TABLE III. Action of Saliva on Activity of 1-hydroxy-2(1H)pyridinethione.

Organism	M.I.C. in μg/ml: In basal medium	M.I.C. in μg/ml: Basal medium + 30% saliva
M. pyogenes var. *aureus* 209P	.15	3.5
Strep. pyogenes C-203	.3	.6
K. pneumoniae	2	3
Ps. aeruginosa	5	6

TABLE IV.
Effect of Serum on Activity of Several Concentrations of MC 3277 against *Saccharomyces cerevisiae.*

Concentration, μg/ml	M.I.C. in μg/ml at 0 hr	1 hr	3 hr	24 hr
A) 50% Bacto beef serum at 37°C				
1000	.0261	.0230	.0245	.0285
500	.0277	.0238	.0238	.0245
100	.0230	.0223	.0277	.0277
5	.0328	.0384	.0396	.0592
B) Distilled water at 37°C				
1000	.0277	.0238	.0253	.0245
500	.0253	.0260	.0268	.0317
100	.0245	.0238	.0245	.0223
5	.0306	.0317	.0357	.0317

coccus neoformans in both media. The M.I.C. of 0.15 μg/ml for *M. pyogenes* var. *aureus* and 0.10 μg/ml for *C. neoformans* was unchanged by the presence of CSF after 2 days incubation at 37°C.

C. *Serum.* Weighings of MC 3277 were made up in 50% sterile Bacto beef serum and sterile distilled water. The levels made up were 1,000 μg/ml, 500 μg/ml, 100 μg/ml, and 5 μg/ml. Aliquot portions were drawn off and assayed immediately and the samples then placed at 37°C. At one hour, 3 hours, and 24 hours, portions were drawn off and assayed by the method described above. The data (Table IV) indicate that at concentrations of 1,000 μg/ml, 500 μg/ml, and 100 μg/ml, there is no apparent inactivation. At 5 μg/ml in 50% Bacto beef serum, however, there appears to be some inactivation after 24 hours.

Summary and conclusions. An antibacterial and antifungal spectrum for the sodium salt of 1-hydroxy-2(1H)pyridinethione as well as an assay method for the same is presented. *In vitro* the substance is partially inactivated by saliva and mucin, by serum at low concentration, but not by human cerebrospinal fluid. The inactivation by saliva, mucin, and serum however may be of relatively minor importance in view of the extraordinarily high activity displayed by this compound.

1. Shaw, E., Bernstein, J., Losee, K., and Lott, W. A., *J. Am. Chem. Soc.*, 1950, v72, 4362.
2. Thomas, B. G. H., *et al.*, to be published.
3. Donovick, R., Hamre, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, v50, 623.

Received December 8, 1952. P.S.E.B.M., 1953, v82.

Antibiotic Combinations and Resistance to Antibiotics: Penicillin-Erythromycin and Streptomycin-Erythromycin Combinations *in vitro*.* (20042)

ELMER M. PURCELL, SAMUEL S. WRIGHT, AND MAXWELL FINLAND.†
From the Thorndike Memorial Laboratory, 2nd and 4th Medical Services (Harvard), Boston City Hospital and Department of Medicine, Harvard Medical School, Boston, Mass.

The emergence of penicillin-resistant strains of staphylococci during prolonged exposure to that antibiotic *in vitro* or in the course of therapy is now a well established phenomenon. In many large hospitals where penicillin is used very extensively this phenomenon is probably responsible for the complete reversal from predominantly sensitive to predominantly resistant strains of staphylococci among those isolated from infected materials and carriers in the hospital populations(1-6). It has recently been demonstrated that the new antibiotic erythromycin is highly and equally active against both penicillin-sensitive

* Aided by a grant from the U. S. Public Health Service.

