to 60°C or higher. 2. Filtration of erythromycin through bacterial filters entailed the loss of some activity. 3. The antibacterial action of erythromycin increased progressively with increasing alkalinity of the culture medium, within the pH range of bacterial growth. 4. Many substances which may affect the action of other antimicrobial agents had no important effect on the action of erythromycin. 5. Erythromycin inhibiting substances could not be demonstrated in cultures of erythromycin-resistant bacteria. 6. The size of the inoculum affected the results of sensitivity tests with erythromycin, but there was considerable tolerance within the range of inocula used in clinical testing. 7. The broth-dilution and agar-plate dilution methods generally gave comparable results in tests for sensitivity of bacteria to erythromycin but the values obtained by the agar method often were higher, particularly with some coliform organisms. 8. Tests done on more than 1000 bacterial strains, most of them recently isolated from patients, indicated that erythromycin was most active against the Gram-positive cocci and was quite active against strains of *Neisseria,* diphtheria bacilli and *Hemophilus,* but for practical purposes it could be considered inactive against most coliform and enteric bacilli. 9. Concentrations of erythromycin in plasma after single oral doses varied widely but in general were proportional to the dose. Maximum concentrations were found 1 or 2 hours after a dose, and no activity was demonstrated at 6 hours except following doses of 1.0 g. Significant concentrations were maintained in the plasma with oral doses of 250 mg or more every 3 or 4 hours. 10. The amounts of erythromycin activity recovered in the urine appeared to be small and erratic after ingestion of single doses or after repeated small doses, particularly early in the course of therapy; however, on continuous therapy with divided doses, up to 15% of the amount ingested daily could be demonstrated in active form in the urine. Results of studies on the mode of action of erythromycin and on the resistance of bacteria to that agent are presented in the succeeding papers.

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Resistance of Bacteria to Erythromycin.* (19816)

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In the course of studies on some of the antibiotic and other important biologic properties of erythromycin, a number of which were presented in a previous paper(1), observations were also made on the occurrence or development of resistance of bacteria to erythromycin. These included a search for resistant variants in cultures of "sensitive" strains, with and without exposures to the antibiotic. The development of resistance in bacteria during treatment of patients was also observed. Some of the details of these observations are presented here. The methods and materials

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were, for the most part, similar to those used in the preceding paper(1).

Search for Highly Resistant Variants in "Sensitive" Strains. Subcultures were made in broth from isolated colonies of the following organisms: *Streptococcus* C203, *Streptococcus* 98, a type 3 and a type 7 pneumococcus, *Staphylococcus* 193 and *Staphylococcus* 195. In the usual 2-fold broth-dilution tests the strains of streptococci and pneumococci were completely inhibited by 0.02 μg of erythromycin per ml and the staphylococcal strains by 0.2 μg per ml. The centrifugated sediment from 10 ml of each of these fully grown cultures was resuspended in a minimum volume of the supernatant fluid; a 2 mm loopful of each suspension was then streaked on the surface of a series of blood-agar plates, one of which contained no antibiotic while the others contained 2-fold dilutions of erythromycin from 800 to 100 μg per ml, inclusive. Each of these cultures grew luxuriantly on the antibiotic-free agar but no growth was discernible on any of the erythromycin-containing plates.

Variations in Resistance After Single Subcultures in Erythromycin. Two recently isolated strains of *Staphylococcus aureus* (No. 192 and 197) were used for this study; they were each completely inhibited by 0.4 μg of erythromycin per ml in the usual plate-dilution test. Isolated single colonies were picked from pour-plates of these cultures and they were subcultured in broth. When fully grown each was used for 2 further subcultures, one in the antibiotic-free broth and the other in broth containing 16 μg of erythromycin per ml, employing a 10% inoculum. After incubation for 48 hours, pour plates were made from these 4 cultures and 8 individual colonies were picked from each. These colonies, in turn, were subcultured in fresh broth and tested for sensitivity to erythromycin by the plate-dilution method.

