# THE ABILITY OF GROUP A STREPTOCOCCI KILLED BY HEAT OR MERCURY ARC IRRADIATION TO RESIST INGESTION BY PHAGOCYTES

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PLATE 1

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It is widely believed that the virulence or pathogenicity of many bacterial species, particularly among the Gram-positive cocci, is due in large part to their ability to resist destruction by host phagocytes. The group A streptococci possess two elements that have been implicated in virulence, namely the M substance and the capsule; and of these the M substance is thought to be the more important (1). Nevertheless, there are certain observations which suggest that there may be other factors involved in streptococcal virulence. For example, strains freshly isolated from acute human streptococcal infections may be of low virulence for mice, even though they contain abundant M substance and produce good capsules. Serial intraperitoneal passage of such a strain in mice often causes its mouse virulence to be increased many fold. This increase is associated with an increase in the M substance, when the strain has relatively little M substance to begin with, but the problem of why unpassed strains with abundant M and well developed capsules should be relatively avirulent for mice, continues unsolved. Speculation has been offered that there may be other as yet unrecognized structural elements in the streptococcus involved in virulence. It has been suggested also that certain undefined metabolic activities of the cocci may play a role in virulence, and this idea is supported by the practice of using young, actively proliferating and actively metabolizing cocci in virulence and phagocytosis studies. No specific indication has been offered of the nature of such metabolic functions other than those concerned with the synthesis of M and the capsule.

In 1898 Marchand (2) published a classic work showing that streptococci retained their ability to resist ingestion by phagocytes after they had been killed by heat or several chemical agents. This work has attracted little notice, although Todd (3) in 1927 and Hare (4) in 1931 each published similar views without giving experimental data.

This seemed to us an important observation that deserved reinvestigation. If the factors in the streptococcal cell that enable it to resist phagocytosis sur-

vive after death of the cell, support is given to the idea that these factors are heat-resistant structural elements of the cell. It then seems unlikely that metabolic activities, for example those involved in the energy metabolism of the cocci or those essential for cellular proliferation, could be responsible in any direct way for the resistance to the phagocyte. This observation thus would point to those aspects of the streptococcal cell most deserving of investigation from the standpoint of its virulence, at least as far as that is reflected in capacity to resist phagocytosis.

In this study the conditions by which streptococci can be killed by heat or mercury arc irradiation with minimal change in phagocytosis resistance have been investigated, together with studies of the persistence of this resistance in the killed cells. Consideration is given to the question of whether the heat treatment fails to modify the factors responsible for phagocytosis resistance or acts by releasing substances into the medium which interfere with the phagocytic capacity of the neutrophils. The effect of heat on the M substance and the capsule is given limited consideration and the implications of the observations in the problem of streptococcal virulence is discussed.

## *Materials and Methods*

The method adopted for these studies was an *in vitro* test in which mixtures of leukocytes and streptococci were rotated in test tubes, stained smears being prepared after 15 and 30 minutes' rotation. The percentage of neutrophils containing streptococci was computed, which reflected the susceptibility of the cocci to ingestion. This method of reading was preferred to counting the number of cocci or chains ingested, because it is often difficult to decide whether contiguous cocci constitute one or several chains and because direct observation of phagocytosis on glass slides has shown that long chains may break up into two or more shorter chains shortly after phagocytosis is completed. The density of leukocytes and the concentration of cocci used in the test mixtures were standardized by counts and volume adjustments, and we feel this assured uniformity in leukocyte:chain ratio is responsible for the relatively high degree of reproducibility we have observed in this test.

The test used in these studies measures essentially the ability of the cocci to resist ingestion by neutrophils. Unlike observations of phagocytosis in the intact animal it is not necessary to wait for inflammatory cells to accumulate at the site of the injection. Since contact between cocci and leukocytes is assured by the turbulence of the mixture in the rotated tube, chemotaxis is not a factor in determining whether phagocytosis will occur. The large bacterial inoculum used, in relation to the number of leukocytes, gives each leukocyte an opportunity to ingest chains if it is capable of doing so. Whether the ingested cocci are killed or not by the phagocytizing leukocyte is not a factor, since the reading of the test is on a morphologic basis rather than by culture for surviving cocci. Thus the test is constructed to encourage as much phagocytosis as possible by the leukocytes. If they fail to phagocytize it is primarily because of an intrinsic capacity of the cocci to resist ingestion under the conditions of the test rather than to other factors that may influence phagocytosis. Egestion of streptococci from leukocytes, which has recently been described by one of us (5), may occur in the rotated tubes, but there does not seem to be a method of determining how frequently that happens, and it probably is a minor factor in the results. We have chosen to determine phagocytosis in the presence of plasma rather than with washed cells, because we feel it to be closer to conditions in the host. The test mixture is an excellent medium, and proliferation occurs rapidly in the tubes

during tests with living cocci. There is, therefore, a difference in the number of cocci that become available for phagocytosis when living organisms are used compared with experiments in which killed cocci are used, with effects that will be shown.