† With technical assistance of Clare Wilcox and Marilyn K. Broderick.

and penicillin-resistant staphylococci(7-10). It was also shown that staphylococci and other erythromycin-sensitive organisms may rapidly acquire high degrees of resistance to erythromycin during repeated subcultures in the presence of that agent(11). Several cases have been reported in which there was a change from sensitive to resistant strains of staphylococci and streptococci isolated from blood cultures during the course of treatment with erythromycin(8,9). Previous studies have also shown that repeated subcultures of various bacteria in the presence of streptomycin results in rapid development of high degrees of streptomycin-resistance in strains originally sensitive to that antibiotic(12,13) and clinical counterparts of this phenomenon have been noted in the rapid emergence of highly resistant strains during the course of treatment with streptomycin(14). The present study was undertaken to determine whether the development of resistance of staphylococci to penicillin and erythromycin *in vitro* could be suppressed by exposure of sensitive strains to both agents simultaneously. Similar studies were also made to determine whether the development of resistance of staphylococci and of enterococci to streptomycin and erythromycin could be prevented or suppressed *in vitro* by the use of these 2 antibiotics in combination.

Materials and methods. 6 coagulase-positive strains and 1 coagulase negative strain (No. 1) of *Staphylococcus aureus,* all of them hemolytic and each sensitive to both penicillin and erythromycin, were chosen for the first part of this study; their sources are listed in the upper part of Table I. These strains had each been obtained from single colony cultures, stored at −25°C and subcultured in broth prior to use in this study. Crystalline potassium penicillin G was obtained commercially; the erythromycin (Ilotycin, Lilly) was furnished in a purified crystalline form by Dr. John W. Smith of Lilly Research Labs. and contained 890 μg per mg. A stock solution of erythromycin was prepared and kept frozen at −25°C when not in use. Stock solutions of penicillin were freshly prepared each week and stored at 5°C when not in use. The method used for increasing resistance to antibiotics was similar to that previously employed in this laboratory(11,13,15); in this part of the study subcultures were made daily on graded concentrations of antibiotics incorporated in blood agar and the inoculum each time consisted of a broth suspension made from the surface growth of the plate containing the highest concentration of antibiotic(s) on which the growth was maximal or nearly so. When the 2 antibiotics were used together, a constant ratio of 16 times as much erythromycin as penicillin was used, this ratio having proved optimum in preliminary tests. The strains used in the studies with streptomycin and erythromycin were all recently isolated from patients with severe illness; their sources are listed in the lower portion of Table I. None of the patients had previously received treatment with either streptomycin or erythromycin. The organisms had undergone very few subcultures, most of them only 1 or 2, prior to the isolation of the single colony cultures with which these studies were made. In each instance the original colony chosen was 1 of 10 picked from a pour plate of a fully grown culture so diluted as to yield widely separated and discrete colonies; each of the 10 colonies was tested for sensitivity to the antibiotics under study and all were found to be identical within the limits of the 2-fold dilution method. In this part of the study the inoculum for agar plates was obtained by subculturing to antibiotic-free broth

TABLE I. Sources of Strains Used.

Strain		Source	Diagnosis
Penicillin-erythromycin series			
Staphylococcus	1	Pus	Abscess
	2	Throat	Pneumonia
	3	Pus	Furuncle
	4	Urine	Pyelonephritis
	5	Sputum	Pneumonia
	6	Pus	Paronychia
	7	Sinus	Osteomyelitis
Streptomycin-erythromycin series			
Staphylococcus	H	Blood	Endocarditis
	S	Sinus	Osteomyelitis
	W	Blood	Peritonitis
	M	Urine	Pyelonephritis
	U	Blood	Septicopyemia
	A	Urine	Pyelonephritis
	2	Sinus	Osteomyelitis
	197	Sputum	Pneumonia
Enterococcus	2	Urine	Pyelonephritis