The results are shown in Table I. The sensitivity of the cultures derived from the antibiotic-free media were all the same, within the limits of error of the method. The colonies derived from the growth in broth containing 16 μg of erythromycin per ml varied in their sensitivity over a range of concentrations from that which inhibited the parent strains to essentially that to which they had been exposed.

TABLE I. Sensitivity of Cultures of Single Colonies from Antibiotic-Free Broth and from Broth Containing Erythromycin.

Colony	Min complete inhibiting conc., μg/ml — From antibiotic-free broth — Staph. 192	Staph. 197
1	.4	.4
2		
3		
4		
5	.2	.4
6	.4	.8
7		
8		
	From broth containing erythromycin, 16 μg. ml	
1	.4	25
2	6.3	12.5
3	.4	.4
4	6.3	.4
5	12.5	12.5
6	12.5	.4
7	.2	12.5
8	6.3	12.5

Test done by plate-dilution method.

Effect of Repeated Subcultures in Graded Concentrations of Erythromycin. An attempt was made to develop strains with increased resistance to erythromycin by a method similar to that previously employed in this laboratory with other antibiotics(2, 3). Eight strains of "sensitive" bacteria, recently isolated from patients, were subcultured serially at 48-hour intervals on the surface of blood-agar plates containing 2-fold concentrations of erythromycin up to and including 100 μg per ml. The plates were freshly prepared at the time of each transfer and the inoculum for each subculture was a 2 mm loopful of a broth suspension of the growth from the surface of the plate containing the highest concentration of the drug on which the strain had previously shown good growth. The sensitivity of each strain at the time of each subculture is shown in Fig. 1.

The 4 strains of *Staphylococcus aureus,* one of which (No. 195) was subcultured serially on 2 occasions, all increased in resistance very rapidly so that they grew profusely on agar containing 100 μg/ml after only 3 to 5 subcultures on the erythromycin-containing medium. The strain of enterococcus also increased in resistance in about the same man-

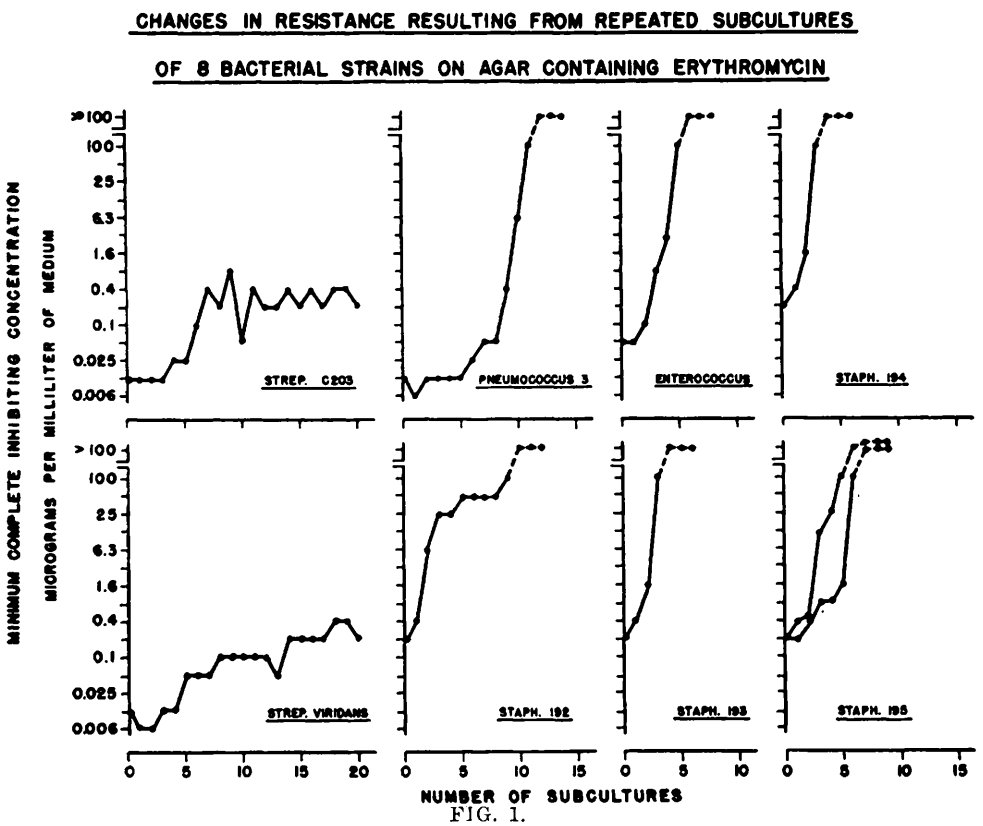

FIG. 1.

