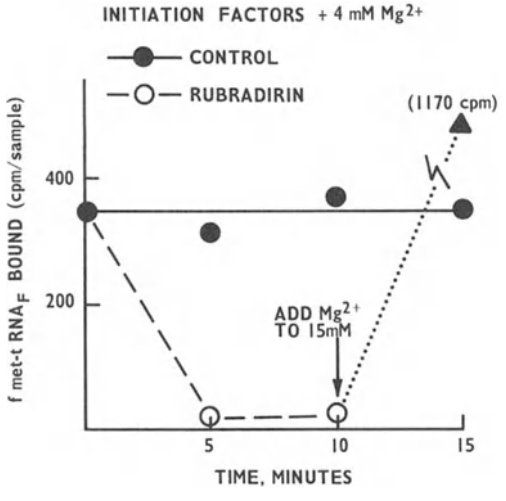

Fig. 3. Effect of rubradirin on the stability of the enzymatic 30S initiation complex. Basic reaction mixtures are described under Table 4. Preincubation before addition of rubradirin (0.05 m*M*) was 15 min

L. Effect on Ribosomal Functions Associated with T- and G-Factors

Rubradirin inhibited the T-factor-stimulated binding of phenylalanyl-tRNA to washed 70S ribosomes only negligibly, and G-factor-dependent polyphenylalanine formation marginally.

M. Stability of Rubradirin in Aqueous Solution

Rubradirin solutions of various ages showed one or the other of the following properties in terms of their mode of action: Fresh solutions specifically inhibited protein synthesis only in cell-free test systems; older ones also inhibited RNA polymerase functions.

The stability of rubradirin was thus investigated upon storage in aqueous solution. A stock solution was assayed periodically in specific cell-free systems to assess protein and RNA synthesis. The ability of the solution to inhibit protein synthesis decreased gradually with storage time while the ability to inhibit RNA synthesis increased accordingly. Apparently an unknown reaction caused the slow and gradual conversion of rubradirin into another or several other compounds.

The eventual determination of the structure of rubradirin and the availability of key rubradirin fragments allowed us to interpret this phenomenon (REUSSER, 1979). The specific fragments studied are defined in Fig. 4.

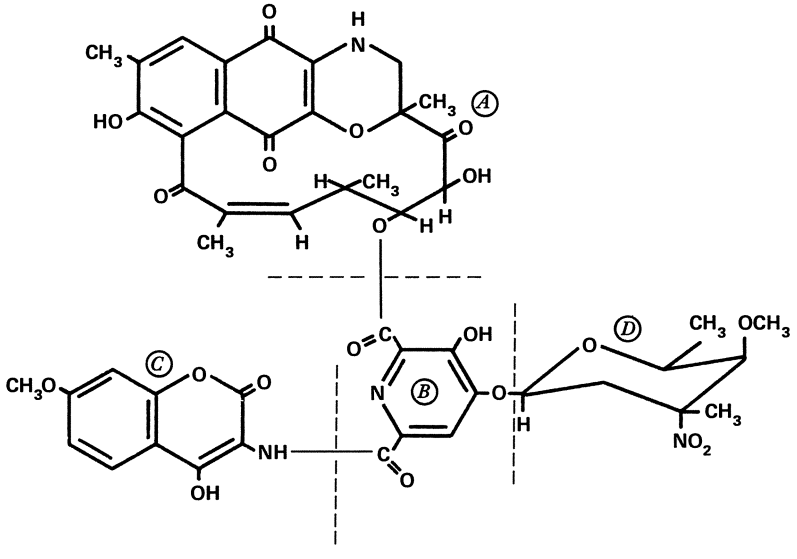

Fig. 4. Definition of rubradirin fragments tested. A = Rubransarol; $A+B+C$ = Rubradirin aglycone; $B+C$ = Picolinic acid-coumarin moiety; $B+C+D$ = Picolinic acid-coumarin-rubranitrose moiety; D = Rubranitrose

N. Effect of Rubradirin Fragments on Cell-free Macromolecular Biosynthetic Systems

Peptide synthesis was carried out in a Nirenberg-type system measuring poly(U)-directed incorporation of [^{14}C]·phenylalanine. Intact rubradirin strongly inhibited this system as expected. On the other hand, rubransarol A, the picolinic acid-coumarin, and the picolinic acid-coumarin-rubranitrose moieties inhibited the reaction moderately. This might have been due to the presence of small traces of intact rubradirin contaminating these preparations. The aglycone of rubradirin significantly inhibited the test system although the extent of inhibition was clearly inferior to that of rubradirin itself.

Intact rubradirin did not impair RNA synthesis effected by *E. coli* RNA polymerase. The aglycone of rubradirin, on the other hand, caused complete cessation of RNA synthesis. The remaining fragments showed marginal inhibition, which might be attributable to trace impurities present in these preparations as reasoned above. The only exception was the picolinic acid-coumarin moiety, which did inhibit the reaction slightly. It was also found that rubradirin aglycone prevents the attachment of RNA polymerase to template DNA and dissociates preformed DNA-RNA polymerase complexes.

Aged rubradirin solutions as discussed above showed a decrease of inhibitory activity against protein synthesis concomitant with the emergence of inhibitory activity against RNA polymerase. This phenomenon is obviously attributable to the slow deglycosylation of rubradirin to its aglycone.

Rubradirin contains a coumarin residue similar to the one present in the novobiocins. The coumarin moiety is essential in novobiocin for its inhibition of DNA replication (REUSSER, unpublished results). Hence the effect of the rubradirin fragments on replicative DNA synthesis in toluenized *Escherichia coli* cells was investigated. None of the fragments inhibited this system significantly.

As discussed, rubradirin acts as a strong inhibitor of enzymatic peptide chain initiation and thus prevents the formation of ternary fMet-tRNA$_f$-mRNA-ribosome complexes. The rubradirin fragments were also tested in such an initiation system. Rubradirin strongly inhibited this reaction as expected. The aglycone of rubradirin acted also as a strong inhibitor of the reaction. All the other fragments did not affect initiation to any drastic extent, thus corroborating the results obtained in the poly(U)-directed phenylalanine incorporation system.

V. Discussion

Rubradirin prevents the formation of a stable 30S initiation complex formed with *E. coli* 30S ribosomal subunits, A-U-G and fMet-tRNA$_f$, if the reaction is carried out in low Mg^{2+} concentrations and depends on initiation factors. If the factor-induced 30S initiation complex is formed in the absence of rubradirin, subsequent addition of this antibiotic rapidly dissociated the complex.

The rubradirin-dissociated complex reaggregates upon adjustment of the Mg^{2+} concentration to 15 m*M* to a form now resistant to rubradirin. Initiation-factor-induced 70S initiation complex are also unstable in the presence of rubradirin. Again an increase of the Mg^{2+} concentration to 15 m*M* results in the reassociation of the 70S complex into a form resistant to rubradirin.

These results indicate that rubradirin functions as a selective inhibitor of the initiation-factor-dependent peptide chain initiation process (enzymatic initiation). Rubradirin has no effect on the Mg^{2+}-induced nonenzymatic process. The products of these two types of initiation differ, therefore, in some significant way from each other since rubradirin interacts only with the enzymatically formed complex.

Rubradirin contains an ansamycin-like moiety and is in this respect related to that family of antibiotics. Most members of this group (rifamycins, streptovaricins, tolypomycins) act as very potent inhibitors of bacterial RNA polymerase (RINEHART et al., 1974). Intact rubradirin, however, does not impair the function of RNA polymerase, but instead inhibits bacterial protein synthesis.

By contrast, the aglycone of rubradirin retains some inhibitory activity toward protein synthesis but proves to be an extremely potent inhibitor of RNA polymerase. The specific mode of action of rubradirin aglycone on RNA polymerase differs significantly from the one exerted by streptolydigin or rifamycins. These latter agents block initiation or chain elongation of de novo RNA synthesis but do not prevent the binding of the polymerase to its initiation sites on the DNA template (REUSSER, 1976). On the other hand rubradirin aglycone prevents the attachment of RNA polymerase to its promoter sites on the DNA template and dissociates preformed DNA-RNA polymerase complexes.

The highly methylated nitrosugar present in rubradirin must play a pivotal role in the ability of rubradirin and its aglycone to affect either protein or RNA synthesis. In the glycosylated form the antibiotic inhibits protein synthesis only, as an aglycone it primarily inhibits RNA synthesis and inhibits protein synthesis to a lesser extent.

Rubradirin B was also isolated from rubradirin fermentations in small quantities (HOEKSEMA et al., 1978b). It lacks the hydroxyl group at position 4 of the dipicolinic acid which is glycosylated in rubradirin. Consequently, it lacks rubranitrose. In addition, rubradirin B differs slightly from rubradirin itself in the stereochemistry at Δ^7 of the ansachromophore which is *trans* in the case of rubradirin B (Fig. 1). Surprisingly, rubradirin B does not impair the function of RNA polymerase at all, but exclusively affects ribosomal functions and that to a much lesser extent than rubradirin. This observation suggests that the nonglycosylated hydroxyl group in rubradirin aglycone is probably essential for interaction with RNA polymerase. The significance of the minor stereochemical difference between rubradirin and rubradirin B within the ansamycin chromophore is more difficult to assess.

Rubradirin is an extremely potent growth inhibitor of whole bacterial cells. The drug can thus penetrate the bacterial cell envelope. The aglycone of rubradirin, although extremely potent in cell-free systems, modestly inhibits growth of whole cells. This compound is thus an inefficient penetrator of the cellular envelope.

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Silver Sulfadiazine

M.S. WYSOR

I. Introduction

Silver sulfadiazine (AgSD) was synthesized by LOTT (1947) and found by FOX (1967, 1968a, 1968b, 1973, 1975, 1977b, 1979), FOX et al. (1969a, 1969b, 1970), and others (STANFORD et al. 1969; GROSSMAN 1970; WITHERS 1970; DICKINSON 1973; BURKE 1973) to be topically effective in the prevention and treatment of burn infections due to *Pseudomonas aeruginosa* and other microorganisms. Although AgSD is a sulfonamide drug, its mode of action differs from that of other sulfonamides since its antibacterial effectiveness is not reversed by p-aminobenzoic acid (FOX, 1969a). The original concept of FOX was to combine the oligodynamic heavy-metal antimicrobial action of silver ions (Ag^+) with the antimicrobial effect of sulfadiazine. When suspended in a 1% water-miscible ointment base, AgSD was effective topically in reducing the development of invasive, early burn wound sepsis (FOX, 1967).

Interest in the topical therapy of burns was greatly stimulated by the work of MOYER et al. (1965) and LINDBERG et al. (1965). MOYER used continuous soaks of dilute 0.5% silver nitrate as a topical agent; the latter used mafenide acetate (sulfamylon) ointment. It was discovered that many patients, initially thought to have third-degree burns, did not require skin grafts to cover the wounds. Much of the observed epithelization resulted from preservation of the deep dermal structures which acted as islands from which epithelium could grow, spread out, and heal the burn spontaneously. Without the use of local, topical therapy, these dermal structures were destroyed by infection which converted a deep second-degree burn to a full-thickness skin loss. A burn, thought to be third degree, can epithelize over along enough period of time if sufficient dermal structures remain.

The use of silver nitrate therapy for the treatment of burns is cumbersome and requires extensive and constant nursing care.

Sulfamylon also presented problems in its use. It is absorbed rapidly and is often associated with hyperventilation, metabolic acidosis, and inhibition of renal carbonic anhydrase mechanisms.

FOX (1968) prepared a series of insoluble salts of various antibacterial substances. Particular attention was paid to silver salts because of the oligodynamic anti-bacterial activity of the metal, a phenomenon known since ancient times. As far back as 2000 B.C., man realized that the quality of drinking water

could be improved with the use of copper and silver. Although silver nitrate was described as a pharmacological agent in a pharmacopeia published in Rome in 69 B.C., we cannot be sure that this salt was employed at the time, since Galen specifically rejected all metallic remedies in medicine in 131 A.D. In the writings of GEBER (702–765 A.D.) we have the first clear use of silver nitrate as a medicinal tool. Hence, it is to the Mohammedan school that we must ascribe the first extended use of silver therapeutically.

Avicenna in 980 A.D. employed silver medicinally in several ways, including the silvering of pills and use of silver filings as a blood purifier. The bluish discoloration, characteristic of argyria was described in Avicenna's pathology, the first such description of its kind.

In the late nineteenth century, the Swiss botanist, CARL VON NAGELI, found that small quantities of metal salts in water could kill algae. He did not think that such an extremely dilute solution of silver could produce a chemical effect on bacteria. NAGELI determined that 10^{-8} to 10^{-9} mol per liter of copper could kill spirogyra in less than 1 min. SAXL, THIEL, WOLD, and others of the early 1900's in repeating VON NAGELI'S experiments found that small amounts of silver are also lethal to bacteria.

Silver has been used for sanitation and sterilization of water against water-borne bacteria such a *E. typhi, B. cholerae, S. aureus,* and *E. coli.* Others include *B. Paratyphi, S. dysenteriae, S. paradysenteriae.* Because of the prolonged use of free silver ions in sanitation and its use on eating utensils, many studies have been conducted to determine long-term effects. The literature dealing with the effects of free silver ions in the human organism has revealed symptoms of poisoning and diseases (i.e., argyrosis and tissue hypereansia).

In the early 1960's there was a revival of interest in the use of silver nitrate (0.2%) in the treatment of burn infections, particularly by the organisms *Ps. aeruginosa.* Silver nitrate when applied topically turns black and forms insoluble silver chloride on the surface of the burn wound. Because of this precipitation with chlorides which populate the surface of the burn, silver nitrate's antibacterial activity is limited to the surface of the burn eschar. In some cases, in addition to methemoglobinemia, silver nitrate has precipitated the conversion of second degree burns to third degree burns.

FOX (1967) prepared silver sulfadiazine in the hope that a compound with all the antibacterial advantages of silver nitrate and none of its attendant disadvantages might be achieved. AgSD is stable (insoluble in water, alcohol, and ether) and does not appear to stain or darken like other silver salts such as silver nitrate. Silver sulfadiazine, when exposed to body fluids in burn therapy, appeared to yield the combined properties of the oligodynamic action of silver in addition to the advantages of an antibacterial agent. For use in burns, silver sulfadiazine was suspended in a water-dispersable ointment (Neobase). When used on burns in this manner, silver sulfadiazine presented the advantages of silver and an antibacterial agent without the use of hypotonic solutions and without withdrawing body electrolytes. AgSD appears to react only gradually with the body fluids when used in burn therapy with the result that AgSD when employed in burn wound therapy produces a sustained, effective concentration for as long as 24–72 h after a single application.

When silver sulfadiazine-containing ointment (micronized silver sulfadiazine cream – Silvadene, Marion Laboratories, Inc., Kansas City, Missouri) is applied to a raw wound or an open burn, a soothing sensation is experienced. This may be due to the fact that silver sulfadiazine is insoluble and is in suspension and not in solution and does not appear to dissolve in the body fluids except only gradually. In contrast, normally soluble substances when applied to a raw wound are irritating, probably due to hypertonicity which occurs when a soluble substance is dissolved in a body fluid. The insolublity of silver sulfadiazine which might at first appear to be a disadvantage thus appears to be a distinct therapeutic advantage.

When patients having large burns are dressed with a silver sulfadiazine-containing hydrophilic ointment, no morphine or other pain-relieving drug is needed prior to dressing changes. Indeed, because silver sulfadiazine ointment is painless and even pleasurable, it can readily and generously be applied over large areas of raw tissue in the instance of large burns.

It was observed that second-degree burns epithelize readily in 10 days and do so under the ointment. Deeper burns at 2 to 4 weeks are covered with a thick, yellow exudate which is usually sterile and through which islands of granulation tissue and epithelium can be seen. At this stage, treatment is discontinued and the burn is allowed to dry. A thick crust forms, and as it peels off from the edges of the wound, solid epithelium is found. This crust is similar to the usual burn eschar, but generally is softer and more pliable. It contains high concentrations of silver sulfadiazine and protein debris (Fox, 1969).

II. Chemical Characteristics

The solubility and ionization properties of this compound were reported by Nesbitt and Sandmann (1978) and silver sulfadiazine was characterized as a practically insoluble, white amorphous salt having a solubility product of 8.1×10^{-12} at 25° C and 0.1 M ionic strength. Silver sulfadiazine was first prepared by Fox (1968a) by reacting equimolar aqueous solutions of silver nitrate and sulfadiazine, such as sodium sulfadiazine, to yield a white paste, which is not light-sensitive. When dried, silver sulfadiazine is a fluffy, amorphous solid. A new method (Wysor, 1981a, 1981b, 1982) involves suspending sulfadiazine in water and adding concentrated NH_4OH until solution is effected. A solution of ammoniacal silver nitrate is then added dropwise with stirring to the solution of sulfadiazine. Analytically pure silver sulfadiazine separates immediately upon mixing of the solutions. There is no evidence that one method is superior to the other.

Silver sulfadiazine's molecular formula is $C_{10}H_9N_4O_2SAg$. AgSD decomposes at 277° C. In the presence of a nitrate buffer the silver ion dissociates completely from the sulfadiazine moiety and, therefore, is free to precipitate or complex with any suitable ligand. In the presence of endogenous chloride ion, silver sulfadiazine does not cause a rapid precipitation of silver chloride,

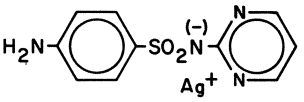


Fig. 1

under physiological conditions, even though the solubility product of silver chloride is exceeded (FOX and MODAK, 1974). Differences between the reactions of other silver sulfonamides and silver sulfadiazine were originally postulated to relate to the ionization constants for the sulfonamide moieties. FOX and MODAK (1974), SANDMANN et al. (1974), NESBITT (1976), and NESBITT and SANDMANN (1978) disagreed, believing AgSD's efficacy was directly related to its unique physical-chemical properties, where the dissociation and solubility are involved in providing the most favorable concentration of the silver ion for anti-infective activity.

Silver sulfadiazine is highly soluble in ammonia. Although elemental analysis has revealed a 1:1 stochiometry, which would tend to yield the structure in Fig. 1, structural studies (BULT and KLASEN, 1978; COOK and TURNER, 1975; STRUSS, 1974; BAENZIGER and STRUSS, 1976) have indicated that the chemical structure of silver sulfadiazine is more complex than previously believed (NESBITT and SANDMANN, 1978). Each sulfadiazine molecule chelates one silver atom through O(2) and N(2) and bridges two others in the pyrimidine grouping N(1) and N(6). The bridged atoms are centrosymmetrically related, the configuration about each silver atom is roughly tetrahedral. Thus the structure appears to be oligomeric with three silver atoms linked to three sulfadiazine molecules.

III. Toxicology and Pharmacology

Silver sulfadiazine's LD_{50} by intraperitoneal administration in mice is 140 mg/kg (FOX et al., 1979), greater than 1000 mg/kg by oral administration (WYSOR, 1975, 1977; HARRISON, 1979), and greater than 1000 mg/kg by subcutaneous administration (WYSOR, 1975, 1977). Toxicity values for intraperitoneally administered silver sulfadiazine tend to parallel the values found for equimolar solutions of silver nitrate (FOX, 1973). Injections of 1050 mg/kg AgSD in water proved lethal, but IP injections of 1050 mg/kg AgSD, when the drug was suspended in peanut oil, was tolerated (WYSOR, 1980). Doses of AgSD administered at 1000 mg/kg/day orally for 30 days proved to be well tolerated with no histological evidence of silver deposition seen in internal organs such as kidney, liver, spleen, intestines or lung (WYSOR, 1975, 1977). When administered orally four times per day for 7 days, AgSD also was well tolerated with no evidence of acute toxicity, behavioral manifestations, neurological symptoms, or diarrhea; there was no histological evidence of argyrosis or deposition of hemosiderin. No evidence of crystalluria or convulsions due to electrolyte depletion (tetany) were seen. HARRISON (1979), using a similar dose of AgSD, found

that approximately 3% of the orally administered dose of AgSD was absorbed systemically. Measureable transfer of radio-labeled $Ag^{110}SD$ occurred from the digestive tract into a number of organs. Peak concentrations occurred at 1 and 3 days postingestion, depending upon the organ (blood and liver, 1 day; kidney and lungs, 3 days). The variations in organ distribution may be an indication that the drug is incorporated by pulmonary and hepatic macrophages. When those systems are normal, complete excretion within 3 weeks may be anticipated.

Toxicity of silver sulfadiazine as judged in burned mice treated with 1% by weight silver sulfadiazine suspension or ointment was not apparent. Absorption was evaluated by excretion of sulfadiazine in the urine after implantation in the cutaneous tissues of dogs. Doses of 100 mg either in 1% suspension or 0.5% ointment resulted in levels of less than 10 mg per 100 ml of urine for 3 days. These low values are in marked contrast to the high levels obtained after implantation of the soluble sodium salts. After application of 300 mg of the 1% by weight AgSD ointment to 1 sq m burned surface in patients the blood levels of sulfadiazine ranged from 3.0 to 3.7 mg%, and from 116 to 336 mg% of free sulfadiazine was excreted in the urine in the succeeding 24 h. This represents 4% to 13% of the total amount applied to the burn surface (Fox, 1973). When silver sulfadiazine is utilized topically on burn wounds, sizeable amounts of sulfadiazine alone are detected in the blood but at levels well below the 4–10 g daily used in conventional systemic sulfonamide threrapy (Fox, 1968a, 1973, 1977b; Delaveau and Friedrick-Noue, 1977). In the treatment of burn wounds involving extensive areas of the body, the serum sulfa concentration may approach adult therapeutic levels (8–12 mg%).

The main toxic manifestation of silver sulfadiazine is an allergic reaction due to the sulfonamide component of the drug. It is not known whether the acute leukopenia sometimes associated with use of AgSD is an allergic manifestation (Chan et al., 1976; Kiker et al., 1977; Jarrett et al., 1978; Valente and Axelrod, 1978; Fraser and Beaulieu, 1979). Berger (1976) presented evidence that the leukopenia was due to the silver component and not the sulfonamide component of AgSD. Linn and Chang (1980) reported toxic effects of silver sulfadiazine against human spermatozoa.

Other toxic manifestations of topical silver sulfadiazine therapy include burning, rash, itching, and a very low incidence of interstitial nephritis (Owens et al., 1974). However, silver sulfadiazine treatment has been discontinued in only about 0.9% of the patient population.

There is a difference of opinion as to whether significant quantities of silver are absorbed with topical use of silver sulfadiazine. Harrison (1979) claims that little silver is absorbed beyond the top layer of epidermal cells, whereas Grabowski and Haney (1972) claim deposits of silver metal in the basement membrane of several organs.

Silver sulfadiazine was shown not to be mutagenic in the Ames test (McCoy and Rosenkranz, 1978). No published studies on the teratogenic potential of silver sulfadiazine are available. Silver sulfadiazine is well tolerated by normal tissues. Various proposals have been advanced to explain its lack of toxicity in normal tissues. Fox (1977a) proposed that AgSD does not interfere with

epithelial regeneration because the DNA content of tissue cells is 100–1000 times greater than that of bacteria. Hence, an inhibitory Ag/DNA ratio cannot be obtained. ROSENKRANZ (1979) claimed that human cells are impermeable to AgSD and that that impermeability is the basis of the drug's selective toxicity.

IV. Growth Inhibitory Properties

A. Bacteria

Silver sulfadiazine, unlike silver nitrate, is bactericidal (FOX and STANFORD, 1971; Fig. 2) with a broad spectrum of activity against both Gram-positive and Gram-negative organisms (WLODKOWSKI and ROSENKRANZ, 1973; CARR et al., 1973; OEPIANESI, 1975; BRIDGES and LOWBURY, 1977; NAGESHA, 1978; BAARS et al., 1980; and YURA et al., 1980). Although CARR et al. reported that AgSD was effective in vitro against bacteria that were resistant to sulfonamides, MCMANNUS et al. (1979) claimed that such was not the case.

Silver sulfadiazine was found to be effective against surface lesions of *Treponema pallidum* (CHANG and WEINSTEIN, 1975a).

Studies by CASON and LOWBURY (1968) found no emergence of silver nitrate resistance among various bacteria including *Pseudomonas*. Resistance to silver sulfadiazine has however been detected both in clinical isolates (ROSENKRANZ

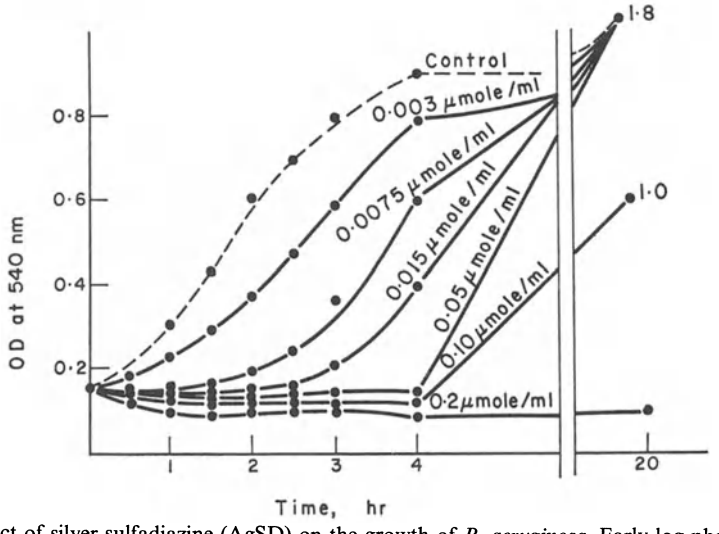


Fig. 2. Effect of silver sulfadiazine (AgSD) on the growth of *P. aeruginosa*. Early log phase cultures at an O.D. of 0.15 were added to tubes containing the concentrations of AgSD. These were incubated at 37° C on a shaker and the O.D. was measured hourly and after 20 h. Drug concentrations of 0.10 μmol/ml or below were bacteriostatic; the concentration of 0.2 μmol/ml was bactericidal. (MODAK SM, and FOX CL Jr, Biochem. Pharm. 22:2393 1978 with permission)

et al., 1974; LOWBURY et al., 1976; HEGGERS and ROBSON, 1978; GAYLE et al., 1978; HENDRY and STEWART, 1979) and in laboratory induced strains (MCMANNUS, 1979). Resistance appears to be plasmid induced (CARR and ROSENKRANZ, 1975; MCMANNUS, 1979). In 1976, MONAFO et al. discovered cerium nitrate to be a topical antiseptic for burns. A synergistic effect between cerium nitrate and silver sulfadiazine was observed (FOX et al., 1977; ROSENKRANZ, 1979a; HEGGERS et al., 1979; HELVIG et al., 1979; SAFFER et al., 1980; MUNSTER et al., 1980; ARTIGAS et al., 1980). FOX (1977a, 1978) subsequently synthesized cerium sulfadiazine. Evaluation of this agent is in progress.

B. Fungi

Silver sulfadiazine is effective topically against strains of surface dermatophytes (SPECK and ROSENKRANZ, 1974) and fungi (WLODKOWSKI and ROSENKRANZ, 1973). It is also effective against most strains of *Candida albicans*.

C. Viruses

TOKUMARU et al. (1974), CHANG (1975), CHANG and WEINSTEIN (1975a), and CHANG and WEINSTEIN (1975b) reported the clinical efficacy of silver sulfadiazine in vitro and against herpes simplex keratoconjunctivitis in rabbits. It was postulated that AgSD's antiviral activity was due to direct inactivation of extracellular virus, a hypothesis consistent with the observation that AgSD does not penetrate cell membranes (ROSENKRANZ, 1979b).

Tests performed by STEPHEN and WYSOR (1980, unpublished) showed AgSD to have activity against Venezuelan equine encephalitis virus and rift valley virus fever; however, no clinical evaluations were made (STEPHEN, 1980).

D. Protozoa

Activity of silver sulfadiazine against *Trichomonas vaginalis* and *Tetrahymena pyriformis* has been reported (FOX, 1973; Marion Laboratories Report, 1973). AgSD is active against *Trypanosoma rhodesiense* (WYSOR, 1980, 1982), *Trypanosoma cruzi* (epimastigotes) (WYSOR and GOTTLIEB, 1982, unpublished), *Leishmania braziliensis* (promastigotes and amastigotes) (WYSOR, 1980), and *Plasmodium falciparum* in vitro (WYSOR, 1980). Silver sulfadiazine is curative of mouse malaria (*Plasmodium berghei*) (WYSOR, 1975) and *Trypanosoma rhodesiense* (WYSOR, 1980; WYSOR and SCOVILL, 1982). In addition, AgSD has demonstrated activity in the mouse malaria prophylactic test (WYSOR, 1980). No synergistic effect was found between cerium nitrate and silver sulfadiazine against *Trypanosoma rhodesiense* in vitro; neither was activity observed for cerium nitrate alone (WYSOR, 1980, unpublished).

V. Mode of Action

Various proposals have been advanced to explain silver sulfadiazine's chemotherapeutic activity. In vitro the drug is effective against eukaryotic and prokaryotic cells. The original concept of FOX (1968b), was that in the presence of bacteria the silver ion was released from silver sulfadiazine. This silver ion subsequently displaced a hydrogen between the base pairs of DNA, distorting the helical structure. This type of binding for silver ions was postulated in 1966 by JENSON and DAVIDSON. Unlike other oligodynamic metals, silver reacts directly with the purine and pyrimidine bases rather than with the phosphate groups. Two types of binding complexes are formed, both of which are chemically and biologically reversible. For guanine-cytosine-rich DNA, type I binding is more prevalent. This consists probably of a sandwiching of silver between the members of a base pair of the substitution of the silver ion. The substitution distorts the DNA helix from 3.0 Å to 3.8 Å. The binding of a positive ion by a nitrogen atom of the ring modifies the electronic state of the base or base pair and must be accompanied by some modification in the ultraviolet spectrum. There will be a change in the n-pi* transition since one of the lone pair of electrons is no longer available. It is likely that the pi-pi* transition is simultaneously affected. The total effect appears to be a red shift of the spectrum (bathochromic effect). Perturbations of the charge distribution in the ring and in the base pair are accompanied by a change of transition temperature and hypochromicity which are qualitatively predictable. These effects will depend on the position of the silver atom, and therefore of base sequence.

The first complex formed with silver and DNA can be viewed as follows: during the first stage, binding of silver to G increases the mobility of the proton in N_1 along the hydrogen bond $(N_1(G))-(N_3(C))$; cytosine is thus partially positively charged, as if it were in an acidic medium. After formation of the first complex, the formation of the second complex begins. For this, the stochiometry is roughly one silver per two nucleotides. The liberation of protons during the second-stage complex formation is observed.

The release of a proton from G and T is accompanied by the rupture of hydrogen bonds in which the NH group is engaged. This escape is favored by sequences A–A or G–G in the same strand. Induced dipoles in this case created by one base pair increase the positive charge of G and T and provoke the liberation of a proton. As silver binds to DNA, one detects an increasing stability of the DNA ligand. This can be interpreted in terms of base–base interaction. According to the calculations of DEVOE and TINOCO (1962), it is possible to assign to each base pair a total free energy, which is the sum of the London van der Waals interactions between nonpaired bases in two adjacent base pairs (stacking energy) and the two bases in a base pair.

A study of silver ion binding by nucleic acids and synthetic ribo and deoxyribo polynucleotides demonstrates that a strong complex between silver and nitrogen atoms of bases is formed *reversibly*. Base reactivity varies considerably with chain length, but a cooperative phenomenon is found in each case. Thermal stabilization occurs simultaneously, and the increase of melting temperature corresponds to calculated changes of stacking energy between base pairs. In

the second complex, a new ordered structure insensitive to temperature change is formed. The silver ion complexing is chemically and biologically reversible. The buoyant density of DNA in Cs_2SO_4 density gradient increases when silver ion interacts with DNA. The buoyant density is about 0.15 g/ml for 0.5 silver bound per nucleotide.

When cyanide is added to silver-DNA solutions in a 10 to 100-fold excess, the ultraviolet absorption of the DNA reverts to that of the original DNA solution, and the buoyant density of the resulting DNA is identical to that of the original DNA. These experiments indicate that the spectral and density changes are not due to an irreversible change in the primary structure or the secondary structure of the DNA. The binding of silver ions to DNA affords protection against any degrading enzymes that might be present. Furthermore, DNA that has been incubated at room temperature for 24 h in the presence of silver ions remains infectious. This same phenomenon can be seen with RNA.

Studies on silver sulfadiazine with a view to the possibility that DNA was the target were begun by Fox (1968a) and continued by Wysor and Zollinhofer (1972, 1973), Modak and Fox (1973) and Fox and Modak (1974). These studies tended to reinforce the view that DNA was the ultimate target of silver sulfadiazine. Early experiments by Fox, utilizing dialysis bags, bacteria, and/or DNA and silver sulfadiazine, showed that silver (and some sulfonamide) were becoming bound to DNA, or other bacterial nucleic acids.

Indications by Rosenkranz (as reported in Fox et al. 1969b) that silver sulfadiazine is relatively firmly bound to DNA and that this bonding was different from that of silver nitrate and other silver salts prompted work by Wysor and Zollinhofer (1972) on the possibility that DNA was the target of the drug. Studies were performed on the effects of silver sulfadiazine on the stability and synthesis of the cellular nucleic acid and protein fractions in *Pseudomonas aeruginosa* and cell-free systems (Fig. 3, 4). To assess the stability of the cellular DNA, RNA, and protein fractions in the presence of silver sulfadiazine, cells were prelabeled for two to three generations in the early log phase with either 20 μCi of thymidine-6-[^{3}H], 3 μCi of [^{14}C]-uracil or 2.5 μCi of [^{14}C]-aspartic acid per 100 ml of culture medium. Silver sulfadiazine (25 μg/ml) was added to one of the groups of medium. Protein synthesis remained unaffected in the presence of silver sulfadiazine during the first 60 min. After this period, partial inhibition of protein synthesis became apparent. Uracil uptake, reflecting RNA synthesis, was not impaired by AgSD during the first 60 min. Even after this period, RNA synthesis did not cease. DNA synthesis was not affected by the antimicrobial during the first 20 min. After this time, DNA synthesis decreased slightly, falling off rapidly after 60 min.

Pseudomonas aeruginosa cells were prelabeled with thymidine for two or three generations and then treated with silver sulfadiazine for 30 min. Cellular DNA was isolated via protoplast formation. Samples of protoplast suspensions were lysed directly on neutral or alkaline sucrose gradients (Fig. 5). The profile of the alkaline gradient showed that most of the DNA originating from the treated cells sedimented lower in the gradient than the controls. The profile suggested that complexes of DNA fragments and bound silver sulfadiazine were sedimenting faster owing to their increased sedimentation coefficient.

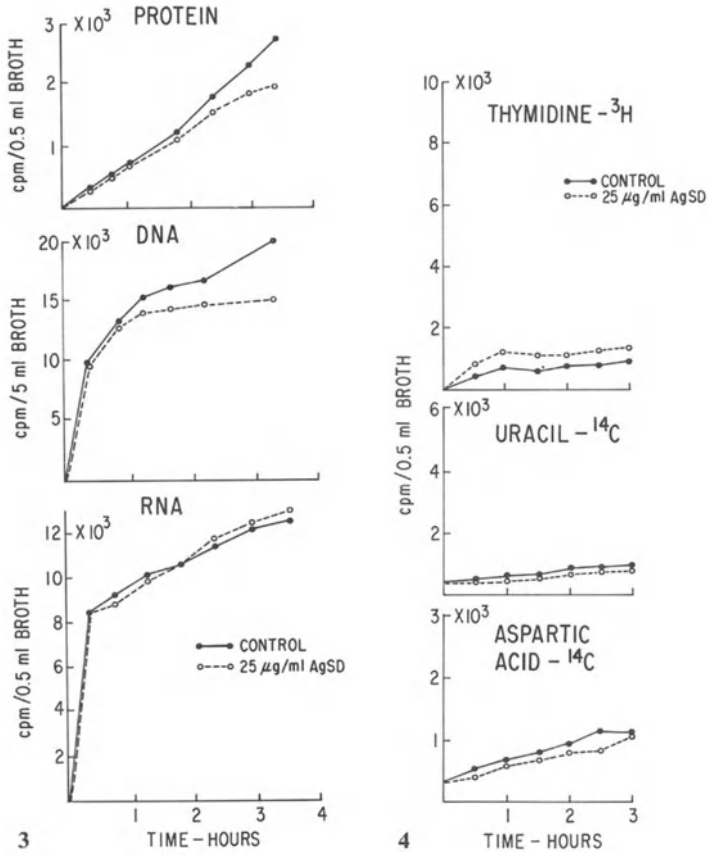
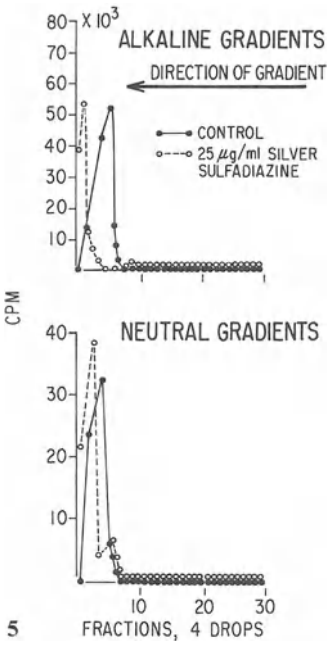
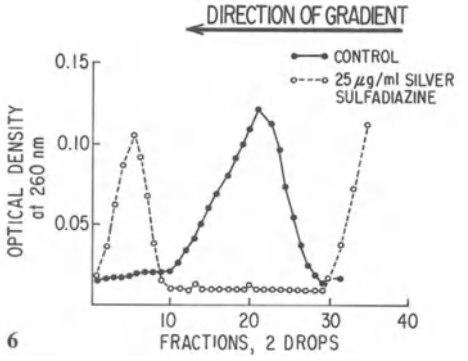


Fig. 3. Effect of silver sulfadiazine on protein and nucleic acid synthesis in *P. aeruginosa*. 2 µCi of ^{14}C-aspartic acid, 2 µCi of ^{14}C-uracil, or 30 µCi of ^{3}H-thymidine was added per 100 ml of culture broth to follow progress of protein, RNA, or DNA synthesis, respectively. Precursors and silver sulfadiazine were added during the early log phase of the growth cycle. Samples were withdrawn from the cultures at appropriate time intervals. The samples were made 5% with cold trichloroacetic acid; the acid insoluble fraction was collected on 0.45 µm membrane filters (Millipore Corp.) and counted by conventional methods. ● = control; ○ = 25 µg/ml AgSD. (WYSOR, M.S. and ZOLLINHOFER, R.E., Path. Microbiol. 38:299 1972, S. Karger, A.G. Basel, with permission)

Fig. 4. Effect of silver sulfadiazine on stability of prelabeled nucleic acid or protein fractions in *P. aeruginosa*. Cells were prelabeled for two or three generations at the early log phase with either 20 µCi of ^{3}H thymidine, 3 µCi of ^{14}C uracil, or 2.5 µCi of ^{14}C aspartic acid per 100 ml of culture medium. After the labeling period, cells from four flasks were recovered by centrifugation, washed and resuspended in 100 ml of fresh culture medium. This suspension was divided into equal portions and silver sulfadiazine (25 µg/ml) was added to one of them. The cultures were incubated and samples were withdrawn at regular time intervals. The cells were removed by centrifugation and 0.5 ml samples of clear broth were counted in 15 ml of diotol. ● = control; ○ = 25 µg/ml AgSD. (WYSOR, M.S. and ZOLLINHOFER, R.E. Path. Microbiol. 38:299 1972, S. Karger, A.G. Basel, with permission)

Isolated DNA of *Pseudomonas aeruginosa* was mixed with silver sulfadiazine and allowed to stand at room temperature for 30 min. The solutions were then rendered alkaline to effect separation of the double-stranded DNA into single strands. The denatured products were sedimented through alkaline sucrose





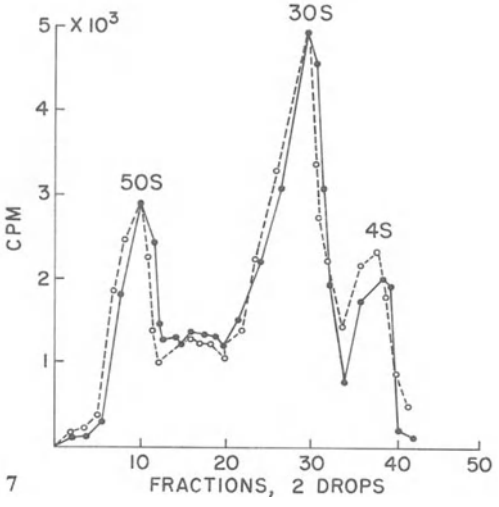


Fig. 5. Effect of silver sulfadiazine on sedimentation patterns of *P. aeruginosa* DNA in vivo. Cells were prelabeled with ^{3}H thymidine for two to three generations. Silver sulfadiazine was then added (25 μg/ml) and the cultures were reincubated for 30 min. Protoplasts were then prepared by the lysosome-ethylenediammine tetraacetic acid technique. Samples of protoplast suspension (0.2 ml) were directly lysed on top of 4.6 ml of alkaline or neutral 15–30% sucrose gradients. The gradients were centrifuged at 30,000 rpm for 60 min at 20° C in an SW-39 rotor. Fractions were collected from the bottom of the tubes. DNA was precipitated with 5% trichloroacetic acid by using tRNA as carrier. The precipitates were collected on membrane filters (Millipore Corp.) and assayed for radioactivity. ●=Control; ○=25 μg/ml silver sulfadiazine. (WYSOR, M.S. and ZOLLINHOFER, R.E. Path. Microbiol. 38:300 1972, S. Karger, A.G. Basel, with permission)

Fig. 6. Effect of silver sulfadiazine on sedimentation patterns of isolated *P. aeruginosa* DNA on alkaline sucrose gradients. A 300 μg/ml amount of *P. aeruginosa* DNA was applied per gradient. Concentration of silver sulfadiazine was 25 μg/ml. The gradients were 4.6 ml of 5–20% sucrose containing 0.1 N NaOH and 0.9 M $NaNO_3$. The gradients were centrifuged at 39,000 rpm for 6 h at 4° C in the SW-39 rotor. Fractions were collected from the bottom of the tubes and processed as described in Materials and Methods. ●=Control; ○=25 μg/ml silver sulfadiazine. (WYSOR, M.S. and ZOLLINHOFER, R.E. Path. Microbiol. 38:301 1972, S. Karger, A.G. Basel, with permission)

Fig. 7. Effect of silver sulfadiazine on sedimentation pattern of *P. aeruginosa* cell extracts. Cells were prelabeled with ^{14}C-uracil for two to three generations. Silver sulfadiazine (25 μg/ml) was then added, and the cultures were reincubated for 30 min. Cells were disrupted mechanically, and 0.2 ml portions of cell extract were applied to 4.5 ml of 5–20% sucrose gradients containing 0.01 M tris-hydrochloride (pH 7.4) and 10^{-4} M magnesium acetate. The gradients were centrifuged at 39,000 r.p.m. for 180 min at 4° C. Acid precipitable material was colleced and assayed as described in legend to Fig. 3. (WYSOR, M.S. and ZOLLINHOFER, R.E. Path. Microbiol. 38:302 1972, S. Karger, A.G. Basel, with permission)

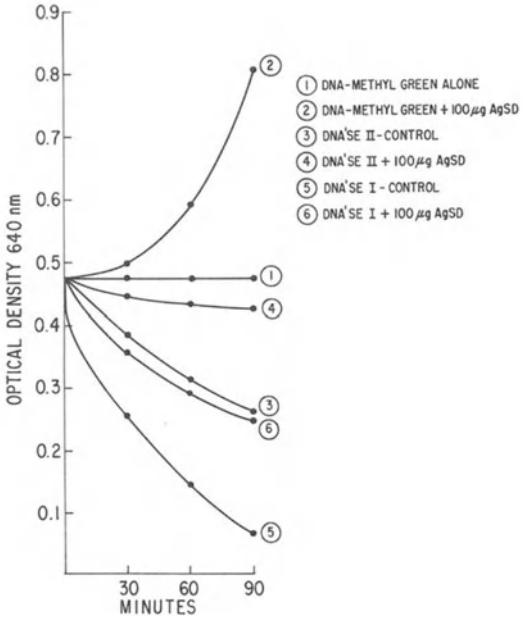


Fig. 8. Effect of silver sulfadiazine on stability of methyl green DNA complex and deoxyribonuclease I and II activities. Reaction mixtures contained in a total volume of 6 ml: 5 ml of methyl green reagent, pH 4.6; 60 units of deoxyribonuclease I or II; 100 µg of silver sulfadiazine. (WYSOR, M.S. and ZOLLINHOFER, R.E. Path. Microbiol. 38:305 1972, S. Karger, A.G. Basel, with permission)

gradients (Fig. 6). The sedimentation profiles of the experimental run, as compared with that of the control, showed an increased sedimentation coefficient for the experimental samples.

