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Effects of Macrolides on Peptide-Bond Formation and Translocation*

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ABSTRACT: The formation of *N*-formylmethionylpuromycin and the synthesis of dilysine in the absence of translocation were inhibited by niddamycin, carbomycin, spiramycin, and tylosin, the former two being more potent than the latter two. Erythromycin and oleandomycin had no significant effect on these reactions. In the complete reaction mixture in which oligolysine was synthesized, niddamycin and carbomycin inhibited dilysine synthesis, spiramycin and tylosin caused the accumulation of dilysine but inhibited trilysine synthesis, and erythromycin and oleandomycin caused the accumulation of di-, tri-, and even tetralysine on some occasions, but inhibited the synthesis of longer peptides. Similar results were obtained with polyphenylalanine synthesis. The G- and T-dependent hydrolysis of GTP and enzymic binding of aminoacyl-tRNA were not inhibited by the macrolides. A strict interpretation of these data is that carbomycin and niddamycin inhibited peptide-bond formation, spiramycin and tylosin inhibited peptide-bond formation as well as translocation, and that erythromycin and oleandomycin neither inhibited peptide-bond formation nor translocation but inhibited some other unknown step. This interpretation involving diversified actions of antibiotics in a single family is rather unattractive. Since their structures are similar and they compete for the same binding site on the 50S ribosomal subunit, an alternative hypothesis is proposed. All macrolides inhibit peptide-bond synthesis.

However, the formation of earlier peptide bonds is less sensitive to the macrolides than later peptide bonds, and thus, accumulation of short peptides is caused by less potent macrolides.

The macrolide antibiotics have been shown to inhibit protein synthesis in intact cells and in cell-free systems of bacteria (Brock and Brock, 1959; Taubman *et al.*, 1964; Vazquez, 1966; Mao and Wiegand, 1968; Ahmed, 1968). The specific step affected by the macrolides has not been identified, although the general consensus is that they inhibit some step after the binding of aminoacyl-tRNA to the ribosomes. Two mechanisms of inhibition have been proposed. The first one is based on the inhibition of the formation of poly(Lys)-puromycin (Čërnà *et al.*, 1969), on the addition of a single lysine unit to the ribosomal-bound poly(Lys)-tRNA (Jayaraman and Goldberg, 1968), and on the inhibition of the release of peptides of CpA-Gly[1] (Rychlík *et al.*, 1967). These data implied that the macrolides inhibit the peptide-bond formation step. The second mechanism is based on the lack of inhibition of the puromycin-induced release of nascent peptides in the presence of chlorotetracycline (Cundliffe and McQuillen, 1967), on the inhibition of Ac-triPhe but not Ac-diPhe synthesis (Corcoran and Oleinick, 1969), and on inhibition of tRNA release (Igarashi *et al.*, 1969). These data suggested that erythromycin inhibits translocation. However, D. Schlessinger (1969, personal communication) observed that ribosomes could move on mRNA in erythromycin-treated *Escherichia coli* cells even though peptide synthesis had ceased. A further complication arises by noting that although the macrolides are structurally similar, their action on protein synthesis seems to be different. The formation of F-Met-puromycin from F-Met-hexanucleotide, puromycin, and 50S ribosomes, a stringent test for peptide-bond formation, was inhibited by carbomycin and spiramycin, but not by erythromycin and oleandomycin (Monro and Vazquez, 1967).

It has been proposed that since dipeptide synthesis is a function of peptidyl transferase activity and the synthesis of longer peptides involves translocation, the ability of an antibiotic to inhibit peptide-bond formation or translocation can be determined by quantitative analysis of dipeptide and tripeptide synthesis. Based on this hypothesis, the mechanisms of action of several antibiotics were studied by Pestka (1970b).

In this communication, synthesis of dilysine in the absence of G factor and GTP, or in the presence of fusidic acid, and the F-Met-puromycin reaction were used as tests for the peptide-bond formation step, and synthesis of tri- and longer peptides

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[1] Abbreviations used are: Lys_2, dilysine; Lys_3, trilysine, etc. Lys-tRNA, lysyl-tRNA; poly(Lys)-tRNA, poly(lysyl-tRNA); Phe-tRNA, phenylalanyl-tRNA; Ac-Phe-tRNA, *N*-acetylphenylalanyl-tRNA; Ac-diPhe-tRNA, *N*-acetylphenylalanylphenylalanyl-tRNA; F-Met-tRNA, *N*-formylmethionyl-tRNA; CpA-Gly, cytidylyl-(3′→5′)-2′(3′)-*O*-glycyladenosine; GMPPCP, 5′-guanylylmethylenediphosphonate; 1 optical density unit is the amount of material which in 1.0 ml would yield a value of 1.0 for the optical density measured at 260 mμ in a cuvet with a path length of 1.0 cm.