ner, while the type 3 pneumococcus reached the same level of resistance after 12 subcultures on erythromycin-agar. However, a group A hemolytic streptococcus and a strain of *Streptococcus viridans*, the latter from a patient with subacute bacterial endocarditis, increased only slightly in resistance after 20 transfers on the antibiotic-containing agar. The parent strains all retained their sensitivity unaltered throughout a parallel series of subcultures on similar plates without antibiotic. The results are summarized in Table II.

Cross-Resistance. The final subcultures of the 8 parent strains and of the 9 strains which had been transferred serially on erythromycin-agar were then all tested for sensitivity to penicillin, streptomycin, aureomycin, chloramphenicol, terramycin, bacitracin, polymyxin and neomycin. The results are shown in Table III. In almost every instance the strain which had been transferred without antibiotic and the corresponding one which had been repeatedly exposed to erythromycin were inhibited by the identical concentration of each of the other antibiotics. There were a few minor exceptions: the parent strains of type 3 pneumococcus and enterococcus appeared to be slightly more sensitive to penicillin than the corresponding erythromycin-resistant strains; the parent strain of *Staph.* 195 was inhibited by 1.6 μg/ml of terramycin and 25 μg/ml of chloramphenicol, while the erythromycin-derived strain of this organism was inhibited by 3.1 and 50 μg/ml of these antibiotics, respectively; and the type 3 pneumococcus which had not been exposed to erythromycin was inhibited by 400 μg/ml of neomycin while the corresponding erythromycin-resistant strain was inhibited by 100 μg/ml of neomycin. Except in the latter instances, the differences were only 2-fold (*i.e.*, one serial dilution) and were considered to be within the limits of error of the method; the sensitivity of the pneumococcus to neomycin, however, may have been significantly in-

TABLE II. Development of Erythromycin Resistance *In Vitro*.

Organism	Min complete inhibiting conc. of erythromycin (μg/ml)				
	Before subcultures	After serial subcultures on blood-agar		After 10 additional subcultures in antibiotic-free broth	
		Without antibiotic (Series P)	Containing erythromycin (Series R) †	Series P	Series R‡
Pneumococcus, type 3	.02	.04	>100 (13)	.02	400
Streptococcus C203	"	"	.4 (20)	"	.2
Streptococcus viridans	"	"	.4 (20)	"	.4
Enterococcus	.1	.4	>100 (9)	.2	>1600
Staphylococcus 192	.4	"	" (11)	.4	1600
Staphylococcus 193	"	"	" (5)	.2	>1600
Staphylococcus 194	"	"	" (5)	.4	
Staphylococcus 195	"	"	" (9)	"	
Staphylococcus 195(a)*	"	"	" (7)	"	

* A second series of subcultures with the same strain.

† The highest concentration of erythromycin used in this test was 100 μg/ml. The number of subcultures is given in parentheses and is the same for the corresponding strain in Series P.

‡ The highest concentration used in this test was 1600 μg/ml.

creased by the exposure to erythromycin.

Effect of Further Subcultures in Antibiotic-Free Broth. The final subcultures of each strain on the erythromycin-agar and the corresponding parent strains after they had been subcultured without antibiotic in the previous experiment were all subsequently transferred serially 10 additional times in antibiotic-free broth. They were then all tested again for sensitivity to erythromycin by the plate-dilution method. There was no significant change in the resistance of any of these strains as a result of the 10 additional subcultures. The "resistant" strains at this time were tested in concentrations up to 1600 μg/ml; the pneumococcus was found to be inhibited by 400 μg/ml, the enterococcus and 4 of the staphylococcal strains were only slightly inhibited by 1600 μg/ml, while *Staph.* 192 was completely inhibited by the latter concentration.