*Streptococcal Strains.*—The streptococcal strains used in these experiments are presented in Table I. They were typable by the precipitin method (6) with the exception of Type 1, which gave a 1 plus reaction, and 327W which was typable by agglutination only. Grouping and typing of strains by the capillary precipitin method was done with antisera furnished by the Communicable Disease Center of The Public Health Service, by Dr. Rebecca C. Lancefield, or by preparation in this laboratory and by the slide agglutination method (7) with sera kindly sent to us by Dr. R. E. O. Williams of the Streptococcal Reference Laboratory, Colindale, England. All strains had been preserved in the lyophilized state. For recovery they were

TABLE I

*List of Group A Streptococci*

| Strain | Type | Source |
|---|---|---|
| AD240 | 6 | Dr. Charles H. Rammelkamp, Jr., from acute upper respiratory infections, Warren Air Base, Wyoming, 1951 and 1952 |
| AD242 | 14 | |
| AD245 | 26 | |
| AD454/15 | 14 | |
| D58/47 | 3 | Dr. Rebecca C. Lancefield, strain "Richards" |
| Type 28 | 28 | Dr. Rebecca C. Lancefield, derived from original type strains of F. Griffith |
| Type 28op | 28 | |
| Type 28/143/Rb10 | 28 | |
| Type 1 | 1 | |
| Type 1/119 | 1 | |
| 327W | 1 | Food-borne epidemic, United States Navy, 1945 |

The first figure following a diagonal indicates the number of mouse passages. The figure following Rb indicates the number of rabbit passages.

taken up in neopeptone—sheep blood broth, incubated at 37°C. for 18 hours, and subcultured once in blood broth. This subculture was refrigerated and served as a stock culture. Approximately every 2 to 4 months each strain was subcultured in fresh blood broth which in turn served as the new stock culture. Strains handled in this manner gave uniform results in the ingestion test over long periods of time. Two strains during a 2½ year period showed evidence of reduced resistance to phagocytosis when tested with leukocytes from donors used previously, and in those cases fresh subcultures were obtained from lyophilized stock or the strain was given a mouse passage.

*Media.*—Three media were used in these tests: *1. Autoclaved beef heart infusion broth* was prepared by adding to beef heart infusion, at pH 7.0, 1 per cent neopeptone, 0.2 per cent glucose, 0.2 per cent sodium bicarbonate, 0.2 per cent sodium chloride, and 0.1 per cent disodium phosphate. The pH was adjusted to 7.8 and the medium boiled for 20 minutes. It was then filtered through paper and sterilized by autoclaving for 20 minutes at 15 pounds' pressure. *2. Filtered beef heart infusion broth* was similar in composition to the autoclaved broth described above, except that the sodium bicarbonate was omitted from the initial mixture. Instead of being boiled, the medium was heated for 30 minutes in a 56°C. water bath, filtered

through paper to remove precipitate and sterilized by filtration through porcelain filters. Sodium bicarbonate in a 4 per cent solution sterilized by filtration was added immediately before use. Preheating to 56°C. prevented further precipitation when the medium was exposed to that temperature during the heat-killed experiments to be reported. *3. Donor's serum broth* was prepared by adding 1 volume of serum to 4 volumes of filtered beef heart infusion broth. The serum was always obtained from the same donor whose leukocytes were used in the ingestion test. Sodium bicarbonate was omitted from the filtered medium when serum was included. Usually serum 1 to 5 days old was used. However, serum as old as 15 days has been used, and there was no detectable difference in its action.

*Ingestion Test.*—The test was carried out in plain glass test tubes, measuring 7 × 70 mm., to which were added 0.1 ml. of bacterial suspension, 0.05 ml. of 0.85 per cent NaCl solution, and 0.2 ml. of leukocyte suspension in that order. Details of preparing these constituents are given below.

The tubes were stoppered with paraffin-coated rubber stoppers and then rotated about the short axis at 8 R.P.M. for 15 minutes in a 37°C. incubator, when a loopful of the mixture was withdrawn for preparing cover-slip smears. The tubes were rotated for an additional 15 minutes and coverslip smears were again made. The smears were stained with Wright's stain and 200 neutrophils were observed to determine the percentage that had accomplished phagocytosis.

Experiments were conducted to determine whether the use of siliconed glassware had any advantage over plain glassware in the ingestion test. Strain 327W was used. Significant differences in the amount of ingestion were not observed between tests in which all surfaces with which the blood, plasma, and leukocytes had contact were siliconed and those conducted with unsiliconed glassware. The siliconed equipment was difficult to handle, made quantitative measurements more difficult and necessitated the use of tubes of larger diameter for rotation. In the absence of compensating advantages for siliconed equipment, plain glass has been used in all the experiments reported here. Sterile equipment and reagents were employed throughout.

*Leukocyte suspensions* were prepared by a modification of the method of Boerner and Mudd (8). Blood, containing heparin in a final dilution of 1:11,000, was centrifuged at 700 to 800 R.P.M. for 10 minutes. One-half of the semipacked erythrocyte layer was withdrawn and discarded. The remaining artificially anemic blood was placed in rubber-stoppered glass tubes, 6 × 250 mm., which were incubated at a 45° angle for 30 minutes at 37°C. A plasma-leukocyte layer formed which contained few erythrocytes. This was withdrawn, mixed with a pipette, and a leukocyte count was performed. On the basis of that count the suspension was adjusted to contain 10,000,000 leukocytes per ml. by adding heparinized plasma from the same donor. If the count were below 10,000,000 the suspension was concentrated by centrifuging and drawing off some of the supernatant plasma, recounting and readjusting the volume with plasma. Thus 2,000,000 leukocytes in 0.2 ml. of suspension were used in the test. The suspension was transferred to a rubber-stoppered tube in which it remained at room temperature until used in the ingestion test. These suspensions have given uniform results in the ingestion test for as long as 5 hours after the blood was drawn. Suspensions older than 5 hours have not been used.