TABLE I: Effect of Macrolides on Dilysine Synthesis in the Absence of GTP and G factor.[a]

Antibiotics	Concn (M)	Lys_2 cpm	Lys_2 % Control	Lys_3 (cpm)
None		730	100	40
Erythromycin	10^{-3}	788	108	27
	10^{-4}	838	115	33
	10^{-5}	794	109	19
	10^{-6}	771	106	22
Niddamycin	10^{-5}	73	10	5
	10^{-6}	164	22	10
	10^{-7}	547	76	13
	10^{-8}	621	85	22
Spiramycin	10^{-3}	341	47	23
	10^{-4}	374	51	20
	10^{-5}	415	57	14
	10^{-6}	505	69	19

[a] The reaction mixture of 0.2 ml contained washed ribosomes (5.3 optical density units), poly(A) (50 μg), [^{14}C]Lys-tRNA (3.1 optical density units, 2676 cpm/optical density unit), and antibiotics. Incubation was at 35° for 17 min. Lysine peptides were separated by the paper chromatography procedure (Waley and Watson, 1953).

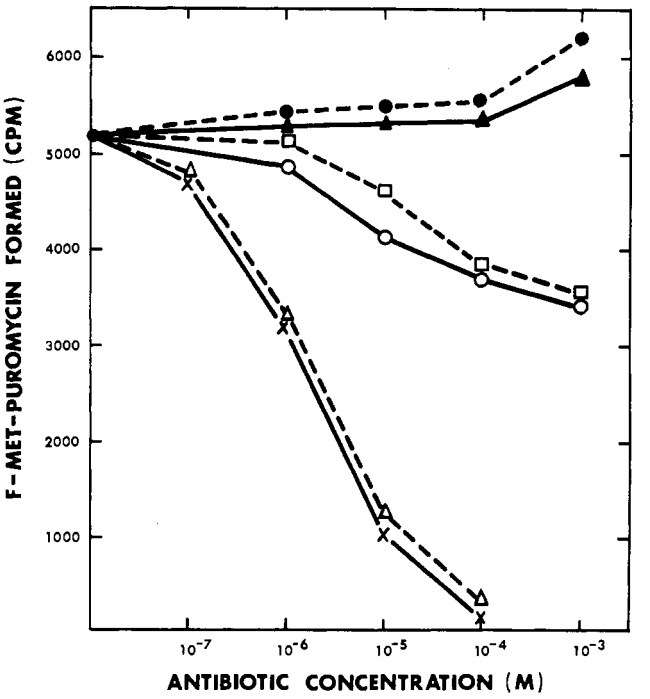

FIGURE 1: The reaction mixture of 0.05 ml contained ribosomes (5.5 optical density units), ApUpG (0.66 optical density unit), F-Met-tRNA (2.6 optical density units, 3823 cpm/optical density unit), GTP (25×10^{-9} mole), and antibiotic, and it was incubated for 5 min at 30°. Then puromycin (5×10^{-8} mole) was added and the incubation was continued for 20 min. F-Met-puromycin was analyzed according to Leder and Bursztyn (1966b): ▲, erythromycin; ●, oleandomycin; □, tylosin; ○, spiramycin; △, carbomycin; ×, niddamycin.

were used as an indicator for the translocation step. Kinetic studies of lysine peptide synthesis resolved much of the puzzling data. Two alternative hypotheses for the mode of action of the macrolides are presented.

Materials and Methods

The procedures for the preparation of cell fractions from *E. coli* Q13 have been described previously (Mao, 1967). Ribosomes were washed three times with 1 M NH_4Cl. F-[^{14}C]-Met-tRNA (3823 cpm/optical density unit, 187 Ci/mole of [^{14}C]methionine), [^{14}C]Lys-tRNA (4031 cpm/optical density unit, 264 Ci/mole of [^{14}C]lysine), and [^{14}C]Phe-tRNA (3800 cpm/optical density unit, 376 Ci/mole of [^{14}C]phenylalanine) were prepared according to Leder and Bursztyn (1966a), ApUpG was prepared by the method of Leder *et al.* (1965), and transfer factors T and G were prepared by the methods of Ertel *et al.* (1968).

Reaction mixture components and conditions are given in the legend of each figure or table. The standard buffer solution contained 0.01 M Tris (pH 7.6), 0.05 M NH_4Cl, 0.016 M $MgCl_2$, and 0.001 M dithiothreitol. In the experiments in which there was separation of lysine peptides, the reactions were stopped by adding KOH to 0.3 N and incubated at 35° for 30 min. The reaction mixtures were then neutralized with HCl and diluted to 3 ml, and unlabeled dilysine and trilysine were added as markers. $HClO_4$ to 0.3 N was added, and the reaction mixtures were allowed to stand for a period of time to precipitate protein and RNA. They were then centrifuged, the supernatant was recovered, adjusted to pH 4–5 with KOH, and further diluted to 12 ml. The chromatographic procedure for the separation of lysine peptides on a cellulose phosphate column was done according to Smith *et al.* (1965) except that it was run at pH 5.0. In some experiments lysine peptides were separated by descending paper chromatography. After the lysine peptides were cleaved from tRNA by KOH, 0.1-ml samples were plotted onto paper strips (2.2 × 58 cm), and eluted with 1-butanol–acetic acid–pyridine–water (36:6:20:24 by volume, Waley and Watson, 1953) for 5 days. Phenylalanine peptides were separated by Sephadex G-15 column chromatography (Bretthauer and Golichowski, 1967). Formation of F-Met-puromycin was measured by the ethyl acetate extraction method (Leder and Bursztyn, 1966b). Radioactivity was measured by the liquid scintillation method with the counting efficiency about 75%.

