sorption and elution by the erythrocytes are clearly indicated in Fig. 5. It may be seen that for each concentration of virus in the adsorption-elution curves, the titer before adsorption is considerably lower than after elution. That packing of the virus during sedimentation causes aggregation is borne out by the electron micrographs which were made after centrifugation and before adsorption and elution by the erythrocytes. The micrographs showed most of the virus particles in a state of aggregation.

Conclusions. The electrophoretic mobility of human type O erythrocytes was found to decrease from 1.32 to 0.5-0.6 μ/sec./v./cm in the presence of 0.311 to 16.9 μg/ml of a highly purified preparation of the PR8 strain of influenza virus.

Adsorption and elution of the virus, and agglutination of the erythrocytes was found to be concurrent with the decrease in electrophoretic mobility of the erythrocytes.

The time of intimate contact of virus with cells and the pattern of decrease of electrophoretic mobility are not only interdependent but also dependent upon the concentration of added virus.

At the optimal ratio of virus to erythrocytes, as determined by the mobility and titration curves, it has been calculated that only 1/80 of the erythrocytic surface is covered by virus particles and that this value corresponds to about 298 PR8 virus particles to one erythrocyte.

16495

Relation of Induced Sulfonamide Resistance to Biochemical Properties in Alpha Streptococci.

WILLIAM E. CLAPPER AND MARY E. HEATHERMAN.
(Introduced by Richard Thompson.)
From the Department of Bacteriology, University of Colorado Medical School, Denver, Colo.

A decrease in the production of hydrogen peroxide accompanying an induced increase in resistance to sulfonamide bacteriostasis was first observed in a strain of pneumococcus by MacLeod.[1] Since some streptococci produce hydrogen peroxide, it seemed desirable to determine whether or not these organisms would produce less peroxide when they were made resistant to sulfonamide, whether other biochemical changes would be produced, and what relation these changes might have to the mechanism of resistance.

Loss of peroxide producing ability by resistant organisms. Five strains of greening streptococci originally obtained from saliva were tested for susceptibility to sulfathiazole by the serial dilution method, and for hydrogen peroxide production by the method of Main and Shinn[2] with slight modifications. They were then made resistant by transferring at intervals of 3 to 5 days in broth containing one-half the inhibiting concentration. All organisms were transferred 15 times. Sulfathiazole susceptibility and hydrogen peroxide production were then determined again. The results of the determination are shown in Table I. The sulfathiazole values represent the average of 3 or more determinations, none of which varied more than one tube from the value given. The peroxide production is expressed in values obtained from 3 or more determinations, none of which varied more than 5 μg/ml from the value given. Ability to produce peroxide was lost by all strains after they became resistant.

Comparison of other biochemical properties. Other biochemical changes were also investigated. Dehydrogenase activity was

[1] MacLeod, Colin M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 215.

[2] Main, Edna R., and Shinn, Laurance, E., *J. Biol. Chem.*, 1939, **128**, 417.

TABLE I.
Relation of Peroxide Production to Sulfathiazole Resistance.

Strain		μg/ml H_2O_2 produced		mg/ml sulfathiazole preventing growth	
		Parent	Resistant	Parent	Resistant
1	No growth on 40% bile agar	25	0	.035	>80
2	" " " " " "	30	0	.05	>80
6	" " " " " "	60	0	.125	>80
12	" " " " " "	30	0	10.00	>80
16	Growth on 40% bile agar	15	0	2.50	>80

TABLE II.
Comparison of Relative Dehydrogenase Activity of Parent and Resistant Strains.

Substrate	Strain	Activity	
		Parent	Resistant
Glucose	2	100	231
	6	100	236
Glycerol	2	100	271
	6	100	154
Pyruvate	2	100	533
	6	100	200
Lactate	2	100	205
	6	100	197

Values represent per cent of activity of the parent strain.

determined by the Thunberg technic. The results for 2 strains are given in Table II. Activity for the parent strain was given a value of 100 and that for the resistant strain expressed as a percentage of this activity. The dehydrogenases of the resistant organisms for all 4 of the substrates were always more active than those of the parent strains. The 4 strains which would not grow on 40% bile agar, grew luxuriantly on it after becoming resistant to sulfathiazole. The resistant strains grew much more profusely in tryptose broth. These variations indicate an overall increase in metabolic activity in the resistant organisms which might account for their greater resistance to sulfonamide. Slower growth rates for sulfonamide[3] and penicillin[4] resistant organisms and loss of physiological functions for penicillin[5] resistant strains have been reported. MacLeod's sulfapyridine resistant pneumococcus[1] grew at the same rate as the parent strain and showed no change in the activity of its glucose dehydrogenase. The parent strain had active dehydrogenases for glycerol, lactate, and pyruvate, but no activity for any of these substrates was shown by the resistant strain. Apparently, the resistance to sulfathiazole developed by the streptococci in our investigation involves a different mechanism than that found by any of these workers. It is a relatively stable characteristic, since all strains have maintained their high levels of resistance for more than 8 months while being subcultured on blood agar.