*Streptococcal Suspensions.*—0.5 ml. of an 18 hour blood broth culture was delivered to 5 ml. of the particular medium desired and incubated at 37°C. for 2 to 3 hours. The culture was then centrifuged, the supernatant broth withdrawn, and the cocci resuspended in 5 ml. of fresh medium. Wet india ink preparations were made for capsule studies, when desired, and bacterial density counts were made in the Petroff-Hauser counting chamber. The suspensions were kept in an ice bath to retard proliferation while counts were being done. Accuracy of the counts was facilitated by mixing 1 ml. of the streptococcal suspension with an equal volume of 70

per cent glycerol in water before adding to the chamber. This, because of increased viscosity, largely prevented drifting of the cocci. Single cocci, diplococci, and chains were each counted as units. The concentration of organisms desired for a given test was obtained by adding the calculated necessary quantity of fresh medium of the type in which the cocci had been grown. Throughout this article the bacterial density or *"chain count"* is expressed as the number of cocci, diplococci and chains in 0.1 ml. of adjusted suspension, which was the amount of suspension used in the ingestion test. The ratio of chain-units to neutrophils, assuming the latter constituted about 50 per cent of the total leukocyte count, was thus roughly 1.5:1 when the chain count was 1,500,000 and 7:1 when the chain count was 7,000,000.

*Selection of Donors.*—The problem of differences in the capacity of various donor's leukocytes to phagocytize a particular streptococcal strain grown in a particular way is not the one we are primarily concerned with in this work, and we have tried to minimize the effect of donor differences by using the blood of a single donor for all but one of the experiments reported here. The exception donated blood for work reported in Table VII, and it can be seen that she phagocytized strain AD242 to an extent greater than the usual donor, shown in Table X. That the usual donor was not atypical in his capacity to ingest the streptococci studied here is shown in experiments not reported in detail in which these same strains have been tested with plasma-leukocyte mixtures from two to six other donors. In general it may be said that strains that were highly resistant to phagocytosis by cells of our usual donor were also resistant to phagocytosis by cells of the other donors and a corresponding situation was found with readily phagocytized streptococci. Strain D58/47 was highly resistant to the blood of 5 donors, including our usual donor, but was readily phagocytized by the cells of a 6th donor. We believe on the basis of plasma-absorption tests, which will be reported in detail elsewhere, that the blood of this donor contained type-specific antibodies. The amount of type-specific antibody present in the blood of the other donors must be very small if, indeed, there was any at all, because each serological type of streptococcus studied has included a virulent representative which the donors were able to phagocytize only to a very limited extent.

TABLE II

*Variations in Ingestion Tests*

| Strain | No. of experiments | No. of tubes in all experiments | Maximum ingestion in any single tube | | Minimum ingestion in any single tube | | Range of variation in tests done on different days | | Range of variation in duplicate tubes | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 15 min. rotation | 30 min. rotation | 15 min. rotation | 30 min. rotation | 15 min. rotation | 30 min. rotation | 15 min. rotation | 30 min. rotation |
| | | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| AD242/1* | 13 | 26 | 2 | 3 | 0 | 0 | 2 | 3 | 2 | 2 |
| D58/48A* | 13 | 26 | 3 | 5 | 0 | 1 | 3 | 4 | 3 | 2 |
| Type 1/119 | 13 | 26 | 32 | 59 | 17 | 45 | 15 | 14 | 4 | 8 |
| Type 1 | 13 | 26 | 98 | 100 | 84 | 99 | 14 | 1 | 5 | 1 |
| 327W | 12 | 24 | 100 | 100 | 92 | 100 | 8 | 0 | 3 | 0 |

Donor: G.G.W.; inoculum: 7.0 million; medium: donor's serum broth.

* These strains received one mouse passage before these experiments were begun.

*Reproducibility of the Ingestion Test.*—This was studied by analyzing the results of replicate tests on 5 different strains, using the same donor, type of medium, and inoculum size on different days. The medium used was donor's serum broth and the inocula were from suspensions

containing a chain count of 7,000,000. All tests were performed in duplicate, in which portions of the same plasma-leukocyte suspension and the same streptococcal suspension were added to the two tubes. In this way, the error involved in counts of slides from duplicate preparations made on the same day was determined, as well as the day-to-day variability of the test. The data are presented in Table II. The maximum day-to-day variability observed among 52 tubes in 26 tests with two strains which were highly resistant to ingestion was 3 and 4 per cent after 15 and 30 minutes, respectively. The maximum differences observed between any two duplicate tubes of the 26 sets was 3 and 2 per cent after 15 and 30 minutes, respectively. With three strains which were moderately or readily ingested the maximum day-to-day variability observed among 76 tubes in 38 tests was 15 and 14 per cent after 15 and 30 minutes, respectively. The maximum differences observed between any two duplicate tubes of 38 sets were 5 and 8 per cent after 15 and 30 minutes, respectively. During one experiment with 327W, a strain usually readily phagocytized by the donor, the amount of ingestion after 15 minutes' rotation was only 39 per cent, but reached 100 per cent after 30 minutes' rotation. This strain had been used in many other experiments as well as those reported in Table II, and such a low value for the 15 minute period had never been encountered. It is clear that some disturbing factor was operating in that reading, although we were unable to discover what the nature of it may have been. The low value was considered to be of such rare occurrence as to warrant its being dropped from the data in Table II, although it means that exceptional results can be encountered and must be guarded against or recognized when they occur. The results of these studies indicate that the test has a gratifying degree of reproducibility considering the complexity of the biological systems involved. Uniformity in results of repeated tests is higher when the streptococcal strain is highly resistant to phagocytosis than when the strain is highly susceptible or of intermediate degrees of susceptibility.