Results

Effect of the Macrolides on Dilysine Synthesis in the Absence of Translocation. To test the effect of the macrolides on the peptide-bond formation step alone, G factor and GTP were omitted from the reaction mixture. As the results in Table I show, only negligible amounts of Lys_3 were synthesized. Niddamycin strongly inhibited Lys_2 synthesis. The inhibition by spiramycin was rather weak. Erythromycin had no significant effect on Lys_2 synthesis, although it may have been slightly stimulatory. Another way to uncouple peptide-bond formation from translocation was to add fusidic acid to the complete reaction mixture. The results, shown in Table II, are essentially similar to the above experiment, *i.e.*, without the involvement of translocation, niddamycin, carbomycin, spiramycin, and tylosin inhibited the synthesis of Lys_2, but erythromycin had no significant effect.

Effect of the Macrolides on F-Met-puromycin Formation. Synthesis of Lys_2 in the above experiments may be an artificial

TABLE II: Effect of Macrolides on Lysine Peptide Synthesis in the Presence of Fusidic Acid.[a]

Antibiotics	Lys_2		Lys_3 (cpm)	Lys_4 (cpm)	Lys_5 (cpm)	Lys_6 (cpm)
	cpm	% Control				
None	3344	109	2349	1864	1161	561
Fusidic Acid (FA)	3056	100	76	0	0	0
FA + Niddamycin, 10^{-5} M	294	10	0	0	0	0
FA + Carbomycin, 10^{-5}	380	12	0	0	0	0
FA + Erythromycin, 10^{-3}	3354	110	0	0	0	0
FA + Spiramycin, 10^{-3}	1510	49	0	0	0	0
FA + Tylosin, 10^{-3}	1192	39	0	0	0	0

[a] The reaction mixture contained washed ribosomes (5 optical density units), [^{14}C]Lys-tRNA (10 optical density units, 4031 cpm/optical density unit), poly(A) (50 μg), T factor (18 μg), G factor (6.2 μg), GTP (0.5 μmole), and 1 mM fusidic acid was present in samples as indicated in the table. The lysine peptides were separated by the use of cellulose phosphate columns (Smith *et al.*, 1965).

test of peptidyl transferase activity because *in vivo* the action of this enzyme involves the transferring of an N-blocked (F-Met or peptidyl) rather than an N-free aminoacyl moiety as was the case in the previous experiments. However, the results of these experiments were made more valid by employing the F-Met-puromycin reaction. The data are shown in Figure 1, and the results are parallel to those of dipeptide synthesis. Niddamycin, carbomycin, spiramycin, and tylosin inhibited the formation of F-Met-puromycin, the former two being more potent than the latter two. On the other hand, erythromycin and oleandomycin had no significant effect on the formation of F-Met-puromycin. The effect of the above mentioned macrolides on the binding of F-Met-tRNA to the ribosomes was also tested, and no inhibition was observed.

Effect of the Macrolides on Lysine Peptide Synthesis. The data in Table III, experiment 1, in which washed ribosomes and purified G and T factors were used, show that erythromycin caused the accumulation of Lys_2 but inhibited synthesis of $Lys_{>2}$. Tanaka and Teraoka (1968), using reaction mixtures containing unwashed ribosomes and S-100 fraction, reported that erythromycin caused accumulation of not only Lys_2 but also Lys_3 and Lys_4. We ran a similar experiment and, indeed, Lys_3 and Lys_4 were also accumulated (Table III, experiment 2). The amount of accumulation did not respond in a fashion linear to the concentration of erythromycin. Niddamycin and carbomycin consistently inhibited synthesis of Lys_2 as well as $Lys_{>2}$ peptides (Table III, experiments 3 and 5). The effect of spiramycin and tylosin on Lys_2 synthesis was variable (Table III, experiments 4–6). In 13 experiments with spiramycin or tylosin accumulation of Lys_2 was observed in 8 experiments and inhibition in 5 experiments, but inhibition of $Lys_{\geq 3}$ synthesis was consistently noted.