Biologic Effects of Exposures to Erythromycin. All the 8 strains which were included in the preceding study were tested for some of the principal biologic properties by which they are usually characterized; these tests were done before and at intervals during and after the serial subcultures both with and without erythromycin. The pneumococcus retained its ability to produce capsular swelling with type 3 antipneumococcus rabbit serum and its colonial characteristics and morphology also were unchanged. The enterococcus retained its colonial and microscopic morphology and remained heat-resistant after it had become erythromycin-resistant. The group A hemolytic streptococcus and the strain of *Streptococcus viridans* each retained its own colonial and microscopic morphology and its characteristic hemolytic property after the repeated subcultures on erythromycin-containing media. The strains of *Staphylococcus aureus* were all hemolytic and produced coagulase at the start of the experiment. However, as the resistance of these strains to erythromycin increased, their coagulase-producing activity first became poor and then was completely lost while the hemolytic property was retained throughout. Coagulase production was not regained by the subsequent serial transfers of the resistant staphylococci in antibiotic-free broth but it remained intact in the organisms which had been subcultured without erythromycin. The resistant staphylococci in this study also showed other biochemical changes in that they coagulated milk poorly and failed to ferment mannitol.

Development of Resistance During Therapy. In the preliminary clinical studies(4) there was an opportunity to observe strains of the same offending organism before erythromycin was given and again after several days of therapy with this antibiotic. In 2 patients with scarlet fever, hemolytic streptococci which were isolated from nasopharyngeal cultures after one week of treatment with erythromycin were as sensitive as the pretreatment strains in each instance. On

TABLE III. Effect of Repeated Subcultures on Erythromycin-Containing Agar on Sensitivity of Bacteria to 8 Other Antibiotics.

Organism	Min complete inhibiting conc., μg/ml															
	Penicillin		Aureomycin		Terramycin		Chloramphenicol		Streptomycin		Neomycin		Bacitracin		Polymyxin B	
	P*	R*	P	R	P	R	P	R	P	R	P	R	P	R	P	R
Pneumococcus, type 3	.04	.08	.4	.4	3.1	3.1	6.3	6.3	100	100	400	100	3.1	3.1		
Streptococcus C203	"	.04	.8	.8	"	"	"	"	200	200	200	200	1.6	1.6	>400	>400
Streptococcus viridans	.1	.1	"	"	"	"	"	"	100	100	"	"	3.1	3.1		
Enterococcus	6.3	12.5	6.3	6.3	12.5	12.5	50	50	"	"	"	"	6.3	6.3		
Staphylococcus 192																
" 193	>400	>400	100	100	>400	>400	12.5	12.5	"	"	100	100	3.1	3.1	400	400
" 194																
" 195	.8	.8	.8	.8	1.6	3.1	25	50	"	"	200	200	"	"	"	"
" 195(a)†	"	"	1.6	1.6	3.1	3.1	12.5	12.5	"	"	100	100	"	"	>400‡	>400‡

* Series P—Subcultured repeatedly on blood agar without antibiotic. Series R—Subcultured repeatedly on blood agar containing graded concentrations of erythromycin. The numbers of these subcultures are shown in Table II.

† Second series of subcultures of Staphylococcus 195.

‡ Partial inhibition in 400 μg/ml, the highest concentration tested.

the other hand, increases in resistance were observed in strains of *Staphylococcus aureus* isolated after unsuccessful erythromycin treatment in 2 patients with acute bacterial endocarditis. In one of these patients the staphylococcus obtained from the blood culture before erythromycin therapy was started was completely inhibited by 0.4 μg/ml of that antibiotic while a strain of the same organism isolated from a blood culture made on the tenth day of erythromycin therapy and tested at the same time required 100 μg/ml for complete inhibition. In the second patient, strains of *Staphylococcus aureus* isolated on 4 occasions during the 8 weeks before erythromycin was started were each inhibited by 0.4 μg/ml while 2 strains isolated after 7 and 8 days of therapy, respectively, and tested at the same time as the others required 12.5 μg/ml of erythromycin for complete inhibition. Interestingly enough the resistant strains of staphylococci which were obtained from the blood of these 2 patients retained all of the biologic characteristics (including coagulase production) possessed by the sensitive organisms which were isolated before erythromycin therapy was started.