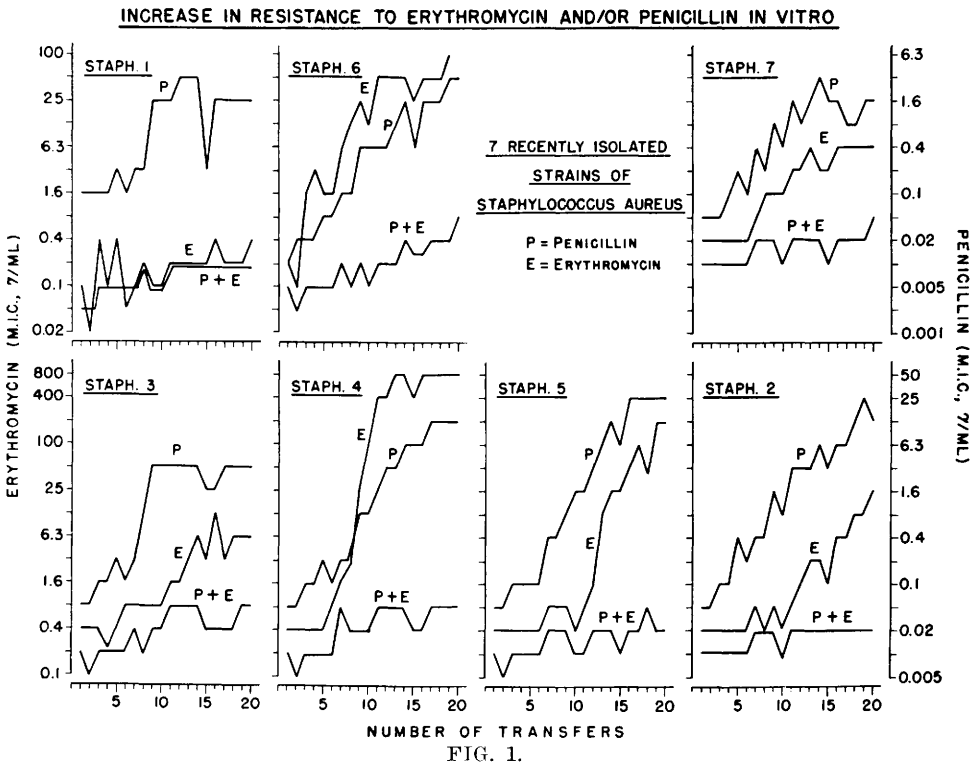

FIG. 1.

from the plate containing the maximum concentration of antibiotic which permitted good growth at 18 hours; the broth was allowed to incubate for 6 hours and the resulting growth used for the next transfer on antibiotic agar. Three parallel series of transfers were carried out in each instance:—one in erythromycin, a second in streptomycin and a third with both antibiotics combined. For the third series, carried out with staphylococcal strains H, 2 and 197 and Enterococcus 2, a constant ratio of 16 parts of streptomycin to 1 of erythromycin was used throughout and for the remaining strains this ratio was 256 to 1; these ratios were found to be optimal in preliminary tests with the parent strains. In part of this study the serial daily transfers were made in increasing concentrations of antibiotics incorporated in broth, each transfer being essentially a test for sensitivity done with serial 2-fold dilutions in broth. Streptomycin sulfate was used and was obtained commercially. Throughout these studies the sensitivity of a strain is defined as the minimum concentration of antibiotic producing complete inhibition of growth in 24 hours; it is expressed in μg/ml and 1 unit of penicillin was taken as equal to 0.6 μg. Coagulase tests were carried out daily or on alternate days with all of the strains of staphylococci in each series.

Results with penicillin and erythromycin. The changes in resistance of the 7 strains during the daily subcultures on agar containing penicillin (P), erythromycin (E), or both (P + E) are shown graphically in Fig. 1. In each instance there was a more or less steady increase in resistance to penicillin and, except in the case of *Staph. 1,* similar increases in resistance occurred during repeated subcultures on erythromycin. Considering the crudeness of the method, the rates of increase in resistance of each strain to the individual agents were remarkably similar. When the subcultures were made on agar containing both antibiotics, only minor increases or none at all developed in the resistance of the staphylococci to the combination of penicillin and erythromycin.