Mechanism of resistance. The reduction of peroxide production associated with increased resistance raises interesting questions concerning the mechanism of acquired resistance. Is this loss of ability to produce hydrogen peroxide related directly to a change in the oxidative enzymes normally used by the organism or is it a loss of function that occurs concomitantly with other changes, the other changes being the important ones for resistance? Davies and Hinshelwood[6] state that if the inhibited enzymes are not essential they will suffer gradual elimination. The enzymes bringing about the production of peroxide may be of this kind, and if so, would not be directly associated with the ability to resist sulfonamides. However, after consideration of the following findings, we feel that resistance is directly related to changes in the respiratory system involving riboflavin.

It seemed possible that the resistant organisms might be producing catalase. This would account for the decreased peroxide production. Since the peroxide is bacteriostatic, destruction

[3] Yegian, Diran, Budd, Vera, and Middlebrook, Gardner, *J. Bact.*, 1946, **51**, 479.

[4] Seligmann, Erich, and Wasserman, Michael, *J. Immunol.*, 1947, **57**, 351.

[5] Bellamy, Dexter W., and Klimek, John W., *J. Bact.*, 1948, **55**, 153.

[6] Davies, D. S., and Hinshelwood, C. N., *Trans. Faraday Soc.*, 1947, **43**, 257.

by the catalase in the resistant organisms might allow growth at higher concentrations of sulfathiazole. However, catalase determinations were negative, and the addition of 60 μg/ml of hydrogen peroxide to broth did not make the resistant strains more susceptible to sulfathiazole. Hydrogen peroxide is produced by the transfer of hydrogen from a substrate through the flavoprotein enzymes directly to oxygen, without the aid of the cytochrome system. Since the resistant organisms no longer produced hydrogen peroxide, it might be postulated that they had shifted to an energy producing mechanism that did not involve flavoprotein or riboflavin. To test this hypothesis we attempted to grow strains 1, 2, 6 and 12 (all but the enterococcus) in a riboflavin-free broth.* This broth was made for riboflavin assays using *Lactobacillus casei,* and would not allow growth of this organism unless riboflavin was added. None of the 4 organisms grew in the riboflavin-free broth, but usually grew when 20 μg/ml of riboflavin was added. Growth did not take place as readily as in tryptose broth. The resistant strains from these parent organisms grew luxuriantly in the riboflavin-free broth. This indicated that the resistant organisms were either synthesizing their own riboflavin, or were growing without it. Assays of the resistant organisms grown in the riboflavin-free medium showed the presence of riboflavin in the cells and a small amount in the broth. This is similar to McIlwain's findings[7] that organisms capable of synthesizing pantothenic acid are insensitive, while those incapable of synthesizing it are sensitive to pantoyltaurine and that organisms capable of synthesizing a-aminocarboxylic acids were insensitive to the inhibitory action of a-aminosulfonic acids. However, riboflavin in amounts up to 100 μg/ml did not reverse the action of the sulfathiazole when added to broth in which the susceptible organisms had been seeded. This may mean that the sulfathiazole interferes with the utilization of riboflavin rather than with the synthesis which, according to Sevag,[8] is much more difficult to overcome by an antagonist. Cytochromes introduced between the flavoprotein and oxygen in the respiratory system of an organism bring about the production of water instead of hydrogen peroxide. The decreased peroxide production in the resistant organisms might be due to a hitherto unused cytochrome system being put into active use. Heavy suspensions of the resistant organisms would not oxidize p-phenylene diamine. This means that there is not a complete cytochrome c-cytochrome oxidase system present, but does not rule out the possibility of the presence of other cytochromes.[9] Experiments to further explain this mechanism, and to attempt to reverse the biochemical changes observed in the resistant organisms are in progress.

It is recognized that in the absence of single cell isolation there is the possibility that the sulfathiazole is selecting a resistant enterococcus initially present. This would appear most unlikely, however, as no growth was observed with any of the susceptible organisms on 40% bile agar; the trained organisms were repeatedly reisolated by picking from single colonies; and the development of resistance was a gradual one. If an enterococcus were present, it would be likely to grow out as a resistant culture in the first few transfers.

Summary. Peroxide production by 5 strains of greening streptococci was reduced with the acquisition of resistance to sulfathiazole. Other biochemical changes indicated an overall increase in metabolism in the resistant cells. The resistant cells did not acquire the ability to produce catalase, nor a cytochrome c-cytochrome oxidase system, but did acquire the ability to synthesize riboflavin. The possible relation of these changes to respiration is discussed.

* Generously supplied by the Difco Laboratories.

7 McIlwain, H., *Brit. J. Exp. Path.*, 1943, **24**, 212.

8 Sevag, M. G., *Enzyme Problems in Relation to Chemotherapy. "Adaptation," Mutations, Resistance, and Immunity,* Advances in Enzymology 6, 109, New York, Interscience, 1946.

9 Sevag, M. G., and Shelburne, M., *J. Gen. Physiol.*, 1942, **26**, 1.