Reaction Conditions and Action of Spiramycin. Due to the varying effects of spiramycin and tylosin on Lys_2 synthesis, several reaction conditions were tested in hopes of finding a specific condition that would consistently cause either inhibition or accumulation of Lys_2. It seemed that NH_4^+ concentration was the most important factor on the action of spiramycin. At low concentration of NH_4^+, spiramycin inhibited Lys_2 synthesis, and at a high concentration of NH_4^+, it caused accumulation of Lys_2 (Table IV, A). Other factors, such as the concentration of Mg^{2+}, age of ribosomes, and pH of the reaction mixtures, changed the degree of accumulation (Table IV, B–D).

Kinetics of Inhibition of Lysine Peptide Synthesis. Results of lysine peptide synthesis from the above studies, in which only the final products were analyzed, indicated that carbomycin and niddamycin are peptide-bond formation inhibitors since they always inhibited dipeptide synthesis whether the reactions involved translocation or not. The action of spiramycin, tylosin, erythromycin, and oleandomycin is not as clear. The kinetic data, shown in Figure 2, cleared up much of the confusion. At the early stage of the reaction, spiramycin inhibited Lys_2 synthesis slightly, but caused accumulation of Lys_2 at the later stage (Figure 2A). Synthesis of tri- and longer oligolysine was inhibited by spiramycin (Figure 2B–D). This phenomenon can be explained by postulating that if a reaction after the first peptide-bond formation was more strongly inhibited than the first peptide-bond formation, then the intermediate synthesized prior to that step would be accumulated. Erythromycin initially caused an accumulation of Lys_2 which became greater at the later stage (Figure 2A). This also indicated that a reaction after the first peptide-bond formation was inhibited by erythromycin. Erythromycin also caused an accumulation of Lys_3 in this experiment but to a lesser extent than that of Lys_2 (Figure 2B). Formation of tetra- and longer oligolysine was inhibited by erythromycin (Figure 2C,D). The action of niddamycin was quite clear. It strongly inhibited Lys_2 synthesis immediately, and there was no accumulation at the later stage (Figure 2A).

Effect of the Macrolides on Phenylalanine Peptide Synthesis. Several laboratories have reported that poly(A)-directed synthesis of poly(lysine) is more sensitive to the macrolides than poly(U)-directed synthesis of poly(phenylalanine) (Vazquez, 1966; Wilhelm and Corcoran, 1967; Mao and Wiegand, 1968). The question as to whether various actions of the macrolides on oligolysine synthesis would hold true for other types of oligopeptide synthesis was resolved by analyzing phenylalanine peptides in the poly(U)-directed system. Results shown in Table V are similar to those concerning oligolysine in that erythromycin caused accumulation of Phe_2, Phe_3, and Phe_4. Spiramycin caused accumulation of Phe_2 but inhibited Phe_3 synthesis, and niddamycin inhibited both Phe_2 and $Phe_{>2}$ synthesis. Peptides longer than Phe_4 were not analyzed because of difficulty in eluting the longer peptides.

Effect of the Macrolides on Other Reactions in Protein Biosynthesis. It has been shown that nonenzymic binding of aminoacyl-tRNA to ribosomes is insensitive to macrolides

TABLE III: Effect of Macrolides on Lysine Peptide Synthesis.[a]

			Lys_2		Lys_3		Lys_4		Lys_5		Lys_6		Lys_7	
Expt	Macrolides	Concn (M)	cpm	% Control	cpm	% Control	cpm	% Control	cpm	% Control	cpm	% Control	cpm	% Control
1	None		3966	100	2528	100	2034	100	1039	100	891	100	662	100
	Erythromycin	10^{-3}	5790	146	935	37	0	0	0	0	0	0	0	0
		10^{-4}	6923	175	1128	45	175	9	0	0	0	0	0	0
		10^{-5}	7333	185	1488	59	475	23	0	0	0	0	0	0
		10^{-6}	6904	174	1694	67	710	35	203	19	0	0	0	0
		10^{-7}	4005	101	2150	85	1813	89	995	96	670	75	436	75
2	None		6180	100	2675	100	794	100	735	100	426	100		
	Erythromycin	10^{-4}	8185	132	4454	167	904	113	0	0	0	0		
		10^{-5}	8344	135	4399	164	812	102	62	8	0	0		
		10^{-6}	6257	101	2786	104	710	89	91	12	0	0		
3	None		3466	100	1525	100	757	100	421	100	340	100		
	Niddamycin	10^{-5}	323	9	19	1	0	0	0	0	0	0		
		10^{-6}	570	16	70	5	0	0	0	0	0	0		
		10^{-7}	2565	74	1040	68	345	45	180	43	0	0		
4	None		3924	100	2234	100	2264	100	1907	100	1217	100	690	100
	Spiramycin	10^{-4}	3901	99	88	4	5	0	0	0	0	0	0	0
		10^{-5}	3569	91	102	5	10	0	0	0	0	0	0	0
		10^{-6}	4070	104	451	20	245	11	162	9	87	7	0	0
		10^{-7}	3533	90	1800	81	1661	74	1044	58	718	59	436	60
5	None		4568	100	1247	100	256	100	60	100				
	Spiramycin	10^{-3}	921	20	0	0	0	0	0	0				
	Tylosin	10^{-3}	1295	28	0	0	0	0	0	0				
	Carbomycin	10^{-5}	324	7	0	0	0	0	0	0				
6	None		10308	100	5599	100	1566	100	1151	100	764	100		
	Spiramycin	10^{-3}	15481	150	917	16	62	0	0	0	0	0		
	Tylosin	10^{-3}	12854	125	1437	27	203	0	0	0	0	0		