## RESULTS

Eleven strains of 7 different serological types of group A streptococci were tested for their ability to resist ingestion in the living compared to the heat-killed state.

For these tests the blood of a single donor was used, and the conditions which had been found by preliminary studies to be optimal for preserving ingestion resistance of the cocci were followed. These consisted of growing the bacteria in donor's serum broth, resuspending them in fresh broth of the same composition for heat killing, and using a minimal exposure to heat to kill the cocci, which was found to be 3 or 4 minutes at 56°C. For the purpose of these studies the cocci were considered heat-killed if no growth occurred on 48 hours' incubation of blood broth inoculated with as much of the contents of the rotator tubes containing the heat-treated suspension used in the test as could be removed with a Pasteur pipette.

Ten of the eleven strains showed no loss of resistance to ingestion as a result of heat killing (Table III), and one showed a moderate loss at 15 minutes (Type 1). None of the six strains that were highly resistant to ingestion in the living state showed a loss in resistance on being heat-killed. Two of these and four of the other strains, which in the living state were moderately or highly susceptible to ingestion, showed what appeared to be an actual increase in resistance to ingestion on being heat-killed, in one instance (Type 1/119), to a remarkable degree. That the reduced amount of ingestion of the heat-killed cocci was not due to the release of phagocytosis-inhibiting substances into the

medium in the course of heat treatment will be shown later. It is more likely that it results from proliferation of the living cocci in the course of the test period, giving a more abundant opportunity for phagocytosis to occur, than

TABLE III

*Effect of Heat-Killing on Ingestion of 11 Strains of Group A Streptococcus*

| Strain | Exposure time at 56°C. | Size of Inoculum | Per cent neutrophils containing streptococci | | | |
|---|---|---|---|---|---|---|
| | | | Living cocci | | Heat-killed cocci | |
| | | | 15 min. rotation | 30 min. rotation | 15 min. rotation | 30 min. rotation |
| | | *millions* | | | | |
| AD240 | 3 | 1.5 | 1 | 1 | 0 | 1 |
| AD242 | 4 | 7.0 | 2 | 5 | 1 | 1 |
| AD245 | 3 | 1.5 | 0 | 2 | 1 | 2 |
| AD454 | 4 | 7.0 | 5 | 7 | 2 | 5 |
| D58/48 | 3 | 8.0 | 3 | 10 | 3 | 1 |
| Type 28op | 3 | 7.0 | 2 | 7 | 3 | 7 |
| Type 28 | 3 | 7.0 | 13 | 37 | 10 | 24 |
| Type 28/143 Rb10 | 4 | 7.0 | 70 | 100 | 40 | 72 |
| Type 1/119 | 3 | 7.0 | 17 | 35 | 4 | 4 |
| Type 1 | 3 | 7.0 | 59 | 100 | 73 | 94 |
| 327W | 3 | 7.0 | 100 | — | 81 | 94 |

Donor: G.G.W.; medium: donor's serum broth.

TABLE IV

*Effect of Size of Initial Inoculum and of Proliferation of Living Streptococci on Amount of Ingestion*

| Size of inoculum | Per cent neutrophils containing streptococci | | | |
|---|---|---|---|---|
| | Living organisms | | 56°C. for 3 min. | |
| | 15 min. rotation | 30 min. rotation | 15 min. rotation | 30 min. rotation |
| *millions per ml.* | | | | |
| 1.2 | 37 | 93 | 37 | 52 |
| 1.5 | 61 | 100 | 48 | 71 |
| 2.8 | 69 | 99 | 60 | 82 |

Medium: filtered beef heart infusion broth; strain: 327W, Group A, Type 1.

in the preparations containing non-proliferating cocci. This view is supported by experiments with 327W (Table IV), a readily phagocytized strain. It was shown that as the initial inoculum, either of living or heat-killed cocci, was increased there was an increase in the amount of phagocytosis that occurred. Examination of the stained smears for the concentrations of extracellular cocci in the preparations demonstrated a relative decline in the number of