Summary and Conclusions. 1. Variants with high degrees of resistance to erythromycin were not isolated from small volumes of cultures of erythromycin-sensitive bacteria which had not previously been exposed to that antibiotic. 2. When cultures of erythromycin-sensitive staphylococci (which were completely inhibited by 0.4 μg/ml in the plate-dilution test) were grown from a 10% inoculum in broth in the presence of 16 μg/ml of erythromycin, strains of varying resistance could be recovered after 48 hours; these required concentrations of erythromycin ranging from 0.2 to 25 μg/ml for complete inhibition. 3. By repeated subcultures of erythromycin-sensitive bacteria on blood agar containing graded concentrations of erythromycin it was possible to obtain strains of increased resistance to this antibiotic. The rate at which this resistance increased varied markedly with the strain. Several strains of *Staphylococcus aureus*, an enterococcus and a pneumococcus each increased more than 512-fold in from 3 to 12 such subcultures. Strains of only mod-

erately increased resistance were obtained after 20 similar subcultures of a strain of hemolytic streptococcus and one of *Streptococcus viridans*. 4. The strains of increased resistance resulting from repeated subcultures on the erythromycin-containing agar retained that resistance after 10 further subcultures in antibiotic-free broth. 5. Strains of increased resistance to erythromycin derived by repeated subcultures in that antibiotic were almost invariably similar to their respective parent strains in their sensitivity to 8 other antibiotics; however, an erythromycin-resistant strain of type 3 pneumococcus had apparently increased in sensitivity to neomycin following its repeated subcultures in erythromycin. 6. Staphylococcal strains of markedly increased resistance to erythromycin were obtained from blood cultures of 2 patients with endocarditis following 7-10 days of treatment with this antibiotic. 7. The colonial, morphologic and biochemical characteristics of the erythromycin-resistant strains obtained by repeated subcultures in erythromycin-containing media resembled those of the respective parent strains from which they were derived in almost every instance. The strains of *Staphylococcus aureus*, however, lost their ability to produce coagulase and exhibited other changes in their biochemical properties following their repeated subcultures in the presence of erythromycin. Similar changes were not observed in the 2 strains of *Staphylococcus aureus* which had apparently become resistant during erythromycin therapy.

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Observations on Mode of Action of Erythromycin.* (19817)

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The results of studies carried out in this laboratory on some of the important properties of erythromycin as an antibiotic agent and on the occurrence and development of resistance of bacteria to that agent were presented in the preceding communications(1,2). The original description of the isolation of erythromycin and of its physical, chemical and antimicrobial properties were reported by McGuire *et al.*(3) and additional clinical and laboratory observations were presented by Heilman *et al.*(4). Preliminary results of clinical trials of this agent, chiefly in pneumococcal, streptococcal and staphylococcal infections have also been recorded(3-5). Observations on the mode of action of erythromycin as determined by growth curves of organisms exposed to that agent are described in this paper.

Materials and Methods. The organisms used were: a group A hemolytic streptococcus, No. 98, generally employed in assays of penicillin; a recently isolated strain of a coagulase-positive, hemolytic *Staphylococcus aureus* (No. 194); and the MacLeod strain of *Escherichia coli*. A single, well segregated colony from an agar pour plate of each of these strains was picked into broth and subcultures of these colonies were used for the present studies. The media used were brain-heart infusion broth (Difco) and heart-infusion agar (Difco) each at pH 7.4±. Defibrinated horse blood, 0.5% in broth and 10% in agar, was added for enrichment and as an indicator. Erythromycin in crystalline

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