[a] The reaction mixture, except for experiment 2, contained washed ribosomes (5 optical density units), [^{14}C]Lys-tRNA (10 optical density units, 4031 cpm/optical density unit), poly(A) (50 μg), T factor (18 μg), G factor (6.2 μg), GTP (0.5 μmole), and antibiotics or buffer in a total volume of 0.5 ml. The reaction mixture of expt 2 contained unwashed ribosomes (6 optical density units), [^{14}C]Lys (0.25 μCi, 271 Ci/mole), poly(A) (50 μg), S-100 fraction (800 μg), 1 mM ATP, 0.05 mM GTP, and 5 mM phosphoenolpyruvate in a total volume of 0.5 ml. Incubation was at 35° for 20 min. The analysis of lysine peptides was done by cellulose phosphate column (Smith *et al.*, 1965).

TABLE IV: Effect of Reaction Conditions on the Action of Spiramycin.[a]

	Lys_2			Lys_3			$>Lys_3$		
	Control	Spiramycin	% Control	Control	Spiramycin	% Control	Control	Spiramycin	% Control
(A) NH_4^+ concentration (mM)									
32	674	645	(95)	913	156	(17)	1137	93	(8)
40	702	522	(74)	915	160	(17)	1432	42	(3)
58	776	896	(115)	1071	120	(11)	1610	58	(4)
95	645	842	(130)	1848	37	(2)	2054	65	(3)
(B) Mg^{2+} concentration (mM)									
8	1295	1685	(130)	1001	203	(20)	1522	100	(7)
16	1058	1591	(150)	848	207	(24)	1763	81	(5)
20	1071	1733	(161)	511	165	(32)	1325	197	(15)
(C) Age of ribosomes									
Unwashed ribosomes	317	680	(215)	415	129	(31)	1458	236	(16)
Fresh-washed ribosomes	1009	1182	(117)	438	201	(46)	866	160	(18)
Aged-washed ribosomes	253	511	(202)	120	60	(50)	733	118	(16)
(D) pH of the reaction mixture									
6.4	983	1476	(150)	373	85	(23)	422	119	(28)
7.0	1039	1606	(115)	572	110	(19)	1134	161	(14)
7.8	936	1846	(197)	372	171	(46)	1611	178	(11)

[a] The reaction mixtures were essentially the same as those in the legend of Table II. The components changed are indicated in this table. The concentration of spiramycin was 1 mM. The lysine peptides were separated by paper chromatography (Waley and Watson, 1953).

TABLE V: Effect of Macrolides on Phenylalanine Peptide Synthesis.[a]

	Phe_2		Phe_3		Phe_4		Total Phe peptides	
Antibiotics (M)	cpm	% Control	cpm	% Control	cpm	% Control	cpm	% Control
None	2420	100	2932	100	2361	100	4825	100
Erythromycin (10^{-3})	2934	121	2997	102	2967	126	3764	78
Niddamycin (10^{-5})	941	39	453	15	0	0	434	9
Spiramycin (10^{-3})	6625	274	467	16	0	0	532	11

[a] The reaction mixtures of 0.5 ml contained washed ribosomes (5.1 optical density units), poly(U) (50 μg), [^{14}C]Phe-tRNA (21 optical density units, 3800 cpm/optical density unit), T factor (93 μg), G factor (24.8 μg), GTP (1 mM), and antibiotics or buffer. Incubation was at 37° for 10 min. The phenylalanine peptides in 0.4 ml of the sample were separated by a Sephadex G-25 column (Bretthauer and Golichowski, 1967). The total peptides in 0.1 ml of the sample were precipitated by hot trichloroacetic acid. The column size was 1 × 50 cm, and 90 fractions, each containing 1.8 ml, were collected. At this point, peptides longer than Phe_4 were retained on the column.

(Tanaka *et al.*, 1966; Cĕrnà *et al.*, 1969). Hill (1969) studied enzymic binding of Phe-tRNA to ribosomes in the presence of GTP and reported that spiramycin inhibited 75% of the enzymic binding of Phe-tRNA, but only weakly inhibited nonenzymic binding. In our experiments GTP was replaced by GMPPCP which is effective for the enzymic binding of Phe-tRNA but cannot promote dipeptide synthesis (Skoultchi *et al.*, 1970). Results are shown in Table VI. T factor promoted the binding of Phe-tRNA to the ribosomes which led to a 2.5-fold increase over the nonenzymic binding. The total Phe-tRNA binding, enzymic and nonenzymic, was not significantly altered by erythromycin, niddamycin, or spiramycin.