The changes in resistance to the 2 antibiotics, individually and together, that resulted

TABLE II. Sensitivity of 7 Strains of *Staphylococcus aureus* to Penicillin and Erythromycin before and after 20 Subcultures on Agar Containing One or Both of These Antibiotics.

Strain No.	Minimum complete inhibiting concentration, μg/ml — Penicillin: 0*	P—R	E—R	P+E—R	Erythromycin: 0	P—R	E—R	P+E—R	Penicillin + erythromycin: 0	P+E—R
1	.1	1.6	.1	.1	.4	.4	.4	.2	.002+ .05	.01 + .2
2	"	12.5	"	.05	"	"	25	.8	.01 + .2	.02 + .4
3	"	3.1	"	.2	"	"	6.3	3.1	"	.05 + .8
4	"	12.5	.05	.05	"	"	800	"	"	"
5	"	25	.1	.1	"	"	200	1.6	"	.02 + .4
6	"	3.1	"	.05	"	"	100	>800	"	.1 +1.6
7	"	1.6	"	"	"	"	6.3	.8	"	.05 + .8

* 0 = Parent strain (not previously exposed to antibiotics); P—R = after 20 transfers on penicillin; E—R = after 20 transfers on erythromycin; P+E—R = after 20 transfers on agar containing both penicillin and erythromycin (the first value is for penicillin, the second is for erythromycin).

from the 3 series of transfers are listed in Table II. Increases in resistance to the homologous antibiotics ranged from 16-fold to 256-fold for penicillin alone and (excluding Strain No. 1) from 16-fold to more than 1028-fold for erythromycin alone, as compared with only 2- or 4-fold increases in resistance to the 2 agents in combination (except for Strain No. 6 which increased 8-fold). Cross-resistance between the individual antibiotics did not develop. Moreover, after 20 subcultures on agar containing both penicillin and erythromycin all 7 strains retained their original sensitivity to penicillin and 3 of the strains also retained their sensitivity to erythromycin; the resistance to the latter, however, increased 4-fold or 8-fold in 3 strains and more than 2048-fold in Strain No. 6.

Coagulase production. Strain No. 1 failed to produce coagulase before and at the end of the 3 series of exposures to the antibiotics. The remaining strains all produced coagulase and this property remained unaltered after the transfers with the antibiotics singly or in combination, with 2 exceptions:—after the 20 transfers on penicillin agar, strains 3 and 7 failed to produce coagulase.

Results with streptomycin and erythromycin. Agar plate method. The changes in sensitivity to the homologous antibiotics that occurred during serial transfers on agar containing streptomycin, erythromycin or both were studied with 2 strains of staphylococci (No. 2 and 197) and with Enterococcus 2. The results obtained with the latter are shown in Fig. 2 and those obtained with the staphylococci were very similar. As in other studies employing this crude method, there are irregularities but, in general, each of the strains, after a variable lag, showed a marked increase in resistance to erythromycin and to streptomycin during the successive transfers in the corresponding antibiotic. Resistance to the combined antibiotics developed later and to a lower degree in the staphylococci and was apparently not sustained in the case of the Enterococcus. In the case of all of the organisms used in this study the sensitivity of the original strains to the combination of erythromycin and streptomycin was somewhat more

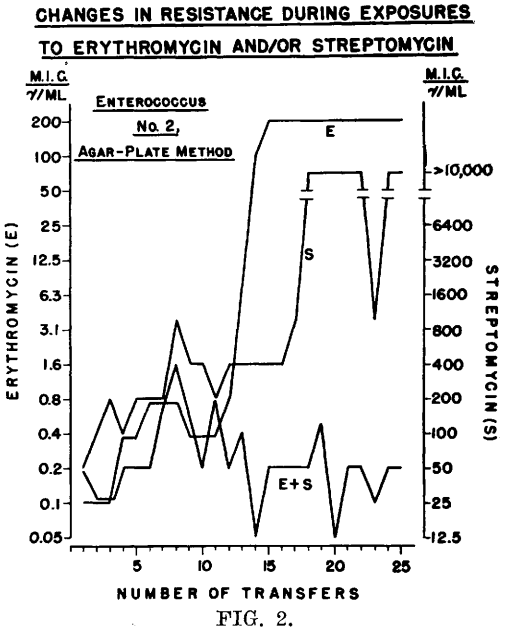

FIG. 2.