Cells were prelabeled with [^{14}C]-uracil for two to three generations and were exposed to AgSD (25 µg/ml) for 30 min (Fig. 7). Extracts prepared from these cells showed no decrease in the radioactive label in the regions of the 50S and 30S areas, as compared with the control gradient. Neither was there a loss of label in the transfer RNA fractions.

The effect of silver sulfadiazine on a cell-free peptide synthesis was studied in a polyuridylic acid (Poly-U) directed phenylalanine incorporation system. Silver sulfadiazine did not inhibit this system to any appreciable extent. DNA polymerase primed with synthetic or natural templates remained largely insensitive to silver sulfadiazine. Experiments were also carried out to determine if silver sulfadiazine might be altering the coding specificity of synthetic polydeoxyribonucleotides in the cell-free DNA polymerase system. No evidence was found in these experiments that AgSD caused incorporation of faulty nucleotides by the enzyme DNA polymerase. AgSD failed to interfere with DNA-directed RNA synthesis.

Silver sulfadiazine increased the T_m of *Pseudomonas aeruginosa* DNA by 25° C, and caused extensive stabilization of double-stranded DNA.

The binding affinity of DNA for methyl green is dependent on the native configuration of DNA. Addition of silver sulfadiazine to DNA–methyl green

complex resulted in the displacement of the dye from the complex (Fig. 8). This indicated that silver sulfadiazine caused substantial changes in the native structure of isolated DNA. In addition, the enzymatic activity of deoxyribonucleases I and II with DNA–methyl green complex serving as substrate was radically altered. Silver sulfadiazine appeared to inhibit DNA replication, while not inhibiting DNA synthesis or RNA and protein synthesis in whole cells of *P. aeruginosa*.

Silver sulfadiazine did not cause degradation of cellular RNA or ribosomes. Since cell-free protein synthesis was not affected by AgSD, it appeared that the antimicrobial did not impair the function of or degrade the ribosomes and synthetic messenger RNA. By the same measure, DNA and RNA synthesis in cell-free extracts was not affected by AgSD and the fidelity of synthetic polydeoxyribonucleotide primers remained unchanged. It was suggested by this work of WYSOR and ZOLLINHOFER (1972) that AgSD inhibited *P. aeruginosa* by preventing DNA replication.

ROSENKRANZ and ROSENKRANZ (1972) demonstrated that silver sulfadiazine interacted with isolated DNA to form nondissociable complexes. They disagreed with the work of FOX et al. (1969b) in that they obtained no evidence that only the silver ion becomes associated with DNA with the subsequent release of the sulfadiazine moiety.

Addition of silver nitrate and silver sulfadiazine to DNA resulted in a shift of the absorption maximum from 257.5 to 263 nm. It was stated that the effects of the two silver compounds were different, as evidenced by differences in hyperchromic shifts and ratios of absorbancies at selected wavelengths. The lack of effect of both compounds on the absorbance in the visible range indicated that no precipitation of DNA occurred when these substances interacted with DNA.

The addition of increasing amounts of silver nitrate to calf thymus DNA resulted in increases in the sedimentation coefficient. However, no such concentration dependence was observed when AgSD was added to DNA. It was consistently found that the maximal effect of AgSD was always smaller than that produced by the addition of equimolar amounts of silver nitrate.

Sedimentation studies on a Spinco model E analytical ultracentrifuge equipped with Schlieren optics were performed on the binding of both silver compounds to DNA (see Fig. 9).

Analysis of the amount of UV-absorbing material sedimenting in mixtures of AgSD and DNA revealed that such mixtures contained UV-absorbing nonsedimenting species, presumably unbound AgSD (AgSD absorbs in UV). As the time of exposure of the DNA to AgSD increased, the amount of unbound UV-absorbing material (AgSD) decreased. It was suggested that the reaction between DNA and AgSD is slow.

ROSENKRANZ found that the presence of silver nitrate had a profound effect on the thermal denaturation profile of calf thymus DNA. Silver sulfadiazine had a similar effect on the thermal denaturation profile of DNA. This effect depended on the AgSD concentration and was influenced by the duration of contact between the DNA and AgSD. Silver sulfadiazine was almost as potent as silver nitrate in modifying the "melting-out" behavior of the DNA. Once

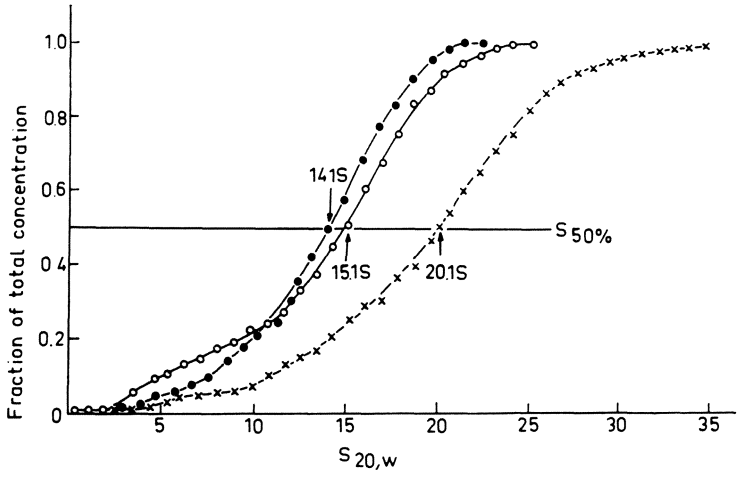


Fig. 9. Distribution of sedimentation coefficients of DNA and silver DNA complexes. ●=Control DNA, o=AgSD DNA (AgSD/DNA-P=1.0); X=$AgNO_3$-DNA ($AgNO_3$/DNA-P=1.0). (ROSENKRANZ, H.S. and ROSENKRANZ, S. Antimicrob. Ag. Chem. 2:375 1972 with permission)

denaturation occurred, it was irreversible indicating that silver sulfadiazine did not cross-link DNA strands.

Studies using cesium chloride density gradient indicated that whereas $AgNO_3$-DNA complexes dissociated in the presence of CaCl due to formation of insoluble AgCl, AgSD-DNA complexes did not dissociate (Fig. 10). Using $CsSO_4$ density gradients, it was found that the $AgNO_3$-DNA complex had a buoyant density higher than that of the control DNA, confirming the earlier findings of JENSON and DAVIDSON (1966). The AgSD-DNA complex showed a decreased buoyant density in $CsSO_4$ (Fig. 11).

The addition of KCN to $AgNO_3$-DNA restored the rate of sedimentation in a sucrose gradient to a level similar to that of unmodified DNA. The addition of KCN to the AgSD-DNA complex did not result in dissociation of the complex.

It was found that NaSD, when added to DNA, did not significantly affect the sedimentation behavior of calf thymus DNA but did greatly influence the thermal helix-coil transition profile. Dialysis of NaSD-DNA reversed the effect of the NaSD, whereas dialysis did not remove AgSD. Exposure of DNA to mixtures of NaSD and AgSD revealed that NaSD competed with AgSD for the polydeoxynucleotide. On the other hand, addition of NaSD after AgSD had no effect on the sedimentation coefficent. The results with NaSD suggested that the sulfadiazine moiety was involved in the binding of AgSD to DNA.

Therefore, ROSENKRANZ postulated that the reaction between AgSD and DNA occurred in two stages: (1) a weak and reversible interaction (intercalation) between DNA and the sulfadiazine moiety, and (2) a tight binding involving the silver atom. In the first stage, sodium sulfadiazine would compete with the AgSD for the DNA. It was claimed, however that this mechanism of AgSD

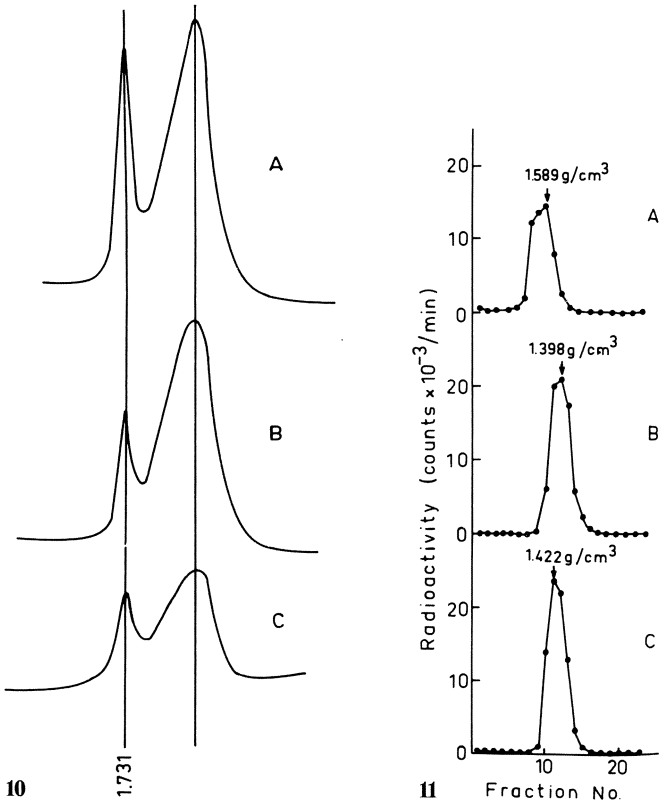


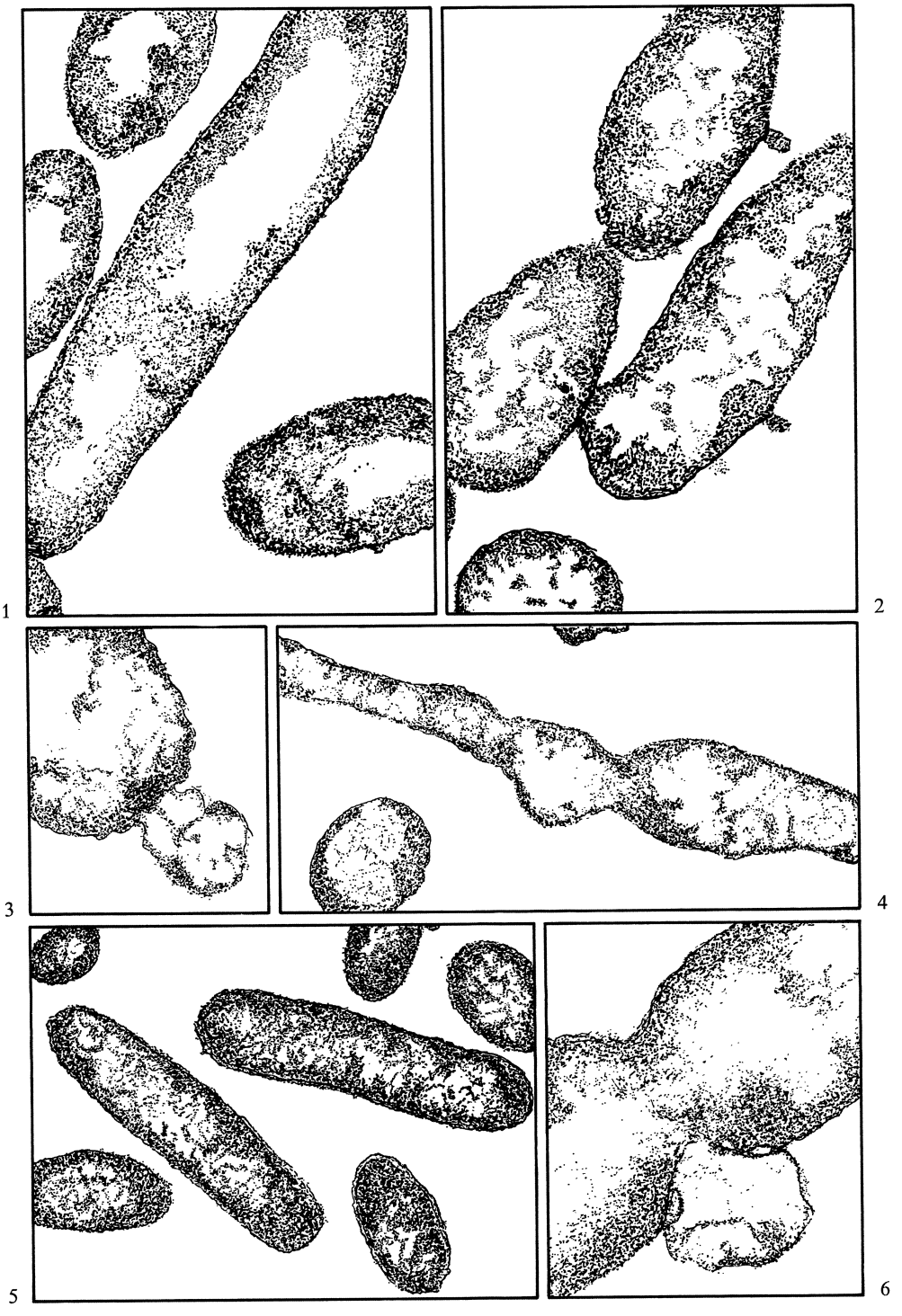
Fig. 10. Buoyant densities in CsCl of *A* normal calf thymus DNA; *B* $AgNO_3$-DNA, $AgNO_3$/DNA-P = 1.0; and *C* AgSD-DNA, AgSD/DNA-P = 1.0. The band at the *extreme left* represents the position of the marker DNA (*Micrococcus lysodeikticus* DNA: 1.731 g/cm^3). (ROSENKRANZ, H.S. and ROSENKRANZ, S. Antimicrob. Ag. Chem. 2:378 1972 with permission)

Fig. 11. Banding peoperties of silver-DNA complexes in Cs_2SO_4. *A* $AgNO_3$-DNA ($AgNO_3$/DNA-P = 1.0); *B* AgSD-DNA (AgSD/DNA-P = 1.0); and *C* control ^{3}H-labeled DNA from chicken embryos. (ROSENKRANZ, H.S. and ROSENKRANZ, S. Antimicrob. Ag. Chem. 2:378 1972 with permission)

binding to DNA in vitro was *not* the mechanism of action of AgSD in vivo (ROSENKRANZ and CARR, 1972).

COWARD et al. (1973) presented data from electronmicroscopic examination of *Pseudomonas* bacterial cells treated with silver sulfadiazine, showing that a defect was created by the drug in the cell membrane and/or cell wall, (see Fig. 12). Indeed, later electron microscopic studies on *Enterobacter cloacae* tended to reinforce this view (ROSENKRANZ et al., 1974).

Studies by MODAK and FOX (1973), and FOX and MODAK (1974), using doubly labeled silver sulfadiazine indicated that the sulfadiazine moiety was released at the membrane of the bacteria and that the silver bound to the nucleic acids in vivo. This work was clearly in contrast with the results of ROSENKRANZ and CARR (1972). FOX and MODAK (1974) proposed that silver,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and not sulfadiazine, was bound by bacteria. During inhibition with AgSD, no bacterial binding of SD occurred. Inhibition by NaSD, however, requires considerably higher concentrations of drug and utilizes a different mode of action, i.e., pABA antagonism, and the binding of sulfadiazine (SD) might occur. Against *Pseudomonas* and *Staphylococcus* strains, measurements of the inhibitory concentration of SD were made. For both organisms, 5 µmol/ml was partially inhibitory, and 15 µmol/ml was completely inhibitory. The binding at the inhibitory concentration was less than 1% of the amount of the drug present, representing a 100-fold increase over the minute uptake when inhibition did not occur. During inhibition with AgSD (0.1 µmol/ml) no SD uptake occurred, although as much as 20% of the [^{110}Ag] was taken up by the cells.

Studies to indicate the dissociation of AgSD prior to binding with DNA were performed by three different methods: (1) equilibrium dialysis, (2) separation in sephadex columns, (3) UV absorption of DNA. Equilibrium dialysis results showed that no SD became bound to DNA either from Ag^{35}SD or Na^{35}SD, in contrast to a binding of 25% of [^{110}Ag] from [^{110}Ag]SD (see Table 1). Separation by Sephadex columns showed that in the presence of Ag^{35}SD-DNA, there was no elution of radioactivity from ^{35}SD. The elution patterns of [^{110}Ag]-DNA complexes from [^{110}Ag]SD and [^{110}Ag]NO_3 showed that DNA reacted more with soluble [^{110}Ag]NO_3, acquiring more radioactivity than with slightly soluble [^{110}Ag]SD (Fig. 13).

The UV absorption of the Ag-DNA complex formed from AgSD was similar to that formed from $AgNO_3$ (see Table 2). The UV absorption of the DNA was not increased by either silver compound until the Ag/DNA-P ratio in the reaction mixture was 0.25. At this ratio, the Ag-DNA complex formed from $AgNO_3$ showed an increase in absorbance, and higher ratios showed proportionately greater increases (agreeing with the report of JENSON and DAVIDSON, 1966, which indicated an increased absorbance at a ratio of 0.26 or higher). The Ag-DNA complex formed from AgSD showed no such increase until the Ag/DNA-P ratio reached 1.0. The complexes resulting from $AgNO_3$ attained consistently higher Ag/DNA ratios than those from AgSD, due to the greater solubility of $AgNO_3$. Because of the agreement in all three experimental approaches, FOX and MODAK (1974) concluded that AgSD dissociates upon combining with DNA, and that the SD moiety does not combine.

Fig. 12. Ex. **1**. Untreated *P. aeruginosa* ×40,000. Ex. **2**. *P. aeruginosa* 686 after exposure to AgSD (2.8×10^{-5} M) for 1 h. The cytoplasm is almost completely devoid of ribosomes. Survival rate was 0.027% ×100,000. Ex. **3**. "Bleb" arising from the cell surface of *P. aeruginosa* treated with AgSD for 1 h. Fibrillar components within lighter-staining nuclear area are clearly defined, ×100,000. Ex. **4**. A typical elongated form of *P. aeruginosa* after 1 h of exposure to AgSD, ×40,000. Ex. **5**. *P. aeruginosa* treated with $AgNO_3$ (3.7×10^{-5} M) for 1 h. Survival rate was 0.038%. Central aggregate of nuclear material is fibrillar in appearance, ×40,000. Ex. **6**. *P. aeruginosa* R-1, a strain resistant to AgSD, after exposure to AgSD (2.8×10^{-5} M) for 1 h. Survival rate was 73%. A few small "blebs" are seen at cell surface, ×40,000. (Drawings taken from original electron photomicrographs which appeared in COWARD, J.E., CARR, H.S., and ROSENKRANZ, H.S. Antimicrob. Ag. Chem. 3:622–633 1973 with permission)

Table 1. Binding of silver and sulfadiazine to DNA by equilibrium dialysis[a]

Compounds	Radioactivity associated with DNA (% of total radioactivity)
$Na^{35}SD$	0
$Ag^{35}SD$	0
$^{110}AgSD$	25

[a] Dialyzing bag contained 3 ml of DNA at a concentration of 300 µg/ml. Dialysis was carried out for 20 h against 200 ml of 0.5 µmol of the above compounds in ammoniacal solution per ml. pH 8.0 (total radioactivity corresponds to 500,000 counts/min). The radioactivity of the solutions inside and outside the bag was measured. (Fox, C.L., Jr. and Modak, S.M. Antimicrob. Ag. Chem. 5:584 1974 with permission)

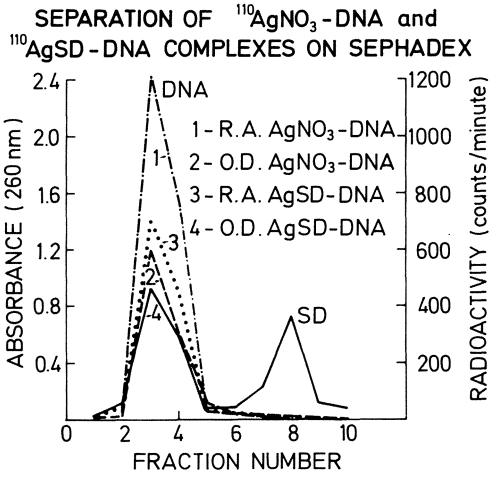

Fig. 13. DNA solutions (35 µg/ml) were mixed with $^{110}AgSD$ or $^{110}AgNO_3$ (silver/DNA-P = 1.0) and incubated for 20 h. After centrifugation, a portion of the clear supernatants was loaded on a Sephadex column RA. Radioactivity. (Fox, C.L., Jr., and Modak, S.M. Antimicrob. Ag. Chem. 5:584 1974 with permission)

It was further proposed that the efficacy of the SD combination with the Ag might be due to a difference in dissociation and/or reaction rates under physiological conditions. The ionization constants of the sulfonamides tested and other compounds appeared to contribute importantly to the differences in the antibacterial activity of their silver salts.

Fox and Modak concluded that silver sulfadiazine functions as a reservoir of obtainable silver ions in the burn wound. The slow liberation of silver ions does not cause the rapid and extensive depletion of chloride ion experienced

Table 2. Effect of silver binding on ultraviolet absorption of DNA. (Fox, C.L., Jr. and Modak, S.M. Antimicrob. Ag. Chem. 5:585 1974 with permission)

Ag/DNA-P in reaction mixture	Binding ratio[a] (μmol of Ag/100 mg of DNA)	OD_{260}	OD_{260} after correction for SD absorption[b]	Increase in OD caused by Ag	Binding ratio (μmol of Ag/100 mg of DNA)	OD_{260}	Increase in OD caused by Ag
0.0	0	0.68	0.68	0.0	0	0.68	0.0
0.125	22	0.88	0.66	0.0	38	0.67	0.0
0.25	33	1.0	0.67	0.0	64	0.71	0.03
0.5	48	1.15	0.68	0.0	96	0.79	0.11
1.0	60	1.23	0.70	0.02	120	0.81	0.13

[a] The binding ratios were estimated from the ^{110}Ag taken up by DNA.
[b] Free SD determined in the supernatant as described in Results.

with continuous silver nitrate soaks, and hence systemic electrolyte withdrawal is avoided. Since only a small part of the silver in silver sulfadiazine reacted with the chloride, protein and other constituents of body fluids, enough was available to be acquired by the microorganisms, so that many were killed and their growth inhibited.

Synergism experiments between AgSD, SD and other sulfonamides revealed that SD plays a unique role in the topical antimicrobial activity of silver sulfadiazine (see Table 3).

Further studies on the mode of action of silver sulfadiazine were conducted by Modak and Fox (1973). The inhibitory effect of AgSD at various concentrations was studied in its action against *Pseudomonas aeruginosa*. In the initial 4-h period studied, increasing concentrations of AgSD cause a corresponding increase in inhibition, with complete inhibition being attained at a concentration of 0.05 μmol/ml. Concentrations up to 0.05 μmol/ml are bacteriostatic, while at 0.2 μmol/ml a bactericidal concentration is reached.

The uptake of silver from AgSD by a *Pseudomonas* cell culture during incubation determined by the amount of isotopic [^{110}Ag] bound to cells was determined. The amount of silver absorbed reached in 2 h a constant level whose value depends on the drug concentration. Practically all the silver was found in the DNA, RNA and cell residue in fractionated *Pseudomonas* cells (Table 4). The maximum activity was always found associated with the cell residue, consisting primarily of proteins and polysaccharides. This activity reached its maximum during 1–2 h and then remained stable. Even after growth resumed, there was no loss of radioactivity from this residue when examined at 20 h.

The RNA fraction acquired about 3% of the silver at the concentration, causing complete inhibition; the silver present was almost negligible at the lower concentrations of AgSD. In the DNA fractions, which attained a maximum within 2 h and receded with further incubation, peak values were 3%, 4.8% and 12% of the total silver absorbed under the conditions of mild, moderate, and complete inhibition, respectively. Growth did not resume until the Ag/DNA ratio fell to around 2, with more time required for the higher ratios to fall

Table 3. Synergism of silver sulfadiazine and sodium sulfadiazine against *P. aeruginosa*

Compounds (μmol/ml)	Effect on growth[a]	Compounds (μmol/ml)		Effect on growth
		AgSD	NaSD	
AgSD				
0.004	+	0.001	0.5	+
0.006	+	0.001	0.6	−
0.008	+			
0.01	−	0.002	0.1	+
		0.002	0.2	+
NaSD		0.002	0.4	−
		0.002	0.6	−
0.2	+	0.002	1.0	−
0.4	+			
0.5	+	0.004	0.1	−
0.6	−	0.004	0.2	−
1.0	−	0.004	0.4	−
		0.004	0.6	−
NaST		0.004	1.0	−
0.025	+	0.006	0.1	−
0.05	+			
0.1	−	0.008	0.025	−
0.2	−	0.008	0.05	−
		0.008	0.1	−
		AgSD	NaST	
		0.002	0.025	+
		0.002	0.05	−
		0.002	0.1	−
		0.002	0.2	−
		0.004	0.05	−
		0.006	0.05	−

[a] +, Growth; −, no growth; NaST, sodium sulfathiazole. The inoculum was 0.2 ml of 1/2,500 dilution of an overnight Trypticase soy broth culture in 5 ml of broth containing the drugs. The cultures were incubated for 20 h. (FOX, C.L., Jr. and MODAK, S.M. Antimicrob. Ag. Chem. 5:586 1974 with permission).

to this value. After 20 h, when the cells had completely overcome the bacteriostatic effect, the binding was about 0.10.

The relative affinity of silver for the three cell fractions (DNA, RNA, cell residue) was measured by placing equal weights of each fraction into dialysis bags and dialyzing for 20 h against an ammoniacal solution of [^{110}Ag]SD. The DNA showed 10 times more affinity than the cell residue and 40 times more affinity than RNA.

Comparison of the miniminal inhibitory concentrations (MIC) of the two silver compounds and sulfadiazine was performed (Table 5). The MIC for sulfadiazine was found to be 2.0 μmol/ml, while it was only 0.005 μmol/ml for each

Table 4. Relative Affinity of AgSD to DNA, RNA, and cell residue prepared from *P. aeruginosa*[a]

Macromolecule	μmol Ag/100 mg
RNA	1.1
DNA	40.0
Cell residue	4.5

[a] Dialyzing bags were presoaked in a solution of radioactive AgSD (1.0 μmol/ml). Dialysis of 3 ml of the above macromolecules at a concentration of 20 μg/ml was carried out for 20 h against 100 ml of 1.0 μmol/ml of ^{110}AgSD in ammoniacal solution. The radioactivity inside and outside the bag was measured after the dialysis. The DNA content inside the bag after dialysis was estimated by the diphenylamine reaction (MODAK, S.M. and FOX, C.L., Jr. Biochem. Pharm. 22:2396, 1973 with permission).

Table 5. Comparative action in vitro of sulfadiazine and silver compounds. (MODAK, S.M. and FOX, C.L., Jr. Biochem. Pharm. 22:2397 1973 with permission)

Drug tested	MIC[a] (μmol/ml)
Sulfadiazine	2.0
Silver sulfadiazine	0.005
Silver nitrate	0.005
Sulfadiazine + *p*-aminobenzoic acid[b]	No inhibition
Silver sulfadiazine + *p*-aminobenzoid acid[b]	No growth

[a] MIC = minimum inhibitory concentration. The inoculum was 0.1 ml of a 1/1000 dilution of an overnight broth culture in 5 ml broth containing the drugs. The endpoint for silver compounds was the lowest concentration of drug showing no turbidity after 24 h of incubation and no growth on subculture; sulfadiazine was bacteriostatic only.

[b] *p*-Aminobenzoic acid was added in equimilar concentration to SD to AgSD.

of the two silver salts. The addition of pABA to SD and AgSD had no effect on the latter, while it completely reversed the inhibition by sulfadiazine.

MODAK and FOX concluded that the silver ion appears to be of central importance in the antibacterial effect of AgSD because (1) AgSD dissociates in the culture medium and only silver is bound to the cells — no binding of sulfadiazine occurs; (2) the antibacterial effect of AgSD in vitro against various organisms is practically the same as that of $AgNO_3$; and (3) while the MIC of AgSD is near or identical to that of $AgNO_3$, the MIC of sulfadiazine is considerably (200×) higher. Growth proceeds in spite of absortion of silver by the cell residue (proteins and polysaccharides). This bound silver remains constant during and after the inhibitory phase. The evidence for a direct effect on DNA is: (1) silver binds to DNA with an affinity that is 10× the cell residue and 40× that of RNA; (2) in the inhibitory period there is a rise in silver bound to DNA which proceeds to a peak value and then recedes

– with growth not resuming until the bound silver has fallen to a defined level; (3) a similar rise and fall of DNA-bound silver is seen with silver nitrate; (4) after cell proliferation at 20 h, the DNA-bound silver has fallen to a very small value, in contrast to the cell-residue-bound silver; (5) at those higher concentrations of AgSD in which the DNA-bound silver has not fallen to a small value at 20 h, no growth has occurred.

The rise of DNA-bound silver to a peak value in vivo followed by a decline to a low value, agrees with the finding of JENSON and DAVIDSON (1966) that the silver ion complexing reaction is chemically and biologically reversible. The hypothesis that in growing cells the silver-containing DNA segments are selectively cleaved by repair mechanism was confirmed by the work of WYSOR and ZOLLINHOFER (1973).

A study on the mechanism of "repair synthesis" after the administration of sublethal doses of silver sulfadiazine was attempted. This was accomplished by the simultaneous study of UV-induced repair synthesis and silver sulfadiazine administration, preferably at equal levels of cell survival, by using procedures in which the incorporation of 5-bromouracil (5-BU) into DNA was avoided, even though PETTIJOHN and HANAWALT (1964) reported evidence of the resynthesis of excised regions, using BU as a density marker. This was observed because of the well-established toxic and synergistic effects attributable to BU. Therefore, a D_2O-[^{15}N]-[^{13}C] system as a density marker to examine repair synthesis following both UV radiation and silver sulfadiazine exposure was employed.

Density gradient equilibrium sedimentation analyses of isotope distribution were performed using $CsSO_4$ gradients (substituted for CsCl). This experiment was a variant of the MESELSON and STAHL experiment (1958) utilizing "heavy" and "light" strands of DNA. Although the details of this study are too complex to be presented in their entirety in this review, a few pertinent points can be discussed.

It was observed that both silver sulfadiazine-treated and UV-irradiated showed repair synthesis. This was indicated by the appearance of [^3H] label in the DD-DNA as well as the DL-DNA. Since resolution of the DNA fragments was incomplete in the cell lysates, and in an attempt to quantify the extent of repair synthesis, the banded DD- and DL-DNA fractions were separately pooled and rebanded for further purification. The resulting DD- and DL-DNA fractions were dialzyed against $NaSO_4$-citrate buffer to remove the $CsSO_4$ and the DNA was then denatured by immersion in a boiling water bath for 15 min, followed by rapid cooling. The results of rebanding and "renaturation" were then determined (the extent of "denaturation" of the DNA treated with AgSD is a function of excision and repair synthesis) (Fig. 14). It was determined that the DNA from both the UV-irradiated and silver sulfadiazine-exposed cells contained the tritiated label in the parental D-DNA strands, whether obtained from the native DD or DL-DNA, as would be expected from the "excision repair" synthesis model (Fig. 15).

During the 15 min of incubation, following a UV dose of 159 erg/mm^2, approximately 4% of the thymine incorporated into the chromosome via a repair synthesis, and about 96% by semiconservative replication. After a dose

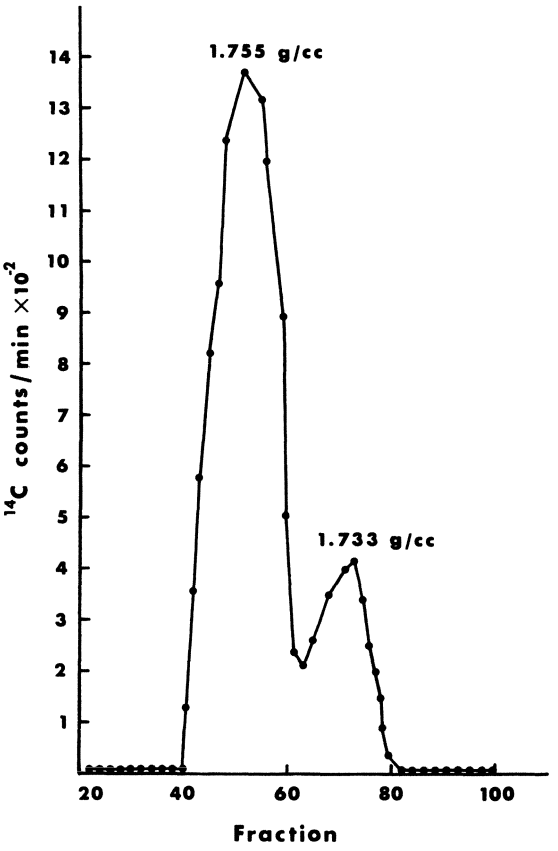

Fig. 14. Density distribution of DNA fragments isolated from *P. aeruginosa* (ATCC 15693) after a "light" chase. The cells were first grown in D, ^{15}N, ^{13}C medium containing ^{14}C-thymine. They were then shifted to a H, ^{14}N, ^{12}C medium for 30 min. The cell lysate was then subjected to Cs_2SO_4 gradient analysis. Approximately 25% of the ^{14}C counts were in the newly synthesized DNA (buoyant density of 1.733 g/cm^3). Two drops were collected per fraction. (WYSOR, M.S. and ZOLLINHOFER, R.E. Path. Microbiol. 39:438 1973, S. Karger, A.G. Basel, with permission)

of 4 μg/ml of silver sulfadiazine, approximately 9% of the thymine incorporated into the chromosome via repair synthesis, and about 91% by semiconservative replication. It should be noted that in both cases 20% or less (depending on the rate of pool clearance) of the DNA present at the time of irradiation was the L-DNA synthesized during the 30 min "light" phase. After UV exposure, repair synthesis as measured by [^{3}H]-thymidine uptake in this L-DNA could not be differentiated from the [^{3}H]-thymidine uptake into the L-DNA due to semiconservative replication (Figs. 16, 17, 18).

In summary, the results of this study showed that when cells of *P. aeruginosa* were exposed to sublethal doses of silver sulfadiazine and ultraviolet radiation in an exponential growth phase, repair, as well as semiconservative synthesis of DNA, occurred in the initial 15 min period. At the doses used, most of

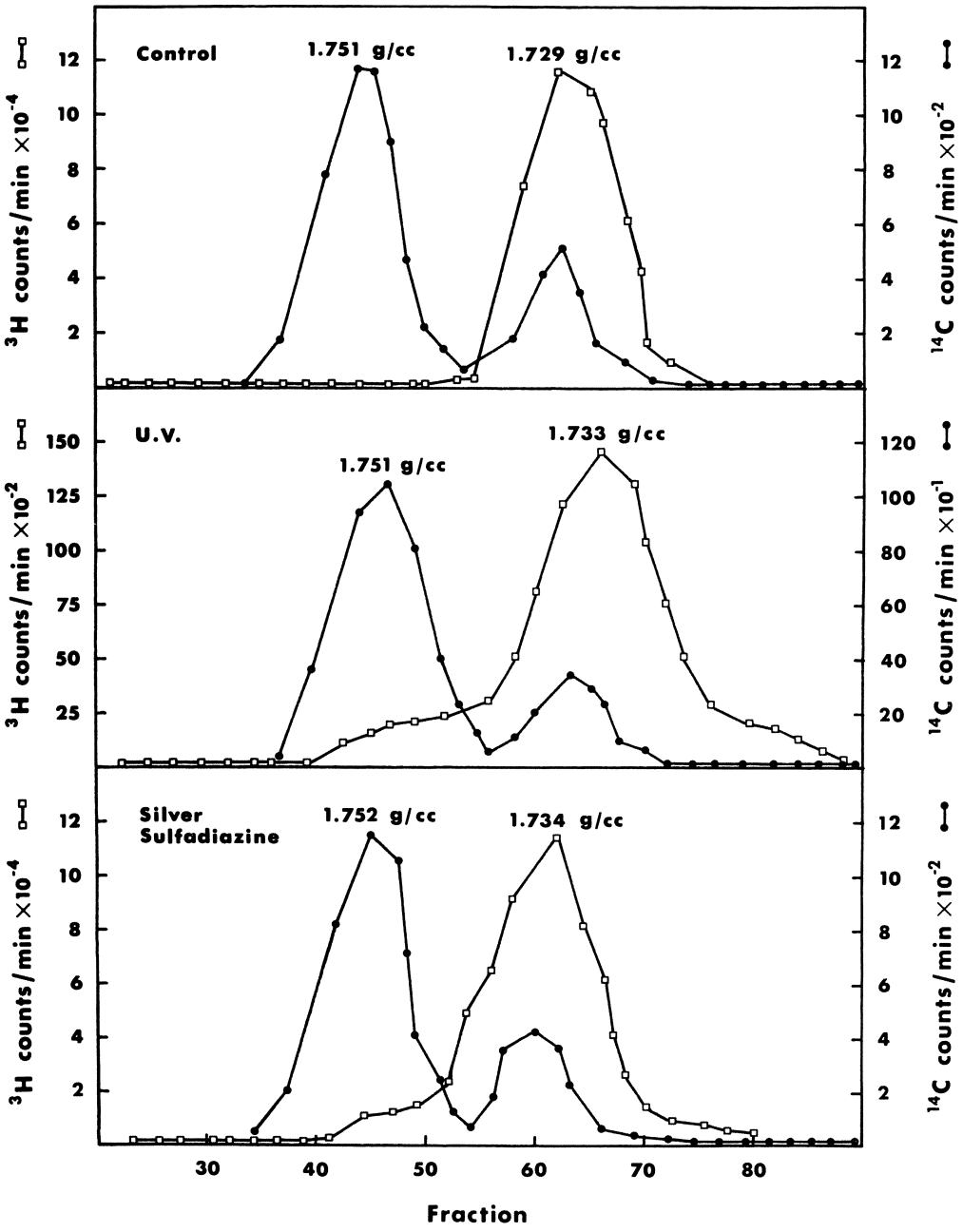

Fig. 15. Cs_2SO_4 density gradient profile of lysates of *P. aeruginosa* (ATCC 15693). Doses of irradiation and survival are given in Table 1. Two drops collected per fraction. (WYSOR, M.S. and ZOLLINHOFER, R.E. Path. Microbiol. 39:439 1973, S. Karger, A.G. Basel, with permission)

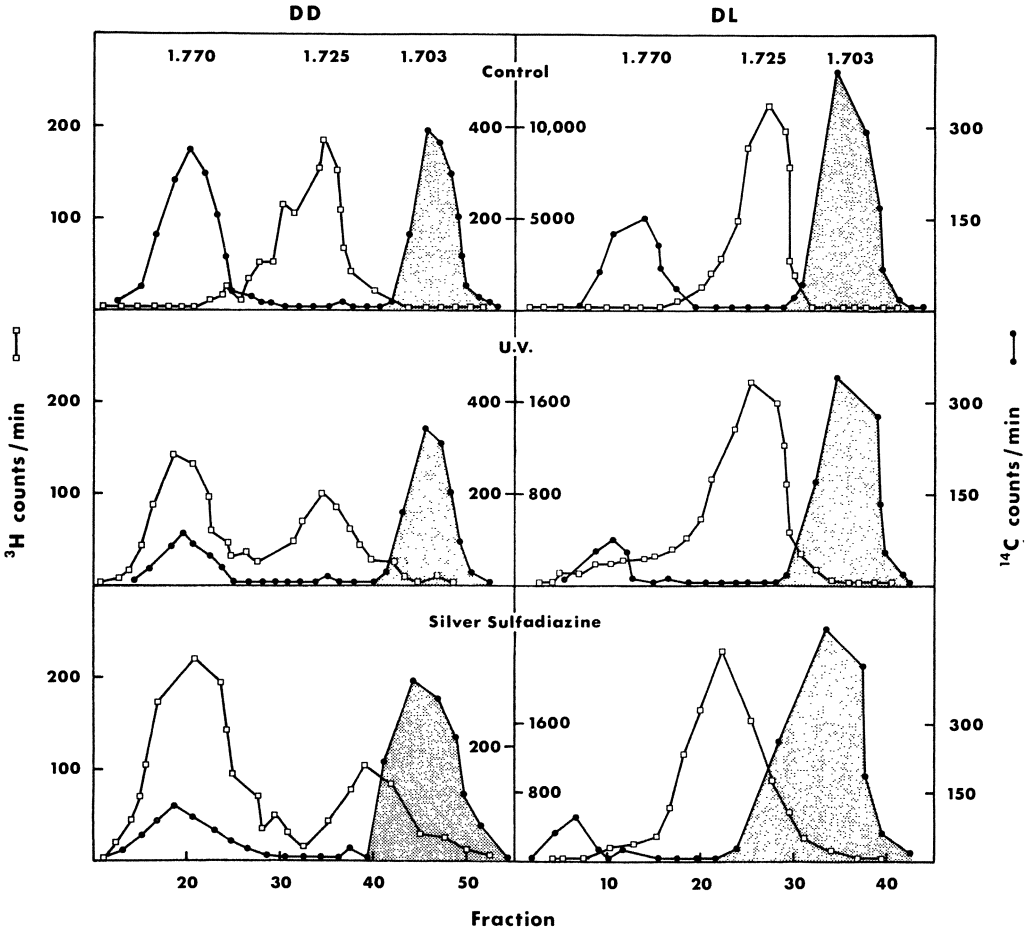

Fig. 16. Semi-conservative versus repair synthesis in *P. aeruginosa* (ATCC 15693) after UV-irradiation and silver sulfadiazine treatment. The DNA fractions shown were obtained by combining all the DNA from the experiment in Fig. 15 that banded higher than 1.740 g/cm^3, to form the DD-DNA sample; whereas all the DNA fractions banding at lower densities were pooled and constituted the DL-DNA sample. The DD-DNA and DL-DNA samples were rebanded again and subjected to the same pooling procedure. The DD- or DL-DNA finally obtained was divided into two fractions, one of which was denatured and then rebanded. The other half of the sample was banded as native DNA (results not shown) to ascertain the purity of the pooled DNA. The left hand column (DD) represents the Cs_2SO_4 gradient profile of the "purified" denatured DD-DNA. The right hand column (DL) represents the Cs_2SO_4 profile of "purifed" denatured DL-DNA (The numbers over the bands given the density as determined by refractive index. Five drops were collected per fraction). *Stippled band* is a ^{14}C-labeled *B. subtilis* DNA used as a density marker (P=1.703 g/cm^3). (WYSOR, M.S. and ZOLLINHOFER, R.E. Path. Microbiol. 39:440–441 1973, S. Karger, A.G. Basel, with permission)

the thymine uptake exhibited by cells in the initial 15 min of posttreatment resulted from semiconservative DNA replication. Since a D, [^{15}N], [^{13}C] density system was employed in these studies, repair synthesis was not an artifact caused by the added insult of BU substitution for thymine during or after irradiation. A similar type of synthesis was not observed in the untreated cells.