Tanaka *et al.* (1969) have shown that erythromycin did not inhibit G-dependent GTP hydrolysis. We confirmed this observation, and further showed that other macrolides had no significant effect on this step (Table VII). The T-dependent GTP hydrolysis which was tested in the presence of 1 mM fusidic acid to inhibit residual G factor, and the formation of GTP-T-Lys-tRNA complexes also were not inhibited by the macrolides (Table VII).

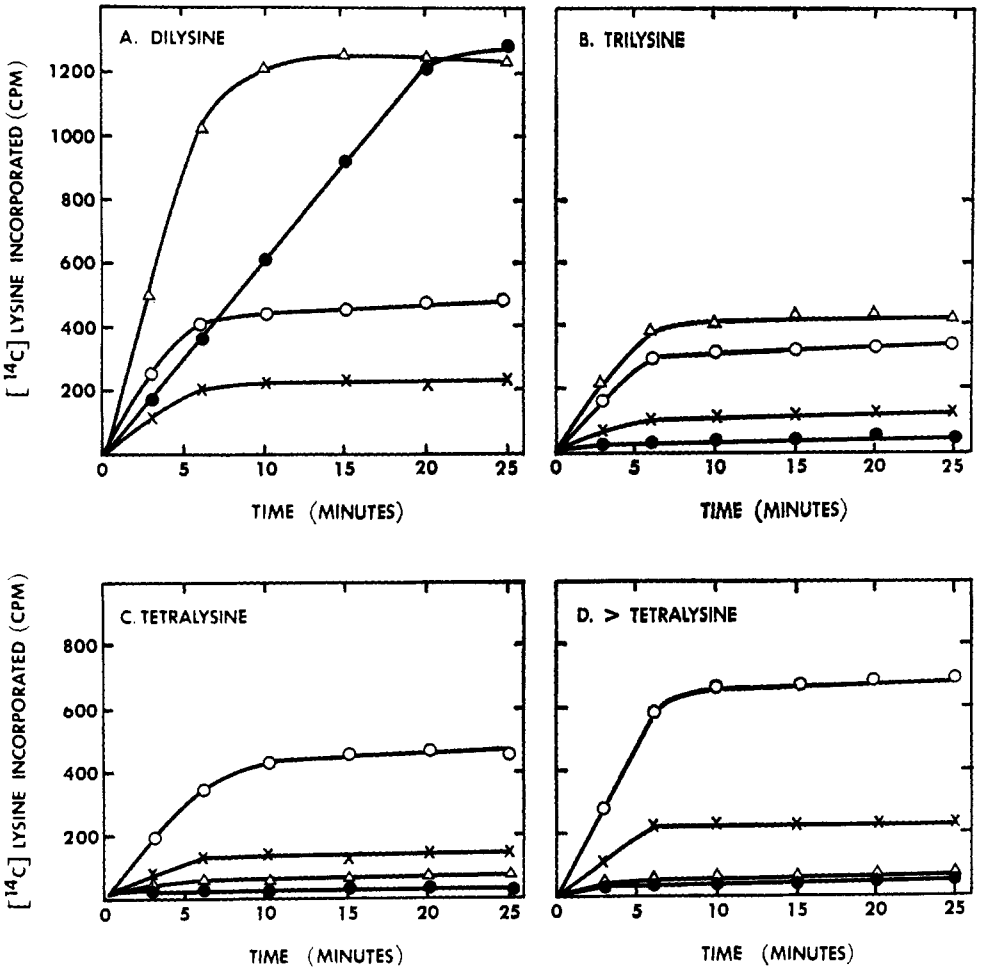

FIGURE 2: Kinetics of lysine peptide synthesis in the presence or absence of macrolides. The total reaction volume was 1.5 ml containing washed ribosomes (16 optical density units), poly(A) (250 μg), S-100 fraction (1.6 mg), [^{14}C]Lys-tRNA (25 optical density units, 5954 cpm/optical density unit), GTP (1.5 μmoles), and macrolides or buffer. Samples of 200 μl were removed at timed intervals and the reaction was stopped by adding 40 μl of 1.8 N KOH. Lysine peptides were separated by paper chromatography as described in the text. A, dilysine; B, trilysine; C, tetralysine; and D, lysine peptides longer than tetralysine. △—△, 1×10^{-3} M erythromycin; ●—●, 1×10^{-4} M spiramycin; ×—×, 1×10^{-6} M niddamycin; and ○—○, control.

Discussion

The action of six macrolide antibiotics on protein biosynthesis has been examined in a stepwise fashion. The results showed that they have no significant effect on the enzymic or nonenzymic binding of aminoacyl-tRNA (Table VI) nor on the G- or T-dependent GTP hydrolysis (Table VII). They appear to act on a step after the formation of the ternary complex and cause a variety of responses. Accordingly, they may be classified into three groups.