TABLE V

*Effect of Medium on Resistance to Ingestion of Heat-Killed Streptococci*

| Strain | Medium | Size of Inoculum | Per cent neutrophils containing streptococci | | | |
|---|---|---|---|---|---|---|
| | | | Living cocci | | Heat-killed cocci | |
| | | | 15 min. rotation | 30 min. rotation | 15 min. rotation | 30 min. rotation |
| | | *millions* | | | | |
| AD245 | Autoclaved | 1.5 | 2 | 2 | 3 | 7 |
| | Filtered | 1.5 | 1 | 2 | 1 | 2 |
| | Serum broth | 1.5 | 0 | 2 | 1 | 2 |
| AD240 | Autoclaved | 1.5 | 17 | 27 | 9 | 17 |
| | Filtered | 1.5 | 2 | 5 | 4 | 17 |
| | Serum broth | 1.5 | 1 | 1 | 0 | 1 |
| D58 | Autoclaved | 1.5 | 0 | 1 | 15 | 25 |
| | Filtered | 1.5 | 3 | 3 | 5 | 7 |
| | Filtered | 7.0 | 3 | 12 | 13 | 29 |
| | Serum broth | 1.5 | 0 | 3 | 1 | 2 |
| | Serum broth | 8.0 | 3 | 10 | 3 | 1 |
| Type 1/119 | Autoclaved | 7.0 | 6 | 17 | 20 | 33 |
| | Filtered | 1.5 | 1 | 4 | 4 | 10 |
| | Filtered | 7.4 | 8 | 26 | 10 | 26 |
| | Serum broth | 7.0 | 17 | 35 | 4 | 4 |
| AD242 | Autoclaved | 1.5 | 5 | 10 | 10 | 13 |
| | Filtered | 7.0 | 1 | 2 | 13 | 28 |
| | Serum broth* | 7.6 | 2 | 5 | 1 | 1 |
| Type 28op | Filtered | 7.0 | 4 | 22 | 34 | 75 |
| | Serum broth | 7.0 | 2 | 7 | 3 | 7 |

Donor: G.G.W.

* Exposure to heat: 56°C. 4 minutes; all others 3 minutes.

TABLE VI

*Effect of 3 and 45 Minutes' Exposure to 56°C. on Ingestion of Strain AD240*

| Amount of exposure to heat | Per cent neutrophils conaining streptococci | |
|---|---|---|
| | 15 min. rotation | 30 min. rotation |
| Unheated* | 1 | 1 |
| 56°C. 3 min. | 0 | 1 |
| Unheated‡ | 1 | 1 |
| 56°C. 45 min. | 10 | 23 |

Strain: AD240, group A, Type 6; medium: donor's serum broth; donor: G.G.W.; inoculum: 1.5 million.

* Kept at room temperature while treated suspension was being heated.

‡ Kept in ice bath while treated suspension was being heated.

cocci available for ingestion in the heat-killed preparations. In the living controls, although extensive ingestion had taken place, there were many more extracellular cocci than in the killed preparations, a result of proliferation of the cocci that had escaped ingestion. The effect of proliferation in the living preparations was thus equivalent to giving a larger initial inoculum, resulting in more ingestion of living than of killed cells.

TABLE VII

*Effect of Variable Exposure to Heat on Ingestion of Strain AD242*

| Amount of exposure to heat | Per cent neutrophils containing streptococci | |
|---|---|---|
| | 15 min. rotation | 30 min. rotation |
| Unheated (room temperature 10 min.) | 15 | 25 |
| Unheated (ice bath 30 min.) | 17 | 27 |
| 56°C. 10 min. | 15 | 34 |
| 56°C. 30 min. | 27 | 48 |
| 100°C. 10 min. | 33 | 65 |
| 100°C. 30 min. | 64 | 78 |

Strain: AD242, group A, Type 14; medium: autoclaved beef heart infusion broth; donor: E.M.T.; inoculum: 1.5 million.

*Effect of Medium on Ingestion of Heat-Killed Streptococci.*—

Three media were used for growing and resuspending the cocci during the heat-killed studies. In the order of their growth-promoting properties they were: (*a*) autoclaved beef heart infusion broth; (*b*) filtered beef heart infusion broth; (*c*) donor's serum broth. Five strains were studied using all three media and one strain using only the filtered medium and serum broth. (Table V).

From these studies it was concluded that serum broth was the optimal medium, because cocci of none of the 5 strains lost resistance to ingestion after exposure to 56°C. for 3 to 5 minutes, if they had been grown and resuspended in it, whereas with the autoclaved medium, 2 strains (D58 and Type 1/119) and with the filtered medium, 4 strains (AD240, D58, Type 28op, and AD242) showed losses of resistance.

*Effect of Degree of Heat on Resistance of Streptococci to Ingestion.*—It has been stated above that killing streptococci by a minimal exposure to heat usually does not cause a loss in their ability to resist phagocytosis. An investigation was made to determine whether exposures to greater amounts of heat, either by increasing the temperature of the bath or by prolonging the period of immersion, would cause a loss in the ingestion resistance of the cocci.

Strains AD240 and AD245 were studied for the effect of duration of exposure to 56°C. on ingestion. Both studies were done with the blood of a single donor (GGW). Donor's serum

broth was used for growing and resuspending the cocci since it did not precipitate at that temperature. The cocci exposed to 56°C. for 3 minutes and 45 minutes were tested in parallel with controls held at room temperature or in the ice bath for corresponding periods. The results were similar for the two strains studied, and data for one of these (AD240) are reported (Table VI).

There was no difference between the amount of ingestion of the living control suspension and that of cocci exposed to 56°C. for 3 minutes, but a marked increase in the amount of ingestion of cocci exposed to 56°C. for 45 minutes was observed compared with the controls.