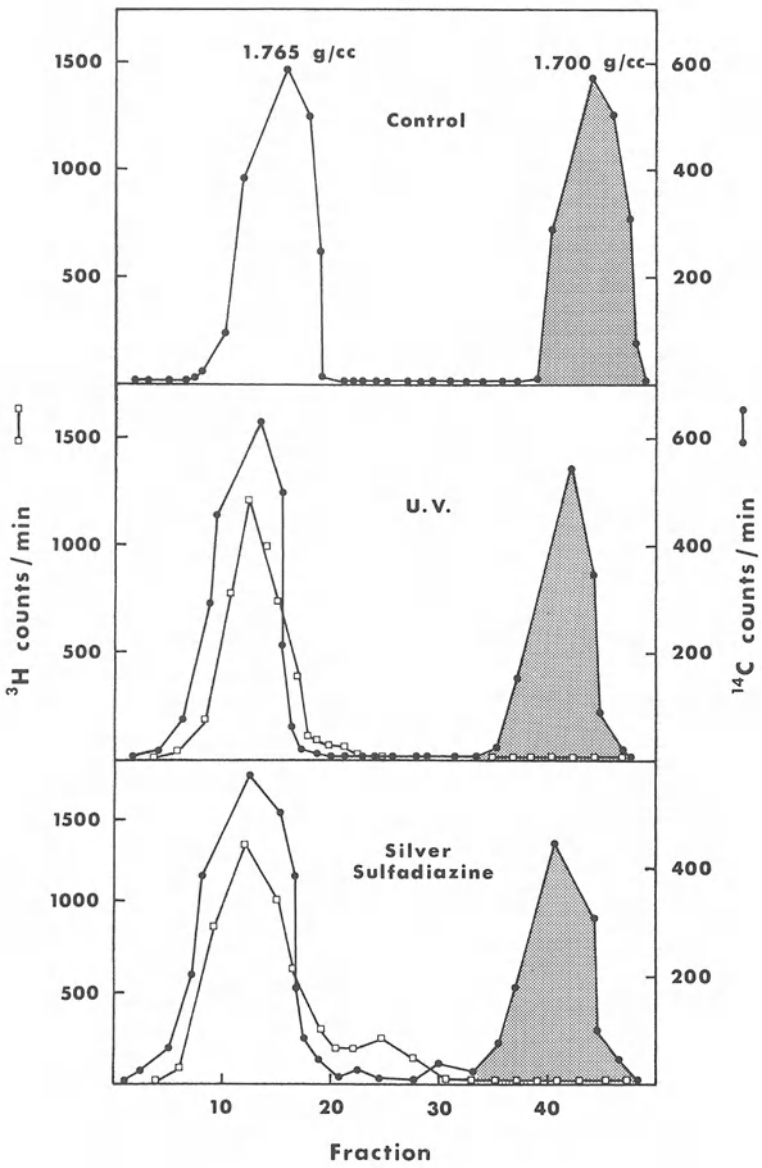

Fig. 17. Cs_2SO_4 gradient profiles of "purified" and then denatured heavy DD-DNA from *P. aeruginosa*. Procedure for preparation was as given in Fig. 16, except that only one purification rebanding was carried out before denaturation of the DNA. *Stippled band* represents the position of a ^{14}C-labeled bacteriophage T2 marker DNA (P = 1.700 g/cm^3). Doses of irradiation and drug as previously stated. Five drops collected per fraction. (WYSOR, M.S. and ZOLLINHOFER, R.E. Path. Micribiol. 39:441 1973, S. Karger, A.G. Basel, with permission)

ROSENKRANZ and CARR (1972) presented evidence that, although AgSD bound to DNA in vitro, this type of mechanism could not explain the drug's activity in vivo. AgSD blocked macromolecular synthesis in treated bacteria, DNA synthesis being slightly more sensitive to this inhibitory action (Fig. 19).

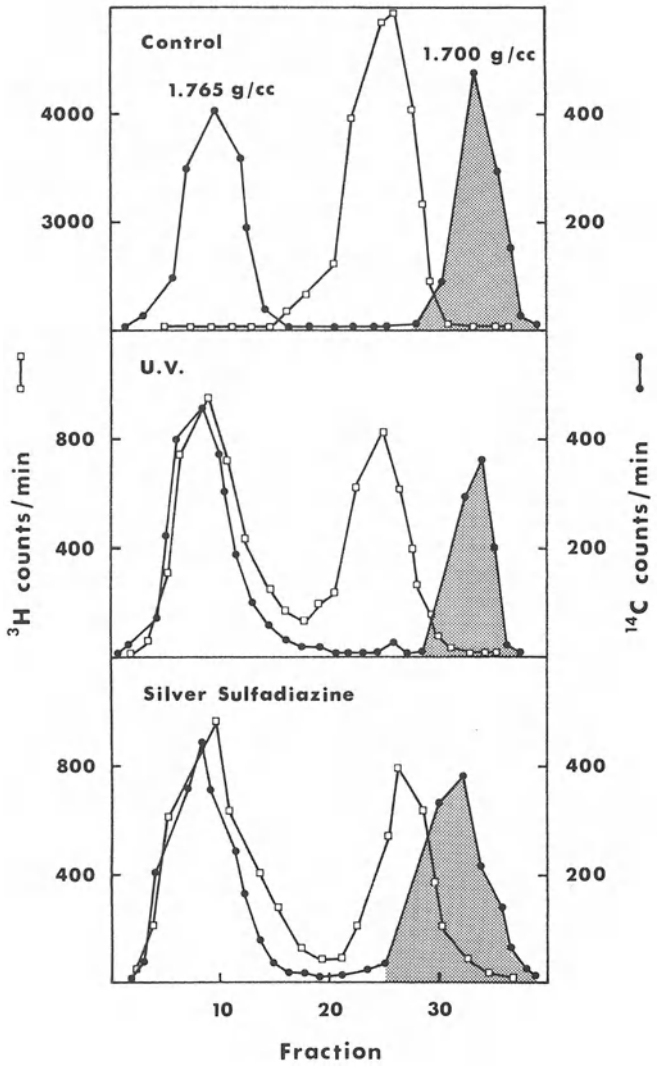

Fig. 18. Cs_2SO_4 gradient of "purified" and then denatured DL-DNA from *P. aeruginosa*. See Fig. 17 for details. (WYSOR, M.S. and ZOLLINHOFER, R.E. Path. Microbiol. 39:442 1973 with permission)

Production of both RNA and DNA was almost completely blocked by AgSD after 15 min of treatment. Protein synthesis continued, but at a reduced rate.

By use of zonal centrifugation in sucrose (Fig. 20A) and density gradient centrifugation in cesium chloride (Fig. 20B) and in cesium sulfate (Fig. 20C), DNA derived from AgSD-treated bacteria was identical to that from untreated bacteria. After prolonged exposure to AgSD, some DNA degradation was observed (Fig. 20A). ROSENKRANZ and CARR (1972) found no presence of material with properties identical to those of in vitro formed AgSD-DNA complexes; i.e., increase in buoyant density in CsCl, decreased buoyant density in Cs_2SO_4, and decreased rate of sedimentation in a sucrose gradient. Rather, it was found

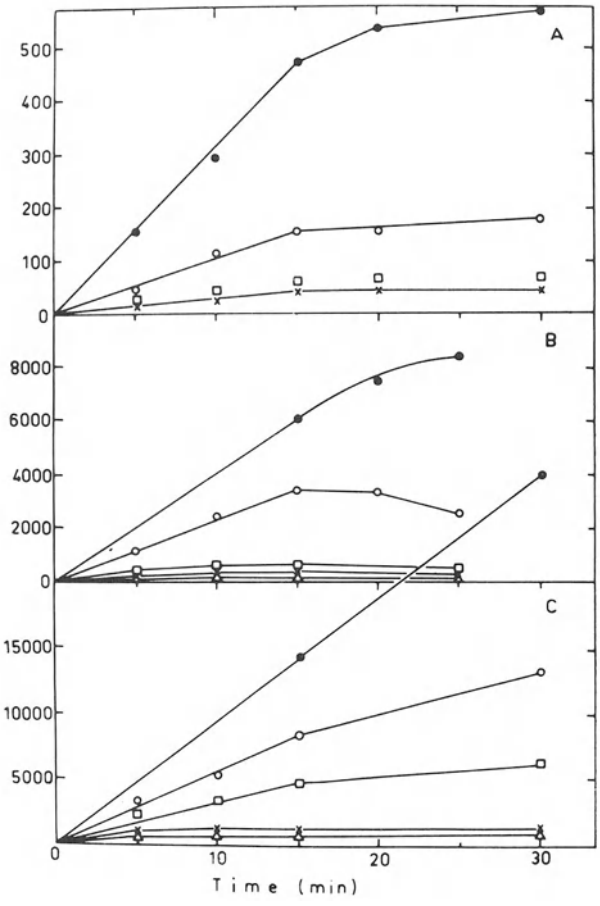

Fig. 19 A–C. Effect of AgSD on macromolecular syntheses. Bacteria were brought to the experimental growth phase at which time portions of the cultures were distributed into flasks containing the radioactive precursors and various amounts of AgSD. At intervals, samples were withdrawn and processed for determination of radioactivity incorporated into acid in soluble form. **A** DNA (^{3}H-thymidine, 6.9×10^8 M, 11.3 Ci/mmol); **B** RNA (5 ^{3}H-uridine, 2×10^4 M, 2 Ci/mmol); **C** Proteins (^{3}H-leucine, 5.4×10^5 M, 0.7 Ci/mmol). ● = Control; ○, □, X, and △ 2.5, 5, 7.5, and 10 μg of AgSD per ml, respectively. (ROSENKRANZ, H.S. and CARR, H.S. Antimicrob. Ag. Chem. 2:368 1972 with permission)

that radioactive AgSD was localized mainly on the cytoplasmic membrane fraction of treated cells.

The studies reviewed are of two types. The first school of thought suggests that, although silver sulfadiazine binds to DNA in vitro, the true target in vivo is the bacterial membrane. Since DNA metabolism is connected with the cell membrane, this might explain the bactericidal action of the drug. The second school of thought postulates that the in vivo target is DNA. The silver reaches the DNA by being selectively released from the sulfonamide with the sulfonamide moiety remaining behind.

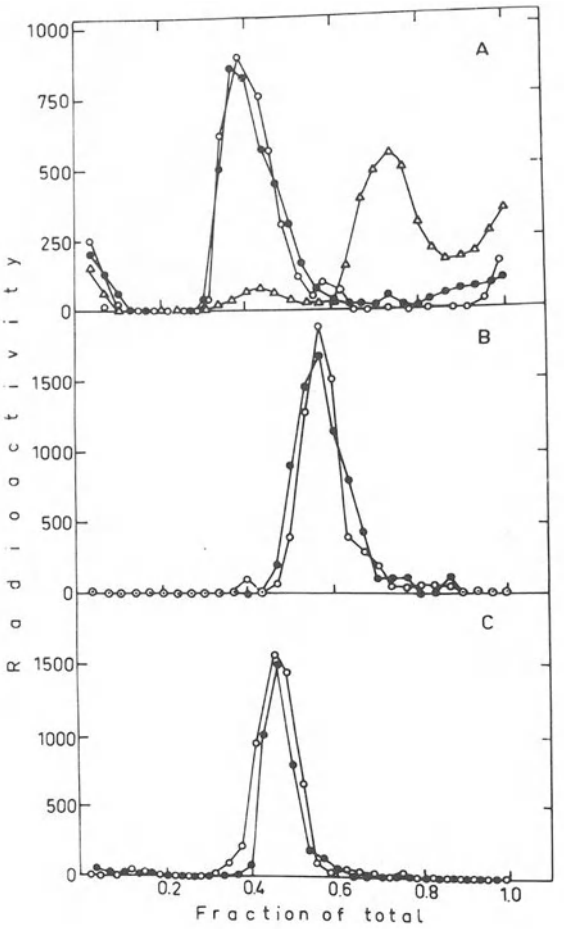

Fig. 20. **A** Sucrose density gradient centrifugation of DNA derived from normal and AgSD (5 μg/ml) treated cells. ○ = DNA from untreated cells; ● and △ = DNA from cells exposed to AgSD for 1 and 3 h, respectively. **B** Cesium chloride density centrifugation of DNA from ○ = normal and ● AgSD-treated cells (5 μg/ml, 1 h). **C** Cesium sulfate density gradient centrifugation of DNA from ○ = normal and ● AgSD-treated cells (5 μg/ml, 1 h). (ROSENKRANZ, H.S. and CARR, H.S. Antimicrob. Ag. Chem. 2:370 1972 with permission)

A possible reconciliation of these two views is a mechanism that would allow for an initial lesion on the cell membrane resulting in "blebs" in the membrane and eventual leakiness to the drug. The previously impermeable membrane would now allow the entry of silver sulfadiazine into the bacterial cell with a second lesion expressed as binding of silver ion to the nucleic acids.

As stated previously, silver sulfadiazine is also active against many other cellular types. Its activity against *Plasmodium berghei* (malaria) is not inhibited by p-aminobenzoic acid (WYSOR, 1973, 1975). Silver sulfadiazine's activity against epimastigotes of *Trypanosoma cruzi,* amastigotes and promastigotes of *Leishmania braziliensis* manifests itself as a lysis of the membranes of these

cellular types (WYSOR, 1981, unpublished) while its activity against *Trypanosoma rhodesiense* appears to be a direct effect on the nucleic acids (WYSOR, 1982).

There have been no studies on the antiviral mechanism of action of silver sulfadiazine. The observation that silver nitrate exerts its antiviral effect against herpes viruses by inactivation of sulfate sulfhydral groups on the capsid membrane of the virus (COLEMAN et al., 1973) does not appear to explain silver sulfadiazine's anti-herpes virus activity. AgSD, unlike other metal-containing compounds was ineffective in vitro in inhibiting the sulfhydral component of the glucose oxidase enzyme of *Aspergillus niger* (WYSOR and ZOLLINHOFER, 1970). Further support for this type of mechanism of action is dampened by the failure of silver sulfonamides to inhibit ^{14}C-leucine uptake by trypanosomes (WYSOR, 1982).

VI. Structure–Activity Relationships

Data on the activities of synthetic relatives of silver sulfadiazine are limited in extent and numerical precision. They do not afford the formation of structure/activity rules. Most silver sulfonamides have demonstrated antimicrobial activity in vitro; however, only silver sulfadiazine was active topically in vivo in controlling burn wound sepsis (FOX, 1968a, 1973). Although various studies have indicated that the ionization constants for sulfonamide, with a resulting difference in stability constants and silver release, play a major role (NESBITT and SANDMANN, 1978), it is clear that the complex character of silver sulfadiazine's structure plays the primary role (BULT and KLASEN, 1978; COOK and TURNER, 1975; BAENZIGER and STRUSS, 1976). Unlike bacteria, which are uniformly susceptible to silver compounds in vitro, trypanosomes are only killed by a few silver sulfonamides (WYSOR, 1980, 1982). MODAK and FOX (1973) believe that the sulfadiazine moiety plays a role in the topical efficacy of AgSD by possibly localizing the action of the drug to microbial cells and by the ionization characteristics of sulfadiazine itself. AgSD's characteristics allow enough silver to reach microbial cells in spite of high chloride and other ion concentration in the burn wound. Whether such is the case systemically is not at all apparent. Reaction kinetics systemically are not clear and the problem of AgSD's reactivity with microorganisms in a systemic milieu is much more complex than its reactivity in the burn wound. Against *Trypanosoma rhodesiense* systemically other silver compounds, such as silver metachloridine, are effective (WYSOR, 1980, 1981a; 1982) yet silver metachloridine is ineffective topically in controlling burn wound sepsis (FOX, 1981, unpublished)

VII. Concluding Discussion

Silver sulfadiazine has received worldwide acceptance as a topical agent to control bacterial infection, especially in burn wounds. Although initially de-

veloped as a topical agent, recent studies have demonstrated a wide spectrum of activity against several types of organisms. Its activity against parasites, and *Trypanosoma rhodesiense* in particular, is interesting in that no new drug has been introduced for the treatment of African trypanosomiasis in the last 30 years (WILLIAMSON, 1970). Although some attempt has been made in this review to discuss the mode of action of AgSD topically, a discussion of its systemic activity is much more complex and difficult.

Silver sulfadiazine, along with Cis-diamminedichloroplatinum (II) (and certain arsenical and antimonial compounds), represents that rare example in medicine of a heavy metal compound with an acceptable chemotherapeutic index. The belief of EHRLICH (1911) that the toxicity of heavy metals could be altered by combination with carrier molecules appears, at least in the case of silver sulfadiazine, to have been confirmed.

Note Added in Proof:

Silver sulfadiazine possesses potent activity against *Legionella pneumophilia,* the causative agent of legionnaire's disease, in vitro (WYSOR and LOWRY, 1982).

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Sporamycin

K. KOMIYAMA and I. UMEZAWA

Sporamycin is an antitumor antibiotic of unknown structure isolated from cultures of *Streptosporangium pseudovulgare* strain No. PO-357 (UMEZAWA et al., 1976; KOMIYAMA et al., 1977a). The antibiotic is a basic polypeptide antibiotic with a very small amount of the nonprotein component which can be dissociated from the protein moiety using methanol extraction as reported with neocarzinostatin (KOIDE et al., 1980; NAPIER et al., 1979) and auromomycin (SUZUKI et al., 1980). The nonprotein component possesses remarkable cytotoxicity and antimicrobial activity. Although the physicochemical properties of the nonprotein component are unknown, this substance is quite unstable when separated from the protein moiety. The protein moiety consists of at least 12 kinds of amino acids.

Sporamycin is partially active against Gram-positive bacteria but not Gram-negative bacteria and fungi. The antibiotic has a strong inhibitory effect on the in vitro growth of a number of cell strains. According to morphological studies, cell damage induced by sporamycin is mainly karyopycnosis at higher concentrations, and many polynuclear giant cells appear at lower concentrations of the antibiotic (KOMIYAMA et al., 1977b).

Antitumor Activity on Experimental Tumors

Sporamycin has a strong inhibitory effect on murine ascites tumors and solid tumors, such as Ehrlich ascites carcinoma, sarcoma 180 or Meth-A. The antibiotic also induces a significant increase in survival time in mice bearing L1210 or P388 leukemia. The growth of sarcoma 180 was inhibited much more effectively by a combination of sporamycin and PSK, an immunopotentiator (KOMIYAMA et al., 1979), and that of L1210 leukemia by sporamycin and 6TG or Ara C (HOSHINO et al., 1979).

It has been reported that a host-mediated antitumor effect may participate in the antitumor activity of sporamycin. As shown in Fig. 1, moderate tumor growth was observed during the first 2 weeks, in spite of the single treatment, but tumor regression began thereafter, and the tumor regressed completely in six out of seven treated mice after 4–5 weeks (KOMIYAMA et al., 1979). According to histopathological findings of this effect, tumor cells were damaged by the

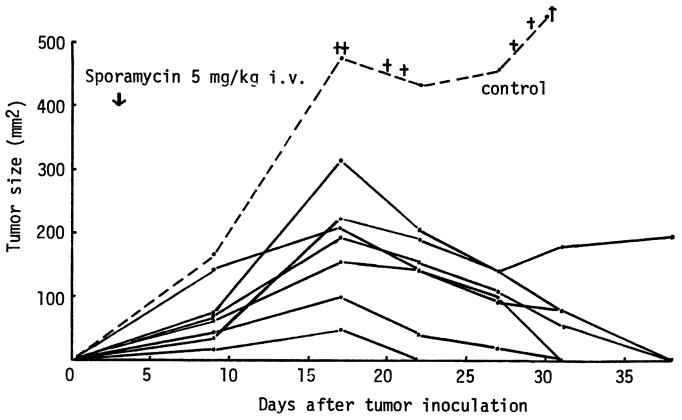

Fig. 1. Antitumor effect of sporamycin on sarcoma 180 tumors. Male ddY mice were inoculated sc with 1×10^5 tumor cells. Three days later, a group of seven mice was treated with a single dose of 5 mg/kg of sporamycin iv. The *broken line* indicates the mean tumor size of seven untreated mice

Table 1. Winn's tumor cell neutralization test. BALB/c mice were inoculated sc with 1×10^5 Meth-A cells on day 0 and were treated with 4 mg/kg of sporamycin iv on day 3. Sixteen days after tumor inoculation spleen cells were collected, and the tumor neutralization activity of the spleen cells was compared with that of spleen cells from normal, tumor-bearing, and drug-treated mice by Winn's method

Source of spleen cells	Ratio of spleen cells: tumor cells	Mean tumor size (mm^2)		No. of mice without tumor
		Day 13	Day 19	
–	–	130	199	0/5
Normal untreated mice	200:1	76	160	0/10
Mice treated with sporamycin	200:1	61	140	2/10
Mice bearing Meth-A tumor	200:1	61	152	0/10
Mice bearing Meth-A tumor and treated with sporamycin				
cured	200:1	–	–	10/10
	50:1	–	–	5/5
noncured	200:1	67	134	1/5
Cured mice + anti-Thy 1,2 serum + complement (guinea pig)	200:1	12	44	2/5
Crude mice + complement	200:1	–	–	5/5

direct cytocidal activity of sporamycin for a few days after injection of the antibiotic, and thereafter this damage disappeared with the lapse of time. However, 2–3 weeks after treatment tumor cells were damaged again due to the immunological response of the host to the tumor cells which was characterized by marked lymphoid cell infiltration into the tumor tissue and the surrounding area, and most mice were cured thereafter (KAWAKUBO, et al., 1980).

As shown in Table 1, a marked neutralization activity against tumor cells was observed using Winn's method and spleen cells of tumor-bearing mice, which had been pre- or posttreated with sporamycin (KOMIYAMA, et al., 1979; UMEZAWA et al., 1981). When sarcoma 180 tumor cells were used as an antigen, the macrophage migration inhibitory reaction by spleen cells derived from tumor-bearing mice treated with sporamycin was positive on day 7–14 after the medication. The delayed hypersensitivity tested by the foot-pad reaction was positive in tumor bearing mice treated with sporamycin, and no decrease in the footpad reaction was observed, whereas this reaction was decreased remarkably in nontreated tumor-bearing mice. To determine the effect of sporamycin on macrophage activity, ddY mice were treated with 5 mg/kg of sporamycin ip. Macrophages were collected 5 days later from the intraperitoneal cavity, and the inhibitory activity of macrophages on the incorporation of [^{3}H]-TdR into DNA of EL4 tumor cells in vitro was measured. The incorporation was remarkably suppressed when compard with the control group. All these findings suggested that sporamycin may, at least partially, act by modulation of cellular immune response directed at a tumor-associated antigen.

Toxicology and Pharmacology

Examination of blood and organ concentrations of sporamycin in normal mice showed a rapid decrease of sporamycin from the peripheral blood and a high level of the antibiotic in urine 10 min after intravenous injection of the antibiotic. At the same time, the highest levels were found in the lungs and spleen (KOMIYAMA, 1978).

Sporamycin inhibited tritiated thymidine incorporation into DNA of normal tissues in vivo, but a different pattern on inhibition and recovery of the incorporation of [^{3}H]-TdR into DNA was observed among mouse organs (UMEZAWA et al., 1978). It was noted that the antibiotic may damage normal tissues in spite of a rapid excretion and inactivation of sporamycin in mice, but this damage cured rapidly within 1–2 days after treatment.

Mode of Action

Sporamycin primarily inhibited DNA synthesis, while RNA and protein synthesis were not significantly affected in HeLa S3 cells. The antibiotic also caused strand scission of cellular DNA (Fig. 2). However, these effects were not observed when the cells were incubated at 0° C before washing and subsequently incubated at 37° C. The Tm of calf thymus DNA decreased when incubated with sporamycin in vitro. Sporamycin did not affect DNA synthesis in

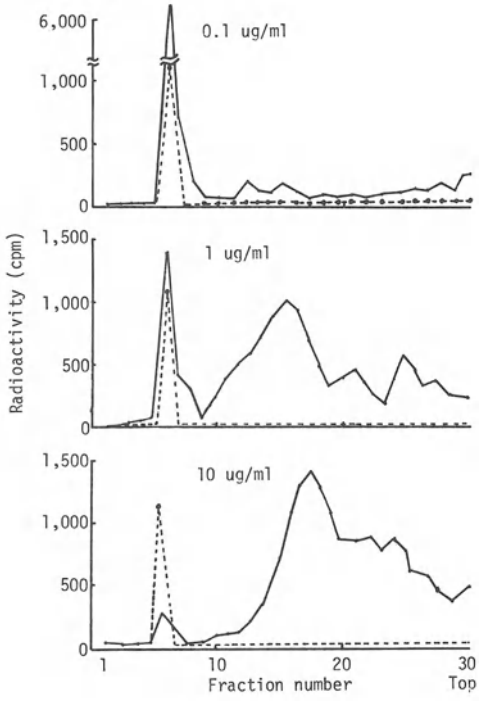

Fig. 2. Alkaline sedimenation patterns of cellular DNA from HeLa cells treated with sporamycin. HeLa cells prelabeled with [^{3}H]-TdR were incubated with 0.1, 1, or 10 μg/ml of sporamycin for 4 h (*solid line*). Cells prelabeled with [^{14}C]-TdR served as nontreated cells (*broken line*)

vitro catalyzed by partially purified DNA polymerase α, β, and γ derived from Ehrlich ascites cells (OKAMOTO et al., 1979).

In conclusion, sporamycin acts as an immunomodulator in the host in addition to its direct cytotoxic activity caused by strand scission of cellular DNA.

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Streptothricin F

J. JONÁK and I. RYCHLÍK

Streptothricin F (Fig. 1), the first member of the streptothricin family of antibiotics, was discovered by WAKSMAN and WOODRUFF (1942). It is a basic water-soluble compound, produced by *Streptomyces lavendulae*. Shortly after its discovery, many other similar antibiotics, produced by a number of *Streptomyces* strains, were reported, namely streptolin, geomycin, phytobacteriomycin, yazumycin, racemomycin, pleocidin, polymycin, etc. (see KHOKHLOV and SHUTOVA, 1972). All these antibiotic preparations were mixtures of closely related compounds and soon the different components were isolated and identified (HOROWITZ and SCHAFFNER, 1958; VAN TAMELEN et al., 1961; JOHNSON and WESTLEY, 1962; BRADLER and THRUM, 1963; KHOKHLOV and RESHETOV, 1964; TANIYAMA et al., 1971a, b; KHOKHLOV and SHUTOVA, 1972; BORDERS, 1975; GRÄFE et al., 1977). The general structural formula of the antibiotics of the streptothricin group is shown in Fig. 1 (KHOKHLOV and SHUTOVA, 1972). The individual members differ from each other by the number of L-β-lysine

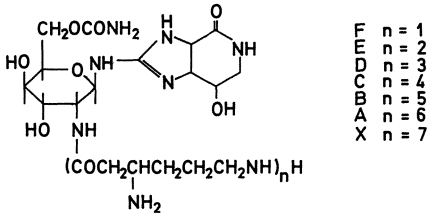

Fig. 1. Structural formula of the streptothricin group

Table 1. Synonyms of streptothricins

Number of β-lysine residues	Streptothricin	Racemomycin	Yazumycin
1	F	A	A
2	E	C	C
3	D	B	
4	C	D	
5	B		
6	A		
7	X		

residues present in the peptide moiety. The nomenclature and synonyms of individual streptothricins are given in Table 1.

In addition to the typical streptothricins, other streptothricin-like antibiotics were isolated, in which the β-lysine is replaced by another amino acid, i.e., glycine (BORDERS et al., 1970; SAWADA et al., 1977) or its derivatives N-formiminoglycine (BORDERS et al., 1970) or N-methylglycine (TSURUOKA et al., 1968; BORDERS et al., 1970).

Structure/Activity Relationship

Chemical modification of the β-lysine moiety indicates that both the β-amino and ε-amino groups are important for exhibiting antibiotic activity. Thus, selective acetylation of either the β-amino or ε-amino group of streptothricin F decreased its activity against *Staphylococcus aureus* about 100- and 500-fold, respectively (SAWADA et al., 1974; SAWADA and TANIYAMA, 1974a).

Also, substitution of the ε-amino group of streptothricin F by different amino acid residues leads to preparations which have lower antibacterial activity than the original compound (SAWADA and TANIYAMA, 1974b; TANIYAMA et al., 1974).

Upon acid hydrolysis the lactam ring of streptothricin F (I) is opened (Fig. 2), yielding an antibiotically inactive compound, streptothricin F acid (streptothricinic F acid) (II) (TANIYAMA et al., 1971c), and finally the compound

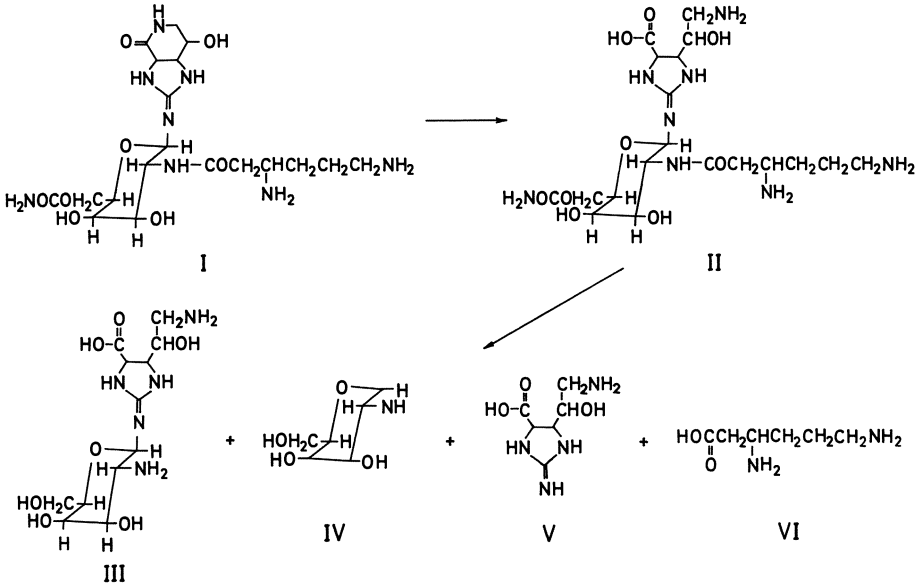

Fig. 2. Acid hydrolysis of streptothricin F

is converted into the characteristic degradation products: N-*guan*-streptolidyl-β-D-gulosaminide (III), gulosamine (2-amino-2-deoxy-α-D-gulose) (IV), streptolidine (V) and β-lysine (VI) (BORDERS et al., 1970; KHOKHLOV and SHUTOVA, 1972; TANIYAMA and SAWADA, 1972; BORDERS, 1975).

Toxicity

Streptothricin antibiotics are highly toxic compounds. Their antibiotic activity as well as their toxicity are directly related to the number of β-lysine residues in their molecule. In mice the LD_{50} for streptothricin F (n=1), streptothricin E (n=2), streptothricin D (n=3), and streptothricin C (n=4) is 300 mg/kg, 26 mg/kg, 10 mg/kg, and 8 mg/kg body weight, respectively, when injected intravenously as a single dose (UMEZAWA, 1967; TANIYAMA et al., 1971a, b). Similar results were obtained in mice also by MELNIKOVA et al. (1980). When given perorally, streptothricin B and C were more toxic than streptothricin D and F. A detailed study of acute and delayed toxicity of streptothricin F and streptothricin C was performed by Japanese investigators (INAMORI et al., 1978; INAMORI et al., 1979a, b). Both streptothricins were administered intravenously in a single dose: streptothricin F 200 mg/kg and streptothricin C 40 mg/kg. The antimicrobial activity levels in the blood serum declined very rapidly and no antibiotic activity of streptothricin F could be detected 2 h after administration (INAMORI et al., 1978). Labeled streptothricin F ([^{14}C]-glycyl-streptothricin F) was found to be present in high concentrations in the kidney as late as 48 h after administration (INAMORI et al., 1979a). Mice given streptothricin F showed a gradual decrease in body weight, the weights of spleen, pancreas, and liver were also decreased; the lung, adrenals showed congestion and the intestine was bleeding. The kidneys had a white appearance. Microscopic examination of renal tissues from rats revealed lesions, such as pronounced degradation and necrosis of the proximal and distal renal tubular epithelium 48 h after injection. However, there were no significant changes in the renal glomeruli, even 96 h after administration. The histological findings suggest that the nephrotoxicity of the streptothricin antibiotics involves a mechanism resembling more that of kanamycin than that of puromycin. Biochemical serum tests showed that blood urea nitrogen and creatinin nitrogen levels (indicators of renal damage) remained virtually unaltered during the first 24 h after injection, but both showed a sharp increase from about 48 h onward.

Electrophoretic patterns of serum and urinary proteins showed the appearance of α_2-globulin together with an increase in β-globulin. The pattern was similar to that observed in renal damage. Urine secretion showed a rapid decrease from about 48 h on (INAMORI et al., 1979a, b). These observations clearly demonstrate the profound nephrotoxic effect of streptothricin antibiotics. Some experiments indicate that the nephrotoxic effect may be caused, not by streptothricin F itself, but by its degradation product streptothricin F acid (II), to which about 80% of the antibiotic are converted in vivo (INAMORI et al., 1979a,

b). This metabolite is devoid of antimicrobial activity, but is about six times as toxic as the parent compound (TANIYAMA et al., 1971c).

In addition to the direct nephrotoxic effect, other adverse effects were observed after administration of streptothricins to mammals. MELNIKOVA (1970a) showed that phytobacteriomycin or polymycin (both mixtures of streptothricins), when given perorally at a 1/10 LD_{50} level, to mice immunized with sheep erythrocytes, inhibited partly the production of hemaglutinins and hemolysins and decreased the weight of the spleen. MELNIKOVA (1971), and MELNIKOVA and RODIONOV (1974) also stressed that cutaneous and intracutaneous administration of phytobacteriomycin to guinea pigs produced signs of allergy and allergic contact dermatitis, and caused anaphylaxia and hypersensitivity to phytobacteriomycin. They questioned the use of streptothricin antibiotics in agriculture since these compounds might have a negative effect on immune resistance of persons, who should be in contact with the preparations. Upon peroral administration of phytobacteriomycin to mice an inhibitory effect on the intestinal enzyme enterokinase and an adverse effect on the microflora of the large intestine were observed (MELNIKOVA 1970b).

Antibiotic Action of Streptothricin F

Antimicrobial activity. Streptothricins are antibiotics with a broad spectrum of antibacterial effects, they are active against Gram-positive and Gram-negative bacteria, mycobacteria, and fungi (Table 2) (GERMANOVA et al., 1965; TANIYAMA et al., 1971b; GANUSHKINA et al., 1980; MELNIKOVA et al., 1980). However, these antibiotics cannot be used clinically because of their acute and delayed toxicity (TANIYAMA et al., 1971a, b, c; INAMORI et al., 1978; INAMORI et al., 1979a, b).

Use in plant protection. Preparations containing streptothricins (phytobacteriomycin, polymycin) have found practical application as plant protection agents against diseases caused by bacteria and fungi, and also in seed protection. They have a strong bacteriostatic and bacteriocidal effect on important plant pathogens of the genera *Xanthomonas, Pseudomonas, Ervinia, Agrobacterium, Corynebacterium,* and others (GANUSHKINA et al., 1980). Good results were obtained in curing gumosis-angular leaf spot disease of cotton, and leaf and pod spot of Leguminosae.

Virostatic activity. Streptothricins are active against viruses. GERMANOVA et al. (1965) reported inhibitory effects of nine streptothricins on the development of influenza virus PR-8 strain in chick embryos. TANIYAMA et al. (1971b) tested streptothricin F, E, D (racemomycin A, C, B) in cell culture of mouse fibroblasts against influenza virus PR-8 strain. The virus inhibitory concentration was 30 µg/ml with streptothricin F and E, and 10 µg/ml with streptothricin D. At these concentrations no or only a marginal cytotoxic effect was observed. There was some correlation between the antiviral activity and the number of β-lysine residues in the streptothricins. With phage MS 2, 1 µg/ml of streptothri-

Table 2. Antimicrobial spectrum of streptothricin F (Racemomycin A)[a]

Test organism	M.I.C.[b] (μg/ml)
Staphylococcus aureus Terajima	1
Staphylococcus albus	25
Staphylococcus epidermidis	10
Diplococcus pneumoniae Type 1	0.78
Staphylococcus SMST-R	100
Bacillus subtilis PCI-219	3.12
Bacillus subtilis ATCC-6633	3.12
Bacillus anthracis No. 119	100
Salmonella typhi 0-901-W	3.12
Escherichia coli K-12	3
Aerobacter aerogenes	100
Pseudomonas aeruginosa IAM-1007	3.12
Pseudomonas aeruginosa Thuchijima	100
Sarcina lutea	1.56
Mycobacterium tuberculosis $H_{37}Rv$	50–10
Mycobacterium phlei	1
Mycobacterium avium	1
Shigella sonnei	10
Proteus vulgaris	100
Aspergillus fumigatus	30
Nocardia asteroides	30
Cryptococcus neoformans	1
Microsporum gypseum	30
Trichophyton interdigitale	3
Trichophyton asteroides	50
Epidermophyton floccosum	10
Sporotrichum schenchii	3
Candida albicans ATCC-10257	3
Trichophyton mentagrophytes	3
Trichomonas vaginalis 4F	100

[a] TANIYAMA et al., (1971 b).
[b] Median inhibitory concentration μg/ml of streptothricin F (sulfate).

cin F had no effect on phage development while 3 μg/ml blocked bacterial growth.

Insecticidal effects. Phytobacteriomycin is highly toxic for mosquito larvae of the genera *Culex, Aedes,* and *Anopheles* in laboratory as well in field experiments. This killing effect is mainly due to the presence of streptothricin C and D in the preparation; streptothricin F is the least active (GOLDBERG et al., 1975; CHAGIN et al., 1976; GANUSHKINA et al., 1980).

Taeniacidal effects. Streptothricin F and also other members of the streptothricin group were effective against various tapeworms in dogs, cats, sheep, and mice (SZANTO et al., 1975, 1976).