The first group of macrolides, carbomycin and niddamycin, strongly inhibited the first peptide-bond formation as shown in three limited reactions, namely (1) Lys_2 synthesis in the absence of G factor and GTP (Table I), (2) Lys_2 synthesis in the presence of fusidic acid (Table II), and (3) F-Met-puromycin formation (Figure 1). The results clearly suggested that the action of carbomycin and niddamycin was on the peptide-bond formation since they inhibited the first peptide-bond formation to the extent of 90% at 10^{-5} M. The 50% inhibition concentration, estimated from Table I, is between 10^{-6} and 10^{-7} M which is also the 50% inhibition concentration against whole cells (Mao and Wiegand, 1968).

In the complete reaction mixtures in which homopeptides of lysine or phenylalanine of various lengths were synthesized, carbomycin and niddamycin inhibited dipeptide synthesis, and the inhibition became progressively stronger for the longer peptides synthesized (Tables III and V). There was no accumulation of short peptides on any occasion. The kinetic experiment (Figure 2) showed that niddamycin inhibited Lys_2 and other oligolysine synthesis immediately, and that there was no accumulation of Lys_2 at a later stage of the reaction. The lack of accumulation of Lys_2 suggested that niddamycin had little or no effect on a reaction beyond the peptide-bond formation step.

Spiramycin and tylosin, members of the second group of macrolides, were weaker inhibitors of the first peptide-bond formation than the first group (Tables I, II, and Figure 1), the inhibition reaching only 50–60% at 10^{-3} M in our experiments (Table I). Results from other laboratories (Cĕrnà *et al.*, 1969; Pestka, 1970a,b) showed that spiramycin and tylosin were much more potent inhibitors of the first peptide-bond formation. This was probably due to the difference in the reaction conditions. Analysis of individual peptides in the complete reaction mixture showed that spiramycin and tylosin could cause either accumulation or inhibition of dipeptide synthesis, but tripeptide synthesis was consistently inhibited. The different reaction conditions such as NH_4^+, Mg^{2+} concentrations, pH of the reaction mixture, and the age of the

TABLE VI: Effect of Macrolides on the Enzymic Binding of Phenylalanyl-tRNA.[a]

Antibiotics (M)	[^{14}C]Phe-tRNA Bound to Ribosomes (cpm)
None	6865
Erythromycin (10^{-3})	6801
Niddamycin (10^{-5})	6801
Spiramycin (10^{-3})	6678
−T factor	2703
−T factor and poly(U)	133

[a] The reaction mixture for the binding of [^{14}C]Phe-tRNA to ribosomes contained washed ribosomes (5.5 optical density units), poly(U) (50 μg), [^{14}C]Phe-tRNA (9 optical density units, 3894 cpm/optical density unit), T factor (10 μg) GMPPCP (9×10^{-9} mole) in a total volume of 0.46 ml. It was incubated at 37° for 15 min. Binding of [^{14}C]Phe-tRNA to the ribosomes was determined by the membrane filtration method (Nirenberg and Leder, 1964).

TABLE VII: Effect of Macrolides on Other Reactions in Protein Synthesis.

Antibiotics (M)	G-Dependent GTP Hydrolysis[a] (cpm)	T-Dependent GTP Hydrolysis[b] (cpm)	Formation of GTP-T-Lys-tRNA[c] (pmole)
None	44,665	10,860	39.4
Erythromycin (10^{-3})	42,807	11,240	39.1
Niddamycin (10^{-5})	46,142	10,012	38.3
Carbomycin (10^{-5})	45,320		
Spiramycin (10^{-3})	41,438	10,184	
Tylosin (10^{-3})	43,580		
−G factor	346		
−T factor		3,122	

[a] The reaction mixture for the G factor assay contained washed ribosomes (7 optical density units), G factor (31 μg), and [γ-^{32}P]GTP (0.24 μmole, specific activity 4.56 Ci/mole) in a total volume of 0.3 ml. It was incubated at 30° for 10 min. The [^{32}P]P_i was analyzed by the procedure of Conway and Lipmann (1964). [b] The reaction mixture for T-dependent GTP hydrolysis contained washed ribosomes (3.3 optical density units), T factor (25 μg), poly(U) (5 μg), [^{12}C]Phe-tRNA (19.5 μg), [γ-^{32}P]GTP (4×10^{-8} mole, sp act. 8.9 Ci/mmole), and fusidic acid (0.25 μmole) in a volume of 0.25 ml. It was incubated at 30° for 10 min. The [^{32}P]P_i was analyzed by the procedure of Conway and Lipmann (1964). [c] The reaction mixture for the formation of [^{3}H]GTP-T-[^{14}C]Lys-tRNA contained T factor (200 μg), [^{14}C]Lys-tRNA (26 optical density units, 4031 cpm/optical density unit), and [^{3}H]GTP (10^{-9} mole, specific activity 1.09 Ci/mmole) in a total volume of 1.0 ml. It was incubated for 30 min at 0°. The ternary complex was isolated by Sephadex G-25 column (Ravel *et al.*, 1967).