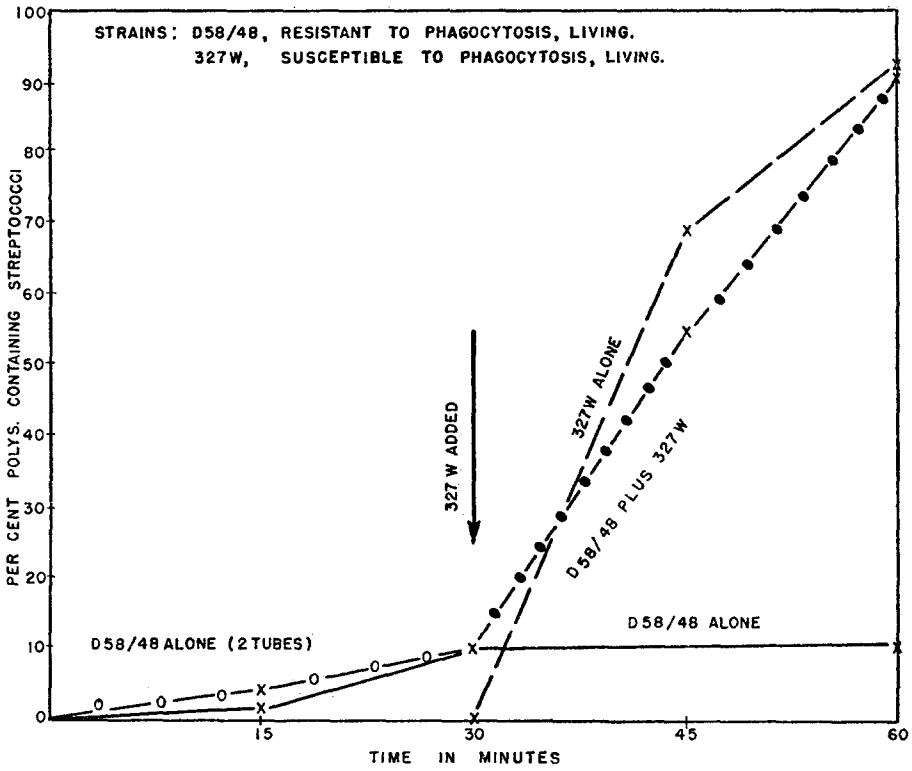
Studies of exposure to higher temperatures were complicated by the fact that serum-containing and filtered media formed a precipitate when immersed in the boiling water bath, and since it was feared the precipitate might be phagocytized, causing "blockade" of the leukocytes, autoclaved broth, which did not precipitate, had to be used for studies at that temperature. Furthermore, some of the strains showed marked agglutination when exposed to the higher temperatures and the strain (AD242) which showed the least tendency to agglutinate was used in these studies.

Studies with this strain were done with the plasma-leukocyte suspensions of two donors and since the results were similar in both cases, only those of one experiment are given here (Table VII). To each of six rotator tubes was pipetted 0.1 ml. of a suspension adjusted to contain 15,000,000 cocci and chains per ml. One tube was left at room temperature for 10 minutes and one was placed in an ice bath for 30 minutes, two were immersed in a 56°C. water bath for 10 minutes and 30 minutes, respectively, and two were placed in a boiling water bath for 10 and 30 minutes, respectively. After completion of these treatments the suspensions were tested for resistance to ingestion by phagocytes.

There was no difference in the amount of ingestion of the living cocci that had stood at room temperature for 10 minutes compared to the cocci that were held in an ice bath for 30 minutes. The difference in the amount of ingestion of the living cocci allowed to remain at room temperature for 10 minutes and that of cocci exposed to 56°C. for 10 minutes was within the experimental error of the method. Cocci exposed to 56°C. for 30 minutes and to 100°C. for 10 and 30 minutes, respectively, showed a loss of resistance to ingestion, and the loss was greater at the higher temperature and with the larger exposures.

*Tests for an Ingestion-Inhibiting Substance Released from the Streptococci during Heat Killing.*—The failure of leukocytes to phagocytize more heat-killed than living cocci could be interpreted to mean that, although the essentials for streptococcal proliferation were destroyed by heat, no significant change was made in those elements of the cell responsible for phagocytosis-resistance. Another possibility, however, was that in the course of heat treatment some substances were liberated from the cocci which injured the leukocytes and interfered with their ability to phagocytize. The fact that readily phagocytized strains were phagocytized somewhat less when heat-killed than when

living was compatible with this idea, although as we have shown above the factor of proliferation will account adequately for that difference. Experiments were done to discover whether rotating leukocytes with heat-killed, phagocytosis-resistant cocci that were suspended in the medium in which heat-killing had taken place, and which contained any hypothetical leukocyte-