Mode of Action of Streptothricin F

Interaction with nucleic acids. In a detailed study ZIMMER et al. (1967) investigated the effect of streptothricin F and of a mixture of streptothricins C and D on the melting and sedimentation properties of bacterial DNA. They found that streptothricins stabilize the DNA helix structure against thermal denaturation and acid denaturation by forming complexes at low ionic strength with the negatively charged phosphate groups of DNA by electrostatic interaction.

This effect was found to be the stronger, the greater the number of aliphatic basic β-lysyl groups in the streptothricin molecule. The bonding of the antibiotic with the phosphate groups can be reversed by increasing the ionic strength.

At very high antibiotic concentrations aggregates were formed, as indicated by scattering effects in the ultraviolet spectrum of DNA.

Other cationic aminoglycoside antibiotics (streptomycin, dihydrostreptomycin, paronomycin, kanamycin, neomycin) also produced a marked stabilization of the DNA helix and affected the sedimentation behavior in a qualitatively similar manner as streptothricins.

The authors concluded that the formation of such complexes might be associated with their antibiotic action.

Although studies on a nucleic acid model may be a valuable tool for elucidating the mechanism of action in vivo, other investigations indicate that the formation of complexes with DNA might not be the sole target system.

Effect on protein biosynthesis — whole cell systems. MISRA and SINHA (1971) studied the effect of boseimycin — an antibiotic of the streptothricin group containing β-lysine — on the growth and biosynthesis of protein, RNA and DNA in *Bacillus subtilis*. Cells in an early logarithmic phase of growth were used for incorporation of [^{14}C]-leucine and [^{32}P]-phosphate as precursors for the synthesis of protein and nucleic acids. At a concentration of 0.2 µg/ml, boseimycin depressed the growth rate and the synthesis of protein. The synthesis of RNA was reduced slightly after 30 min of incubation, while synthesis of DNA remained almost unaffected up to 3 h of incubation. At 2 µg/ml, biosynthesis of DNA was also partly depressed. The normal growth of bacteria was restored immediately after removal of the drug which suggested only a transient inhibition of the macromolecular synthesis. The antibiotic was found to cause no damage to the bacterial cell wall or cell membrane. The authors have concluded that the primary effect of boseimycin appears to be directed to a site or sites closely associated with the protein synthesis, most probably affecting the translational level of the protein synthesizing system. Similar results with respect to the site of action were obtained by HAUPT et al. (1978), who studied the effect of streptothricin F on DNA, RNA, and protein synthesis on intact cells of *Escherichia coli*. The synthesis of macromolecules was measured by incorporation of labeled precursors, such as thymidine, uridine, and leucine. Streptothricin F at 0.03 mM inhibited protein synthesis almost completely within 15 min, while the synthesis of DNA and RNA was unaffected.

Effect on protein biosynthesis — cell-free systems. KHOKHLOV and BLINOV (1970) and TELESNINA et al. (1973) were the first to study the effect of streptothri-

cins on protein biosynthesis in cell-free systems derived from *E. coli*. They compared the effect of streptothricin B, D, and E, and of their hydrolysis products (the respective streptothricinic acids), on protein biosynthesis directed either by endogenous messenger RNA or by poly(U) messenger. Both systems gave similar results. The antibiotic effect of streptothricins was closely related to their ability to inhibit protein biosynthesis at the ribosomal level. Both the antibiotic effect on the whole bacterial cell and the direct inhibitory effect on protein biosynthesis increased with increasing length of the β-lysine chain in the molecule. Thus, a 90% inhibition of protein biosynthesis was attained at 0.4 μmol/ml streptothricin B, 0.8 μmol/ml streptothricin D, and 3.2 μmol/ml streptothricin F. Inactivation products of streptothricin B, D, F, prepared by mild acid hydrolysis (the respective streptothricinic acids) were also tested. These compounds inhibited cell-free protein biosynthesis only slightly less than the parent streptothricins, whereas these degradation products were almost inactive in antibiotic tests on bacteria. The degradation product of streptothricin F, the streptothricin F acid (II), is about 200-fold less active than the parent compound. To explain this discrepancy, the authors suggest that mild acid hydrolysis may affect those properties of the streptothricin molecule which are connected with the penetration into the bacterial cell, but leaves intact the structures responsible for inhibition of protein biosynthesis at the level of the ribosome. As stressed by HAUPT et al. (1978), the effect of streptothricin F on cell-free protein biosynthesis depends on the composition of the homopolynucleotide added as messenger RNA. With poly(U), streptothricin F, at 0.01 mM concentration, inhibited polyphenylalanine synthesis to about 50%, which correlates with its growth inhibitory action. In contrast, streptothricin F stimulated the synthesis of polylysine or polyproline directed with poly(A) and poly(C) messenger, respectively. In this respect, the action of streptothricin F is very similar to that of streptomycin. The inhibitory effect of streptothricin F is Mg^{2+}-dependent. Poly(U)-directed polyphenylalanine synthesis was inhibited only at Mg^{2+} concentrations optimal for cell-free protein biosynthesis, whereas at less-than-optimal Mg concentrations the drug had no effect.

Miscoding activity. Streptothricin F causes errors in reading the genetic message. In polypeptide synthesis directed by homopolynucleotides streptothricin F stimulates the incorporation of amino acids, which are normally not coded by the polymer (Table 3). With poly(U), misreading was observed for isoleucine, leucine, serine, and tyrosine. The extent of miscoding of poly(U) with different amino acids increased with increasing streptothricin F concentrations. Furthermore, misreading was also demonstrated with poly(A) for glutamic acid and with poly(C) for serine. In the poly(A) and poly(C) systems, streptothricin F stimulates not only the incorporation of the incorrect but also of the correct amino acid (HAUPT et al., 1978).

Miscoding activity is a characteristic feature of aminoglycoside antibiotics with the streptamine or deoxystreptamine moiety (DAVIES et al., 1965; DAVIES and DAVIS, 1968). Streptothricin F does not contain this aminoglycoside structure, similarly to negamycin, a peptide-like antibiotic with miscoding properties (MIZUNO et al., 1970). However, UEHARA et al. (1972) have shown that there is similarity in the three-dimensional structure of negamycin and the aminogly-

Table 3. Miscoding activity of streptothricin F in polypeptide synthesis (*E. coli*) directed by synthetic homopolynucleotides[a]

Polynucleotide and amino acid		cpm incorporated		Ratio B/A
		A −Streptothricin F	B +Streptothricin F (0.1 mM)	
Poly(U)	Ileu	122	1256	10.3
	Leu	4715	7983	1.7
	Ser	247	1425	5.8
	Tyr	380	3672	9.7
Poly(A)	Glu	281	636	2.3
Poly(C)	Ser	368	877	2.4

[a] HAUPT et al. (1978).

coside antibiotics with miscoding activities. Whether such similarity in three-dimensional structure also exists in streptothricins remains to be decided.

With regard to the inhibition of polypeptide synthesis and miscoding pattern, streptothricin F induces similar effects as do other miscoding antibiotics, streptomycin, kanamycin, and megamycin.

The effect of streptothricin F on proteosynthetic activity of the ribosome was tested in the factor-free and factor-dependent synthesis of polyphenylalanine (HAUPT et al., 1980). The system is based on the observation of GAVRILOVA and SPIRIN (1974) and PESTKA (1974) that ribosomes programed with poly(U) can synthesize polyphenylalanine in the absence of elongation factors EF-Tu and EF-G. This indicates that the addition of elongation factors does not activate any new mechanism in protein synthesis but stimulates the mechanism already intrinsic to the ribosome. Streptothricin F very effectively suppresses both types of translation. However, factor-free translation is more sensitive to streptothricin F than is the factor-dependent translation. Both factors either separately or in a mixture can overcome streptothricin F inhibition at saturation levels.

These results suggested that streptothricin F acts on certain ribosomal components involved in the interaction of both EF-Tu and EF-G. When tested in individual steps of the ribosomal proteosynthetic reaction, streptothricin F inhibited the EF-Tu-dependent binding of aa-tRNA to the acceptor site of the ribosome and even more strongly the translocation reaction, i.e., the transfer of peptidyl-tRNA from the acceptor site to the donor site. The peptide bond formation catalyzed by ribosomal peptidyl transferase was not inhibited by streptothricin F.

Cell-free system from rat liver. In contrast to the results with *E. coli* extracts, cell-free synthesis in rat liver extracts directed by poly(U) or endogenous mRNA was not inhibited up to 1 mM streptothricin F. At 1 mM streptothricin F a slight stimulation of protein biosynthesis was observed (HAUPT et al., 1978).

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Novel Inhibitors of Translation in Eukaryotic Systems

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We have found a number of novel inhibitors of protein synthesis by eukaryotic cells in a screening program including axenomycin B, A201A, bouvardin, cyclopiazonic acid, grandilactones A and B, holacanthone, and 6,6′-dihydroxythionuphlutine (Table 1).

Axenomycin B was originally isolated from culture filtrates of *Streptomyces lisandri* and is a member of a novel family of antibiotics (ARCAMONE et al., 1973), which are potent inhibitors of eukaryotic cell growth (BIANCHI et al., 1974). Axenomycin B (Fig. 1) clearly blocks protein synthesis in intact HeLa cells, rather than either RNA or DNA synthesis (Table 1). Similar results have been reported with yeast cells (SORA et al., 1980). However, neither poly-U-directed polyphenylalanine synthesis nor polypeptide synthesis by cell-free extracts from yeast are particularly sensitive to the action of this antibiotic (Table 2, and SORA et al., 1980). This result might indicate that either the cell-free system does not reflect the in vivo conditions for protein synthesis faithfully, or that the drug must be metabolized in vivo in order to become active or, perhaps, that there is competition with an excess of elongation factors in the cell-free system. EF2- and GTP-dependent translocation of peptidyl-tRNA from the ribosomal A-site to the ribosomal P-site is blocked drastically by axenomycin B (Table 3). Curiously enough, a parallel, although significantly minor, effect is also observed on the enzymic binding of [^{3}H]phe-tRNA to reticulocyte ribo-

Table 1. Effects of inhibitors on protein, RNA, and DNA synthesis by HeLa cells

Inhibitor (M conc.)		Protein synthesis (% control)	RNA synthesis (% control)	DNA synthesis (% control)
Axenomycin B	4×10^{-7}	4	51	50
A201A	10^{-4}	86	100	45
Cyclopiazonic acid	10^{-4}	38	57	86
Grandilactone A	4×10^{-6}	9	118	59
Grandilactone B	4×10^{-6}	8	103	47
Holacanthone	10^{-6}	8	137	87
DOH-thionuphlutine	10^{-4}	5	79	11
Bouvardin[a]	10^{-6}	27	39	70

Experiments were performed as described by MUÑOZ and CARRASCO, 1981 and M. ZALACAIN et al., 1982. Unpublished data provided by E. ZAERA, F. SANTAMARÍA

[a] Data taken from CHITNIS et al. (1981)

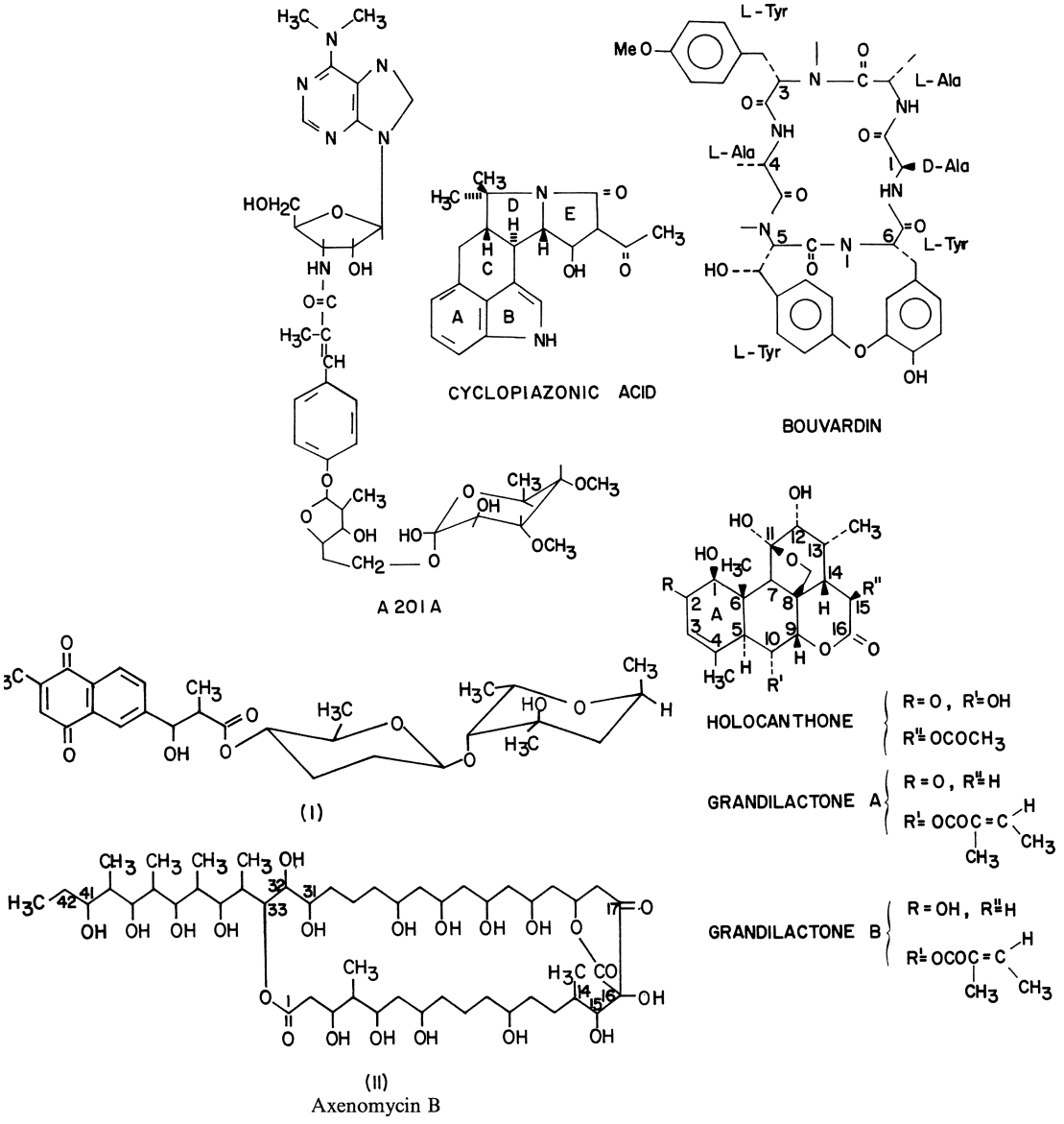

Fig. 1. Chemical structure of several inhibitors of protein synthesis

somes (Table 3). The meaning of this dual effect of axenomycin B is not clear, but similar results have been obtained with some other inhibitors. These findings are not surprising since the binding sites for both EF1 and EF2 elongation factors on the 80S ribosomes appear to be overlapping. Therefore, the binding site for axenomycin B might affect the interaction of both elongation factors with eukaryotic ribosomes. On the other hand, the effect on the binding of aminoacyl-tRNA to 80S ribosomes might be induced by nonspecific interaction

Table 2. Effects of inhibitors on poly(U)-directed polyphenylalanine synthesis and endogenous mRNA-programed polypeptide synthesis by yeast ribosomes

Inhibitor (M conc.)		Polyphenylalanine synthesis (% control)	Polypeptide synthesis (% control)
Axenomycin B	4×10^{-4}	77	100
A 201 A	2×10^{-4}	57	48
Bouvardin	4×10^{-6}	34	43
Cyclopiazonic acid	10^{-3}	64	58
Grandilactone A	2×10^{-5}	16	98
Grandilactone B	2×10^{-5}	60	101
Holacanthone	2×10^{-5}	44	87
DOH-thionuplutine	10^{-4}	44	21

Experiments were performed as described by GONZÁLEZ et al., 1978 and M. ZALACAIN et al., 1982. Unpublished data provided by E. ZAERA, F. SANTAMARÍA

Table 3. Effects of inhibitors on enzymic binding of [^{3}H]phe-tRNA to reticulocyte ribosomes and enzymic translocation by yeast polyribosomes

Inhibitor (M conc.)		[^{3}H]phe-tRNA binding (% control)	Enzymic translocation (% control)
Axenomycin B	10^{-4}	86	30
	4×10^{-4}	54	22
A 201 A	2×10^{-4}	–	45
Bouvardin	2×10^{-5}	90	47
Cyclopiazonic acid	5×10^{-4}	21	110
	2×10^{-3}	0	130
Grandilactone A	2×10^{-4}	36	–
Grandilactone B	2×10^{-4}	38	–
Holacanthone	4×10^{-4}	72	73
DOH-thionuphlutine	2×10^{-4}	–	44

Experiments were performed as described by GONZÁLEZ et al., 1978 and M. ZALACAIN et al., 1982. Unpublished data provided by E. ZAERA, F. SANTAMARÍA

of axenomycin B with ribosomal site(s) involved in the interaction with the elongation factor EF1. Thus, there is a single binding site of high affinity ($Kd = 3\times10^{-8}$ M) for [^{3}H]cryptopleurine on the 40S ribosomal subunit that is responsible for the specific inhibition of enzymic translocation by this alkaloid. In addition, there are multiple binding sites of an unspecific nature and low affinities on the 60S ribosomal subunit, which promote the inhibition of peptide bond formation by high cryptopleurine concentrations (DÖLZ et al., 1982). Yeast mutants resistant to axenomycin B present an altered 60S ribosomal subunit, indicating that this is where the antibiotic acts. Some of the mutants displace cross-resistance to other inhibitors of the large ribosomal subunit, like cycloheximide (an inhibitor of translocation) and sparsomycin, amicetin and gougerotin

(inhibitors of peptide bond formation). This is in agreement with locating the site of action of axenomycin B on the large subunit (SORA et al., 1980). This conclusion agrees with the finding that even at very high concentrations axenomycin B does not affect the binding of [^{3}H]cryptopleurine (a known inhibitor of enzymic translocation at the level of the 40S ribosomal subunit; VÁZQUEZ, 1979) to yeast 80S ribosomes (H. DÖLZ, personal communication).

Antibiotic A201A (Fig. 1) was initially isolated from *Streptomyces capreolus* (HAMILL and HOEHN, 1976) and shown to block protein synthesis by *Escherichia coli* intact cells selectively. This action is not surprising, considering the great similarity that exists between A201A and puromycin (Fig. 1). However, in HeLa cells it appears to inhibit DNA synthesis preferentially as well as partially blocking protein synthesis (Table 1). This result might be explained by considering that, in animal cells, A201A interferes mainly with DNA synthesis and to a less extent with the 80S ribosome. Nucleoside antibiotics have complex modes of action. These may involve inhibiting enzymatic activities in nucleic acid biosynthesis, as well as affecting specific reactions in the process of protein synthesis in the same way (SUHADOLNIK, 1979). In *E. coli* cells this antibiotic induces ribosome "run off," suggesting that it acts on the early rounds of polypeptide chain elongation (EPP and ALLEN, 1976). Indeed, peptide bond formation by the reaction of ribosome-bound Ac-Phe-tRNA with puromycin is severely inhibited in the presence of A201A (EPP and ALLEN, 1976; E. ZAERA, personal communication). However, A201A does not inhibit the formation of initiation complex by *E. coli* cell-free systems (HAMILL and HOEHN, 1976). In eukaryotic cells, the effect of A201A seems to take place at the level of translocation (Table 3), although a weak action on peptide bond formation may also be observed (Table 3).

Ring E of cyclopiazonic acid (isolated from *Penicillium cyclopium;* HOLZAPFEL, 1968) resembles the unique ring structure of tenuazonic acid (Fig. 1). This compound is well established as an inhibitor of protein synthesis in eukaryotic systems, by blocking peptide bond formation specifically (VÁZQUEZ, 1979, review). After comparing the chemical structures of cyclopiazonic acid and tenuazonic acid we expected that both might act in similar ways. Our results showed that, although cyclopiazonic acid inhibited protein synthesis selectively in intact HeLa cells (Table 1), it did not affect the peptide bond formation step by yeast ribosomes (Table 4). The elongation factor EF1- and GTP-dependent binding of Phe-tRNA to 80S ribosomes was clearly inhibited (Table 3) by concentrations of cyclopiazonic acid similar to those required to affect protein synthesis by both intact HeLa cells and yeast cell-free systems (Table 2). Cyclopiazonic acid has been reported as being responsible for outbreaks of cattle diseases (PURCHASE, 1974), and one might expect it to inhibit protein synthesis in intact cells at low concentrations. However, this is not the case for HeLa cells. We might interpret this as the result of a permeability barrier. Similar high concentrations were also required to induce inhibition of both polyphenylalanine synthesis and polypeptide synthesis. This could be explained by a competition of the toxin with the eukaryotic elongation factor EF1. Competition between drugs and elongation factor EF2 has been reported previously for cryptopleurine (JIMÉNEZ et al., 1977).

Table 4. Effects of inhibitors on peptide bond formation by ribosome preparations from yeasts

Inhibitor (M conc.)		Peptidyl-[^{3}H]PM formation (% control)	Fragment reaction (% control)
Axenomycin B	2×10^{-4}	120	112
A201A	10^{-4}	75	80
Bouvardin	2×10^{-5}	–	96
Cyclopiazonic acid	2×10^{-3}	127	101
Grandilactone A	2×10^{-5}	210	36
Grandilactone B	2×10^{-4}	201	57
Holacanthone	2×10^{-4}	90	2
DOH-thionuphlutine	2×10^{-4}	92	100

Experiments were performed as described by FRESNO et al., 1978. Unpublished data provided by E. ZAERA and F. SANTAMARÍA

Several quassinoids, the bitter principles of the *Simaroubaceae* family of plants, have been found to share high antineoplasic acitivy against experimental animal tumors (HARTWELL, 1976, WALL et al., 1976). Indeed bruceantin has been used in clinical trials against certain human cancers. A number of members of this group of compounds, including bruceantin (LIAO et al., 1976, GONZÁLEZ et al., 1978) bruceine B, chaparrinone, glaucarubolone, glaucarubinone, isobruceine A, (PIERRÉ et al., 1980), and brusatol (WILLINGHAM et al., 1981) have been shown to block protein synthesis by eukaryotic cells specifically. Therefore, it was not surprising to find that holacanthone, the active principle from *Holacantha emorgi* (WALL et al., 1976), and the grandilactones A (6α-tigloyloxychaparrinone) and B (6α-tigloyloxychaparrine) (Fig. 1), the active principles from *Simaba cuspidata* and *Ailanthus grandis* (Fig. 1) (POLONSKY et al., 1980), inhibited protein synthesis preferentially to both RNA and DNA synthesis in HeLa cells (Table 1). Also, these three quassinoids block protein synthesis by acting specifically on the peptide bond formation step (Table 4). In order to show this activity, the 80S ribosomes must have the P-site either empty or with a peptidyl tRNA with a very short peptidyl chain moiety. This conclusion is drawn from the fact that the holacanthone, grandilactone A, and grandilactone B have no effect on either protein synthesis (Table 2) or peptidyl-puromycin (Table 4) formation by yeast polysomes. Moreover, they block synthesis of both polyphenylalanine and N-acetyl-leucyl-puromycin by 80S ribosomes in a poly(U)-directed system and in the fragment reaction, respectively, very actively (Tables 2 and 4). On the other hand, holacanthone induces polysome run-off even at high concentrations. These results are similar to those obtained previously with bruceantin (FRESNO et al., 1977). Holacanthone binds at the peptidyl-transferase center of the 60S ribosomal subunit, since it inhibits the binding of [^{3}H]narciclasine to 80S ribosomes. Moreover, yeast mutants resistant to certain inhibitors of the peptidyl-transferase center, such as anisomycin, trichodermin, and narciclasine, are also resistant to holacanthone (ZAERA et al., unpublished). As opposed to holacanthone, both grandilactones A and B are strong inhibitors of the enzymic binding of aminoacyl-tRNA to 80S ribosomes. Comparing the chemical structures of these two drugs with that of holacanthone (Fig. 1), it can be deduced that the substitutes on C_{15} and/or C_{10} must be

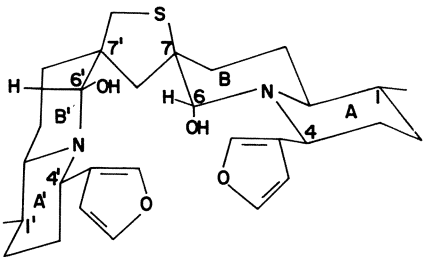

Fig. 2. Chemical structure of 6,6′-dihydroxythionuphlutine

responsible for the differences in activity on the aminoacyl-tRNA binding to 80S ribosomes. These results differentiate grandilactones A and B from the other members that affect the enzymic binding of Phe-tRNA to 80S ribosomes slightly, and only at high concentrations (Table 3, and FRESNO et al., 1977). Of all the known inhibitors of peptide bond formation that affect eukaryotic ribosomes, only the harringtonine group of alkaloids behave like grandilactones A and B in inhibiting the enzymic binding of aminoacyl-tRNA to 80S ribosomes (VÁZQUEZ, 1979, review). This mode of action of the grandilactones seems to be unrelated to that promoted by cyclopiazonic acid, since this compound does not affect peptide bond formation (Table 4).

6,6′-Dihydroxythionuphlutine (DOH-thionuphlutine) (Fig. 2) was initially isolated from the plant *Nuphar luteum* (water lily) and shown to have some activity against infectious fungi (CULLEN et al., 1973, LA LONDE et al., 1973). This effect may be explained by the specific inhibition of the compound on protein synthesis by eukaryotic cells observed in HeLa (Table 1) and yeast cells (ZAERA, personal communication). This inhibitory action is also observed in cell-free systems from yeast (Table 2). From the effects of DOH-thionuphlutine on a number of model reactions of the protein synthesis process (Tables 3 and 4), it can be concluded that this inhibitor blocks the enzymic translocation of peptidyl-tRNA on eukaryotic ribosomes specifically (Table 4). This effect is apparently unrelated to that of cryptopleurine (another inhibitor of enzymic translocation) since polysomes from a yeast mutant, resistant to this alkaloid, are sensitive to DOH-thionuphlutine.

Bouvardin is a cyclic hexapeptide (Fig. 1) which was isolated from extracts of the plant *Bouvardia ternifolia* (*Rubiaceae*). This extract has acted to a certain extent against P388 lymphocytic leukemia and B16 melanotic melanoma test systems (JOLAD et al., 1977). The drug inhibits preferentially protein synthesis in P388 cell suspensions (CHITNIS et al., 1981). However, as occurs with cyclopiazonic acid (Table 1), RNA synthesis is more strongly affected than DNA synthesis, which might indicate that the drug has a secondary effect in the biosynthesis of RNA polynucleotides. Our results showing the effect of bouvardin on cell-free protein synthesis indicate an inhibitory effect of the drug on chain elongation (Table 2), and more precisely on the translocation step (Table 3 and Zalacain et al., 1982). Therefore the drug might inhibit translocation and even at higher concentrations aminoacyl-tRNA binding. Other partial reactions of the elongation cycle were not affected by the drug (Tables 3 and 4).

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Tunicamycin and Related Antibiotics

J.S. TKACZ

In 1971, TAKATSUKI, ARIMA, and TAMURA reported the isolation of a glucosamine-containing antibiotic with antiviral activity from cultures of a soil isolate that they designated *Streptomyces lysosuperificus*. The antibiotic proved to have a wide spectrum of activity, inhibiting not only the replication of Newcastle disease virus in chick embryo fibroblasts but also the growth of Gram-positive bacteria, yeasts, and filamentous fungi as well as uninfected chick embryo cells (TAKATSUKI et al., 1971; TAKATSUKI and TAMURA, 1971a). Initial work on the mechanism of action suggested that the antibiotic interfered with the formation of viral and cellular surface or *coat* components, and for this reason the Latin word *tunica* was chosen as the stem for naming the material.

Interest in tunicamycin and tunicamycin-related antibiotics has grown steadily over the past decade because these compounds form a new class of amphipathic nucleoside antibiotics capable of inhibiting the production of certain "lipid intermediates" which participate in the synthesis of important glycoconjugates in eukaryotic as well as prokaryotic cells. Comprehensive surveys of the literature on tunicamycin have been published in Japanese (TAKATSUKI, 1978; TAKATSUKI and TAMURA, 1979); other reviews have been limited in scope (TKACZ, 1978; SUHADOLNIK, 1979; ELBEIN, 1979, 1981; SCHWARZ and DATEMA, 1980; STRUCK and LENNARZ, 1980). In this article, the molecular properties of the members of the tunicamycin family will be discussed in relation to their biological activities.

A. Physical Properties

The most complete taxonomic descriptions of *Streptomyces lysosuperificus* ATCC 31396 are available in tunicamycin-related patents (ARIMA et al., 1973; TAMURA and TAKATSUKI, 1980).

Tunicamycin is soluble in pyridine, dimethylsulfoxide, dimethylformamide, and methanol, less soluble in ethanol and butanol, and insoluble in benzene, chloroform, ethyl acetate, and acetone. In aqueous media it is soluble and stable at alkaline pH, and for biological experiments a solution may be prepared in 50 *mM* NaOH and diluted into the appropriate buffer immediately prior to use. Although concentrated aqueous solutions precipitate when the pH falls

below 6, biological experiments at acidic pH values are not precluded by insolubility since no precipitation is encountered when the antibiotic is diluted in acidic buffers to levels that are maximally active in vivo (i.e., 0.1–10 μg/ml). Tunicamycin is rapidly degraded when heated in strong acid or when exposed to periodate, but there is no loss of activity during a 24 h treatment with 2 *N* KOH at 105° C (TAKATSUKI et al., 1971).

By reversed-phase high-performance liquid chromatography the material isolated from *S. lysosuperificus* can be resolved into a series of structurally related components designated tunicamycin I to tunicamycin X according to the order of elution (ITO et al., 1980). Each component exhibits two absorption maxima in the ultraviolet, one at 205 nm and the second at 260 nm, resulting from the presence of a uracil moiety. Extinction coefficients ($E^{1\%}_{1\,cm}$) of methanolic solutions at 260 nm range from 76 to 110 with most components having values near 100 (ITO et al., 1980). The infrared and nuclear magnetic resonance spectra of the tunicamycin complex have been published (TAKATSUKI et al., 1971).

B. Structure

The structures and molecular weights of the ten members of the tunicamycin complex are given in Fig. 1. Each molecule contains the same hydrophilic portion composed of N-acetylglucosamine, uracil, and a novel C_{11}-aminodeoxy dialdose, which has been named tunicamine; the variation among the members of the complex stems from heterogeneity in the hydrophobic moieties.

Elucidation of the structures was accomplished in large part through characterization of the degradation products obtained by acid hydrolysis (3 *N* HCl,

COMPONENT	MOL.WT.	R
III	816	$CH_3(CH_2)_{10}CH{=}CH{-}C$
IV	830	$CH_3(CH_2)_{11}CH{=}CH{-}C$
VIII	844	$CH_3(CH_2)_{12}CH{=}CH{-}C$
IX	858	$CH_3(CH_2)_{13}CH{=}CH{-}C$
I	802	$(CH_3)_2CH(CH_2)_7CH{=}CH{-}C$
II	816	$(CH_3)_2CH(CH_2)_8CH{=}CH{-}C$
V	830	$(CH_3)_2CH(CH_2)_9CH{=}CH{-}C$
VII	844	$(CH_3)_2CH(CH_2)_{10}CH{=}CH{-}C$
X	858	$(CH_3)_2CH(CH_2)_{11}CH{=}CH{-}C$
VI	832	$(CH_3)_2CH(CH_2)_{11}{-}C$

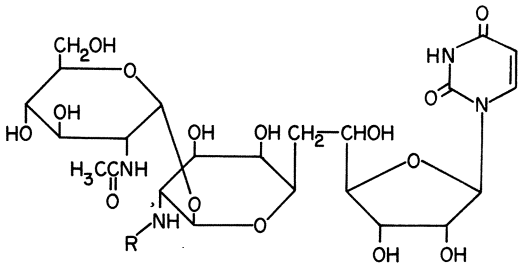

Fig. 1. Structures of the tunicamycins. Variations in the fatty acid substituent differentiate the members of this antibiotic complex. The *numerical designations* of the tunicamycin homologs correspond to the order of elution in reversed-phase column chromatography

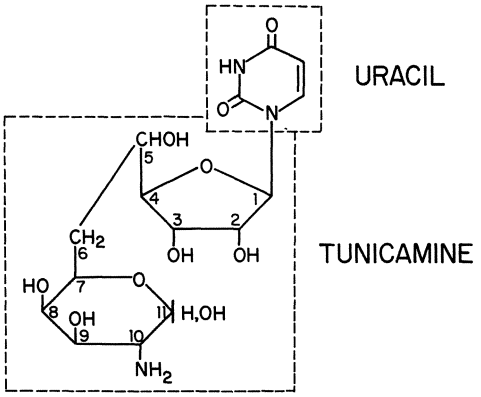

Fig. 2. Structure of tunicaminyl uracil, the UV-absorbing water-soluble acid hydrolysis product of tunicamycin

100° C, 3 h). This treatment released D-glucosamine and a UV-absorbing material, tunicaminyl uracil (Fig. 2), as water-soluble products. The glucosamine was identified by its behavior on ion-exchange and paper chromatography and its IR and NMR spectra; results obtained by NMR analysis of the unhydrolyzed complex and by mass spectrometry of permethylated antibiotic indicated that the glucosamine moiety was N-acetylated in the intact molecule (TAKATSUKI et al., 1971, 1977a). The UV-absorbing hydrolysis product gave a fragmentation pattern by mass spectrometry, which together with the results of elemental analysis established its molecular formula as $(C_4H_3N_2O_2)$ $(C_{11}H_{12}O_2)$ $(OH)_6$ (NH_2). No cleavage was observed between C6 and C7 in the polyol chain, suggesting a methylene unit at C6. From the hydrogen content of the C_{11} chain, two oxygen-containing rings were suspected, and on the basis of [^{1}H] and [^{13}C] NMR spectra, optical rotation, and ORD measurements, D-ribofuranose and 2-amino-2-deoxy-D-galactopyranose centers were assigned at C1 to C4 and C7 to C11, respectively. The presence of a uracil residue as the UV chromophore was inferred from the molecular formula and the value of the molar extinction coefficient; this was confirmed by the finding that this pyrimidine was liberated when the material was refluxed in 6 *N* HCl, conditions which destroyed the tunicamine. Spectral similarities between tunicaminyl uracil and β-D-ribofuranosyl uracil (uridine) led to the conclusion that this fragment of the tunicamycin molecule is composed of uridine linked at C5′ to C6 of 2-amino-2,6-dideoxy-D-galactose by a carbon-carbon bond (ITO et al., 1977, 1979). Verification of the structure through chemical synthesis is under way (SECRIST and BARNES, 1980). Because of its C_{11} sugar, tunicamycin is an unusual, although not unique, natural product; a C_{11} amino sugar has been found as a component of hikizimycin, another streptomycete antibiotic (UCHIDA and DAS, 1973).

The ether-soluble products released by acid hydrolysis of the tunicamycin complex proved to be a series of n-α,βunsaturated-C_{14-17} and iso-α,βunsaturated-C_{13-17} fatty acids. Fully saturated iso-C_{15} fatty acid was found as well. In every unsaturated fatty acid for which spectral analysis has been possible, a *trans* double bond has been detected. The identity of the fatty acid associated

with each component in the tunicamycin complex is given in Fig. 1. By graded acid hydrolysis of tunicamycin, a product composed of tunicaminyl uracil and fatty acid, but no glucosamine, was obtained, the IR spectrum of this product showed the presence of a peptide bond indicating that the amino group of tunicamine is the site for the attachment of fatty acid (TAKATSUKI et al., 1977a, 1979; ITO et al., 1980).

Tunicaminyl uracil and the amphipathic product of graded acid hydrolysis were reducing compounds, whereas the antibiotic before removal of the N-acetylglucosamine residue was not. This established that the anomeric carbon at C11 in tunicamine was glycosidically linked to the C1 of N-acetylglucosamine. From spectral and optical rotation data it was initially proposed that both amino sugars were α-linked, but a reconsideration of the data has led to the conclusion that C11 of tunicamine is β-linked (TAKATSUKI et al., 1977a; ITO et al., 1980).

C. Action on Eukaryotic Cells

I. Initial Observations

Tunicamycin is active on organisms from every part of the eukaryotic kingdom; examples include yeast (TAKATSUKI et al., 1971; KUO and LAMPEN, 1974), filamentous fungi (KATOH et al., 1976, 1978a, and b; LONG and RUDICK, 1979), algae (RAY et al., 1978), protozoa (FRISCH et al., 1976; FRISCH and LOYTER, 1977; KEENAN and RICE, 1980; RICHMOND, 1979; STRICKLER and PATTON, 1980; UDEINYA and VANDYKE, 1979, 1980), slime molds (FRAZIER et al., 1980), insects and other invertebrates (BUTTERS et al., 1981; HEIFETZ and LENNARZ, 1979; SCHNEIDER et al., 1978; AKASAKA et al., 1980; DAN-SOHKAWA et al., 1980), amphibians (ROMANOVSKY and NOSEK, 1980; COLMAN et al., 1981), vertebrates (TAKATSUKI and TAMURA, 1971a; ARIMA et al., 1973; SURANI, 1979; ATIENZA-SAMOLS et al., 1980), and plants (SCHWAIGER and TANNER, 1979; MELLOR et al., 1980; JAMES and ELBEIN, 190). The activity results from an inhibitory effect on the formation of Asn-GlcNAc type glycoproteins, a class of proteins having asparagine residues that are substituted with branched oligosaccharides. The latter characteristically contain mannose and N-acetylglucosamine and, as the name implies, residues of N-acetylglucosamine (GlcNAc) serve to link the oligosaccharides to the asparagine units. These glycoproteins are prominent components of the cell surface and are found among the molecules exported from the cell or sequestered within membrane-bound organelles. As components of membranes, they function as enzymes, receptors for hormones and viruses, structural and transport components, and mediators of immunological specificity. In plants and fungi, they form part of the cell wall structure. Included among the secreted Asn-GlcNAc glycoproteins are enzymes, hormones, toxins, lectins, immunoglobulins, and serum transfer factors (KORNFELD and KORNFELD, 1976).

The first clues to the mode of action of tunicamycin came from examination of the antiviral activity which was the basis of the antibiotic's discovery. The antiviral spectrum included both enveloped RNA viruses (e.g., Newcastle disease virus, fowl plague virus, Semliki forest virus, Sindbis virus, and vesicular stomatitis virus) and enveloped DNA viruses (e.g., herpes virus) but did not extend to nonenveloped viruses such as the encephalomyocarditis virus (TAKATSUKI et al., 1971; SCHWARZ et al., 1976; LEAVITT et al., 1977a). Inhibition of replication was independent of host cell type, and with infection of chick embryo fibroblasts by Newcastle disease virus it was demonstrated that the antibiotic did not prevent adsorption, penetration, or uncoating (TAKATSUKI and TAMURA, 1971a). These findings focused attention upon the synthesis and assembly of viral components. especially envelope components (i.e., lipids and Asn-GlcNAc glycoproteins), as likely targets for tunicamycin action, and in experiments measuring the formation of macromolecules and lipids from radioactive precursors in cells infected with Newcastle disease virus, TAKATSUKI and TAMURA (1971c) found that the antibiotic markedly suppressed [^{14}C]glucosamine incorporation, had less pronounced effects upon labeling with [^{14}C]amino acids or [^{3}H]thymidine, and did not perturb the incorporation of [^{14}C]choline or [^{14}C]uridine.

These indications that the pathway for the synthesis of Asn-GlcNAc type glycoproteins was the metabolic site of tunicamycin's activity were corroborated by studies with yeast; KUO and LAMPEN (1974) reported that the formation of the active forms of two glycosylated cell-wall enzymes (invertase and acid phosphatase) was curtailed by tunicamycin, whereas the synthesis of a nonglycosylated cytoplasmic enzyme (α-glucosidase) and [^{14}C]amino acid-labeled protein was unaffected. As observed in the virus-infected cells, the antibiotic reduced the incorporation of [^{3}H]glucosamine into acid-insoluble products, but it did not interfere with the uptake of glucosamine by the cells or with any of the metabolic steps leading to the formation of UDP-GlcNAc (KUO and LAMPEN, 1976).

The observation that most of the tunicamycin bound by a cell is associated with cellular membranes (TAKATSUKI and TAMURA, 1972; KUO and LAMPEN, 1976) led to an erroneous conclusion regarding the manner in which the antibiotic interfered with glycoprotein formation; TAKATSUKI and TAMURA (1972) proposed that tunicamycin was incorporated in place of sugars into precursors of cell-membrane glycoproteins to generate defective molecules which could not be further glycosylated. Unambiguous evidence in support of this concept was not presented and, subsequently, it was shown that virtually all the antibiotic bound by membranes could be extracted with organic solvents (KUO and LAMPEN, 1976), suggesting that its association with this subcellular fraction was a consequence solely of its amphipathic character which enabled it to intercalate among the lipids of the bilayer. Once within the membrane, tunicamycin is not easily washed out with aqueous buffers or EDTA, a point which is often overlooked by those using the antibiotic as a tool to explore glycoprotein function in vivo. Protection from the antibiotic is afforded by lipid micelles (KUO and LAMPEN, 1976; TAKATSUKI and TAMURA, 1978), and although washing cells with micelle preparations results in the removal of a major part of the cell-bound tunicamycin, that which remains is sufficient to inhibit the cells (KUO

and LAMPEN, 1976). Partial protection is apparently also given by several N-acetyl and N-acyl hexosamines, but the basis of this effect is not clear at present (TAKATSUKI and TAMURA, 1971 b).

The biological activity of tunicamycin is apparently not dependent upon metabolic alteration of the molecule, for the material extracted from membranes of cells that had been incubated in its presence behaved as authentic tunicamycin by thin-layer chromatography (KUO and LAMPEN, 1976).