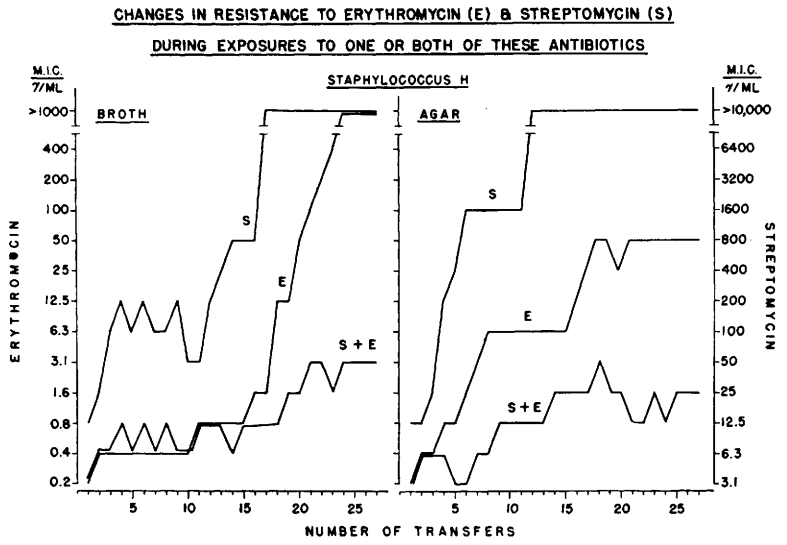

FIG. 3.

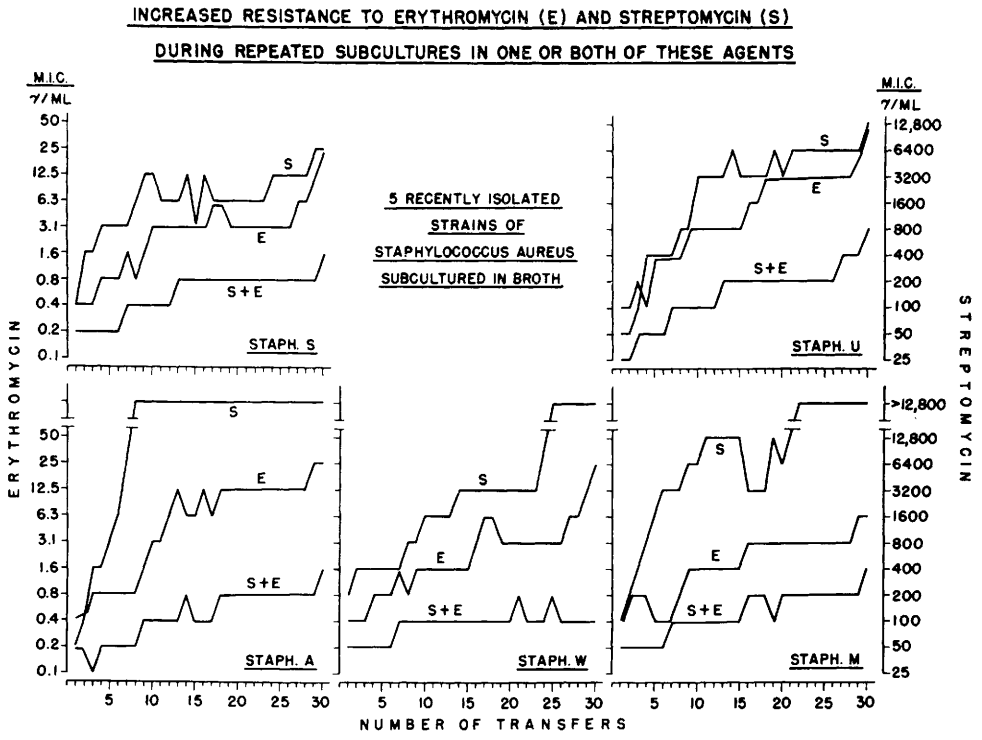

FIG. 4.

than additive.