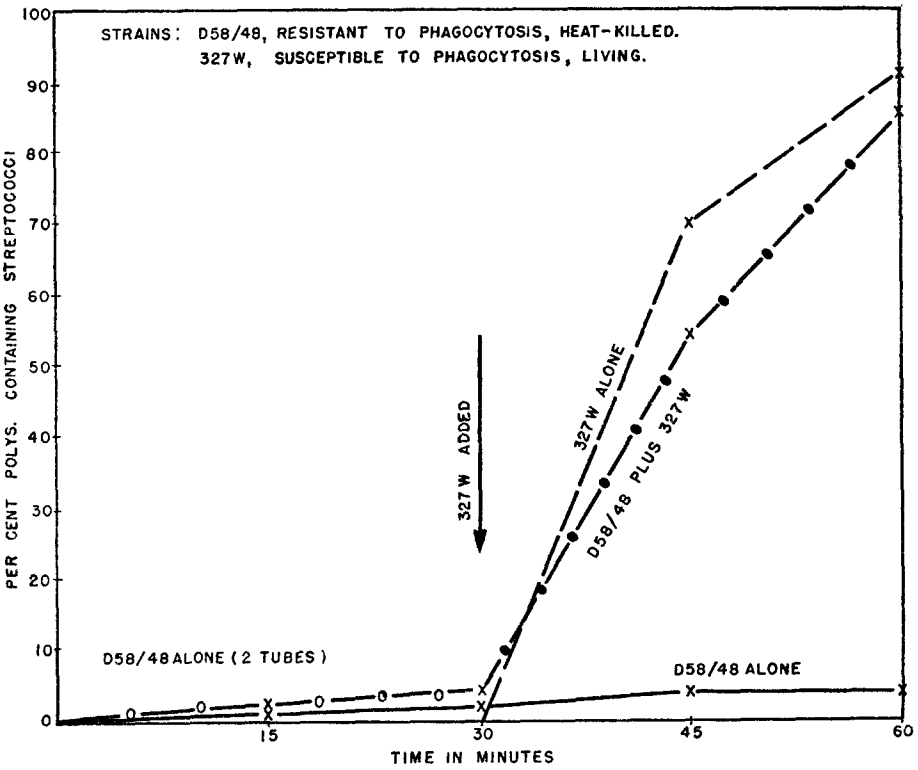



TEXT-FIG. 1. Failure of a living streptococcal suspension to inhibit the phagocytic ability of neutrophils.

injuring substances liberated during the heat treatment, would affect the ability of the leukocytes to phagocytize readily ingestible cocci added later. For purposes of comparison, other tubes were rotated containing mixtures of leukocytes and living cocci of the phagocytosis-resistant strain, to discover whether the proliferating, metabolizing cocci liberated any substances that interfered with the ability of phagocytes to ingest a susceptible strain added later.

A suspension of a 2 hour serum broth culture of the ingestion-resistant strain D58/48 was prepared which contained a chain count of 24,000,000. A similar suspension of the ingestion-susceptible strain 327W was prepared, containing a chain count of 54,000,000. To each of

four rotator tubes was delivered 0.1 ml. of the D58/48 suspension. Two of these tubes were submerged in a 56°C. water bath for 3 minutes. Saline and plasma-leukocyte suspension were added to all four tubes, which were then rotated for 30 minutes, coverslip preparations being made after 15 and 30 minutes' rotation. It was shown (Text-fig. 1) that no more than 10 per cent of the leukocytes in any tube had accomplished phagocytosis. To one tube containing heat-killed cocci, and to one tube containing living cocci, 0.1 ml. of the suspension 327W was added. To the other two tubes, which served as controls, 0.1 ml. of saline was added to equalize




TEXT-FIG. 2. Failure of a heat-killed suspension of streptococci to inhibit the phagocytic ability of neutrophils.

the volumes. To a fifth tube containing 327W and plasma-leukocyte mixture, but no D58/48 suspension, 0.15 ml. of saline was added to make the volume comparable to the other four tubes. All five tubes were then rotated for 30 minutes, coverslip preparations being prepared at the usual times. (Text-figs. 1 and 2).

The results showed that after 15 minutes' rotation the living susceptible cocci were ingested slightly less in the presence of both heat-killed and living resistant cocci than in their absence, the differences being 15 and 14 per cent, respectively, but this difference was no longer present after 30 minutes' rotation. These results indicate that substances capable of inhibiting ingestion to

a marked degree were not present in or liberated by suspensions of heat-killed or of living cocci of strain D58/48.

The absence of an ingestion-inhibiting substance was indicated also by testing another way.

Two 5.0 ml. cultures of D58/47 were prepared in donor's serum broth and incubated for 2 hours at 37°C. The cultures were distributed in four, 15 ml. centrifuge tubes which were rubber-stoppered and submerged in a 56°C. water bath for 5 minutes. The cocci were then washed three times with filtered broth and the sedimented organisms were resuspended in fresh broth, giving a chain count of 5.4 million. In an ingestion test the washed cocci were phagocytized only 1 and 2 per cent after 15 and 30 minutes' rotation, respectively.

These results indicate that the failure of large amounts of ingestion to occur was not due to any materials released into the medium in which the cocci

TABLE VIII

*Persistence of Resistance to Ingestion of Refrigerated, Heat-Killed Cocci.*

| Time after heat killing | Per cent neutrophils containing streptococci | |
|---|---|---|
| | 15 min. rotation | 30 min. rotation |
| 5 min. | 2 | 2 |
| 1 day | 2 | 1 |
| 6 days | 3 | 1 |
| 14 " | 3 | 4 |
| 23 " | 3 | 7 |

Strain: D58/48, heat-killed at 56°C. for 3 minutes and stored at 2°–4°C.; donor: G.G.W.; medium: donor's serum broth.

were suspended for heat treatment, for washing the cocci and removing any such possible materials failed to lead to an increase in phagocytosis.

*Persistence of Resistance to Ingestion of Stored Heat-Killed Streptococci.*—In the experiments described above, the heat-killed streptococci were tested within a few minutes of the heat treatment. To discover whether they would retain their ingestion-resisting ability for longer periods of time, the following tests were carried out.