II. Identification of the Enzymatic Target

The pathway for the glycosylation of an asparagine residue in a protein is a multistage process involving the assembly of an oligosaccharide upon a lipid carrier, the transfer of the oligosaccharide to the asparagine residue while the latter is still part of a nascent polypeptide, and the maturation of the oligosaccharide through hydrolytic trimming and, in some cases, further glycosylation. Although the oligosaccharide transferred from the lipid is composed of N-acetylglucosamine, mannose, and glucose, the latter sugar is associated only transiently with the glycoprotein product. Like the intermediates in prokaryotes which participate in the formation of various cell-surface glycoconjugates, the lipid carriers utilized for protein glycosylation are linear polyprenyl alcohols (known as dolichols). The pathway is initiated by the action of a phosphotransferase which removes GlcNAc-1-P from UDP-GlcNAc and couples it with dolichyl phosphate to produce the pyrophosphate derivative. The resulting glycolipid then serves as the acceptor for a series of glycosyl transferases which assemble the remainder of the oligosaccharide. The saccharide donors in these reactions are either sugar nucleotides or dolichyl glycosyl monophosphates which are the products of glycosyl transferase reactions involving sugar nucleotides and dolichyl phosphate. Further details regarding the pathway may be found in recent reviews by PARODI and LELOIR (1979), STRUCK and LENNARZ (1980), and HUBBARD and IVATT (1981).

The influence of tunicamycin on the incorporation of glucosamine into glycoprotein results from its ability to interfere with the dolichol pathway. It is a potent inhibitor for the synthesis of dolichyl N-acetylglucosaminyl pyrophosphate (Fig. 3), the initial intermediate in oligosaccharide assembly. This inhibition was first demonstrated with membrane preparations from liver (TKACZ and LAMPEN, 1975) and chick embryos (TAKATSUKI et al., 1975) and was subsequently verified with membranes from several other sources including plants (ERICSON et al., 1977; LEHLE and TANNER, 1976; STRUCK and LENNARZ, 1977; WAECHTER and HARFORD, 1977). The antibiotic does not inhibit the glycosyl transferases responsible for the synthesis of dolichyl mannosyl phosphate (KUO and LAMPEN, 1976; STRUCK and LENNARZ, 1977; TKACZ and LAMPEN, 1975), dolichyl galactosyl phosphate (TAKATSUKI et al., 1975), or dolichyl di-N-acetylchitobiosyl pyrophosphate, the second intermediate in the pathway (LEHLE and TANNER, 1976). Nor does it block the transfer of the peripheral GlcNAc residues to the oligosaccharide during the maturation process (STRUCK and LENNARZ, 1977; WAECHTER and HARFORD, 1977). It does suppress the formation of doli-

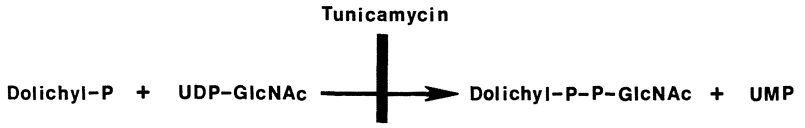

Fig. 3. The enzymatic target of tunicamycin in eukaryotic cells. Dolichyl N-acetylglucosaminyl pyrophosphate is a key intermediate in the formation of the oligosaccharide portions of Asn-GlcNAc type glycoproteins

chyl glucosyl phosphate but only at a concentration a 100-fold higher than that required to curtail dolichyl N-acetylglucosaminyl pyrophosphate synthesis (ELBEIN et al., 1979); this activity is discussed further in Sect. F. Thus, tunicamycin is not a general inhibitor of glycosyl transferase reactions and does not act as an analog of UDP-GlcNAc for all GlcNAc transfer reactions.

Kinetic studies with partially purified preparations of the UDP-GlcNAc:dolichyl phosphate GlcNAc-1-P transferase from aorta and oviduct have shown that tunicamycin behaves as a "tight binding" inhibitor (HEIFETZ et al., 1979; KELLER et al., 1979). Structural characteristics which make the antibiotic a bisubstrate analog account for this behavior. The hydrophilic part of tunicamycin, containing an α-linked GlcNAc residue and a uridine-like moiety, bears a resemblance to UDP-GlcNAc, and the fatty acid portion imbues the molecule with a hydrophobic property mimicking the polyprenyl substrate (for a structural comparison see Fig. 5 of KELLER et al., 1979). Because of the "tight binding" character of the inhibitor, classical kinetic analysis to establish the competitive or noncompetitive nature of the inhibition is not possible (KELLER et al., 1979).

III. Physiological Consequences

STRUCK and LENNARZ (1977) established that tunicamycin blocks protein glycosylation in vivo by the same mechanism demonstrable in vitro. It is generally found that cells incubated with tunicamycin produce the unglycosylated forms of glycoproteins. Although this argues against a regulatory link requiring a functional glycosylation system for continued synthesis of the protein portion of a glycoprotein, regulation of this sort has been proposed (HASILIK and TANNER, 1976, 1978; SCHWAIGER and TANNER, 1979; SEAGAR et al., 1980). Based upon what is currently known regarding the behavior of the carbohydrate-free forms of glycoproteins, it would not be surprising to find that the methodologies employed in the studies which led to the idea of a regulatory link were incapable of detecting the production of the unglycosylated protein species.

The consequences of not being glycosylated seem to vary from one protein to the next. In many instances, the deficiency markedly alters the fate of the molecule. For example, as unglycosylated proteins, IgA, IgE, and IgM are inefficiently secreted (HICKMAN and KORNFELD, 1978; HICKMAN et al., 1977). The migration of the envelope proteins of Sindbis virus and vesicular stomatitis virus from the site of synthesis in the endoplasmic reticulum of the host cell to the plasma membrane is impaired if the proteins are not glycosylated (LEAVITT

et al., 1977b). Moreover, the envelope protein of the vesicular stomatitis virus becomes much less soluble when it is not glycosylated (LEAVITT et al., 1977b; GIBSON et al., 1979). The carbohydrate-free forms of chick fibronectin and the hemagglutinin of fowl plague virus are unusually susceptible to proteolytic digestion in vivo (OLDEN et al., 1978; SCHWARZ et al., 1976), but the polypeptide precursor of the envelope protein of Rauscher murine leukemia virus, on the other hand, is not proteolytically processed unless it is glycosylated (SCHULTZ and OROSZLAN, 1979). In the presence of tunicamycin, the α and β subunits of thyroid-stimulating hormone are produced in mouse pituitary cells, but the subunits appear unable to associate to form active hormone (WEINTRAUB et al., 1980). Although there is more than one physiological consequence of tunicamycin action, dysfunctions, such as altered solubility, changes in sensitivity to proteolytic attack, modified intermolecular interactions, and inability to pass through the membrane compartments of a cell, could plausibly have a single biochemical basis: all could be manifestations of alterations in protein conformation. This reasoning, together with the finding that the glycosylation of asparagine residues in many cases is a co-translational event, has prompted the suggestion that a major role of the glycosylation process is to influence the folding of a peptide chain as it emerges from the ribosome so that it may assume a biologically active conformation (GIBSON et al., 1980).

Of course, folding is a complex process involving many intramolecular interactions, and glycosylation may not necessarily be a crucial determinant in every case. In fact, there are several examples of glycoproteins for which carbohydrate deficiency is apparently inconsequential. The "signal" peptides of placental prelactogen and of the pre-α subunit of human chorionic gonadotropin are proteolytically removed whether or not the molecules are glycosylated (BIELINSKA et al., 1978). Yeast alkaline phosphatase synthesized in the presence of tunicamycin is enzymatically active even though it lacks carbohydrate (ONISHI et al., 1979). The secretion of ovalbumin, liver transferrin, α_1-acid glycoprotein, the apoprotein B of very low density lipoprotein, interferon, or IgG proceeds quite normally when the protein is not glycosylated (EDWARDS et al., 1979; FUJISAWA et al., 1978; HICKMAN and KORNFELD, 1978; KELLER and SWANK, 1978; MIZRAHI et al., 1978; STRUCK et al., 1978). Thus, although it is not possible at present to predict the outcome of carbohydrate deficiency for a specific glycoprotein, it is clear from the inhibitory action of tunicamycin on eukaryotic cells that the formation of certain Asn-GlcNAc glycoproteins is obligatory for continued growth and maintenance of viability.

Several groups of investigators have observed that exposure of certain animal cell lines to tunicamycin causes a moderate reduction of the incorporation of radioactive amino acids into proteins, as well as the marked inhibition of the incorporation of labeled glucosamine into complex saccharides. MAHONEY and DUKSIN (1979) claimed to have separated by preparative reversed-phase chromatography two fractions of tunicamycin which differed with respect to this effect on amino acid incorporation; regrettably, a characterization of these fractions (e.g., by analytical h.p.l.c.; MAHONEY and DUKSIN, 1980) was not provided. In the recent study of KEENAN et al. (1981), little inhibition of leucine incorporation in canine kidney cells was observed with any of the individual members of the complex at concentrations that blocked glycosylation, but at higher levels

some inhibition was found with each component. Despite references to this effect of tunicamycin as an inhibition of protein synthesis, there are two reasons to view the phenomenon as a secondary result of impaired membrane function (i.e., amino acid uptake) rather than as an inhibition of the machinery of protein synthesis per se. Firstly, HICKMAN et al. (1977) found that the antibiotic had no influence upon a cell-free protein synthesizing system. And secondly, the exposure of chick embryo fibroblasts to tunicamycin has been shown to have a detrimental effect upon the uptake of certain nutrients by the cells (OLDEN et al. 1979b; ZALA et al., 1980). Possibly when produced as nonglycosylated species, some permeases cannot achieve biologically active conformations or, like the envelope protein of vesicular stomatitis virus, cannot reach the plasma membrane.

Oligosaccharides composed of mannose and glucosamine are found attached to asparagine residues in the peptide extensions of procollagen, and because tunicamycin is capable of blocking the addition of these sugars (DUKSIN and BORNSTEIN, 1977b; HOUSLEY et al., 1980), it appears likely that these oligosaccharides, just as those of Asn-GlcNAc type glycoproteins, are synthesized via dolichol intermediates. As a result of tunicamycin action the migration of procollagen to the cell surface is impeded (HOUSLEY et al., 1980), and its proteolytic conversion to collagen is diminished (DUKSIN and BORNSTEIN, 1977b; DUKSIN et al., 1978b). Oligosaccharides containing mannose and glucosamine are also present in corneal keratan sulfate, where they serve to link the sulfated polysaccharide chains to protein. HART and LENNARZ (1978) found that tunicamycin abolishes the synthesis of this glycosaminoglycan, and suggested the involvement of polyprenyl intermediates in the formation of these oligosaccharide linkage assemblies as the basis for the inhibition. In other sulfated glycosaminoglycans, the polysaccharide portions are linked to protein through xylose rather than oligosaccharides composed of mannose and glucosamine. Nevertheless, the synthesis of these glycosaminoglycans is also sensitive to tunicamycin (HART and LENNARZ, 1978; PRATT et al., 1979; TAKATSUKI et al., 1977c, 1978). For heparan sulfate and chondroitin sulfate, the antibiotic causes substantial inhibition of the production of protein-bound polymers but has little effect on β-xyloside-dependent polysaccharide formation (HART and LENNARZ, 1978). This suggests that polyprenyl intermediates are not utilized in the synthesis of the polysaccharide chains, and in this connection it is noteworthy that other studies have failed to produce compelling evidence for their involvement (SUGAHARA et al., 1979; TAKATSUKI et al., 1978; TURCO and HEATH, 1977). The data presented by HART and LENNARZ point to the formation of functional protein acceptors as the aspect of the assembly process influenced by tunicamycin. By learning how and why the antibiotic can affect this process, new insight into the structure or biogenesis of this important class of glycoconjugates may be gained.

D. Action on Prokaryotic Cells

Tunicamycin is a potent growth inhibitor for Gram-positive bacteria, notably members of the genus *Bacillus* (TAKATSUKI et al., 1971; WARD, 1977),

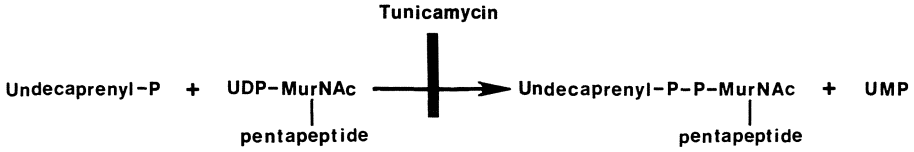

Fig. 4. The tunicamycin-sensitive step in the peptidoglycan pathway

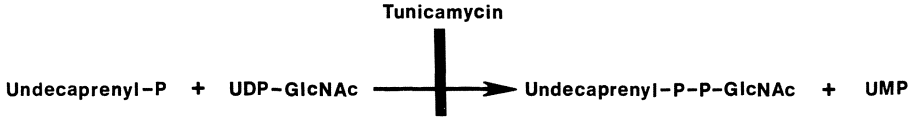

Fig. 5. Inhibition of the formation of undecaprenyl N-acetylglucosaminyl pyrophosphate by tunicamycin prevents production of the molecular bridge which links teichoic acid to peptidoglycan in the cell walls of Gram-positive bacteria

even though these bacteria (indeed all eubacteria) are apparently unable to elaborate Asn-GlcNAc glycoproteins (MESCHER, 1981). In *Bacillus* species as in filamentous fungi, tunicamycin induced severe morphological changes (KATOH et al., 1976, 1978a; TAKATSUKI et al., 1972) that implicated the cell wall in the mode of action. Corroborating evidence for this target site was provided by a study of macromolecule synthesis in *Bacillus subtilis,* which revealed a preferential effect of the antibiotic upon [^{14}C]glucosamine incorporation (TAKATSUKI et al., 1972). Subsequent work has shown that tunicamycin is capable of interfering with the formation of two types of bacterial cell-wall polymers: one is peptidoglycan and the other is teichoic or teichuronic acid.

Inhibition of peptidoglycan synthesis was reported by BETTINGER and YOUNG (1975), and the basis for the effect was established by TAMURA et al. (1976) and by WARD (1977) who found that tunicamycin blocked the phosphotransferase that utilizes UDP-N-acetylmuramoyl-pentapeptide and undecaprenyl phosphate to produce undecaprenyl N-acetylmuramoyl-pentapeptide pyrophosphate (Fig. 4), the first membrane-bound "lipid intermediate" in the well-known pathway of peptidoglycan assembly. This was demonstrated with particulate preparations from *B. licheniformis, B. subtilis, Micrococcus luteus,* and *Staphylococcus aureus*. The synthesis of the second intermediate in the pathway which proceeds by the transfer of GlcNAc from UDP-GlcNAc to the first intermediate (a glycosyl transferase reaction) was not sensitive to tunicamycin despite the claim to the contrary (BETTINGER and YOUNG, 1975).

Tunicamycin suppresses the formation of polyribitol phosphate teichoic acid in *S. aureus* and *B. subtilis* (BRACHA and GLASER, 1976; HANCOCK et al., 1976; MCARTHUR et al., 1978; WYKE and WARD, 1977), poly-glycerol phosphate teichoic acid in *B. licheniformis* (WARD, 1977), and a teichuronic acid composed of glucose and N-acetylmannosaminuronic acid in *M. luteus* (WESTON and PERKINS, 1977). Rather than acting at the level of polymer elongation, the antibiotic interferes with the synthesis of a glucosamine-containing unit that ultimately links the polymer to peptidoglycan. This linkage unit is formed upon a polyprenyl phosphate (believed to be undecaprenyl phosphate) by the sequential

transfer of GlcNAc-1-P from UDP-GlcNAc and then one to three glycerol phosphate residues from CDP-glycerol. Subsequently, the product accepts the main teichoic acid chain, and the entire assembly is transferred from the lipid to peptidoglycan (WARD, 1981). It is the enzyme which catalyzes the first step in the formation of the linkage unit that is blocked by tunicamycin (Fig. 5) (BETTINGER and YOUNG, 1975; MCARTHUR et al., 1978; WYKE and WARD, 1977). The sensitivity of this phosphotransferase is approximately fivefold greater than that of the target enzyme in the peptidoglycan pathway (WARD et al., 1980).

Tunicamycin-resistant mutants of *B. subtilis* have been isolated and analyzed genetically (NOMURA et al., 1978; SASAKI et al., 1976). Resistance is conferred by mutations at either of two chromosomal loci, but the physiological basis of resistance has not been elucidated. Mutations at one of the loci also lead to hyperproduction of extracellular α-amylase but apparently no other exoenzymes. Each resistance locus lies close to an amylase structural or regulatory gene (NOMURA et al., 1978). In the presence of tunicamycin, much of the α-amylase synthesized by wild-type or resistant cells is not released into the medium but is retained in the periplasmic space, normally the site of a small pool of the enzyme (GOULD et al., 1975; SASAKI et al., 1980); thus, as a consequence of tunicamycin action, there may be a change in cell-wall porosity sufficient to prevent the passage of proteins the size of α-amylase, viz., 48,800 daltons (JUNGE et al., 1959).

E. Related Antibiotics

Four antibiotics with chemical or biological properties similar to those of tunicamycin have been isolated from *Streptomyces* species; they are mycospocidin, streptovirudin, antibiotic MM19290, and antibiotic 24010.

Mycospocidin was described 14 years before the first publication on tunicamycin appeared (NAKAMURA et al., 1957). It was produced by *Streptomyces bobiliae* and was active against Gram-positive bacteria, mycobacteria, and fungi. Acid degradation gave two ninhydrin-positive products, one of which was thought to be glycine. Although mycospocidin and tunicamycin had virtually identical UV and IR spectra and very similar melting points, TAKATSUKI et al. (1971) concluded that the two were distinct antibiotics on the basis of thin-layer chromatography and the purported presence of glycine in mycospocidin. A more recent examination of mycospocidin by reversed-phase h.p.l.c. revealed that it was an antibiotic complex with components that were indistinguishable from those of tunicamycin on the basis of retention times. The relative proportions of the components in the two complexes differed somewhat, however. Acid hydrolysis gave tunicaminyl-uracil, glucosamine, a series of fatty acids, but no glycine. Mycospocidin did not inhibit the growth of mutants of *Bacillus licheniformis* or *Saccharomyces cerevisiae,* which were selected for resistance to tunicamycin, and in vitro it was capable of blocking the enzymatic synthesis of dolichyl N-acetylglucosaminyl pyrophosphate (TKACZ and WONG, 1978;

TKACZ, 1980). Thus, it appears that mycospocidin and tunicamycin are names which have been applied to the same antibiotic complex.

The streptovirudins are a group of antibiotic compounds synthesized by *Streptomyces griseoflavus* subsp. *thuringiensis* (THRUM et al., 1975). They inhibited the replication of enveloped viruses, such as Sindbis virus, fowl plague virus, Newcastle disease virus, pseudorabies virus, vaccinia virus, and vesicular stomatitis virus, but were not active against nonenveloped viruses including mengo virus, Coxsackie virus, ECHO virus, and polio virus (TONEW et al., 1975; KANG et al., 1981). They also suppressed the growth of Gram-positive bacteria, mycobacteria, and yeast (THRUM et al., 1975). Glucosamine and uracil were among the products of acid hydrolysis (ECKARDT et al., 1975, 1980). The streptovirudins had IR spectra which were similar to each other and to that of tunicamycin, but their UV spectra provided a basis for dividing them into two series of components. Streptovirudins of series I exhibited a single ultraviolet absorption maximum near 210 nm, whereas those of series II had the two maxima typical of tunicamycin (ECKARDT et al., 1975). Reversed-phase h.p.l.c. indicated that most streptovirudins of series II were more polar than the members of the tunicamycin complex, but certain species were common to both complexes (TKACZ, 1980; ECKARDT et al., 1980; KEENAN et al., 1981). By acid hydrolysis and mass spectrometry studies it was established that the hydrophilic portions of tunicamycin and the streptovirudins of series II were identical and that the streptovirudins contained fatty acids which were shorter, on average, than those from tunicamycin (TKACZ, 1980; KEENAN et al., 1981). Mass spectrometry also showed that the streptovirudins of series I differ from their counterparts in series II by 2 mass units as a consequence of the presence of dihydrouracil moieties in place of uracil residues (ELBEIN et al., 1981). The formation of dolichyl N-acetylglucosaminyl pyrophosphate in membrane preparations from animal and plant tissues was prevented by streptovirudin (ELBEIN et al., 1979; JAMES and ELBEIN, 1980; TKACZ, 1980) and, as expected from this, glycosylation of proteins could be suppressed in vivo (JAMES and ELBEIN, 1980; KANG et al., 1981).

Antibiotic MM19290 is one of two antimicrobial products formed by a strain of *Streptomyces clavuligerus* (KENIG and READING, 1979). The antibiotic contained glucosamine and gave IR and UV spectra that indicated its similarity with tunicamycin. Reversed-phase h.p.l.c. demonstrated that the antibiotic is a complex containing all the components of the tunicamycin complex as well as more polar forms analogous to streptovirudins of series II.

Antibiotic 24010 was isolated from an unclassified streptomycete on the basis of its activity against phytopathogenic fungi (MIZUNO et al., 1971). Like tunicamycin, it induced mycelial swelling and was active against Gram-positive bacteria and yeast. Its UV, IR, and NMR spectra closely resembled those of tunicamycin. Additional studies showed that antibiotic 24010 blocked the formation of polyprenyl N-acetylglucosaminyl pyrophosphates in membrane preparations from animal and plant tissues (ELBEIN et al., 1979; JAMES and ELBEIN, 1980) as well as bacteria (MURAZUMI et al., 1979, 1981; YAMAMORI et al., 1978). From these chemical and biological findings it is clear that antibiotic 24010 is a member of the tunicamycin family of antibiotics; however, no evidence is available at present to indicate which member it most closely resembles.

F. Structure–Activity Relationships

Although the enzymes which are subject to inhibition by tunicamycin are involved in the synthesis of three quite distinct types of glycoconjugates in phylogenetically diverse organisms, several common features can be recognized (Fig. 6). All are phosphotransferases which catalyze the translocation of an N-acetylhexosamine-α-1-phosphate from a UDP precursor. The acceptor utilized in the reaction is a polyprenyl phosphate, and the hexosamine is glucosamine or a glucosamine derivative (N-acetylmuramoyl-pentapeptide may be considered a peptide-substituted 3-O-lactyl ether of N-acetylglucosamine). Having these characteristics, the enzymes are members of the Enzyme Commission class 2.7.8.

Not all the phosphotransferases identified by this numerical index are targets for the antibiotic, however. The enzyme which synthesizes undecaprenyl galactosyl pyrophosphate, the first lipid intermediate of O-antigen synthesis in *Salmonella,* is not inhibited in vitro even at high tunicamycin concentrations (TKACZ, unpublished); similarly, the formation of undecaprenyl N-acetylgalactosaminyl pyrophosphate, which occurs in membranes from *Bacillus licheniformis,* is insensitive (WARD et al., 1980). This implies that the interaction of tunicamycin with an enzyme involves recognition of the α-linked GlcNAc moiety in the antibiotic. There is evidence that the interaction involves other facets of the structure as well. For example, the fact that a streptovirudin species from series II is slightly more effective than an homologous species from series I, both as a growth inhibitor (ECKARDT et al., 1975) and as an enzyme inhibitor (ELBEIN

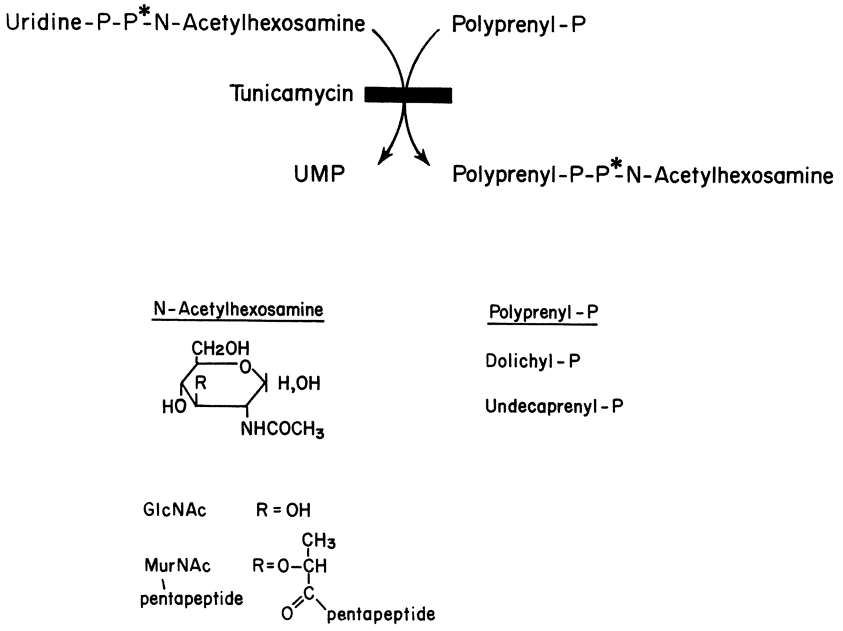

Fig. 6. Mechanism of action of tunicamycin

et al., 1981), suggests that the pyrimidine moiety plays a role in binding: the comparison demonstrates that modification of the pyrimidine, even a relatively minor change, such as replacement of the uracil (series II) with dihydrouracil (series I), leads to a decrease in biological activity. Similar comparisons of the individual components of the tunicamycin complex and the streptovirudins of series II show that activity is influenced by the length of the fatty acid residue, the most potent components being those with the longest fatty acid chains (ECKARDT et al., 1975; ELBEIN et al., 1981; KEENAN et al., 1981). The picture which emerges is one in which the inhibitor occupies the active site of the enzyme by virtue of its affinity for portions of the active site normally involved in binding the two substrates for the reaction. This view is consistent with the conclusions drawn from the kinetics of inhibition (HEIFETZ et al., 1979; KELLER et al., 1979).

TAMURA and TAKATSUKI (1980) have claimed that removal of N-acetylglucosamine or the fatty acid and N-acetylglucosamine from the tunicamycin molecule generates derivatives with inhibitory activity against coccidiosis and viruses but with only weak antibacterial activity. In light of the importance of these molecular groupings for the interaction of tunicamycin and its target enzymes, it is tempting to speculate that the activity of the derivatives involves a mechanism distinct from that of tunicamycin itself. The levels required to inhibit viral replication were very high (1.5–3 mg/ml).

At concentrations 20 to a 100-fold higher than those sufficient to block completely the formation of dolichyl N-acetylglucosaminyl pyrophosphate, tunicamycin depresses the activity of chitin synthetase (DURAN and CABIB, 1978; SELITRENNIKOFF, 1979) and the enzyme that synthesizes dolichyl glucosyl phosphate (ELBEIN et al., 1979, 1981; KEENAN et al., 1981). For these enzymes, both of which are glycosyl transferases (E.C. 2.4.1), the antibiotic behaves as a competitive inhibitor with K_i values several orders greater than that estimated for the UDP-GlcNAc:dolichyl phosphate GlcNAc-1-P transferase; on the basis of this differential it is quite unlikely that inhibition of these glycosyl transferases is a significant element in the overall biological effect of the antibiotic in eukaryotes. A clear illustration of the relative potency of a tunicamycin-like antibiotic as an inhibitor of phosphotransferases and glycosyl transferases is provided by studies on the action of antibiotic 24010 in membranes of *B. subtilis,* which are capable of producing three polyprenyl derivatives of GlcNAc. The antibiotic strongly inhibited the phosphotransferase which formed undecaprenyl α-N-acetylglucosaminyl pyrophosphate, but only weakly depressed the glycosyl transferase responsible for the synthesis of undecaprenyl α-N-acetylglucosaminyl phosphate. The third enzyme, a glycosyl transferase producing undecaprenyl β-N-acetylglucosaminyl phosphate, was not inhibited showing that there is anomeric specificity exerted in the inhibition of glycosyl transferases (YAMAMORI et al., 1978; MURAZUMI et al., 1979). As expected, the pyrimidine moiety of the antibiotic also serves as a determinant for specificity, and its influence appears to be greater in the case of glycosyl transferases than in the inhibition of phosphotransferases. ELBEIN et al. (1981) compared homologous components from the two streptovirudin series as inhibitors of dolichyl glucosyl phosphate synthesis and found that the K_i of the series II homolog (containing uracil) was approxi-

mately a 100-fold lower than that of the species from series I (containing dihydrouracil).

G. Chemotherapy

Despite the antibacterial, antifungal, and antiviral activities of tunicamycin and related antibiotics, these compounds have little or no potential for application to infectious diseases, because of their high toxicity. By intraperitoneal administration to mice the compounds gave the following LD_{50} values: mycospocidin, 1–2 mg/kg (NAKAMURA et al., 1957); tunicamycin, less than 5 mg/kg (ARIMA et al., 1973); streptovirudin, 3–17 mg/kg (THRUM et al., 1975). This toxicity is almost certainly a consequence of the mechanism of action, for although not all normally glycosylated proteins produced by an organism need to be glycosylated to achieve biologically active conformations, some which are crucial for membrane function will by synthesized as nonfunctional products when glycosylation is blocked. Without proper membrane function viability cannot be sustained. By the same token, chemical derivatives with reduced toxicity may be expected to be less effective inhibitors of microbial growth and viral replication.

Interest in tunicamycin for cancer therapy has been generated by reports that a variety of chemically or virally transformed cells are more sensitive than their normal counterparts to the action of the antibitotic (DUKSIN and BORNSTEIN, 1977a; DUKSIN et al., 1978a; KOHNO et al., 1979; OLDEN et al., 1979a; TAKATSUKI et al., 1977b). Animal tests are currently under way to determine whether the differential observed in tissue culture can be exploited for therapy. Tunicamycin has also been used to alter the surface antigens of cancer cells in the hope of enhancing their immunogenicity. BERNACKI et al. (1980) found that the administration of leukemia cells that had been cultured in the presence of tunicamycin had a protective effect in mice when the animals were subsequently challenged with untreated leukemia cells. Further work is required before a proper assessment of the promise of this approach can be made.

H. Conclusion

Tunicamycin, mycospocidin, streptovirudins (series II), antibiotic 24010, and antibiotic MM19290 constitute a group of streptomycete antibiotics with common biological activities. From the structural data that have been reviewed it is evident that the organisms producing these antibiotics share the ability to synthesize the nucleoside structure composed of tunicaminyl uracil and N-acetylglucosamine. However, the series of fatty acids they use in the acylation of the tunicamine moiety varies from one strain to the next. Those incorporated into tunicamycin and mycospocidin are longer on average than the ones attached

to streptovirudins; the spectrum of fatty acids present in the antibiotic MM19290 complex, in contrast, seems to encompass the range found in both the tunicamycins and the streptovirudins. The streptovirudins of series I represent a variation on this general theme in that their nucleoside moieties contain dihydrouracil in place of uracil. At present, no specific information is available regarding the intermediates or enzymatic steps in the pathway of tunicamycin biosynthesis, and in view of the novel structure of tunicamine, elucidation of the mechanism by which this amino sugar is formed will undoubtedly become a primary goal of future work in this area.

As an inhibitor of the production of polyprenyl intermediates, tunicamycin has proven to be very useful in the analysis of the synthesis of glycoconjugates in both prokaryotes and eukaryotes. For example, tunicamycin inhibition constituted one of the major bits of evidence in support of the role of undecaprenyl N-acetylglucosaminyl pyrophosphate in the formation of the structure which links teichoic or teichuronic acid to peptidoglycan in Gram-positive bacteria (BRACHA and GLASER, 1976; HANCOCK et al., 1976). Its use with eukaryotic cells helped establish that dolichol intermediates were in fact involved in the biogenesis of all Asn-GlcNAc type glycoproteins, whether membrane-bound or soluble. The antibiotic has also provided a means of exploring the biological function of protein glycosylation in vivo, and as a result, the long-held view that glycosylation is a requisite for the passage of a protein through a membrane has been replaced by a new hypothesis that emphasizes the importance of glycosylation in the folding of the peptide portion of the molecule (GIBSON et al., 1980; ROTHMAN et al., 1978; WIRTH et al., 1979). Because of the specific nature of its action, tunicamycin should continue to be an invaluable tool in future research to assess the role of carbohydrate moieties in the intracellular sorting and export of glycoproteins (BERGMAN et al., 1981; DAMSKY et al., 1979; LOH and GAINER, 1979; MILLER et al., 1980; VON FIGURA et al., 1979), in the function of membrane glycoproteins as receptors and antigens (BHARGAVA and MARKMAN, 1980; ERTL and ADA, 1981; ITOH and KUMAGAI, 1980; PLOEGH et al., 1981; PRIVES and OLDEN, 1980; ROTUNDO and FAMBROUGH, 1980), and in membrane-related phenomena, such as cell fusion, aggregation, and differentiation (BORDY et al., 1979; BUTTERS et al., 1980; DAN-SOHKAWA et al., 1980; FRAZIER et al., 1980; GILFIX and SANWAL, 1980; HEIFETZ and LENNARZ, 1979; KOHNO et al., 1980; NAKAYASU et al., 1980; WATANABE et al., 1979).

Addendum

During the preparation of this volume, several pertinent publications have appeared. An English-language monograph on tunicamycin edited by TAMURA (1982) has been published. ECKARDT et al. (1981) found by mass spectrometry that the members of the streptovirudin complex, unlike the tunicamycins, are acylated only with unsaturated iso and anteiso fatty acids. There has been considerable progress toward the chemical synthesis of the carbohydrate nucleus

of tunicamycin (FUKUDA et al., 1981, 1982a, 1982b; SUAMI et al., 1982). Glycolipid toxins produced by *Corynebacterium rathayi* in seed heads of annual ryegrass, causing a potentially lethal syndrome in grazing animals, have proven to be members of the tunicamycin group of antibiotics (EDGAR et al., 1982; VOGEL et al., 1982). Beyond demonstrating that the capacity to synthesize tunicamycin-like antibiotics is not restricted to streptomycetes, this intriguing observation adds an unexpected source of toxicological information to what was already known regarding this facet of tunicamycin.

Continuing their work on the biological activities of individual members of the tunicamycin complex, DUKSIN and MAHONEY (1981, 1982) have extended their observations on inhibition of protein glycosylation versus suppression of protein formation to nine homologues; they report (DUKSIN et al., 1982) that component VI (B_3 in their nomenclature; see Fig. 1), the only tunicamycin species containing a saturated fatty acid, has the highest activity in vivo as an inhibitor of protein glycosylation and is the most selective in its toxicity for transformed cells. Chinese hamster ovary cell mutants have been isolated which are resistant to tunicamycin as the result of an overproduction of the target enzyme (CRISCUOLO and KRAG, 1982). When protein glycosylation in mammalian cells is blocked with tunicamycin, newly synthesized hydrolytic enzymes that would otherwise be delivered to the lysosome are exported from the cell and are not subject to recapture (PARENT et al., 1982; ROSENFELD et al., 1982). In yeast, where the vacuole is the functional equivalent of the lysosome, hydrolases are transported to this subcellular compartment whether or not they are glycosylated (CLARK et al., 1982; SCHWAIGER et al., 1982). The yeast cell evidently does not use the same oligosaccharide-based addressing system that is employed during the sorting of glycoproteins in mammalian cells. The role of glycosylation in the association of acetylcholine receptor subunits (MERLIE et al., 1982) and cell recognition by thymic lymphocytes (HART, 1982) has been investigated with tunicamycin. OLDEN et al. (1982) have established that interferon does not act by mimicking tunicamycin. The insensitivity of *Micromonospora* species to tunicamyin permits the use of the antibiotic in a selective culture medium for their isolation (WAKISAKA et al., 1982).

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Viral Translation Inhibitors

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The present chapter will focus on the action of compounds that affect translation and have a certain specificity in their action against cells infected with animal viruses.

In general, viruses use most of the components of the cellular translation machinery to synthesize their own proteins (LURIA et al., 1978; SMITH, 1975; REVEL and GRONER, 1978; SMITH and CARRASCO, 1978; CARRASCO and SMITH, 1980). Thus, it is not surprising that most of the known translation inhibitors are equally active in blocking protein synthesis in both virus-infected and uninfected cells (CONTRERAS and CARRASCO, 1979). However, there are a number of distinct differences between the translation of host and viral mRNA's, particularly at the level of mRNA structure, which can be exploited to design specific antiviral agents. For instance, it is known that mRNA from picornaviruses does not possess a cap structure at the 5′ end of the polynucleotide. This structural variation is indicative of a differential requirement for initiation factors in the translation of viral and cellular mRNA (HEWLETT et al., 1976; NOMOTO et al., 1976; FERNÁNDEZ-MUÑOZ and DARNELL, 1976). Such differences in (1) the formation of the cap structure on both viral and cellular mRNA (SHATKIN, 1976; REVEL and GRONER, 1978), and (2) the initiation of viral mRNA translation (REVEL and GRONER, 1978; CARRASCO and SMITH, 1980), are potentially exploitable to allow the design of compounds that selectively interfere with viral protein synthesis.

A further approach, which will be considered in this chapter, is to obtain selective inhibition of viral translation by using inhibitors that do not block protein synthesis in intact mammalian cells because they fail to permeate the membrane barrier (CARRASCO, 1978; VÁZQUEZ, 1979; CARRASCO et al., 1981). Indeed, the infection of animal cells by viruses can render the cell membrane permeable to a great variety of compounds (KOHN, 1979; CARRASCO and SMITH, 1980). Thus, a number of antibiotics that fail to enter uninfected cells show a selective action against virus-infected cells. Selectivity in this instance is attained not because the protein synthesizing machinery of the virus-infected cell is more sensitive to antibiotic inhibition, but results from the accumulation of the compound within the cell following virus infection.

Finally, in this chapter some attention will be given to the mechanism of action of interferon since, at least for some viruses, the impairment of viral development in interferon-treated cells is controlled at the level of viral mRNA translation (BAGLIONI, 1979; STEWART, 1981).

Sinefungin and Ribavirin–Antiviral Compounds that Block the Capping of Viral mRNA

Eukaryotic mRNA species have a 5′ terminal residue (cap) of m7GpppN(m), which is linked 5′ to 5′ with the polynucleotide chain (SHATKIN, 1976; REVEL and GRONER, 1978). There is variable methylation of this cap structure in different messengers and one, two, or even three, methyl groups may be present. The cap structure stabilizes the mRNA against degradation and is also required for efficient binding of mRNA to ribosomes. The various methylations are catalyzed by specific methyl transferases that require S-adenosyl-L-methionine (SAM) as the methyl donor. S-adenosyl-L-homocysteine (SAH) is a structural analog of SAM and acts as an inhibitor of methylation (ZAPPIA et al., 1969; SHATKIN, 1976).

Most viral mRNA's also possess cap structures at their 5′ terminus and methylation of this structure is required for efficient viral protein synthesis (SHATKIN, 1976). Accordingly, SAH and a number for SAM analogs inhibit the reproduction of several animal viruses by blocking methylation of the newly formed viral mRNA's (ROBERT-GERO et al., 1975; JACQUEMONT and HUPPERT, 1977; CHIANG et al., 1978). These analogs inhibit replication of many DNA-containing viruses including herpesvirus, adenovirus, and papovavirus. RNA-containing viruses, such as paramyxoviruses, retroviruses, picornaviruses, and orthomyxoviruses, are also sensitive. In addition, S-isobutyl-adenosine inhibits cell transformation by RNA tumor viruses (LEGRAVEREND et al., 1977; BADER et al., 1978; TERRIOUX et al., 1978).

Sinefungin (A9145) is an antifungal antibiotic produced by *Streptomyces griseolus* (HAMILL and HOEHN, 1973). Both sinefungin and the related metabolite, A9145C (BERRY and ABBOTT, 1978), can be considered as sulfur-modified analogs of SAH (Fig. 1) and are potent inhibitors of NDV and vaccinia virion methyl transferases (PUGH et al., 1978). As a consequence, these compounds inhibit replication of vaccinia virus in L-cells.

Ribavirin is a triazole nucleoside obtained by chemical synthesis and inhibits the replication of a wide variety of viruses, both in cell cultures and in whole

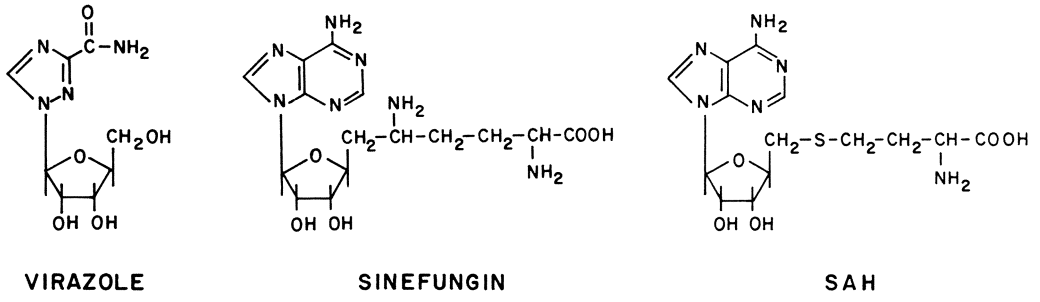

Fig. 1. Molecular structure of virazole, sinefungin, and S-adenosyl-homocisteine (SAH)

animals (HAHN, 1979; review). The compound has had limited use in humans for the treatment of both viral respiratory infections and measles. It is also very active against plant viruses (SHEPARD, 1977; DE FAZIO et al., 1978) but, curiously enough, it is ineffective against polio virus (SIDWELL et al., 1972). The antiviral activity of ribavirin has been attributed to its inhibitory effect on the de novo synthesis of purine mononucleotides. Thus ribavirin-5′-monophosphate is a competitive inhibitor of the enzyme that converts IMP to XMP in route to GMP formation (STREETER et al., 1973). ERIKSSON et al. (1977) found that ribavirin-5′-triphosphate selectively inhibited influenza virus RNA polymerase in a cell-free system. However, these effects together do not account for all the actions observed in vivo, such as the lack of activity against picorna viruses, and it has recently been proposed (GOSWAMI et al., 1979) that ribavirin interferes with the capping mechanisms for viral mRNA's. Ribavirin triphosphate has also been found to be a potent inhibitor of the vaccinia virus mRNA guanylyl transferase, and this property might well account for the selective action of ribavirin against different animal viruses (GOSWAMI et al., 1979).