ribosomes may have had some influence on the results (Table IV). It is known that these factors can change the conformation of the ribosomes, and the action of spiramycin may be accentuated or diminished by ribosomes in different conformations. It was puzzling that although spiramycin and tylosin inhibited Lys_2 synthesis under limited assay conditions allowing only a single peptide-bond formation, yet they could cause accumulation of Lys_2 in the complete reaction mixture. This puzzle was resolved by a kinetic study (Figure 2). Spiramycin at the early stage of the reaction indeed inhibited Lys_2 synthesis; however, this initial inhibition was masked at the later stage of the reaction. This indicated that a step after the first peptide-bond formation was inhibited much more strongly than the first peptide-bond formation which, therefore, resulted in the accumulation of dipeptide.

The question as to which step after synthesis of the first peptide bond was inhibited by spiramycin and tylosin had to be answered. There are three possible steps to be considered. One possibility is inhibition of the binding of a third aminoacyl-tRNA to the ribosomes. Inhibition at this step is unlikely, since both enzymic and nonenzymic binding of aminoacyl-tRNA (Table VI) and the binding of peptidyl-tRNA (Cĕrnà *et al.*, 1969) to the ribosomes were not inhibited by macrolides. A second possibility is inhibition of translocation. The lack of inhibition of G-dependent GTP hydrolysis (Table VII) weakened this hypothesis. However, translocation is a rather complicated step, and the possibility of uncoupling GTP hydrolysis and the movement of peptidyl-tRNA by spiramycin and tylosin cannot be excluded at the present time. A third possibility is inhibition of peptide-bond formation. It is possible that formation of successive peptide bonds is different with respect to antibiotic sensitivity. Therefore, regarding the data above, the inhibition of the second peptide-bond formation was stronger than that of the first peptide-bond formation, thus resulting in the accumulation of dipeptide.

The third group of macrolides, erythromycin and oleandomycin, had no significant effect on the first peptide-bond formation as tested in three different assays as outlined previously. In the complete reaction mixture for oligopeptide synthesis, erythromycin caused the accumulation of dipeptide; but its effect on Lys_3 and Lys_4 synthesis was variable. When washed ribosomes and purified G and T factors were used, erythromycin inhibited Lys_3 synthesis at a concentration as low as 10^{-7} M (Table III, experiment 1). When unwashed ribosomes and the S-100 fraction were used, inhibition did not definitely occur until Lys_5. We did not know what factor(s) caused this difference. Probably the difference in the physical conditions of washed and unwashed ribosomes and the different amount of enzymes in the S-100 fraction and purified G and T factors are related to the variable results. The above results do not give conclusive evidence for stating whether erythromycin and oleandomycin inhibit peptide-bond formation or translocation.

The diversified responses may indeed indicate that macrolides have different modes of action, *i.e.*, carbomycin and niddamycin inhibit peptide-bond formation, spiramycin and tylosin act on both peptide-bond formation and translocation, and erythromycin and oleandomycin act in an unknown manner.

An alternative explanation is that all six macrolides act on the peptide-bond formation but the first peptide-bond formation is less sensitive than the second which in turn is less sensitive than the third. This hypothesis can explain why spiramy-

cin and tylosin inhibited dipeptide synthesis in the limited reactions and yet caused the accumulation of dipeptide in the complete reaction mixture. It also can explain the accumulation of short peptides and the decrease of longer peptides by erythromycin and oleandomycin. The virtue of this hypothesis is that it not only gives a common mechanism to antibiotics which compete for the 50S ribosomal binding site (Vazquez, 1967; Wilhelm *et al.*, 1967) it also accommodates seemingly contradictory data reported from various laboratories. For instance, the puromycin reaction with ac-Phe-tRNA and F-Met-tRNA, which test the synthesis of the first peptide bond, were not inhibited by erythromycin (Chang, 1968; Cĕrnà *et al.*, 1969), but the addition of one lysine unit to polyLys-tRNA (Jayaraman and Goldberg, 1968) and formation of poly(Lys)-puromycin (Cĕrnà *et al.*, 1969) which test peptide-bond synthesis beyond the first bond, were inhibited by erythromycin.

Furthermore, this hypothesis also can explain the unsettled problem of the mode of action of chloramphenicol, another 50S inhibitor (Vazquez and Monro, 1967). Results from many laboratories showed that chloramphenicol acted at the peptide-bond formation step (Traut and Monro, 1964; Goldberg and Mitsugi, 1967; Rychlík *et al.*, 1967; Pestka, 1070a,b), yet caused the accumulation of di- and trilysine (Julian, 1965). The accumulation of di- and trilysine seemed contradictory to the inhibition of the peptide-bond formation. However, if the formation of the second peptide bond was more sensitive to chloramphenicol and the formation of the third peptide bond was even more sensitive than the second peptide bond, the accumulation of di- and tripeptides could be the result.

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