Comparison of the plate and broth methods. Because transfers in broth could be made somewhat more quantitatively than in the agar method as here employed, more uniform changes in resistance might be anticipated if the broth method were used. *Staph. H* was, therefore, studied with parallel transfers in broth and agar; the results are shown in Fig. 3. Resistance appeared to develop somewhat

TABLE III. Sensitivity to Erythromycin and Streptomycin before and after Repeated Subcultures in the Presence of One or Both of These Antibiotics.

	Minimum complete inhibiting concentration, μg/ml									
	Erythromycin (E)				Streptomycin (S)				Erythromycin + streptomycin	
Strain	0	E—R	S—R	E+S—R	0	E—R	S—R (×1000)	E+S—R	0	E+S—R
Strains transferred in broth										
Staph. H	.4	>1000	.8	3.1	12.5	12.5	>10	50	.2 + 3.1	1.6 + 25
S	"	25	.4	"	100	100	.64	400	.2 +50	3.1 +800
W	"	12.5	.2	.8	200	50	>12.8	"	"	.4 +100
M	"	6.3	.4	6.3	100	100	"	1600	"	1.6 +400
U	"	50	.2	3.1	50	25	12.8	800	.1 +25	3.1 +800
A	"	25	.4	1.6	50	50	>12.8	"	.2 +50	1.6 +400
Strains transferred on blood-agar										
H	.2	50	.2	3.1	12.5	50	>10	50	.2 + 3.1	1.6 + 25
2	.4	12.5	.2	.2	6.3	25	"	25	"	"
197	"	200	.2	1.6	25	12.5	"	"	.4 + 6.3	"
Entero. 2	.1	"	.04	.2	50	100	.64	100	.1 + 1.6	.2 + 50

0 = Parent strain (not previously exposed to antibiotics); E—R = after repeated subcultures with erythromycin; S—R = after repeated subcultures with streptomycin; E+S—R = after subcultures with both erythromycin and streptomycin; Staph. H was transferred 36 times in antibiotic broth and 27 times in antibiotic agar; the other strains were transferred 30 times in antibiotic broth and 20 times on antibiotic agar.

more uniformly and a higher degree of resistance to erythromycin was attained when the broth method was used. Qualitatively, however, the results with the 2 methods were similar.

Broth-dilution method. An additional group of 5 strains of staphylococci were each carried through the 3 series of 30 transfers in broth; the results are shown in Fig. 4. In each instance, resistance to erythromycin increased more slowly, and in some of the strains the total change in resistance achieved (in terms of "fold increase") was less than with streptomycin. For each of the 5 strains, however, the resistance resulting from the transfers in the combination of erythromycin and streptomycin was slower in developing and was of a significantly lower order of magnitude than that which resulted from exposures to each antibiotic separately.

Cross-resistance. At the completion of the study all of the strains resulting from the various transfers were tested for sensitivity to streptomycin and erythromycin by the plate-dilution method. The results are shown in Table III. The parent strains in this study had been maintained in the frozen state at −25°C during the course of the study and were subcultured and tested at the same time for comparison. It is seen that, within the limits of the method, no cross resistance to erythromycin or streptomycin resulted from the subcultures in the presence of these agents individually. The organisms which had been transferred in the combination of the 2 antibiotics did show increases in resistance when tested with each antibiotic separately; these increases in resistance were, in general, of the same order of magnitude as the increases in resistance that occurred to the 2 agents used in combinaton. Additional tests for *sensitivity* to penicillin, bacitracin, aureomycin, terramycin, chloramphenicol, polymyxin B (Aerosporin) and neomycin were carried out with staphylococcal strains W, S, M, U and A, using the parent cultures and the final transfers of the 3 antibiotic series in each instance. The parent strains were each resistant to > 400 μg/ml of penicillin and to >200 μg/ml of polymyxin and were inhibited by 6.3 μg/ml of bacitracin; no changes in resistance were noted with respect to these 3 antibiotics in any of the 5 strains. Four of the 5 strains showed no change in resistance to aureomycin and terramycin (both grew in >400 μg/ml of each agent) as a result of the exposures to erythromycin and/or streptomycin; *Staph. M,* however, was initially resistant to >400 μg/ml of both aureomycin and terramycin but the erythromycin-resistant vari-