Translation Inhibitors that Selectively Penetrate Virus-infected Cells

The cytoplasmic membrane forms an impermeable barrier against many antibiotics. In particular, hydrophilic drugs or those possessing a macromolecular structure are effectively excluded from the cell. An increasing number of compounds fall into this category since, although they may actively inhibit translation in cell-free systems, they fail to inhibit protein synthesis in intact cells (VÁZQUEZ, 1979; CONTRERAS and CARRASCO, 1979).

The infection of animal cells by viruses induces modifications at the membrane level (KOHN, 1979; CARRASCO and SMITH, 1980; PASTERNAK and MICKLEM, 1981). These alterations include changes in the composition of several membrane components, i.e., new proteins appear on the cell surface after viral infection (NICOLSON, 1974; BURNS and ALLISON, 1977), and modifications of the physicochemical properties of the membrane have also been described (KOHN, 1979). Hence, the fluidity of the membrane increases after viral adsorption (LEVANON and KOHN, 1978), and a drop in membrane potential is induced after infection with Sendai virus (FUCHS et al., 1978; IMPRAIN et al., 1980). It is perhaps a consequence of such changes that the permeability of the plasma membrane to a number of very different compounds is altered after virus infection (YAMAIZUMI et al., 1979; IMPRAIN et al., 1980; WYKE et al., 1980; FOSTER et al., 1980; BENEDETTO et al., 1980). The increase in membrane permeability to ions, low molecular weight compounds, and even macromolecules is induced by a great number of animal viruses and is observed both very early during infection and late when most of the viral structural components are being synthesized.

Modification in Membrane Permeability to Translation Inhibitors During Early Virus Infection

Viral infection commences with the attachment of virion particles to membrane receptors and is followed by internalization of the virus into the cell where decapsidation occurs (DALES, 1973; LURIA et al., 1978). Attachment of mengovirus to ascites cells induces changes in the distribution of membrane proteins in such a way that patching and capping of membrane receptors occurs soon after infection (GSCHWENDER and TRAUB, 1979). Using the indirect immunofluorescence technique, GSCHWENDER and TRAUB (1979) have demonstrated the redistribution of mengovirus particles that were first located in small clusters and evenly distributed on the cell surface when mengovirus was allowed to adsorb at 0° C. An increase in the temperature to 20° C induced the redistribution of the viral antigens into a patchy pattern within 20 min. The authors suggested that this redistribution of components on the plasma membrane could influence membrane permeability, as previously observed (see also CARRASCO, 1978). During viral entry the permeability of the membrane to a number of translation inhibitors is indeed altered (FERNÁNDEZ-PUENTES and CARRASCO, 1980; CARRASCO, 1981 a). Thus gougerotin, edeine, $GppCH_2p$, hygromycin B,

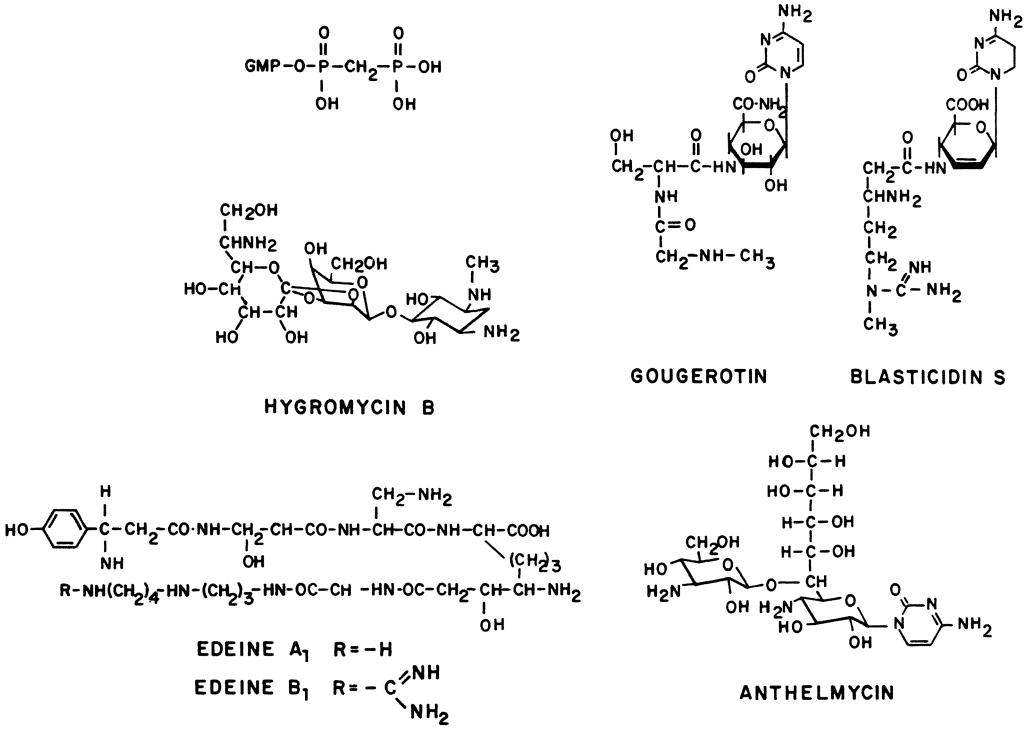

Fig. 2. Molecular structure of several translation inhibitors that show selectivity against animal virus-infected cells. $GppCH_2p$ = GDPCP (a GTP analog)

Table 1. Translation inhibitors that exhibit a higher inhibition of protein synthesis in animal virus-infected cells versus noninfected cells

Compound	Source	Chemical structure	MW
Edeine A_1	*Bacillus brevis*	Peptide	738
GDPCP	Chemical synthesis	Nucleotide analog	521
Gougerotin	*Streptomyces gougeroti*	See Fig. 2	443
Blasticidin S	*Streptomyces globifer*	See Fig. 2	422
Anthelmycin	*Streptomyces longissimus*	See Fig. 2	583
Hygromycin B	*Streptomyces hygroscopicus*	Aminoglycoside	527
Alpha-sarcin	*Aspergillus giganteus*	Protein	16,800
PAP	*Phytolacca americana*	Protein	31,000
Mitogillin	*Aspergillus restrictus*	Protein	16,200
Restrictocin	*Aspergillus restrictus*	Protein	16,300
Ricin A chain	*Ricinus communis*	Protein	32,000
Abrin A chain	*Abrus precatorius*	Protein	30,000

destomycin A, and blasticidin S (Fig. 2) selectively blocked translation in encephalomyocarditis virus-infected cells when these compounds were present during virus adsorption (CARRASCO, 1981a; CARRASCO et al., 1981). Entry of inhibitors into infected cells required neither viral replication, nor the expression of the viral genome and was observed even when low multiplicities of infection were used. Presumably, therefore, viral attachment and penetration per se induces the drastic increase in membrane permeability that allows entry of these hydrophilic translation inhibitors. This early leakiness of the plasma membrane was not blocked by inhibitors of cellular metabolism, such as Na_3N, or by compounds that disrupt the cell cytoskeleton, suggesting that the patching but not the capping of membrane receptors is involved in the permeability change. Both enveloped and nonenveloped viruses, that do not possess a lipidic membrane, are able to increase membrane permeability, indicating that this is a rather widespread phenomenon (CARRASCO, 1981a). Table 1 summarizes the viruses which are able to induce permeability changes early during infection.

Recent findings indicate that during early virus infection not only low molecular weight compounds, but also certain protein toxins can pass through the cellular membrane (YAMAIZUMI et al., 1979; FERNÁNDEZ-PUENTES and CARRASCO, 1980). Some protein toxins are effective inhibitors of protein synthesis (VÁZQUEZ, 1979), and the mechanism of action of these compounds normally involves two steps (OLSNES and PIHL, 1976): (1) the attachment of the toxin to a surface receptor mediated by the haptomer moiety of the molecule, followed by (2) the entry of the effectomer moiety into the cell and subsequent action on a specific translation component (ribosomes, elongation factors). The effectomers of several plant toxins specifically block translation in virus-infected cells if they are present during the early steps of virus infection (FERNÁNDEZ-PUENTES and CARRASCO, 1980).

The molecular mechanisms by which virus adsorption promotes the entry of low molecular weight inhibitors and protein toxins into cells is still puzzling. A parallelism has been found between the changes in permeability induced

by viral infection and the action of some ionophores on the membrane. Thus, treatment of mammalian cells with nigericin also promotes the entry of translation inhibitors and toxins into the cells (ALONSO and CARRASCO, 1980; ALONSO and CARRASCO, 1981).

The antiviral potency of these translation inhibitors is high, because, once the compound has entered the cell along with the infecting virus, virus replication is blocked and the formation of the progeny viruses is strongly diminished (CARRASCO, 1981 a). The antiviral action of some of these compounds in animals has, however, yet to be tested.

Modification in Membrane Permeability to Translation Inhibitors During Late Virus Infection

After entry of a virus into the cell with the associated alteration in membrane permeability the membrane gradually reseals (LACAL et al., 1980; CARRASCO, 1981 a). The extent and speed of this resealing depends on the multiplicity of infection. Thus, with low multiplicity the permeability properties of the membrane are restored sooner than after higher virus doses (CARRASCO, 1981 a; CARRASCO and ESTEBAN, 1981). As viral infection proceeds, however, the permeability properties of the membrane again change at a time when most viral structural components have been synthesized (CARRASCO and SMITH, 1976; EGBERTS et al., 1977; CARRASCO, 1978; NAIR et al., 1979). For picornaviruses this second modification of the membrane becomes apparent just at the beginning of the synthesis of viral proteins. This late modification of permeability toward translation inhibitors was first discovered in encephalomyocarditis virus infected cells (CARRASCO, 1978). Thus, addition of the translation inhibitor $GppCH_2p$, a GTP analog, to uninfected cell cultures did not cause any inhibition of protein synthesis. However, if this compound was present in the medium of EMC-infected cells at a time when the bulk of EMC virus proteins had been synthesized, an almost complete inhibition of translation was observed. This blockade was selective for virus-infected cells (CARRASCO, 1978, 1981 b) (Fig. 3).

The late induction of this membrane leakiness which allow entry of translation inhibitors is dependent on virus multiplication. Thus inhibitors that prevent the expression of the viral genome or UV-inactivated virions induce the early, but not the late leakiness of the membrane. The exact viral component(s) involved in permeability modification has not as yet been identified.

A number of translation inhibitors have now been tested against picorna virus infected cells and some of them exert a selective inhibition (Table 1) (CONTRERAS and CARRASCO, 1979; LACAL et al., 1980). Hydrophilic inhibitors generally preferentially inhibit translation in virus-infected cells, since they will not permeate easily into normal cells. Theoretically, therefore, any translation inhibitor could be rendered selective for inhibition of virus-infected cells (CARRASCO, 1979). Certain inhibitors could be chemically modified by the introduction of

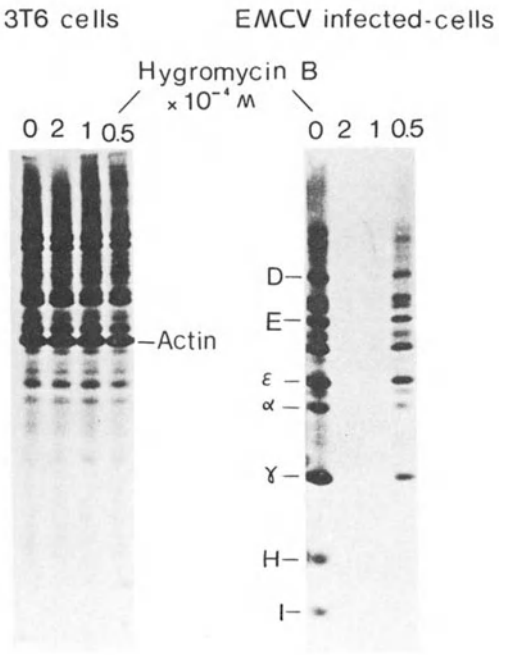

Fig. 3. Analysis by polyacrylamide gel electrophoresis of the proteins synthesized in uninfected mouse 3T6 cells and EMC virus-infected cells. Effect of the antibiotic hygromycin B

polar groups to prevent entry through membranes of uninfected cells, without loss of the relevant inhibitory property. It should also be stressed that, to date, studies on selective inhibition of virus-infected cells have employed translation inhibitors only and to our knowledge nucleic acid inhibitors that do not penetrate into normal cells have not been studied in the above way for antiviral activity.

Most of the studies on membrane permeability changes have been carried out on either Sendai virus or picornavirus infected cells (for reviews see KOHN, 1979; CARRASCO and SMITH, 1980). However, the induction of membrane leakiness toward translation inhibitors after viral infection is a rather widespread phenomenon in animal viruses (BENEDETTO et al., 1980; LACAL et al., 1980). Table 2 summarizes the different animal viruses that, to date, are known to induce modification in membrane permeability toward translation inhibitors. Both HSV 1 and HSV 2 infected cells show an increased permeability to hygromycin B 6 h after infection (BENEDETTO et al., 1980; LACAL and CARRASCO, unpublished observations). Similarly, a more profound study carried out early after infection of HeLa cells with vaccinia virus shows an increased membrane permeability toward hygromycin B (CARRASCO and ESTEBAN, 1981). Adenovirus type V, another DNA-containing virus, produces a drastic change in permeability toward hygromycin B, starting about 8 h after infection (LACAL and CARRASCO, unpublished observations), and also SV40 infection of monkey cells results in permeability modification at the time when massive amounts of the

Table 2. Animal viruses that induce alterations in membrane permeability to translation inhibitors after infection. N.D. = Experiment not done

Animal virus	Family	Leakiness to inhibitors	
		Early	Late
Encephalomyocarditis virus	Picornaviridae	+	+
Polio virus	Picornaviridae	+	+
Mengo virus	Picornaviridae	N.D.	+
Semliki Forest virus	Togaviridae	+	+
Vesicular stomatitis virus	Rhabdoviridae	+	+
Rous sarcoma virus	Retroviridae	N.D.	−
Sendai virus	Paramyxoviridae	+	+
SV 40	Papovaviridae	N.D.	+
Adeno virus type V	Adenoviridae	N.D.	+
Herpes virus type 1 and 2	Herpesviridae	N.D.	+
Vaccinia virus	Poxviridae	+	+

coat proteins VP1, VP2, and VP3, are synthesized (CONTRERAS and CARRASCO, 1979). With respect to RNA-containing viruses membrane leakiness in infected cells has been clearly established for picornaviruses (EMC virus and poliovirus), togaviruses, paramyxoviruses, and rhabdoviruses (CARRASCO, 1978, 1979; BENEDETTO et al., 1980; LACAL et al., 1980). Possible changes in permeability toward inhibitors as induced by retroviruses, reoviruses, or myxoviruses have not yet been explored.

The presence of inhibitors in the medium of virus-infected cells during the late phase of infection strongly reduced virus replication even at drug concentrations that do not alter protein synthesis in normal cells. Hygromycin B at a concentration of 0.1 mM inhibited production of infectious EMC virus by over 95% (CARRASCO, 1978; LACAL et al., 1980). At 1 mM hygromycin B reduced the production of vesicular stomatitis virus, and when present in a plaque assay the drug reduced the diameter of the VSV plaques on L-cells (BENEDETTO et al., 1980). Hygromycin B concentrations of 0.5 mM inhibited the production of HSV 1 in monkey 37RC cells by over 99%, even when added 7 h after infection. However, no inhibitory effect was observed on virus replication when the inhibitor was present from 18 h postinfection, since most of the infectious HSV 1 virus is already formed by this time (BENEDETTO et al., 1980). The inhibition of Sendai virus by hygromycin B was also apparent when the compound was present 1 h after infection. However, as yet no data are available concerning the antiviral action of these compounds in experimental animal systems.

Selective Inhibition of Translation in Virus-transformed Cells

The infection of susceptible cells by some retroviruses does not lead to a cytolytic effect of the cell, but instead causes transformation. Similarly, infection

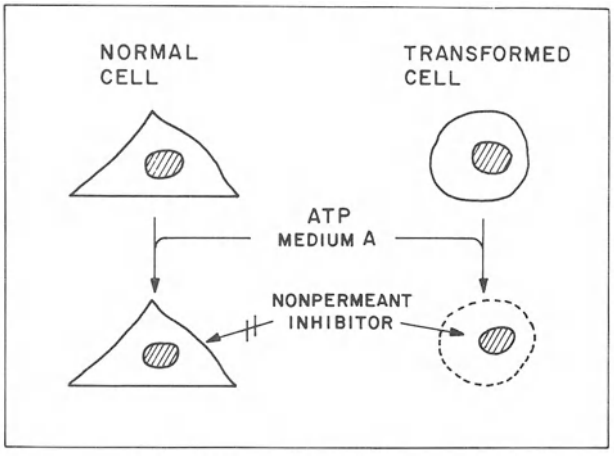

Fig. 4. Schematic representation of the selective permeabilization of transformed cells by medium A and ATP and subsequent killing by an inhibitor nonpermeant to normal cells (CARRASCO, 1980)

of certain cell types by DNA oncogenic viruses induces cellular transformation with no cell death (LURIA et al., 1978). Attempts to find translation inhibitors that are more active on transformed cells than on normal cells have been unsuccessful, since the permeability of both kinds of cells toward translation inhibitors is very similar. However, in some types of transformed cells more receptors for some toxins could be present, and hence susceptibility to the inhibitor is increased. A report that diphteria toxin shows a selected toxicity for certain malignant cells (IGLEWSKI and RITTENBERG, 1974) has been disproved (PAPPENHEIMER and RANDALL, 1975).

Incubation of cultured transformed cells in a particular medium that contains ATP, can induce specific permeabilization of these cells toward low molecular weight compounds (ROZENGURT et al., 1977; MAKAN, 1978; DICKER et al., 1980). This phenomenon is general for all cell lines tested after transformation by either animal viruses or chemicals. Normal cells subjected to similar treatments did not become permeabilized. The treatment was fully reversible in that neither normal nor transformed cells lost their viability when they were first permeabilized, and then returned to a normal culture medium. Under these conditions the integrity of the membrane of transformed cells is rapidly restored (ROZENGURT et al., 1977). The molecular basis for the permeabilization of transformed cells is related to a specific phosphorylation of membrane proteins (DICKER et al., 1980; MAKAN, 1981). This phosphorylation somehow promotes the formation of a hydrophilic channel in the membrane. Permeabilization can take place at normal pH provided that a phosphatase inhibitor is present, which prevents removal of the phosphate group from the membrane proteins (MAKAN, 1978). Compounds, such as rutamycin and oligomycin, that lower intracellular ATP levels act synergistically with external ATP in this permeabilization of transformed cells (ROZENGURT and HEPPEL, 1979). It was reasoned, based on these findings, that selective permeabilization could provide a method for selectively killing virus-transformed cells by addition of those translation inhibitors toward which normal cells are impermeable (CARRASCO, 1980; KITAGAWA,

1980). This technique has indeed been used successfully in culture cells since mouse 3T3 cells, which are transformed by Kirnsten sarcoma virus and then incubated in the presence of the translation inhibitor hygromycin B during the permeabilization period, are killed by the antibiotic (CARRASCO, 1980). Similar treatment of normal cells did not block protein synthesis because these cells are not permeable to the inhibitor. Although these methods can not yet be applied in chemotherapy, they nevertheless provide a new rationale in the search for selective inhibitors of tumor cells. In principle, it will be necessary to find methods that permeabilize transformed cells under physiological conditions in whole organisms. There is also a need for translation inhibitors that do not penetrate into and are devoid of toxicity toward normal cells.

Interferons

The interferons form a family of cellular proteins that are synthesized in response to certain inducers (FRIEDMANN, 1977; BURKE, 1977; STEWART, 1981) including animal viruses, microorganisms, and some chemicals. Although the induction mechanism in still far from clear, it appears that the interferon gene is derepressed after interaction of the inducer with the cell surface and the synthesis of interferon mRNA can then take place (HELLER, 1963; WAGNER, 1964). The translation of this mRNA in the cellular cytoplasm gives rise to the protein interferon, which is excreted from the cell to the surrounding medium (TAN et al., 1971; HAVELL and VILČEK, 1975).

Three main classes of interferon molecules have now been described (KNIGHT, 1980), which are distinguished depending on the cell type that synthesizes the molecule and on the kind of inducer employed (GRESSER, 1980). IFN-α, for example, is of leukocyte origin and is synthesized in response to viruses. At least eight different IFN-α genes exist and the differences in the primary structure and the sequence of some of these genes is now known. IFN-β is of fibroblast origin. Several genes of IFN-β exist and it is also induced by viruses (HOUGHTON et al., 1980; KNIGHT et al., 1981; SEHGAL et al., 1981). IFN-γ is of leukocyte origin and its synthesis is induced by mitogens (EPSTEIN et al., 1981; FALCOFF et al., 1981). It is larger than IFN-α and has a molecular weight of around 50,000 (YIP et al., 1981).

The interaction of interferon with the appropriate cell type induces a number of effects. The antiproliferative and antiviral actions of interferon have been of particular interest because of their clinical significance (GRESSER, 1981; MERIGAN, 1981), and we will focus in this chapter on the inhibition of viral translation in interferon-treated cells (METZ, 1975; BAGLIONI, 1979; STEWART, 1981).

The antiviral action of interferon starts with its interaction with cellular surface receptors, and the so-called antiviral state is somehow induced (BERMAN and VILČEK, 1974). Gene expression at both the transcriptional and translational

level is required for the establishment of this antiviral state (TAYLOR, 1964; MUÑOZ and CARRASCO, 1981 b). Although the synthesis of a number of proteins is induced after IFN treatment (KNIGHT and KORANT, 1979), the exact antiviral proteins have not yet been indentified. Infection of interferon-treated cells with an animal virus results in an impairment of virus development and the virus yield is strongly reduced (FRIEDMANN, 1977; BAGLIONI, 1979; STEWART, 1981). The step in virus development that is inhibited depends on the kind of virus used. For instance, in retroviruses one of the steps inhibited occurs late in infection (BILLIAU et al., 1976). In other instances virus transcription or virus translation, and sometimes even both, are inhibited in IFN-treated cells.

Studies performed in cultured cells suggest that in some systems viral transcription is not affected and may even be increased, although viral protein synthesis is inhibited (STEWART, 1981). For instance, no viral protein synthesis takes place in interferon-treated cells infected with reovirus, although transcription is only slightly inhibited (WIEBE and JOKLIK, 1975). Treatment of monkey cells with interferon inhibits the production of T antigens after SV40 infection (OXMAN and BLACK, 1966). If the interferon treatment is performed after infection has taken place, synthesis of coat proteins is inhibited even though the mRNA is present (YAKOBSON et al., 1977).

Infection with vaccinia virus of suspension cells treated with inferferon induces a drastic inhibition of cellular protein synthesis and no viral proteins are synthesized (METZ and ESTEBAN, 1972). Infected cells die even earlier than control cells which have not been treated with interferon (JOKLIK and MERIGAN, 1966; HORAK et al., 1971). The synthesis of early vaccinia transcripts in this system does, however, occur at increased levels, probably as a consequence of the inhibition of viral protein synthesis (METZ and ESTEBAN, 1972). Curiously enough, if the cells are grown in monolayer, early vaccinia protein synthesis takes place at normal levels and the defect in virus production is located at a rather late step of virus multiplication (ESTEBAN, personal communication; MUÑOZ and CARRASCO, unpublished results). A more extreme situation is found for herpes virus where no inhibition of viral protein synthesis is detected in monolayer-infected cells after interferon treatment (MUÑOZ and CARRASCO, unpublished results).

Inhibition of viral protein synthesis in interferon-treated cells is also seen using vesicular stomatitis virus. In this case no viral protein synthesis is detected, even though the viral mRNA is synthesized and is translatable in cell-free systems. For picornaviruses two different situations have been described depending on the input virus used (MUÑOZ and CARRASCO, 1981 a). Infection of interferon-treated cells with picornaviruses at low multiplicities results in cell survival (FALCOFF and SANCEAU, 1979). No viral proteins are detected and the synthesis of viral RNA is also inhibited, probably as a consequence of the inhibition of the viral replicase which is necessary for synthesis of the viral nucleic acid. Under high multiplicities of infection the suicide phenomenon, that takes place also in vaccinia virus infected suspension cells, is observed (KERR et al., 1973; MUÑOZ and CARRASCO, 1981 a). Thus, neither viral proteins nor RNA are synthesized, host protein synthesis is inhibited, and the cytopathic effect develops quicker than in control cells not treated with interferon. For Semliki Forest

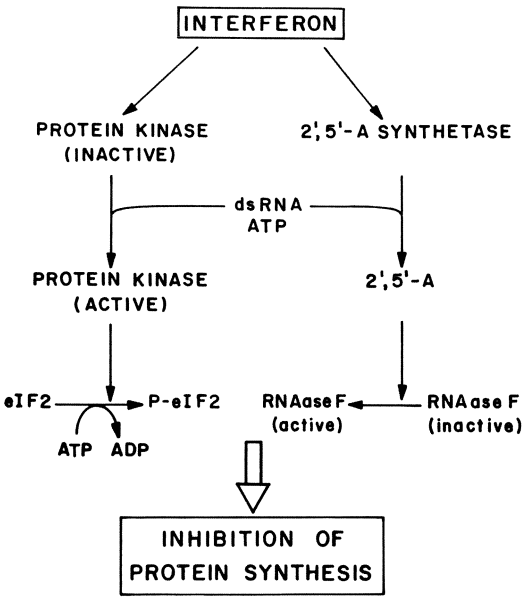

Fig. 5. Pathways followed to inhibit protein synthesis in interferon-treated cells after virus infection

virus (a togavirus) the defect seems also to be located at the translational level (FRIEDMANN, 1968).

The molecular basis underlying the impairment in the ability of interferon-treated cells to translate viral mRNA is complex and evidence from cell-free systems suggests that multiple mechanisms may be involved. Cell-free systems obtained from IFN-treated cells are more susceptible to inhibition by dsRNA (KERR et al., 1974), and two inhibitory reactions are triggered by this compound. In the first place, an enzyme, the so-called 2′-5′ A synthetase, makes oligo A from ATP, the product being linked by the unusual 2′,5′ bond (KERR et al., 1977; KERR and BROWN, 1978). This enzyme is activated by the presence of dsRNA. In turn, the 2′,5′ oligo A stimulates an endonuclease activity that degrades RNA (BAGLIONI et al., 1978; CLEMENS and WILLIAMS, 1978; SCHMIDT et al., 1978). No selectivity has been found in this endonuclease activity and both cellular and viral mRNA are equally susceptible to degradation (RATNER et al., 1977). Although some 2′,5′ oligo A has been found in interferon-treated cells after virus infection, the physiological significance of this system in vivo remains obscure. Certainly, though, in most systems both the viral transcription and host mRNA are present in undegraded and translatable form even though viral translation, and in some instances host protein synthesis, are inhibited (VAQUERO et al., 1981).

dsRNA also activates a protein kinase that phosphorylates some proteins in cell-free systems, with two proteins of molecular weights 67,000 and 30,000 respectively being particularly susceptible (LEBLEU et al., 1976; ZILBERSTEIN et al., 1978). The latter protein probably corresponds to the small subunit of initiation factor eIF2. The in vivo significance of this phosphorylation mecha-

nism is, however, dubious since no obvious increase in phosphorylation has been found in interferon-treated virus-infected cells.

A lack of methylation activity in cell-free systems from IFN-treated cells has also been described (SEN et al., 1977). Furthermore, the inability of preincubated cell-free systems from IFN-treated cells to translate exogenous mRNA is reversed by the addition of tRNA (GUPTA et al., 1974; CONTENT et al., 1974). In particular, $tRNA_{leu}$ was very active in reversing the inhibition of polyuridylic acid translation (FALCOFF et al., 1976). Again, the significance of these results for the in vivo situation is not clear.

It is obvious that in the next few years much more work at the molecular level will be necessary to understand why IFN-treated cells fail to translate viral mRNA, since none of the the current models has been clearly proved to operate in vivo. It does, however, seem very likely that the observed inhibitory mechanisms will vary depending on the kind of virus used.

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Properties of Virginiamycin-like Antibiotics (Synergimycins), Inhibitors Containing Synergistic Components

C. Cocito

Introduction

Antibiotics inhibiting protein synthesis in prokaryotes can be divided into three groups, according to their targets: (1) 30S ribosomal subunits, (2) 50S subunits; and (3) cytoplasmic factors (the initiation factors IF1, 2 and 3; the elongation factors EF-Tu, EF-Ts and EF-G; and the termination factors RF-1, 2 and 3) (Table 1). Virginiamycin-like antibiotics (synergimycins) belong to the second group. The articles of Weisblum and Davies (1968), Vázquez (1974), Pongs et al. (1974), and Pestka (1971, 1976, 1977), and the volumes of Vázquez (1979) and of Gale et al. (1981) review literature data on antibiotics acting at the ribosome level.

The unique feature of antibiotics of the virginiamycin family is that they contain two types of components, type A and type B, which exert a synergistic inhibitory action on sensitive organisms: hence their designation as "synergimycins." This cooperativity, which operates at both quantitative and qualitative levels, will be stressed in this article, since it represents the distinguishing trait of this group of inhibitors.

Four review articles by Vázquez (1967), Tanaka (1975), Vázquez (1975), and Cocito (1979) summarize the most relevant findings with synergimycins during the period 1955–1978 and provide bibliographic data concerning early publications in this field. The present work essentially updates the previous review by the same author (Cocito, 1979). Reference can be made to the latter article for more detailed study of topics that are just sketched in the present paper, namely: chemistry and physics of synergimycins, the action on different biological systems, and the application in both medicine and animal husbandry.

Structure and Synthesis of Synergimycins

Numerous virginiamycin-like antibiotics have been obtained in laboratories of different countries (cf. Cocito, 1979 for reference), and several products of this kind are commercially available (Table 2). In spite of the large number

Table 1. Antibiotics inhibiting translation in procaryotes[a]

Target Cytoplasmic factors	Target 30S subunit	Target 50S subunit
Fusidic acid	Aminoglycosides[b]	Blasticidin
Kirromycin group[f]	Aurintricarboxylic acid	Bottromycin
	Bacteriocins[c]	Chloramphenicol group[d]
	Edeine	Gougerotin group[e]
	Kasugamycin	Lincosamides[g]
	Pactamycin	Macrolides[h]
	Spectinomycin	Micrococcin
		Multhiomycin
		Negamycin
		Nucleodicin
		Pleuromutilin
		Puromycin
		Sparsomycin
		Synergimycins[i]
		Tetracycline group[j]
		Thiostrepton group[k]
		Viomycin group[k]

[a] Data from COCITO (1979); PESTKA (1971, 1977); and VÁZQUEZ (1979).
[b] Aminoglycosides: Streptomycin group (Amikamycin, Gentamicin, Kanamycin, Neomycin, Paromomycin, Sisomycin, Streptomycin, Tobramycin).
[c] Bacteriocins acting on 30S subunits: Cloacin DF13 and Colicin E3.
[d] Chloramphenicol group: Chloramphenicol, D-AMP-3, D-Thiomycetin, D-Win-5094.
[e] Gougerotin group: Amicetin, Bamicetin, Gougerotin, Plicamicetin.
[f] Kirromycin group: Efrotomycin, Goldinomycin, Kirromycin, Macimycin.
[g] Lincosamides: Celesticetin, Clindamycin, Lincomycin.
[h] Macrolides: Carbomycin group (Carbomycin, Josamycin, Leucomycin, Niddamycin; Erythromycin group (Erythromycin, Neospiramycin, Oleandomycin); Lancamycin group (Chalcomycin, Kujimycin A, Lancamycin); Methymycin group (Forocidin, Methymycin, Narbomycin, Neomethymycin, Picromycin); Spiramycin group (Angolamycin, Relamycin, Spiramycin, Tylosin).
[i] Synergimycins (virginiamycin-like antibiotics): type A and type B.
[j] Tetracycline group: Chlortetracycline, Doxycycline, Oxytetracycline, Tetracycline.
[k] Thiostrepton group: Siomycin, Sporangiomycin, Thiopeptin, Thiostrepton.
[l] Viomycin group: Capreomycin, Viomycin.

of purified compounds, however, synergimycins represent a restricted and homogeneous group. Two basic chemical structures, A and B, are shared by the components of all known inhibitors of this family: various products differ only in minor functional groups (Table 3). Although the two formulas are completely different, yet a similarity exists in the overall architecture of the two molecules: both of them are macrocyclic lactone peptolides.

Compounds of the A group are polyunsaturated cyclic peptolides, which can be considered as highly modified depsipeptides (BYCROFT, 1977). The basic structure of these compounds, which has a M.W. of about 500, was mainly established by X-ray crystallography and mass spectrometry and also by identification of the hydrolysis products (cf. COCITO, 1979 for review). Four antibiotics of type A have been chemically characterized already: griseoviridin (Fig. 1A),

Table 2. Commercial preparations of virginiamycinlike antibiotics

Antibiotic name	Company	Producing organism[a]
Doricin Patricin Vernamycin	Squibb	
Etamycin	Bristol	*S. lavendulae*
Geminimycin Synergistin (PA114)	Chas. Pfizer	*S. olivaceus*
Mikamycin	Kanegafuchi	*S. mitakaensis*
Ostreogrycin (E129)	Glaxo	*S. ostreogriseus*
Plauracin (A2315A) Streptogramin	Eli Lilly	*S. diastaticus*
Pristinamycin (RP7293) Pyostacin	Rhône-Poulenc	*S. pristinaespiralis*
Streptogramin	Merck	*S. graminofaciens*
Vernamycin	Olin Mathieson	*S. loidensis*
Virginiamycin (Staphylomycin)	R.I.T. (Recherche & Ind. Thérapeutiques)	*S. virginiae*
Viridogrisein and Griseoviridin	Parke Davis	*S. griseus* NRRL 2426 *S. griseoviridus*

[a] Streptomyces. (From Cocito, 1979)

Table 3. Type A and B components of antibiotics of the virginiamycin family. (From Cocito, 1979)

Complex antibiotic	Type A components	Type B components
Madumycin (A2315A)	Madumycin II	Madumycin I
Mikamycin	Mikamycin A	Mikamycin B
Ostreogrycin (E129)	Ostreogrycins A, C, D, G, Q	Ostreogrycins B (B1, B2, B3)
Patricin		Patricins A and B
Plauracin	Plauracin II	Plauracin I
Pristinamycin (Pyostacin)	Pristinamycin II (A and B)	Pristinamycins I (A, B, and C)
Streptogramin	Streptogramin A	Streptogramin B
Synergistins (PA114)	Synergistin A	Synergistins B (1 and 3)
Vernamycin	Vernamycin A Griseoviridin	Vernamycins B (α, β, γ, δ) Viridogrisein (Etamycin) (Doricin) (C)
Virginiamycin (Staphylomycin)	Virginiamycins M	Virginiamycins S

ostreogrycins A and G (Fig. 1B), and madumycin II (Fig. 1C). Virginiamycin M1, ostreogrycin A, pristinamycin IIA, streptogramin A, PA 114A1, vernamycin A, and mikamycin A share the same formula ($C_{28}H_{35}N_3O_7$, M.W. 525). The double bond A-2,3 is saturated in virginiamycin M2, ostreogrycin G, and

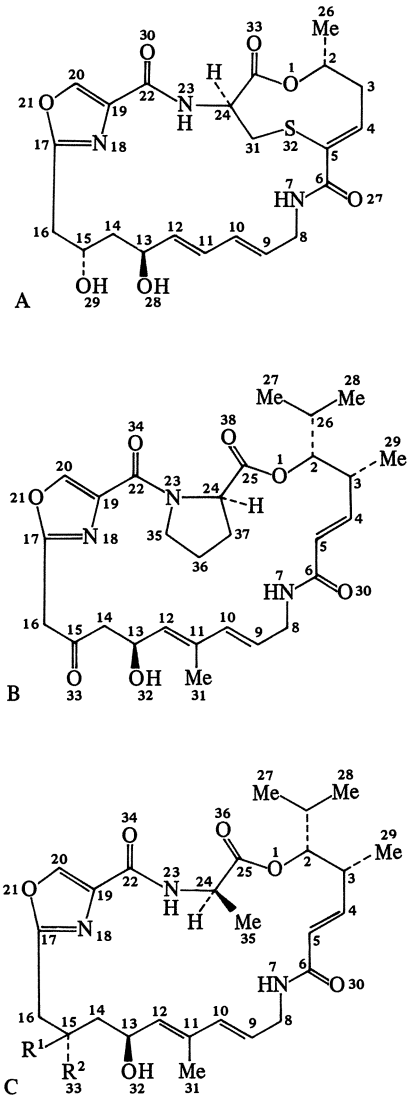

Fig. 1A–C. Structure of type A synergimycins. **A** griseoviridin; **B** ostreogrycin G; **C** madumycin II. (Reproduced from Cocito, 1979)

pristinamycin IIB. All components of the A group contain a substituted aminodecanoic acid and an unusual oxazole system, presumably derived from a cyclized didehydroserine residue.

Very recent studies of nuclear magnetic resonance, mass spectrometry, and X-ray diffraction have allowed the spatial configuration of type A compounds to be unraveled. The crystal conformation of griseoviridin and ostreogrycin A is shown in Fig. 2 (Bycroft, 1977). Low-temperature diffractometry of single crystals of virginiamycin M_1 with one molecule of dioxane has allowed a tridi-

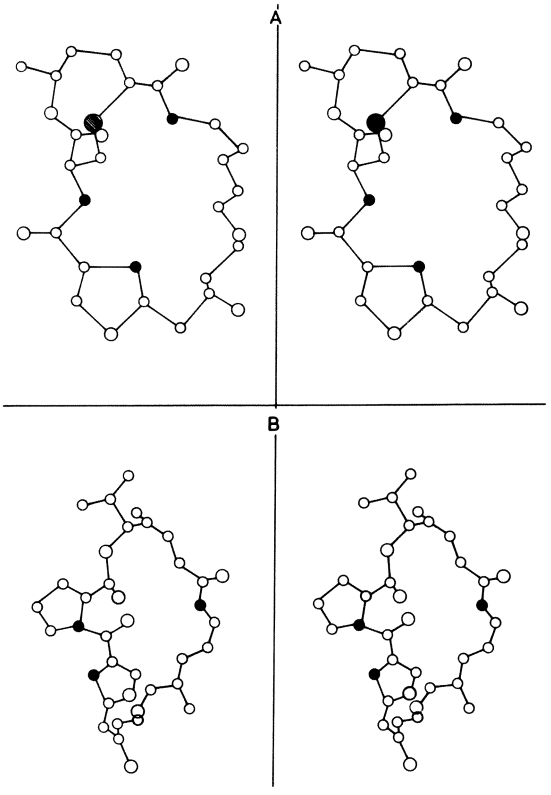

Fig. 2A and B. Crystal conformation of type A synergimycins. Perspective drawing by X-ray analysis of griseoviridin (**A**) and ostreogrycin A (**B**). (From Bycroft, 1977)

mensional model of the antibiotic to be built (Durant et al., 1974) (cf. Cocito, 1979 for review).

Compounds of the B group are cyclic hexadepsipeptides of MW of about 800. The primary structure, which was largely established by chemical identification of the hydrolysis products, is reported in Fig. 3. Most antibiotic preparations contain several components possessing similar structures. Thus, patricin A, PA114B1, doricin, mikamycin IA, ostreogrycins B (B1, B2 and B3), pristinamycins I (IA, IB, IC), streptogramin B, vernamycins B (B_α, B_β, B_γ and B_δ), and virginiamycins S (S1, S2, S3, S4) present minor modifications (hydrogens replaced by alkyl and methylamido groups) of the same basic structure (Compernolle et al., 1972; Crooy and De Neys, 1972) (Fig. 3A). Moreover, while most members of this group contain one molecule of pipecolic acid or its derivatives, this compound is replaced by either aspartic acid or prolin in doricin, patricin A and vernamycin C (Table in Fig. 3). Some of the amino acids in the basic structure of Fig. 3A are replaced by other amino acids in etamycin (Fig. 3B) and plauracin. From the [^{1}H] (at 300 MHZ) and [^{13}C] nuclear magnetic resonance studies of several components of the B group, the Pauling-Corey-Koltrum space-filling models of these antibiotics were constructed (Fig. 4) (Anteunis

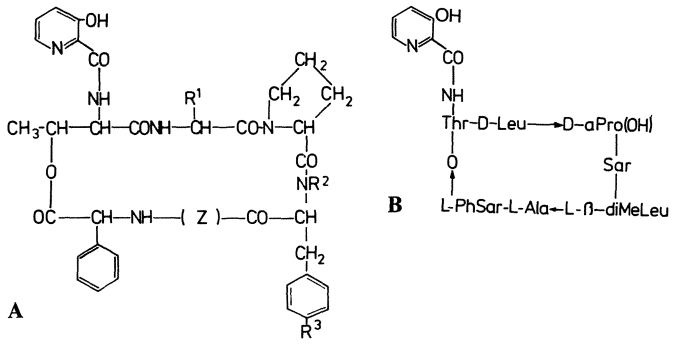

Fig. 3A and B. Structure of type B synergimycins. **A** Streptogramin B, and **B** etamycin (viridogrisein). Most type B synergimycins share the basic structure in **A**: the latter is modified according to the data in the annexed table. (From a work of CROOY and DE NEYS, 1972. Reproduced from COCITO, 1979)

Names	R^1	R^2	R^3	Z
Patricin B	C_2H_5	CH_3	H	Pipecolic acid
Virginiamycin S_1	C_2H_5	CH_3	H	4-Oxopipecolic acid
Virginiamycin S_4	CH_3	CH_3	H	4-Oxopipecolic acid
Virginiamycin S_2	C_2H_5	H	H	4-Hydroxypipecolic acid
Virginiamycin S_3	C_2H_5	CH_3	H	5-Hydroxy-4-oxopipecolic acid
Streptogramin B Mikamycin IA PA 114 B1 Pristinamycin IA Vernamycin Bα Ostreogrycin B	C_2H_5	CH_3	$N(CH_3)_2$	4-Oxopipecolic acid
Pristinamycin 1C Vernamycin Bγ Ostreogrycin B_1	CH_3	CH_3	$N(CH_3)_2$	4-Oxopipecolic acid
Pristinamycin IB Vernamycin Bβ Ostreogrycin B_2	C_2H_5	CH_3	$NHCH_3$	4-Oxopipecolic acid
Vernamycin Bδ	CH_3	CH_3	$NHCH_3$	4-Oxopipecolic acid
Ostreogrycin B_3	C_2H_5	CH_3	$N(CH_3)_2$	5-Hydroxy-4-oxopipecolic acid
Vernamycin C Doricin	C_2H_5	CH_3	$N(CH_3)_2$	Aspartic acid
Patricin A	C_2H_5	CH_3	H	Proline

et al., 1975; CALLENS et al., 1979a, b). Accordingly, virginiamycin S molecule was depicted as the one possessing a polar hydrophilic side and a lipophilic side, the polar function of D-aminobutyric acid carbonyl being screened by hydrophobic structures. The expanded model of the molecule (Fig. 4) shows an extreme conformation around the depside bond 6(CO), which acquires an "inward" or an "outward" orientation according to the solvent. The tridimen-

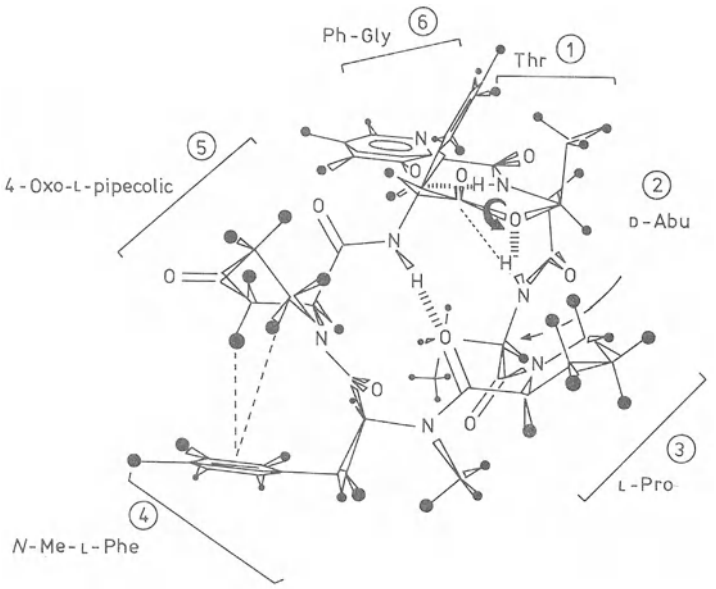

Fig. 4. Tridimensional model of a type B synergimycin. The expanded model of virginiamycin S shows an extreme conformation around the depside bond (CO outward). (From a work of Anteunis et al., 1975. Reproduced from Cocito, 1979)

sional structure of virginiamycin S has been unraveled by X-ray analysis of several crystalline preparations of the antibiotic (Declercq et al., 1971, 1978) (cf. Cocito, 1979 for review): it consists of a macrocyclic ring constrained by a transannular hydrogen bond.

Virginiamycin-like antibiotics are sensitive molecules: most reagents cause a loss of biological activity. However, derivatives of virginiamycin S (Vanderhaeghe et al., 1972; Janssen et al., 1977) and pristinamycin II (Le Goffic et al., 1981) have been recently obtained by restricted reductive and oxydative reactions. These reactions are important for the production of labelled derivatives for experimental work.

Chemical synthesis of two type B components, namely patricin A (Ondetti and Thomas, 1965) and etamycin (Sheehan and Ledis, 1973) has been achieved. Although the total synthesis of type A compounds has not been realized so far, a step toward this goal has been taken recently (Meyers et al., 1980).

The biosynthetic pathways for synergimycin formation are now partly clarified. Concerning type A compounds, a recent work by Kingston and Kolpak (1980) has shown that acetate, methionine, and proline are the best substrates for labeling virginiamycin M in vivo. Scheme in Fig. 5 shows the pathway part known at the present time. Accordingly, methionine is the donor of the 3^a methyl group (transfer of C3 from serine to methionine via N-methyl-tetrahydrofolate), while incorporation of acetate followed by decarboxylation is the source of the $-CH_3$ 10^a, and formation of the oxazole ring is linked to the incorporation of an acylserine precursor.

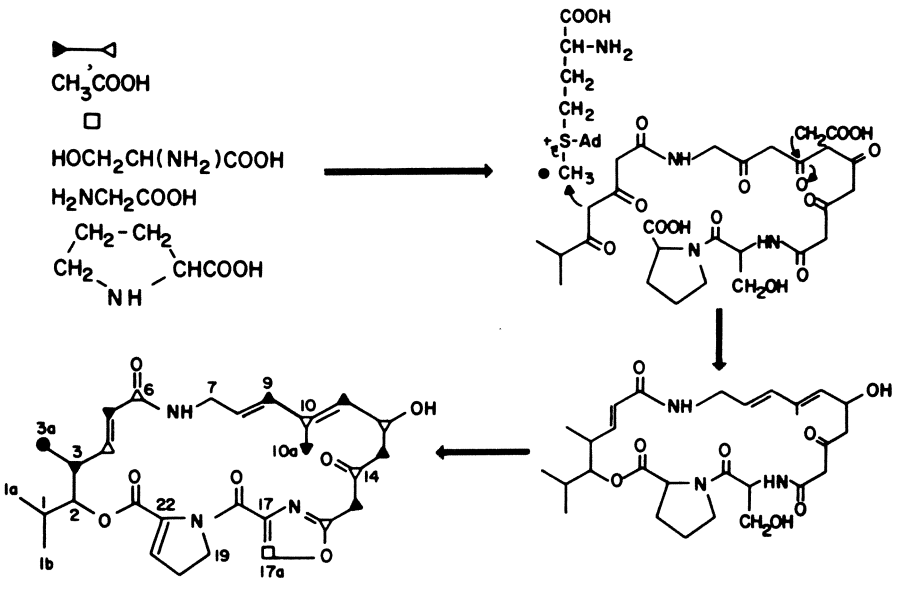

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Fig. 5. Biosynthetic pathway of type A synergimycins. Determination of the labeling pattern of virginiamycin M_1 in *Streptomyces virginiae* by the stable isotope techniques. (From KINGSTON and KOLPAK, 1980)

Biosynthesis of etamycin (type B synergimycin) can be envisaged as follows, according to HOOK and VINING (1973a, b). L-threonine and L-alanine are precursors of the corresponding moieties of the antibiotic, and L-leucine of both the D-leucine and dimethyl-L-leucine moieties (a finding confirmed by KAMAL and KATZ, 1976). Phenylalanine is the precursor of L-phenylsarcosine, and L-lysine of the 3-hydroxypicolinic acid. The methyl groups of sarcosine, phenylsarcosine, and dimethyl-leucine are provided by L-methionine.

The chemical and physical properties of synergimycins are described in an earlier review (COCITO, 1979), where the basic spectra in the visible, ultraviolet, and infrared regions are reported, and the spectrophotometric methods of titration are described.

The Action of Virginiamycin-like Antibiotics on Bacteria

1. Alteration of Growth and Viability

Virginiamycin-like antibiotics inhibit the multiplication of numerous microorganisms, Gram-positive being more sensitive (minimum inhibitory concentration 0.1 to 5 μg/ml) than Gram-negative bacteria (5 to 200 μg/ml). Exceptions to this rule are: *Mycobacteria*, some of which are relatively resistant, and *Hemo-*

Table 4. Minimum concentration of single components inhibiting the growth of different bacteria[a]

Organisms	Inhibitory concentration (μg/ml)								
	Viridogrisein	Streptogramin		Synergistin		Virginiamycin		Mikamycin	
		A	B	A-1	B-1	M	S	A	B
Gram-positive bacteria									
Bacillus megaterium	–	40	3	–	–	–	–	800	20
Bacillus subtilis	2.50	–	–	100	3.12	50	13	800	10
Staphylococcus aureus	0.31	6	10	0.78	6.25	5	125	20	100
Sarcina lutea	–	–	–	–	–	2.5	14	–	–
Streptococcus pyogenes	0.63	–	–	0.19	50	–	–	40	40
Gram-negative bacteria									
Escherichia coli	200	40	100	–	–	–	–	800	800
Hemophilus pertussis	5	–	–	–	–	–	–	4	100

[a] According to Vázquez (1967)

philus and *Neisseria,* which proved quite sensitive to these drugs (Table 4). Divergence in the sensitivity of different bacteria to these antibiotics is due in most cases to permeability, since 70S ribosomes (which are the target of these antibiotics) are equally sensitive irrespective of their source.

A mixture of type A and B components causes a more pronounced inhibition of bacterial growth than do single components separately: 10- to 100-fold increase of growth inhibition was observed in different microorganisms (Table 5). In Fig. 6, the action of virginiamycin on the growth of *B. subtilis* is depicted. Quite high levels (10 to 100 μg/ml) of either components, VM or VS, are required to block completely the increase in turbidity of growing cultures. A like effect is produced, however, by far lower concentration of a VM + VS mixture (0.1 to 1 μg/ml): this means a 100- to 1000-fold potentiation of the antibiotic activity, depending on the microorganism tested (Ennis, 1965a; Cocito, 1969a).

The synergistic action on cell multiplication varies according to the relative proportions of type A and B components in the mixture. When the growth-inhibiting power of different combinations of components A and B on sensitive microorganisms are plotted, asymmetrical bell-shape curves are obtained. In fact, the biological activity sharply decreases when either component is withdrawn, and the type A components appear as the limiting factor. Minimum concentration for activity is 15% of the A component in the mixture, while A/B ratios from 2:1 to 1:1, which are usually found in nature, are the most active.

Single virginiamycins do not reduce viability of most bacteria, except after prolonged incubation. Nonetheless, cells incubated with type A synergimycins show a reduced growth rate when transferred to antibiotic-free medium. This phenomenon, previously described as "bacteriopause" (Chabbert and Courva-

Table 5. Growth inhibition of different microorganisms by mixture of A and B components[a]

Organisms	Inhibitory concentration (μg/ml)				
	Strepto-gramin	Syner-gistin	Virginia-mycin	Mika-mycin	Pristina-mycin
Gram-positive bacteria					
Bacillus megaterium	2	–	–	64	–
Bacillus subtilis	–	0.78	1	32	0.70
Staphylococcus aureus	0.60	0.19	0.20	4	0.20
Sarcina lutea	–	–	0.10	1	–
Streptococcus pyogenes	0.05	0.08	0.07	–	0.10
Streptococcus faecalis	1.49	0.39	0.50	–	0.20
Diplococcus pneumoniae	0.25	3.12	0.07	6	0.15
Corynebacterium diphtheriae	0.04	0.39	–	1	0.02
Mycobacterium sp. 607	11	6.25	–	280	–
Mycobacterium tuberculosis	5	–	20	200	–
Gram-negative bacteria					
Salmonella typhosa	11.80	100	–	1600	–
Escherichia coli	40	100	–	1600	50
Aerobacter aerogenes	–	100	100	–	250
Hemophilus pertussis	0.04	3.12	–	–	–
Neisseria gonorrheae	–	3.12	–	–	0.20
Pseudomonas aeruginosa	50	100	–	1600	250
Yeast					
Saccharomyces cerevisiae	85	–	–	1600	–
Candida albicans	–	100	100	1600	–
Fungi					
Aspergillus niger	85	–	–	–	–
Aspergillus oryzae	–	–	–	1600	–
Protozoa					
Trichomonas vaginalis	490	–	–	–	–
Trichophyton sulfureum	–	100	–	–	–

[a] According to VÁZQUEZ (1967)

LIN, 1971) is now explained (see below) by the lasting damage to ribosomes (PARFAIT and COCITO, 1980).

On the other hand, mixtures of components A and B produce a sharp decrease of colony-forming capacity, not necessarily associated with the lysis of bacteria: 99% of the cells become nonviable within one generation time (cf. Fig. 9). Although a similar effect was observed upon alternated incubation of microorganisms with single virginiamycin components (COCITO, 1969a; COCITO and FRASELLE, 1973), the validity of these findings was questionable owing to the difficulty of removing type A compounds by washing. However, the discovery of the catalytic inactivation of ribosomes by these inhibitors (PARFAIT and COCITO, 1980) explains earlier observations.

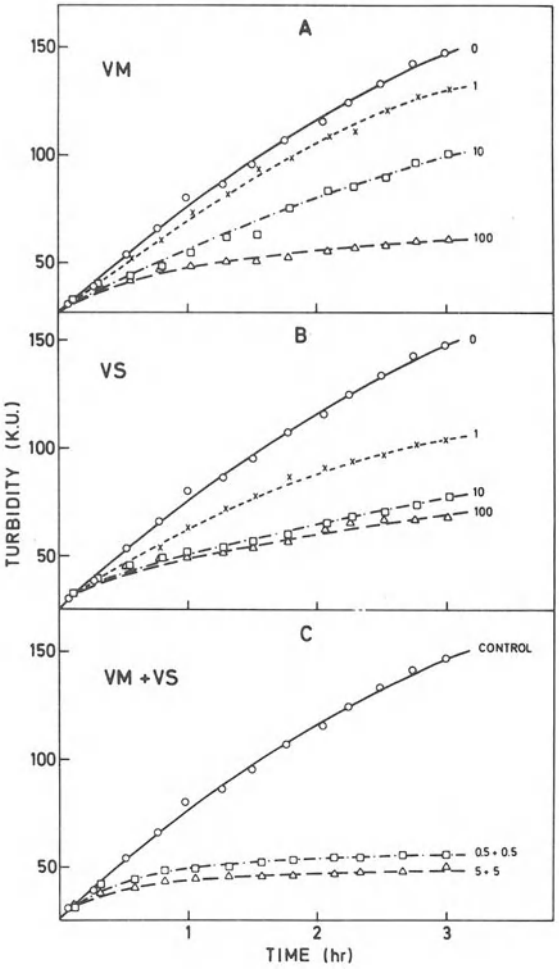

Fig. 6A–C. Growth inhibition of bacteria by single synergimycin components and their mixture. Inhibitors: **A** virginiamycin M (type A) (1 to 100 μg/ml final concentration); **B** virginamycin S (type B) (1 to 100 μg/ml final concentration); **C** mixture of both (0.5 to 5 μg/ml). The inhibitory power of single components is 100-fold lower than that of their mixture. (From Cocito, 1969a)

The two synergistic effects on bacterial growth and viability are quite specific: components of one group increase and render irreversible the inhibitory action of their partners, but not that of other translational inhibitors. Thus, e.g., virginiamycin M and S do not potentiate, at least in *B. subtilis,* the reduction of cell growth and viability caused by several inhibitors acting on the 50S ribosomal subunits, such as chloramphenicol, erythromycin, fusidic acid, and oleandomycin (cf. Cocito, 1979). On the contrary, synergimycins block, under certain conditions, the action of antibiotics. Thus, e.g., mikamycin A was reported to prevent the killing effect of streptomycin, kanamycin, and penicillin (cf. Tanaka, 1975).

2. Macromolecule Formation in the Presence of Synergimycins

When kinetics of incorporation of labeled precursors into DNA, RNA, and proteins are compared, the latter type of synthesis appears blocked without delay, whereas nucleic acid formation is affected only after a considerable lag. It is inferred, therefore, that polypeptide formation is the target of both A and B compounds (cf. Fig. 7), and that the alteration of cell growth and viability is due to a block of this metabolic pathway (cf. COCITO, 1979).

However, early and important alterations of RNA metabolism occur in the presence of synergimycins. Thus, e.g., in *B. subtilis* incubated with these antibiotics, a very early inhibition of 16S and 23S rRNA formation and methylation has been observed: the resulting structural alteration accounts for the metabolic instability of these nucleic acids, otherwise highly conserved and transferred to progeny cells during bacterial multiplication (COCITO, 1969a).

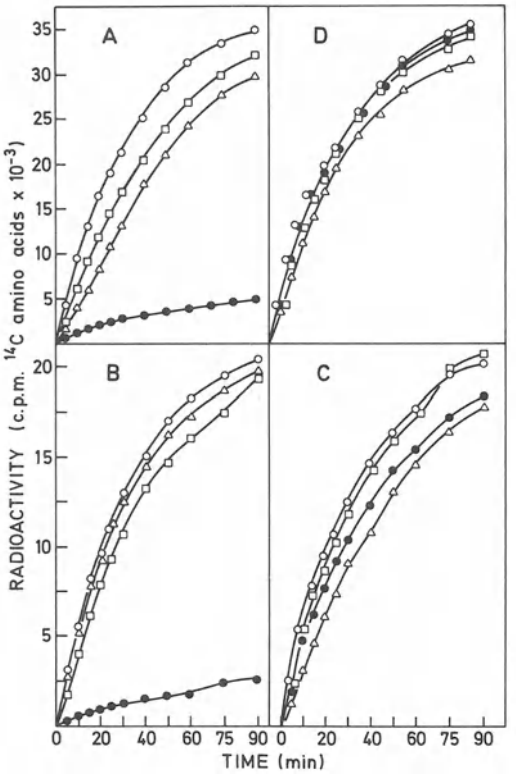

Fig. 7A–D. Reversible and irreversible inhibition of protein synthesis by synergimycins in sensitive and resistant strains. After incubation with either type A (–△–) or type B (–□–) components, or both (–●–) (–○– is the control), bacteria are transferred to antibiotic-free medium and labeled with [^{14}C] amino acids. Mutants: resistant to type A (**B**), to type B (**C**), to both (**D**) (sensitive strain in **A**). Irreversible inhibition of protein synthesis occurs only in sensitive or in type A resistant mutants incubated with a mixture of the two components. (From COCITO and FRASELLE, 1973)

Also, an increase in the half-life of mRNA was observed shortly after the addition of synergimycins to bacterial cultures (Cocito, 1969a). In reality, two contrasting situations develop in bacteria submitted to protein synthesis inhibitors: a puromycin-like effect yielding the dissociation of translational complexes, and a chloramphenicol-like effect freezing polysomes. The half-life of mRNA is decreased in the former type, and increased in the latter type of protein synthesis inhibition (that occurring in the presence of synergimycins) (Cocito, 1971).

3. Metabolism of Polysomes and Ribosomes

Formation of ribosomal subunits is blocked in bacteria incubated with synergimycins (Cocito, 1973 a). This effect is shared by the other inhibitors of protein synthesis, and is due to a block of ribosomal proteins formation. The rRNA, synthesized under these conditions, binds to cytoplasmic proteins already present, thus forming heterogeneous ribonucleoprotein complexes mimicking the "relaxed particles" of relaxed mutants starved for essential amino acids.

When cells incubated with single synergimycin components are transferred to antibiotic-free medium, formation of ribosomal subunits resumes without delay (Cocito, 1973a). Apparently, rRNA molecules within relaxed-like particles dissociate from their protein partners and bind to newly formed rProteins. This recovery process is rather puzzling, for rRNA which accumulates under these conditions of halted protein synthesis is undermethylated (Cocito, 1969a). When sensitive cells are incubated with a mixture of type A and B components, no such recovery occurs upon removal of the drug: this is due to the permanent halt of protein synthesis that is produced under these conditions.

Short incubation of growing cells with virginiamycin "freezes" polysomes (Cocito, 1971), in agreement with the increased half-life of translational complexes (Cocito, 1969a).

When the incubation of bacteria with virginiamycin is prolonged, however, most polysomes disappear and monosomes and ribosomal subunits accumulate (Ennis, 1972; Cocito, 1978). The picture is different in cells treated with compounds of the A and B groups. In the former case, there is an accumulation of ribosomal subunits, a situation mimicking that produced by transcriptional inhibitors. In the latter case, cells are stacked with monosomes, as they do upon treatment with chloramphenicol (a similar situation occurs in auxotrophs starved for an essential amino acid) (Cocito, 1978).

If lysates from cells incubated with type A synergimycins are centrifuged at high speed (100,000 g) in sucrose gradients, an unusual ribosomal peak, the "60S component," appears (Fig. 8B and D) (Cocito, 1973b). Its formation is prevented either by particle fixation with glutaraldehyde (Fig. 8E) or by centrifugation at low speed (50,000 g). Indeed ribosomes, which are produced under certain conditions of halted protein synthesis, dissociate when they reach a certain distance from the axis of rotation while traveling in a dense medium at a critical speed (the steepness of the density and pressure gradients contribute to the sharpness of the peak) (Cocito, 1978). Although the formation of the

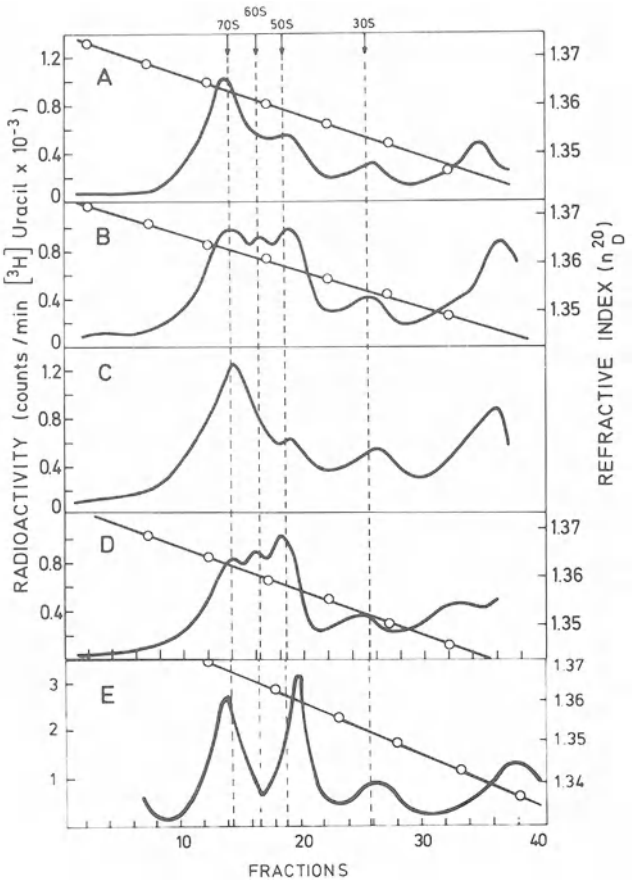

Fig. 8A–E. Production of pressure-labile ribosomes in the presence of type A synergimycins. [^{3}H]uracil-labeled bacteria are incubated with either type A (**B**), or type B (**C**) components and their mixture (**D**) prior to ribosome fractionation by density gradient centrifugation. Sample **A** is the control (no antibiotic), and sample **E** corresponds to sample **B** submitted to glutaraldehyde fixation before centrifugation. Wherever cells are incubated with type A components, pressure-labile ribosomes appear unless they are fixed. (From Cocito, 1973b, 1978)

60S component remains unexplained, it is surprising that no such unusual particles are formed in cells grown in the presence of type B synergimycins (Fig. 8C).

4. Resistance to Synergimycins

Bacterial resistance to antibiotics is due to the following mechanisms: (1) alteration of permeability, (2) modification of the target (the 50S ribosomal subunit, in our case), and (3) inactivation of the drug. Antibiotics can be inactivated either by enzymatic hydrolysis or by derivatization (acylation, adenylation and phosphorylation). Moreover, genetic determinants for antibiotic resistance can be either chromosomal or plasmidic in nature. In most cases, drug inactiva-

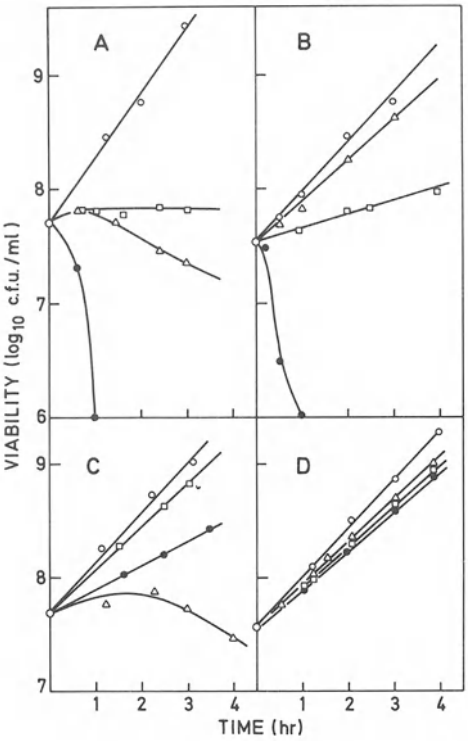

Fig. 9A–D. Viability alteration in sensitive and resistant mutants incubated with synergimycin components. Mutants resistant either to type A (**B**) or type B (**C**) components or to both (**D**) (the sensitive strain is in **A**) are incubated either with type A component (–△–) or with type B component (–□–) or with both (–●–) (–○– is the control) and cell viability is measured. Viability drops only in sensitive and type A resistant strains incubated with a mixture of both components. (From Cocito and Fraselle, 1973)

tion is by extrachromosomal determinants (plasmids), target modification is chromosomal, and permeability loss may have either origin. All these mechanisms were found to be responsible for resistance to synergimycins in different microorganisms.

Ennis (1971 b) has compared the behavior of two bacterial strains (the wild type was sensitive in the case of *B. subtilis* and resistant in the case of *E. coli*), and reached the conclusion that resistance to vernamycin was due to permeability loss.

The situation of chromosomal resistance to virginiamycin in the 168 strain of *Bacillus subtilis* is as follows. Incubation of sensitive strains with a mixture of both A and B components produces a sharp drop in viability (Fig. 9A): this lethal effect still operates in mutants resistant to type A (Fig. 9B), but not in those resistant to type B components (Fig. 9C). Likewise, a mixture of synergimycins A and B produces an irreversible inhibition of protein synthesis in the double-sensitive as well as in type-A-resistant mutants, but not in bacteria resistant to type B compounds (Fig. 7). Consequently, lethality of synergimycins

Table 6. Mapping of type B synergimycin resistance in Bacillus subtilis. (From RON et al., 1980)

Selected donor marker	Unselected donor markers		Total colonies tested	No. of colonies in recombinant class				Marker order deduced[b,c]
100[a]	010	001		100	110	101	111	
cys^+ [a]	VS^R	gfa-3	103	59	4	2	38	cys, VS, gfa-3 or cys, gfa-3, VS
VS^R	cys^+	gfa-3	81	2	0	30	49	cys, gfa-3, VS
VS^R	str^R	gfa-3	67	1	1	40	25	VS, gfa-3, str
VS^R	str^R	ery^R	48	27	4	8	9	str, VS, ery
ery^R	VS^R	str^R	79	14	45	0	20	ery, VS, str
ery^R	VS^R	gfa-3	48	8	4	0	36	ery, VS, gfa-3
str^R	VS^R	gfa-3	48	0	0	4	44	str, gfa-3, VS

[a] Symbols: VS = virginiamycin S (type B); ery = erythromycin; str = streptomycin; fus = fusidic, acid.
[b] Order deduced from the analysis of 3-factor transformation crosses; "cys-str-fus-gfa-3-VS-ery."
[c] Co-transduction obtained in transduction crosses: cys^+-VS^R (49 and 46%); ade^+-VS^R (20% and 15%).

relies on the gene concerned with sensitivity to type B components, and disappears when this mutates to resistance.

Resistance to virginiamycin S (type B component) has been mapped in *B. subtilis* chromosome by transduction and transformation (RON et al., 1980). The corresponding gene was located between the markers of elongation factor G and erythromycin resistance, i.e, within the gene cluster of ribosomal proteins (Table 6). Instead, resistance to type A components has not yet been mapped.

Plasmid-mediated resistance is the main type occurring in staphylococci. A strain of *Staphylococcus aureus* able to inactivate several antibiotics, including pristinamycin IIA, was found to harbor a plasmid (PAC-IIA) directing the synthesis of an acetyl transferase that O-acetylates the drug (LE GOFFIC et al., 1977a, b). Another strain of *Staphylococcus aureus* isolated from humans and resistant to type A components proved able to inactivate the drug via an inducible and strain-specific acetyl transferase, presumably coded for by a plasmid (DE MEESTER and RONDELET, 1976).

A similar plasmid, carrying the genes for resistance to erythromycin, lincomycin, and vernamycin Ba has been isolated from *Streptococcus sanguis*. pAM-77 had a sedimentation coefficient of 25S, a molecular weight of 4.8×10^6, and a contour length of 2.3 μm. Restriction endonuclease segments of this plasmid were used to transfect the transformable Challis strain of Streptococcus (YAGI et al., 1978). Similar studies were recently made by BEHNKE et al. (1979), BOITSOV et al. (1979), MALKE (1979), and EL SOHL et al. (1980).

Inactivation of synergimycins through the cleavage of the lactone ring by lactonases has been observed in *Actinoplanes missouriensis*. The inactivating enzyme had a molecular weight of about 3.5×10^4, a KM value of 3.73×10^{-4} M, and a pH optimum of 7.8 (HOU and PERLMAN, 1970; HOU et al., 1970). Likewise, *Streptomyces mitakaensis* was found to produce a lactonase able to inactivate mikamycin B and to yield the corresponding acid. The purified

enzyme had a molecular weight of 2.9×10^4 and a KM value of 1.43×10^{-5} M (Kim et al., 1974). These enzymes might play the role of physiological regulators of the antibiotic formation, during the growth cycle of the producing organisms.

Several Gram-positive organisms, particularly staphylococci and streptococci are known to acquire an "undissociated" type of resistance to macrolides, lincosamides, and type B synergimycins (MLS), as resistance acquired against one type of inhibitor renders the cell insensitive to the three groups of antibiotics; although "dissociated" patterns entailing resistance to single families have been reported (cf. Weisblum and Demohn, 1969). Bacteria can become unresponsive either because of the acquisition of a genetic defect (constitutive resistance) or as the result of an adaptive process (inducible resistance). The molecular basis of the latter phenomenon has been largely unraveled through a series of remarkable studies.

Staphylococcus strains carrying "dissociated" resistance to erythromycin, in the presence of low concentrations of the latter inhibitor, acquire an inducible "undissociated" resistance (Weisblum and Demohn, 1969; Weisblum et al., 1971; Allen, 1977a, b) owing to the methylation of 23S rRNA. Indeed, 50S subunits harboring N^6-dimethyladenine in their RNA component do not bind antibiotics of the MLS group (Lai and Weisblum, 1971). These findings are confirmed by reconstitution experiments: in fact, hybrid 50S subunits reconstituted with 23S rRNA from either constitutive resistant mutants or induced sensitive cells on one hand, and ribosomal proteins from uninduced wild type on the other hand, are not inhibited by lincomycin when tested in cell-free systems. The fact that 23S rRNA of different strains of erythromycin producing streptomycetes carries N^6-N^6-dimethyladenine (Fusijawa and Weisblum, 1981) suggests a biological role for this methylation process. More recently, the MLS type of resistance in *Staphylococcus aureus* has been related to an inducible plasmid pE194, which is present in 10 to 35 copies per cell during the induction process (Weisblum et al., 1979) and can be transferred to *Bacillus subtilis* by transformation. The nucleotide sequence of the pE194 segment responsible for MLS resistance has now been cloned in *Bacillus subtilis* and sequenced (Horinouchi and Weisblum, 1980). It codes for a 19 amino acid leader peptide and a 243-amino acid protein separated by a spacer containing the promoter region and provided with a stem and a loop. The regulatory model proposed for induction postulates a temporary block of ribosomes at the protein promoter (which is normally hidden within the loop): this leads to destabilization of the stem, opening of the loop, and progression of stalled ribosomes (Horinouchi and Weisblum, 1981). This regulatory process, thus, mimics the attenuator model recently described for the regulation of the tryptophan operon in *E. coli.*

The Action of Synergimycin Components in vitro

The In Vitro Action of Type A Synergimycins

Protein synthesis directed by both synthetic and natural mRNA in cell-free systems from bacteria is strongly inhibited by type A synergimycins (Fig. 10)

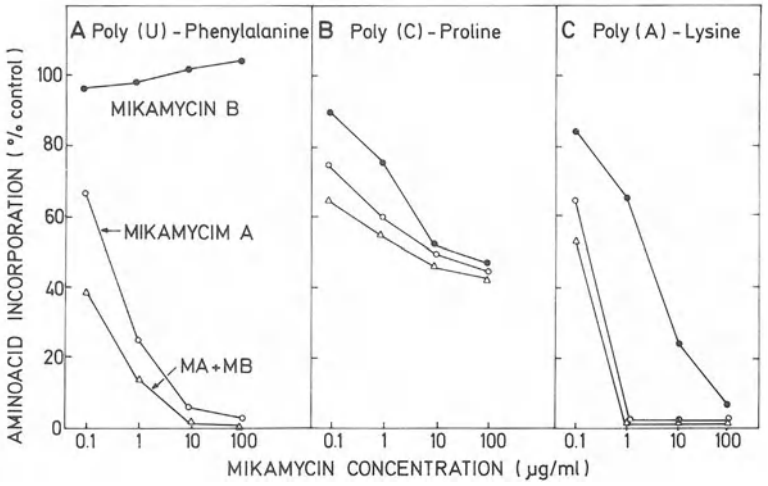

Fig. 10A–C. Inhibition of peptide bond formation in cell-free systems by synergimycin components. Formation of poly(phenylalanine) (**A**), poly(proline) (**B**), and poly(lysine) (**C**) in the presence of mikamycin A (–o–), B (–●–), and their mixture (–▵–). (From TANAKA, 1975)

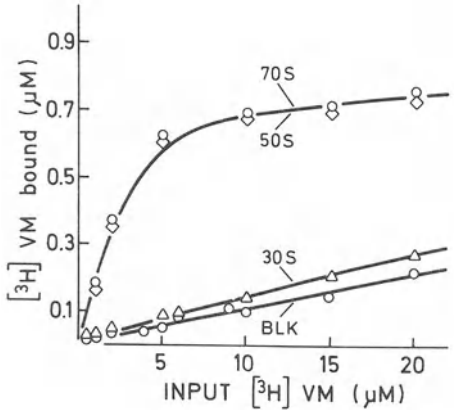

Fig. 11. The binding of a type A synergimycin to ribosomes and to the large subunit. Increasing concentrations of labeled virginiamycin M are incubated with 30S and 50S subunits and 70S ribosomes of *E. coli* (blank without particles), and bound antibiotic is measured by the norite procedure. (From COCITO and DI GIAMBATTISTA, 1978). Type A synergimycins bind to 70S particles as well as to 50S subunits, but have no affinity for the small ribosomal subunit

(LASKIN and CHAN, 1965; ENNIS, 1965b; VÁZQUEZ, 1966a; COCITO and KAJI, 1971). This inhibitory effect occurs at the level of the large ribosomal subunits. In fact, binding of these antibiotics to 50S ribosomal subunits has been demonstrated by different techniques (ENNIS, 1971a; COCITO, 1979). With the norite method (COCITO and DI GIAMBATTISTA, 1978), type A synergimycins appear to bind to 70S monosomes and to 50S subunits, but not to the 30S subunits

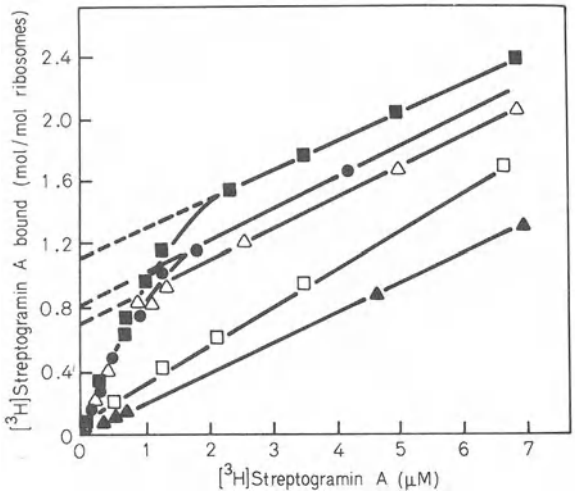

Fig. 12. Binding of type A synergimycins to ribosomes and polysomes. Binding of [^{3}H]streptogramin A to polysomes with (–□–) and without (–●–) nascent peptides, as well as to run-off (–△–) and salt washed (–■–) ribosomes is measured by sedimentation (–▲– is the blank). In contrast to ribosomes, natives polysomes do not bind the inhibitor. (From Contreras and Vázquez, 1977)

(Fig. 11). Moreover, as proven by Contreras and Vázquez (1977), polysomes fail to bind these inhibitors (Fig. 12). The binding reaction, which requires a temperature-dependent ribosome activation, is largely independent (above limiting levels) on both monovalent and bivalent ions, and is not affected by type B components (while the reverse is true, as indicated in a following section). Among the 50S-inhibitors tested, only erythromycin competes effectively with the binding of type A compounds to ribosomes. The association constant of the binding reaction of virginiamycin M to ribosomes, which is monomolecular, is $K_A = 0.32 \times 10^{-6}\ M^{-1}$ (Fig. 13) (Cocito and Di Giambattista, 1978). The closeness between this value and that of chloramphenicol ($K_A = 0.21 \times 10^{6}\ M^{-1}$) could explain the ability of the former drugs to inhibit competitively the binding of the latter to ribosomes (Vázquez, 1966b).

Prior to a detailed analysis of type A synergimycin action in cell-free systems, the main steps in initiation and peptide chain elongation in bacteria are herewith summarized: they are sketched in Fig. 14. The first step of initiation involves the association of 30S particles with mRNA, and the three initiation factors IF-1, 2, and 3. The initiator tRNA is then positioned at the AUG-initiating codon of the messenger. Subsequently, the joining of the 50S subunits results in the hydrolysis of IF-2-bound GTP and in the release of the initiation factors. Complete initiation complexes carrying fMet-tRNA at the P-site are, thus, formed. Elongation, which starts at this point, consists in three main steps that are cyclically repeated at each amino acid addition to the growing peptide: (1) the EF-Tu- and GTP-dependent binding of aminoacyl-tRNA to the A-site, whereby the appropriate aminoacyl-tRNA interacts with the next codon of mRNA as well as with the peptidyl transferase; (2) peptide bond formation

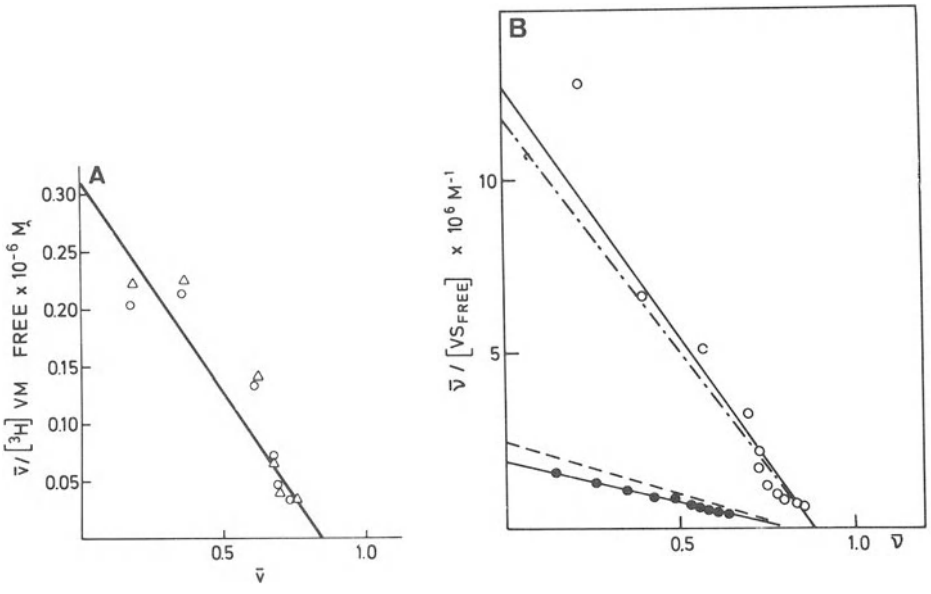

Fig. 13A and B. Scatchard plots of the binding reactions of type A and type B synergimycins to ribosomes. **A** Virginiamycin M binding to ribosomes is followed by the norite method (Cocito and Di Giambattista, 1978), in the presence (–△–) or in the absence of virginiamycin S. **B** The virginiamycin S binding is traced spectrophotometrically (Parfait et al., 1978) in the presence (–○–) or in the absence (–●–) of virginiamycin M. The two binding reactions are monomolecular, however, while the binding of virginiamycin M is not modified by its partner, that of virginimaycin S undergoes a sharp change in the presence of virginiamycin M

between fMet- or peptidyl-tRNA at the P-site and the AA-tRNA at the A-site (reaction catalyzed by the peptidyl transferase center on the 50S particles); and (3) the EF-G- and GTP-dependent translocation of peptidyl-tRNA from the A-site to the P-site with concomitant progression of the ribosome on mRNA.

The action of type A synergimycin on the initiation and elongation steps has been systematically analyzed in *E. coli* cell-free systems. The binding of mRNA, initiation factors, and fMet-tRNA to 30S subunits is not affected by these inhibitors (Fig. 15) (Cocito et al., 1974), as expected by their lack of affinity for the small subunits (the inhibition of fmet-tRNA binding observed by Ennis and Duffy, 1972, remains unexplained). Also, the addition of the 50S subunits to the initiation complexes and the recycling of initiation factors occur normally, thus ruling out a possible interference with the initiation steps (Fig. 16). However, fMet-tRNA positioned at the P-site of the 75S initiation complexes does not react with puromycin in the presence of virginiamycin M (Fig. 16B) (Cocito et al., 1974). Initiation complexes, thus, form normally in the presence of these antibiotics, but they are inactive (Fig. 16). These findings agree with studies in which the reactivity of N-acetyl-aminoacyl-oligonucleotides was explored in the presence of 50S subunits: indeed, the "fragment reaction" with puromycin proved inhibited by virginiamycin A (Fig. 17) (Monro and Vázquez, 1967; Celma et al., 1970, 1971).