ant of this organism was completely inhibited by 25 μg and 6.3 μg/ml of these agents, respectively. All 5 staphylococcal strains increased in resistance to neomycin as a result of the repeated exposures to streptomycin used alone and in combination with erythromycin, but they retained their original sensitivity to neomycin after they had become resistant to erythromycin. The cross-resistance between streptomycin and neomycin was similar to that previously observed in this laboratory(15,16).

Biological reactions. The following observations were made simultaneously on the parent strains and on the final transfers of each of the 3 series of subcultures of the 6 staphylococcal strains done in antibiotic-containing broth: 1) hemolysis on blood agar, 2) pigment production, 3) coagulase reaction, 4) coagulation of milk and 5) fermentation of lactose, mannitol and sucrose. All of these organisms remained hemolytic and coagulase positive and they continued to produce the characteristic yellow pigment but some of the strains which had become resistant to either erythromycin or streptomycin apparently produced the pigment either in smaller amounts or more slowly, and some of them grew less luxuriantly than their parent strains on the surface of blood agar. The fermentation reactions were the same in the parent and derived strains in every instance.

Discussion. These results indicate clearly that the strains of staphylococci used in the first part of this study developed resistance to penicillin and erythromycin rapidly and to significant and high degrees during repeated subcultures in the presence of increasing concentrations of the homologous agent. On the original strains, the combination of these 2 antibiotics had an effect which was somewhat greater than additive. The 20 subcultures on penicillin together with erythromycin left the strains essentially unchanged with respect to their sensitivity to penicillin alone and increased only slightly their resistance to erythromycin alone and to the 2 antibiotics in combination. The reason for the aberrant result in the case of strain No. 6 is not clear at this time and requires further investigation. It was not associated with penicillinase production by this strain after exposure to the 2 agents. Moreover, the reason for the difference of the effects of exposure to erythromycin on coagulase production in this study as compared with previous results(11) is not clear.

The results obtained with the combination of streptomycin and erythromycin were very similar; in the presence of each antibiotic individually, resistance developed rapidly and to a high degree but when both were used together in a fixed and predetermined proportion of each, resistance to the combination of these agents was delayed and depressed. Moreover, following the repeated subcultures in increasing concentrations of the combination of these 2 antibiotics, the resistance to the individual agents increased only to about the same degree as to the combination. There was no evidence, however, that the presence of either of the antibiotics in the 2 combinations studied here reduced in any way the effectiveness of the other agents.

Summary and conclusions. 1. During 20 subcultures on blood agar containing increasing concentrations of penicillin or erythromycin each of 7 strains of *Staphylococcus aureus* increased markedly in resistance to the homologous antibiotic without affecting its sensitivity to the other agent. The combination of penicillin and erythromycin exerted slightly more than an additive effect on the parent strains. During 20 parallel subcultures on agar containing both antibiotics, the organisms retained their original sensitivity to penicillin alone but most of them increased slightly in resistance to the 2 agents in combination and to erythromycin alone, while one strain increased markedly in resistance to the latter agent. Coagulase production by these staphylococci remained unaltered following the exposures to these antibiotics except for 2 strains which lost this property after they became resistant to penicillin. 2. By serial transfers of several strains of staphylococci and of a strain of enterococcus in broth and/or on the surface of blood agar containing increasing concentrations of erythromycin or streptomycin, high degrees of resistance developed against the homologous antibiotic, but cross-resistance to the other agent did not