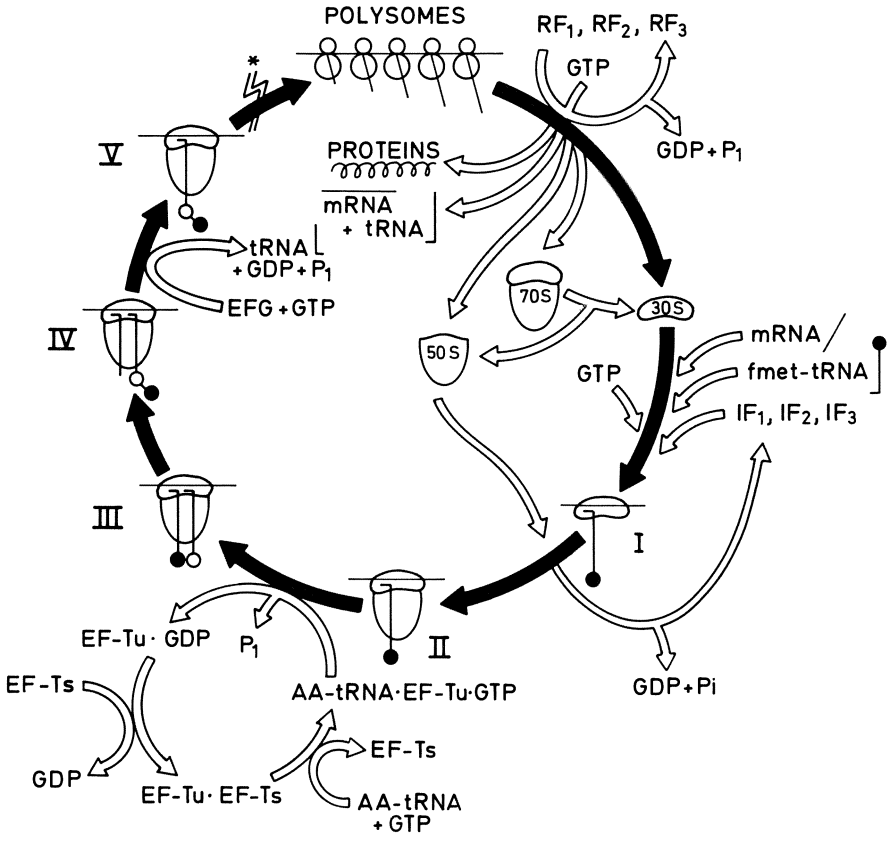

Fig. 14. Metabolic pathway for protein synthesis and ribosomal cycle. *I* 30S initiation complex, *II* 70S initiation complex, *III* Binding (Elongation step I), *IV* Peptidyzation (Elongation step II), *V* Translocation (Elongation step III). (Cocito and Chinali, unpublished)
* repetition of steps III to V

When the elongation steps were examined, the EF-Tu-directed aminoacyl-tRNA binding to the A-site (Table 7) and the reactivity with puromycin of aminoacyl-tRNA positioned at the P-site were found to be prevented by type A synergimycins (Cocito and Kaji, 1971; Cocito et al., 1974). Consequently, it has been proposed that virginiamycin M bound to the A-site prevents peptidyl-tRNA at the P-site from reacting with a suitable acceptor, owing to steric hindrance. Though the simultaneous interference of an inhibitor with both A- and P-sites is not inconceivable, owing to their proximity on the 50S surface, the cited observations have prompted a more detailed dissection of the elongation process. This study has been recently carried out using kirromycin, an inhibitor acting on EF-Tu. The GTP.EF-Tu.kirromycin. aminoacyl-tRNA complex is known to bind to the ribosomal A-site and to trigger GTP hydrolysis. However, GDP.EF-Tu does not leave ribosomes (as it does under normal conditions): the permanent occupation of the A-site by the complex containing kirromycin accounts for the block of protein synthesis induced by this antibiotic

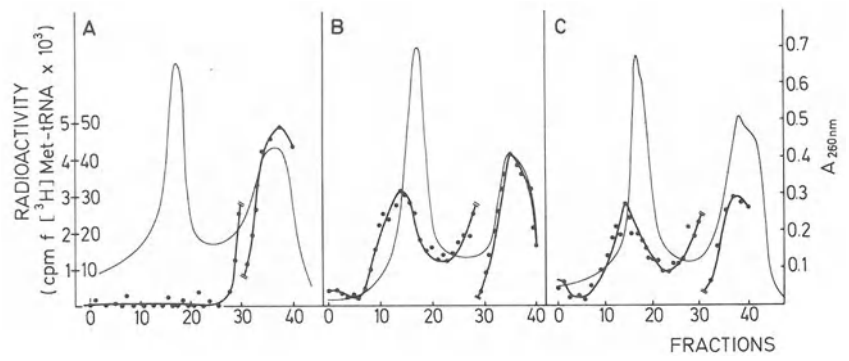

Fig. 15A–C. Formation of 45S initiation complexes in the presence of type A synergimycins. Complexes of 30S ribosomal subunits, mRNA, [^{3}H]fMet-tRNA and IF-1, -2, -3 are assembled either in the presence (**C**) or in the absence (**B**) of virginiamycin M (**A** is the control missing mRNA) and then fractionated by density gradient centrifugation. Initiation complexes are formed irrespective of the presence of the inhibitor. (From COCITO et al., 1974)

(WOLF et al., 1974, 1977; CHINALI et al., 1977). In the presence of kirromycin, virginiamycin M is ineffective. In fact, the rate of EF-Tu-directed binding of Phe-tRNA to poly(U). ribosomes complexes (Fig. 18), and that of the associate stoichiometric hydrolysis of GTP (Fig. 19), are not modified by virginiamycin M. Consequently, irrespective of the presence of the latter inhibitor, the binding reaction results in the final formation of ribosomal complexes carrying Phe-tRNA and EF-Tu.GDP.kirromycin at the A-site (CHINALI et al., 1981). Moreover, in the absence of kirromycin, the release of EF-Tu.GDP complex takes place normally from ribosomes incubated with virginiamycin M (Fig. 19). Such a result indicates that this antibiotic does not affect the initial steps of the binding reaction in elongation. Aminoacyl-tRNA bound enzymatically to the A-site becomes sensitive to the action of virginiamycin M after the release of the EF-Tu.GDP complex from the ribosomes. Indeed, addition of type A synergimycins to poly(U).ribosomes carrying Phe-tRNA^{Phe} bound enzymatically to the A-site causes the detachment of aminoacyl-tRNA (Fig. 20) (COCITO et al., 1974; CHINALI et al., 1981). This alteration of the elongation process may account for both the reduction of EF-Tu-directed binding of aminoacyl-tRNA to ribosomes (Fig. 18 and Table 7) and the stimulation of the coupled EF-Tu. GTPase activity (Fig. 19). These results indicate that type A synergimycins act on the step of the binding process involving the interaction of the aminoacyl end of tRNA with the acceptor site of the peptidyl transferase. The evidence for an interference of virginiamycin M and related antibiotics with the peptidyl transferase has been provided by experiments from several laboratories (cf., e.g., Figs. 16 and 17). The subject has been reviewed (PESTKA, 1977; VÁZQUEZ, 1979). Additional evidence along these lines was gathered by showing that peptidyl-tRNA positioned at the ribosomal A-site is able to protect ribosomes from virginiamycin M (Fig. 21) (CHINALI et al., 1981) in agreement with reports that this kind of inhibitor neither binds to polysomes (CONTRERAS and VÁZQUEZ,

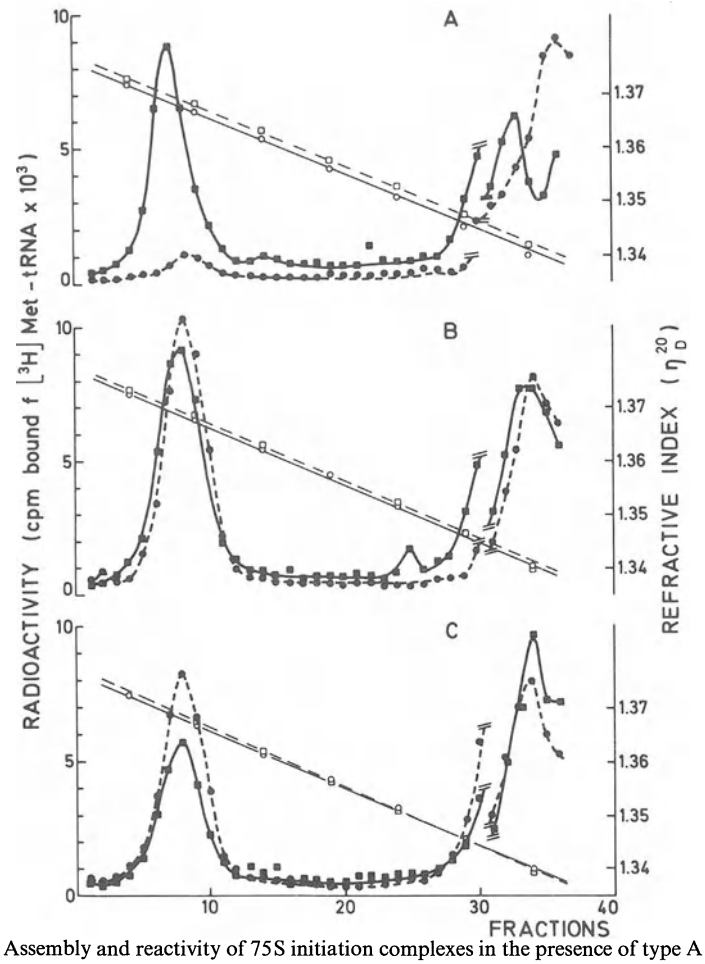

Fig. 16A–C. Assembly and reactivity of 75S initiation complexes in the presence of type A synergimycins. To the initiation complexes assembled as in Fig. 15, 50S ribosomal subunits are added in the presence of either GTP (**A** and **B**) or its nonhydrolyzable analog G-PPCP (**C**): only sample **B** contains virginiamycin M, a type A component. One aliquot of each sample is treated with puromycin (–●–), the other not (–■–): both are fractionated by density gradient centrifugation. Initiation complexes form irrespective of the presence of the inhibitor, but complexes made in the presence of virginiamycin contain unreactive fMet-tRNA. (From Cocito et al., 1974)

1977) (cf. Fig. 12) nor acts on polysomes engaged in protein synthesis (Cundliffe, 1969; Pestka, 1970, 1972, 1974). Consequently, it is proposed that type A synergimycins interact with the acceptor substrate binding center of the peptidyl transferase, which becomes unable to form a stable linkage with the aminoacyl end of tRNA: hence the release of the latter (Chinali et al., 1981).

New impetus to the study of this group of inhibitors was given by the discovery that a transient contact with type A synergimycins produces a lasting damage to ribosomes: this was shown by in vivo and in vitro experiments.

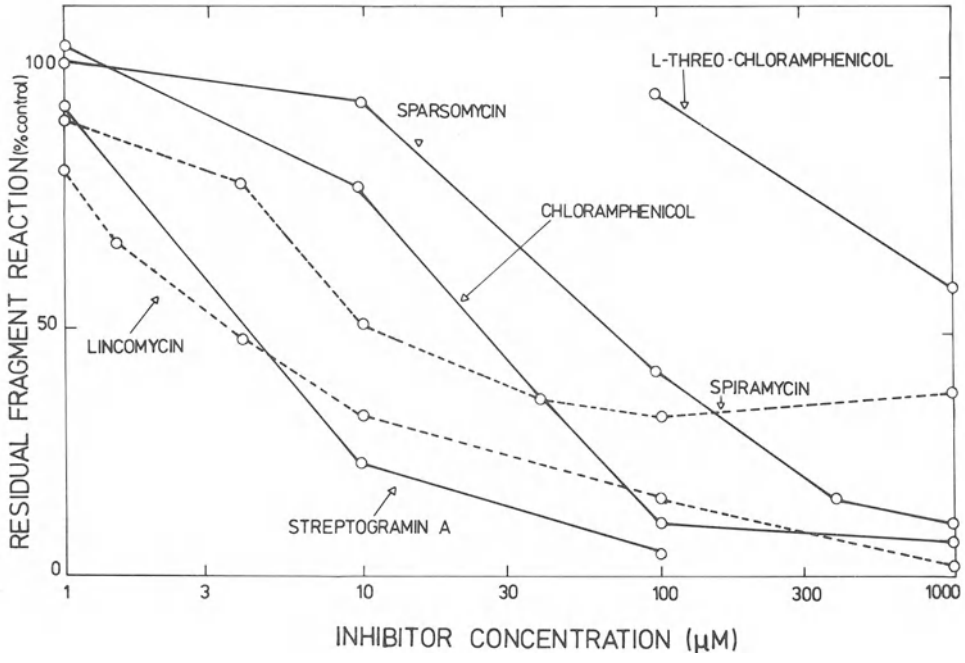

Fig. 17. Inhibition of the "fragment" reaction by type A synergimycins and other 50S-inhibitors. Ribosomes are incubated with the labeled oligonucleotide "fragment" and puromycin, in the presence of different inhibitors including streptogramin A (type A synergimycin). (From MONRO and VÁZQUEZ, 1967)

Table 7. The action of type A synergimycins on the enzymatic and nonenzymatic binding of aminoacyl-tRNA to ribosomes

Experiment (type)	EF-Tu	Virginiamycin M (μg/ml)	Mg^{2+} (mM)	Ribosome-bound AA-tRNA
A	–	–	15	1671
	–	0.5	15	1031
	–	–	5	669
	–	0.5	5	693
B	+	–	7	2.528
	–	–	7	1.823
	+	200	7	0.395
	+	20	7	0.439

A = Formation of elongation complexes of poly(uridylic acid), ribosomes and [^{14}C]phenylalanyl-tRNA in the presence and in the absence of virginiamycin M (dpm/100 μg ribosomes). (From COCITO and KAJI, 1971).

B = Binding of [^{3}H]-alanyl-tRNA to initiation complexes composed of MS2-RNA, 30S and 50S subunits, and fMet-tRNA, in the presence and in the absence of virginiamycin M (pmol bound AAtRNA/sample). (From COCITO et al., 1974)

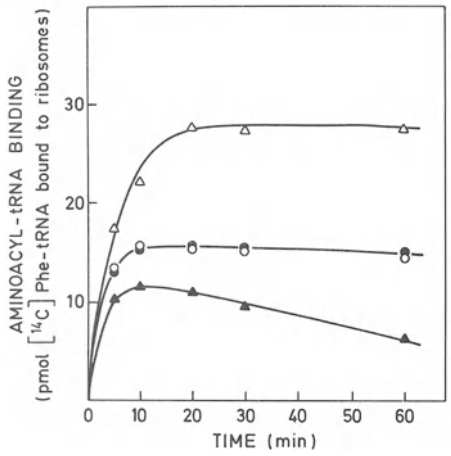

Fig. 18. Kinetics of the enzymatic binding of aminoacyl-tRNA to ribosomes in the presence of a type A synergimycin and kirromycin. The EF-Tu-directed binding of [^{14}C]Phe-tRNA^{Phe} to poly(U)-ribosome complexes carrying deacylated tRNA^{Phe} at the P-site is followed in the absence of inhibitors (control –△–), as well as in the presence of virginiamycin M (–▲–), kirromycin (–○–), and both antibiotics (–●–). Enzymatic binding of aminoacyl-tRNA to ribosomes is strongly inhibited by type A synergimycins only in the absence of kirromycin. (From CHINALI et al., 1981)

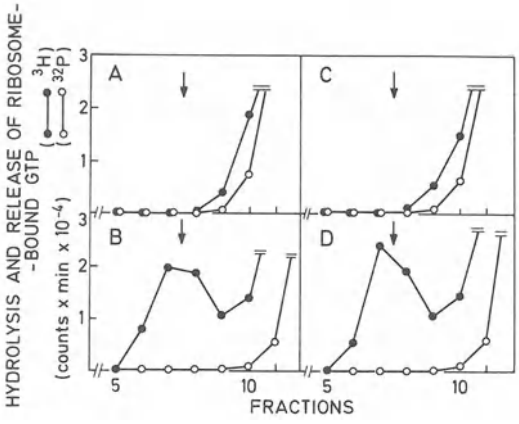

Fig. 19A–D. Hydrolysis and release of GTP from ribosomes, in the presence of a type A synergimycin and kirromycin. Phe-tRNA is allowed to bind to a poly(U)-ribosome complex in the presence of EF-T and GTP which is labeled with [^{3}H] in the base moiety and with [^{32}P] in the γ-phosphate. **A** Control, no antibiotics; **B** + kirromycin; **C** + virginiamycin M; **D** + both antibiotics. GTP hydrolysis (–○– *tracing*) occurs in all cases, but GTP release as EF-Tu.GDP complex (–●– *tracing*) does not take place in the presence of kirromycin, irrespective of whether samples were treated or not with virginiamycin M. (From CHINALI et al., 1981)

Indeed, when either 70S ribosomes of 50S subunits are incubated with type A synergimycins which are subsequently removed before submitting the particles to functional assays, a persisting alteration of ribosomal activity can be demonstrated: such ribosomes with altered 50S subunits (50S*) are unable to catalyze poly(U)-directed poly(phenylalanine) synthesis in cell-free systems (Fig. 22)

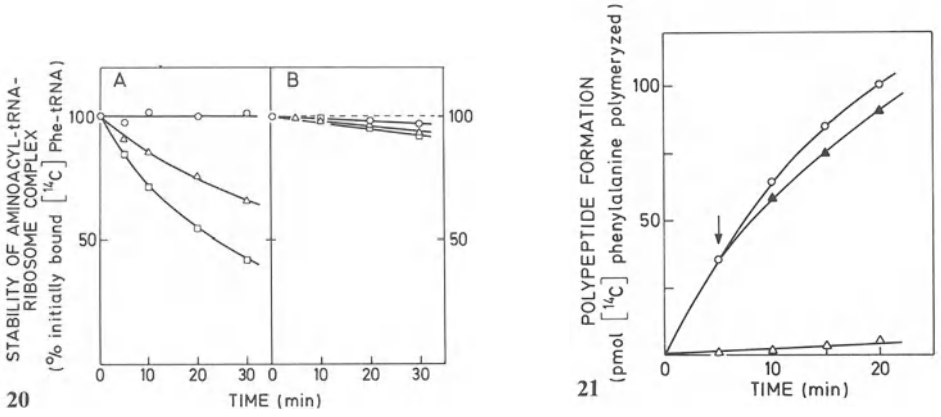

Fig. 20A and B. Stability of enzymatically bound aminoacyl-tRNA at the A-site in the presence of a type A synergimycin and kirromycin. [^{14}C]Phe-tRNA is bound enzymatically to ribosomes in the absence (**A**) or in the presence (**B**) of kirromycin. Some samples are incubated with virginiamycin M (–△– 2 μM, –□– 20 μM) and others are not (controls –○–). The release of aminoacyl-tRNA from the A-site is promoted by virginiamycin M only in the absence of kirromycin. (From CHINALI et al., 1981)

Fig. 21. The action of a type A synergimycin at different steps of the process of peptide chain elongation. Poly(U)-directed poly(phenylalanine) synthesis in a complete cell-free system from *E. coli* is followed in the absence (–○–) or in the presence of virginiamycin M: the latter is added either before (–△–) or after (–▲–) the onset of the polymerisation reaction. The antibiotic prevents protein synthesis if present during translation complex formation, but is ineffective when polymerization is already proceeding. (From CHINALI et al., 1981)

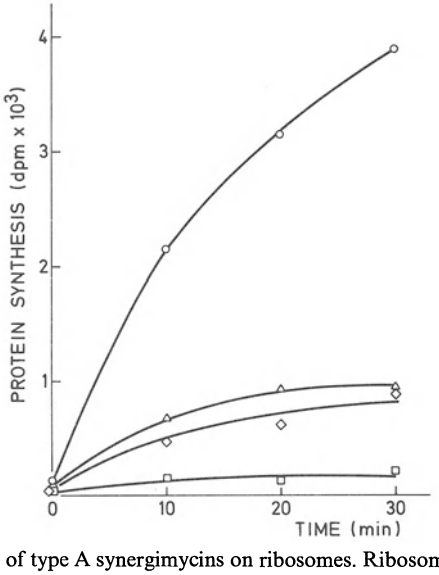

Fig. 22. The lasting action of type A synergimycins on ribosomes. Ribosomes incubated with virginiamycin M and freed from the antibitotic by exclusion chromatography are added to a cell-free system for polypeptide formation. Poly(U)-directed poly(phenylalanine) synthesis catalyzed by the altered particles is followed in the presence (–△–) or in the absence (–◇–) of virginiamycin M. A control of untreated ribosomes (–○–) and a blank (omission of poly(U), –□–) are included. The removal of the antibiotic does not restore the polymerizing ability of ribosomes. (From PARFAIT and COCITO, 1980)

Table 8. The affinity for virginiamycin S of ribosomal subunits preincubated with decreasing concentrations of virginiamycin M

Sample	Preincubation with virginiamycin M (1)		Measurement of bound virginiamycin S (2)			
			− erythromycin		+ erythromycin	
	amount (μM)	removal	$K_a(\mu M^{-1})$	μM	μM	%[a]
1	none	−	2.4	0.56	0.01	1.8
2	0.00105	−	2.3	0.59	0.04	6.8
3	0.0105	−	3	0.65	0.13	20
4	0.105	−	8	0.76	0.39	51
5	1.05	−	13	0.88	0.63	72
6	5.25	−	17	0.92	0.92	100
7	5.25	+	16	0.89	0.83	93
8	5.25	+	18	0.94	0.82	87

[a] % = (bound virginiamycin S after erythromycin/bound virginiamycin S before erythromycin) × 100.

Legend: 50S ribosomal subunits were pre-incubated with different concentrations of virginiamycin M (0.001 to 5 μM) and some of the samples (7 and 8) were submitted to dialysis (to remove bound antibiotic). To all samples virginiamycin S was added, followed by an amount of erythromycin able to displace the former antibiotic: ribosome-bound virginiamycin S was spectrophotometrically measured, and expressed either as real concentration, or as percent of residual complexes, or by the corresponding association constant (K_a). The conclusions drawn are as follows: (1) type A components protect the complex of type B components with ribosome against a competitive displacement by erythromycin; (2) this protection is not sustained by the presence of type A components; (3) the latter substances act catalytically, rather than stoichiometrically. (From Parfait et al., 1981).

(Parfait and Cocito, 1980). Virginiamycin M produces a lasting damage of the 50S subunits by acting catalytically rather than stoichiometrically on ribosomal particles (Table 8). The persistence of type A synergimycin action after their removal could be due to the production of either chemical or conformational changes in the ribosomes. The accumulation of pressure-labile "60S" particles in cells treated with virginiamycin M (Fig. 8) might be an expression of the persistent alteration produced by type A synergimycins on ribosomes.

The Interaction of Type B Synergimycins with Ribosomes

While type A and B components exert comparable inhibitory effects in vivo, the latter type of compounds proved far less active (or inactive) in vitro (Ennis 1965b, 1966; Cocito and Kaji, 1971), although variable degrees of inhibition of polypeptide synthesis in vitro were obtained when polynucleotides were used as messengers (cf. Tanaka, 1975 for review, and Fig. 10). Similar divergence in the puromycin reaction with different donor substrates was reported, for the synthesis of fMet-Ala-puromycin was inhibited by streptogramin B, whereas that of fMet-puromycin was unaffected (cf. Vázquez, 1975). To explain these conflicting results, it was postulated that a critical length of the peptidyl moiety is required for type B synergimycin action: this hypothesis needs additional

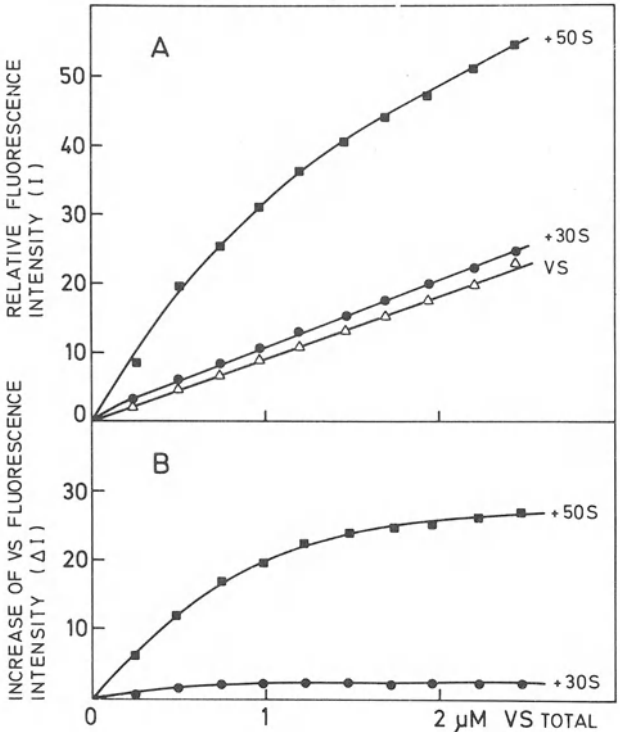

Fig. 23A and B. The binding of a type B synergimycin to ribosomes. 50S (–■–), and 30S (–●–) ribosomal subunits are incubated with virginiamycin S (–△– is a blank of the antibiotic alone): total fluorescence intensity at 416 nm (**A**), and fluorescence variation (ΔI) (**B**) are recorded. The interaction of this antibiotic with ribosomes, interaction that is specific for the large subunit, is measured spectrophotometrically. (From PARFAIT et al., 1978)

experimental confirmation. In conclusion, it would be safe to state that the mechanism of action of type B synergimycins is unknown at the present time, and that the reason for the discrepancy between in vivo and in vitro studies is obscure.

The interaction of type B synergimycins with 50S subunits in vitro is well proven (COCITO, 1971; COCITO and KAJI, 1971; ENNIS, 1974; CONTRERAS and VÁZQUEZ, 1977; DE BÉTHUNE and NIERHAUS, 1978; PARFAIT et al., 1978). With the spectrofluorimetric method it was shown that type B synergimycins have no affinity for the small subunits, but they bind equally well to 50S and to 70S particles (Fig. 23). Unlike type A compounds, type B components bind almost instantaneously to ribosomes, even at low temperature. The two types of components share, however, a lack of affinity for polysomes (Fig. 24) (CONTRERAS and VÁZQUEZ, 1977). Finally, the 1:1 stoichiometry of the antibiotic-ribosome interaction has been assessed, and the corresponding association constant was found to be $K_A = 2.5 \times 10^6\ M^{-1}$ (PARFAIT et al., 1978) (Fig. 13). The latter value essentially agrees with those previously established with several other methods (CONTRERAS and VÁZQUEZ, 1977; and DE BÉTHUNE and NIERHAUS,

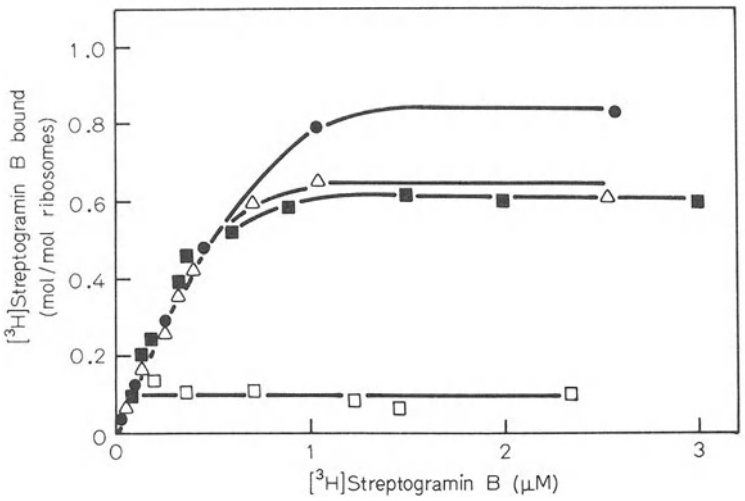

Fig. 24. Binding of type B synergimycins. Binding of [^{3}H]streptogramin B to polysomes with (–□–) and without (–●–) nascent peptides, as well as to run-off (–△–) and salt-washed (–■–) ribosomes is measured by sedimentation. In contrast to ribosomes, natives polysomes do not bind the inhibitor. (From Contreras and Vázquez, 1977)

1978), but not with that recently reported by Le Goffic et al. (1980) ($K_A = 10^8\ M^{-1}$ for dihydropristinamycin I_A).

Competition between type B synergimycins and other antibiotics for binding to ribosomes has been thoroughly explored. Macrolides were found to compete very efficiently (Vázquez, 1967; Ennis, 1974; de Béthune and Nierhaus, 1978; Parfait et al., 1978) (cf. also Fig. 27), whereas chloramphenicol, puromycin and tetracyclines did not (Vázquez, 1966a; de Béthune and Nierhaus, 1978).

The structural requirements for a linkage of type B synergimycins to ribosomes were explored by use of the dissociation–reconstitution technique (Nomura and Held, 1974), whereby ribosomal proteins are gradually stripped off, leaving behind inactive cores: the ability of a protein fraction to restore the missing function of a core can, thus, be assessed. With this method, protein L16 proved able to confer to the 0.8 M LiCl core the capacity to fix virginiamycin S (de Béthune and Nierhaus, 1978). Although this result does not prove that L16 is the binding protein for virginiamycin S, it demonstrates that this protein is stringently required for the attachment of this antibiotic to ribosomes.

The Synergistic Interaction of Type A and Type B Synergimycins In Vitro

The unique feature of the antibiotics of the synergimycin family is the synergistic action of type A and B components in vivo. Unfortunately, no cooperative inhibitory action of type A and B components on a defined biochemical reaction has been so far observed in cell-free systems. After all, such a failure is not

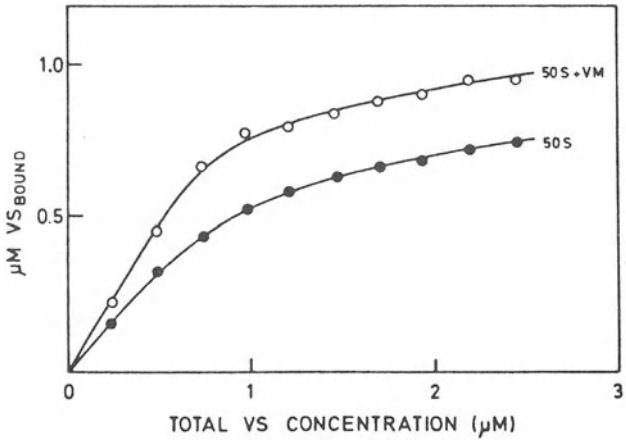

Fig. 25. Type A synergimycins increase the affinity of ribosomes for type B compounds. The binding of increasing concentrations of virginiamycin S to 50S ribosomal subunits in the presence of fixed amount of virginiamycin M is measured spectrophotometrically. (From PARFAIT et al., 1978). The affinity of ribosomes for type B synergimycins undergoes a sixfold increase in the presence of type A components

so surprising, for a synergistic effect between type A and B components can hardly be expected under experimental conditions in which the type B compounds appear inactive when submitted to the usual biochemical tests (in the preceding section, the functional inactivity of type B synergimycins in in vitro systems has been stressed).

Despite the lack of defined in vitro action on protein synthesis, type A components have been found to increase the binding of type B components to isolated 50S and 70S particles. This cooperative effect of the two types of synergimycins has been explored by spectrofluorimetric techniques, allowing a direct evaluation of antibiotic–target interaction (cf. the previous section). Indeed, the affinity of ribosomes for virginiamycin S undergoes a sixfold increase in the presence of virginiamycin M, the corresponding K_A values being $2.5 \times 10^6\ M^{-1}$ in the absence of the latter components, and $15 \times 10^6\ M^{-1}$ in its presence (PARFAIT et al., 1978) (Fig. 25). Likewise, the ethanol-promoted inhibition of binding of vernamycin B to 50S subunits is counteracted by vernamycin A (CONTRERAS and VÁZQUEZ, 1977). The increased stability of the synergimycin B-ribosome complexes toward dilution has been proven as well with experiments of exclusion chromatography (PARFAIT and COCITO, 1980) (cf. also Fig. 26) as by micropore filtration (ENNIS, 1974). A further confirmation of this in vitro synergistic effect between A and B components has been obtained by the binding-competition technique. We have already mentioned the ability of macrolides to prevent the binding of type B synergimycins to ribosomes and to displace the already bound drug from ribosomes. Indeed, the removal of ribosome-bound virginiamycin S by erythromycin is prevented by virginiamycin M (PARFAIT et al., 1981) (cf. also Fig. 27). All these observations point to an increased stability of ribosome-synergimycin B complexes in the presence of type A components.

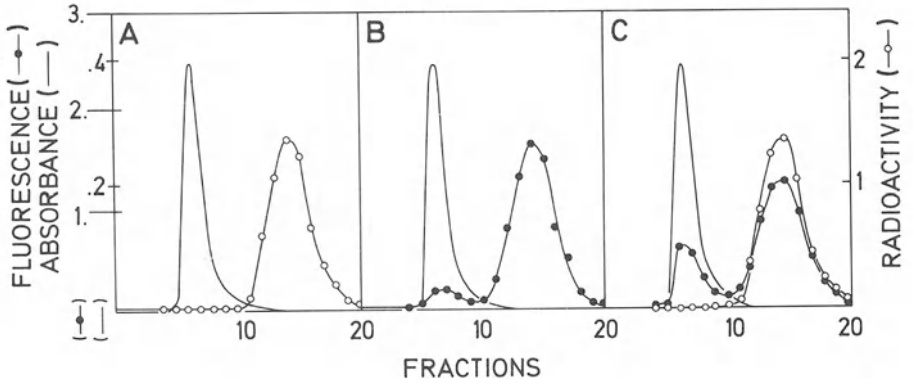

Fig. 26A–C. Increased stability of synergimycin B-ribosome complexes, upon transient incubation with type A components. 50S ribosomal subunits are incubated with either [^{3}H]-labeled virginiamycin M (**A**), or unlabeled virginiamycin S (**B**), or a mixture of both (**C**). Treated particles are submitted to exclusion chromatography and eluates to the following measurements: fluorescence (–●–) (to measure type B component), radioactivity (–○–) (for type A component), and adsorbance (A_{260}, *continuous line*) (to monitor ribosomes). Single virginiamycin components are removed by the dilution effect either completely (**A**) or almost completely (**B**), whereas a considerable amount of type B component is retained by ribosome treated with A + B, and this in spite of the detachment of type A component. (From Parfait and Cocito, 1980)

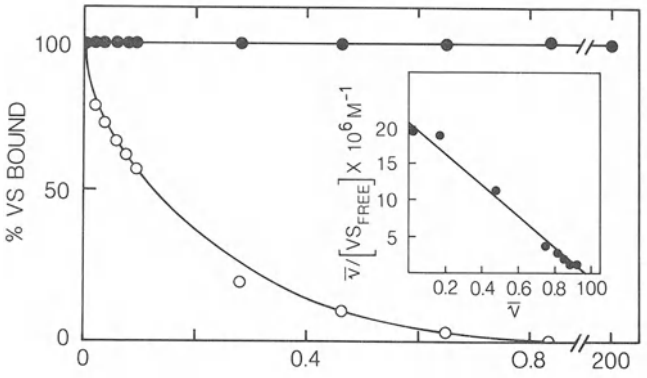

Fig. 27. Protection afforded by type A synergimycins against the competitive displacement of type B compounds by erythromycin. 50S ribosomal subunits are incubated with virginiamycin S in the presence (–●–) and in the absence (–○–) of virginiamycin M. Increasing amounts of erythromycin are added, and ribosome-bound virginiamycin S is measured spectrofluorimetrically. As shown in this figure (the abscisse units are the erythromycin: ribosome ratios), erythromycin completely displaces ribosome-bound virginiamycin S, but is ineffective in the presence of virginiamycin M. (From Parfait, Di Giambattista, and Cocito, 1981)

After the discovery of the lasting inactivation of ribosomes produced by type A synergimycins (cf. the preceding section), the synergistic effect of type A and B compounds in vitro was reinvestigated. It was indeed shown that the affinity for virginiamycin S (type B) of ribosomes transiently incubated with virginiamycin M (type A component) was far higher than the controls

(particles not incubated with type A compounds) (PARFAIT and COCITO, 1980). This conclusion has been warranted by double-labeling experiments, whereby the ribosome-synergimycin B complex has been measured while the complete removal of type A compounds was assessed (Fig. 26). In addition, after fixation and removal of type A synergimycins, altered subunits (50S*) linking type B components in a way not displaceable by erythromycin were obtained (Fig. 27) (PARFAIT and COCITO, 1981).

The Action of Synergimycins on Biological Systems Other than Bacteria

a) Action on Photosynthetic Organisms and Virus-Infected Cells

In the previous sections of this review, evidence has been provided for an interaction of synergimycins with the 50S ribosomal subunit. This type of particle being present as well in cyanophyces as in the cytoplasmic organelles of eukaryotic cells, an inhibitory effect would be expected in all these cases. This is essentially so, albeit there are limitations due to the selective permeability of cellular and organellar membranes (in Euglenoids, e.g., chloroplasts but not mitochondria are inhibited by type A synergimycins, though ribosomes of both organelles are sensitive to these antibiotics).

The pattern of inhibition of synergimycins in *Euglena* is as follows. Type A compounds alone produce a reversible bleaching and inhibition of photoautotrophic growth: this is due to blocking of chlorophyll synthesis and chloroplast multiplication. Type B compounds do not show any appreciable metabolic effects. The association of both components, however, induces a permanent bleaching of algae: leucoplasts doomed to heterotrophic growth are thus obtained with high efficiency (COCITO et al., 1972). These metabolic alterations have been correlated with morphological changes revealed by electron microscopy. In the presence of type A compounds altered chloroplasts capable of recovery are produced, whereas in cells treated with a mixture of the two components chloroplasts disappear and are replaced by reticulated bodies mimicking those present in spontaneous white mutants found in nature (VAN PEL et al., 1973). The primary molecular lesion apparently is at the level of chloroplast ribosomes, which do not multiply in the presence of type A synergimycins owing to the block of both rRNA and rProtein formation (VAN PEL and COCITO, 1973). Reference can be made to the review article of EBRINGER (1972) concerning the bleaching effect of antibiotics in general, and to that of COCITO (1979) concerning the restricted action of synergimycins. The cited studies of synergimycin action on algae can be compared with that on plant chloroplasts. Indeed, the photodependent protein synthesis in isolated plant chloroplasts is inhibited by type A synergimycins. However, type B compounds not only are ineffective with this system, but do not modify the action of their partners (COCITO et al., 1979).

In reality, the work on photosynthetic eukaryotes has pointed to the occurrence of two inhibitory patterns. In bacteria both types of components are active when used separately, whereas in algae only type A compounds display a detectable action per se. An obvious question arising is what pattern applies to cyanophyces, which share the prokaryotic organization of bacteria and the photosynthetic organization of algae. Indeed, cyanophyces show the inhibitory pattern of algae (Cocito and Shilo, 1974), thus implying that type B synergimycins are able to discriminate between the 70S ribosomes of eubacteria and cyanobacteria.

Another very special effect is that produced by type B synergimycins in *Bdellovibrio bacteriovorus,* which multiplies through a complex cycle involving the formation of an elongated body and its fragmentation. It is just the latter step, filament cleavage and vibrios release, that is blocked by the antibiotic (Varon et al., 1976b; Eksztejn and Varon, 1977).

Since viral replication relies on the metabolic machinery of the host cell, the former is expected to be altered by the antibiotics interfering with the latter. Indeed, the inhibitory action of synergimycins on both the multiplication of virulent bacteriophages (Cocito, 1969b, 1974; Cocito and Vanlinden, 1978; Hoet et al., 1981) and the lysogenic cycle of temperate cyanoviruses (Cocito and Goldstein, 1977) has been related (cf. Cocito, 1979 for review). It is sufficient to say that all the effects observed in virus-infected cells can be attributed to inhibition of host protein synthesis, and that synergimycins have proven useful tools for dissecting the replication and lysogenic cycles of viruses.

b) Applications of Synergimycins in Humans and in Animals

Therapeutic applications of synergimycins have been so far restricted for three reasons: (1) poor resorption upon oral administration; (2) low hydrosolubility; and (3) competitive use for stock farming (current regulation proscribes the simultaneous use of a given antibiotic as remedy in man and as growth promoter in animals). Applications in human and veterinary medicine, as well as the use in experimental infections of laboratory animals, have been previously reviewed (Cocito, 1979). It is enough to say that topical applications of these antibiotics (surgery, orthopedics, dentistry and others) and their use in certain microbial diseases (whooping cough) have been quite successful.

On the other hand, synergimycins have proved by far the best antibiotics for stock farming. Negligible toxicity, very low absorption from the intestinal tract, lack of accumulation in animal tissues and biodegradability explain the remarkable success of these products. It can be added that several diseases of the intestinal tract of animals are successfully treated by enteral administration of these antibiotics (cf. Cocito, 1979 for review).

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The Prokaryotes

A Handbook on Habitats, Isolation, and Identification of Bacteria

Editors: **M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, H. G. Schlegel**
1981. XLVIII, 2596 pages. (In two parts, not available separately). ISBN 3-540-08871-7

The Prokaryotes is destined to become the indispensable reference for microbiologists. Prepared under the supervision of a distinguished international editorial board, this comprehensive two volume Handbook will contain 169 contributions by acknowledged experts providing up-to-date, detailed information on the habitats, isolation, and identification of all the prokaryote groups.
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Every researcher working with these organisms will find The Prokaryotes an invaluable and essential source of information.

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Berlin
Heidelberg
New York
Tokyo