develop. Cross-resistance to neomycin was demonstrated in the strains of staphylococci which had increased in resistance to streptomycin following subcultures with the latter agent used either alone or in combination with erythromycin; such cross-resistance to neomycin did not occur in the strains which had become resistant to erythromycin following exposures to that antibiotic alone. The biological characteristics of the staphylococci, including coagulase production, remained unaltered following the development of resistance to either streptomycin or erythromycin, except for decreased production of pigment and less active growth of the resistant strains. The development of resistance to both erythromycin and streptomycin was delayed and depressed when the organisms were repeatedly subcultured in the combination of the 2 antibiotics. 3. It is concluded that the development of resistance to penicillin, streptomycin or erythromycin *in vitro* may be delayed and depressed by the use of a combination of erythromycin with either penicillin or streptomycin.

1. Barber, M., *Brit. M. J.*, 1947, v2, 863.
2. Finland, M., Frank, P. F., and Wilcox, C., *Am. J. Clin. Path.*, 1950, v20, 325.
3. Vogelsang, T. M., *Acta path.*, 1951, v29, 363.
4. Boe, J., and Vogelsang, T. M., *Ibid.*, p. 368.
5. Wilson, R., and Cockcroft, W. H., *Canad. M. A. J.*, 1952, v66, 548.
6. Finland, M., and Haight, T. H., *J. Lab. and Clin. Med.*, in press.
7. McGuire, J. M., *et al., Antibiotics and Chemotherapy*, 1952, v2, 281.
8. Heilman, F. D., *et al., Proc. Staff Meet. Mayo Clin.*, 1952, v27, 285.
9. Haight, T. H., and Finland, M., *New England J. Med.*, 1952, v247, 227.
10. ———, PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 175.
11. ———, *Ibid.*, p. 183.
12. Miller, C. P., and Bohnhoff, M., *J.A.M.A.*, 1946, v130, 485.
13. Murray, R., *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 470.
14. Finland, M., *et al., J.A.M.A.*, 1946, v132, 16.
15. Gocke, T. M., and Finland, M., *J. Lab. and Clin. Med.*, 1951, v38, 719.
16. Haight, T. H., and Finland, M., *Ibid.*, 1952, v39, 637.

Received December 1, 1952. P.S.E.B.M., 1953, v82.

Chloride Exchange between Human Erythrocytes and Plasma Studied with Cl^{36}.* (20043)

WILLIAM D. LOVE† AND G. E. BURCH.

From the Department of Medicine, Tulane University School of Medicine and the Charity Hospital of Louisiana at New Orleans.

It has been demonstrated by the use of isotope technics that in human blood sodium and potassium constantly exchange between erythrocytes and plasma under simulated physiologic conditions *in vitro* (1-6). This exchange is partially controlled by chemical processes which are very susceptible to temperature change and to some metabolic poisons. With this in mind, the exchange of chloride between plasma and erythrocytes has been investigated to determine whether the presence of similar metabolic control of the exchange of this anion could be demonstrated. In these experiments, Cl^{36} was added to whole blood, and the influence of changes in temperature and of several enzyme inhibitors on the speed of chloride uptake by the red cells measured. The inhibitors tested were NaCN, $NaNO_2$, Na-mono-iodoacetate, NaF, $HgCl_2$, Na-pyrophosphate, and sulfanilamide.

Methods. Heparinized venous blood was obtained from healthy donors, and 10 ml placed in paraffined flasks exposed to air at room temperature. After 30 minutes or longer, Cl^{36}, in 0.04 ml of saline solution, was

* Aided by a grant from the Public Health Service, the Life Insurance Medical Research Fund, the Mrs. E. J. Caire Fund for Research in Heart Disease, the U. S. Atomic Energy Commission Grant, and the Medical Research and Development Board, Office of the Surgeon General, Department of the Army.

† Life Insurance Medical Research Fellow 1950-52.