0.1 ml. of a donor's serum broth suspension of D58/48, containing a chain count of 5.8 million was pipetted to a series of rotator tubes. The tubes were rubber-stoppered and submerged in a 56°C. water bath for 3 minutes. One tube was used to test the ability of the killed cocci to resist ingestion as soon as possible after the heat treatment. Half of the remaining tubes were stored at 2°C. and half were allowed to remain at room temperature. Ingestion tests were done at intervals and showed that no loss, or at most slight loss, of phagocytosis resistance occurred as long as the suspensions could be reliably tested. (Table VIII).

By 48 hours the cocci which had remained at room temperature were bare of capsules and marked agglutination had occurred, rendering the suspen-

sions unsuitable for testing. The refrigerated specimens stood up better. Marked agglutination did not occur for 23 days, although the capsule disappeared sometime between 6 and 14 days, as will be described in a later section.

*The Effect on Ingestion of Irradiating Streptococci with the Mercury Vapor Lamp.*—Since heat-killed streptococci retained their ability to resist ingestion, experiments were done to discover whether streptococci killed in another way would behave similarly.

Two ingestion-resistant strains, AD242 and D58/47, and one susceptible strain, 327W, were irradiated with a mercury vapor lamp in the following manner: 2 hour donor's serum broth cultures were centrifuged and resuspended in fresh donor's serum broth. They were counted and adjusted to contain 70,000,000 cocci per ml. The suspensions were kept in an ice

TABLE IX

*Effect of Exposure to Mercury Vapor Lamp on Ingestion Resistance*

| Strain | Per cent neutrophils containing streptococci | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 15 min. rotation | | | | | 30 min. rotation | | | | |
| | Exposure in min. | | | | | Exposure in min. | | | | |
| | Unexposed | 15 | 20 | 25 | 30 | Unexposed | 15 | 20 | 25 | 30 |
| AD242 | 4 | | 3 | 4 | 5 | 11 | | 5 | 7 | 12 |
| D58/47 | 3 | 3 | | 4 | 6 | 9 | 5 | | 7 | 9 |
| 327W | 94 | 73 | | 68 | 66 | 100 | 94 | | 93 | 97 |

Medium: donor's serum broth; inoculum: 7.0 millions; donor: G.G.W.

bath from the time of resuspension until used in the phagocytosis test, approximately 60 to 75 minutes. 3 ml. of suspension was delivered to an open Petri dish, 5 cm. in diameter, which rested on rubber supports in the center of a flat, round, glass dish, 18 cm. in diameter. The dish contained ice and water sufficient to reach about one-fourth the height of the small Petri dish. By rocking the entire system, which rested on a piece of rubber sponge, it was possible to keep both the bacterial suspension mixed and the ice bath changing around the smaller dish, so that the suspension was kept constantly cold. The suspension was irradiated for the times indicated in Table IX with a Hanovia mercury vapor lamp, Type 30600, without a filter. The lowest part of the quartz tube was 5 cm. from the surface of the streptococcal suspension. At desired intervals during irradiation, 0.1 ml. of suspension was pipetted to an appropriately labelled rotator tube. At the end of the irradiation period, the remaining suspension was pipetted to a test tube and incubated at 37°C. 0.1 ml. of the iced, unirradiated suspension was pipetted to a rotator tube and served as the living control. Wet india ink preparations of the suspensions that had been irradiated for 30 minutes showed that all cocci were encapsulated, although the capsules were smaller than those of the unirradiated control.

Although some cocci usually survived the irradiation, the results reported here are based on experiments in which the contents of the rotated tubes

were transferred as completely as possible to tubes of blood broth, and no growth occurred on incubating these tubes for 72 hours at 37°C., indicating that in these particular tubes viable streptococci were not present. As shown in Table IX the irradiated cocci of all three strains suffered no change in ability to resist phagocytosis.

The possibility existed here as with the heat-killed cocci that ingestion-depressing factors were released from the cocci during irradiation. An experiment was done similar in design to that used with the heat-killed cocci and which has been described above in detail. The results were similar, showing that living cocci of the susceptible strain 327W were ingested as well in the presence of an irradiated suspension of strain AD242 as in the control containing only susceptible cocci, which we interpret to mean that the failure of the resistant cocci to be ingested was not due to ingestion-depressing factors released to the medium from the irradiated cocci, or to changes induced in the medium by irradiation.

*Effect of Heat on the M Substance.*—That the M protein is a relatively heat-stable material is shown by the customary procedure of using heat-killed streptococcal suspensions as vaccines for making hyperimmune sera for precipitin typing. This involves immersion of the suspension in a water bath at 56°C. for 20 or 30 minutes. The cocci are killed by this treatment but the antigenicity of the M protein is preserved and appears to be as good as in living occci. Even more drastic treatment is used when making extracts for typing. The streptococcal cells are suspended in N/5 HCl and immersed in a boiling water bath for 10 minutes. The M that appears in the extract reacts well in precipitin tests, but its ability to produce antibodies on injection into rabbits is largely lost.

To get a better idea of the effect on M of the type of heat exposure used in these studies strain AD242 was grown in autoclaved beef heart-infusion broth, using larger amounts of broth and heavier suspension density for heat killing, corresponding to those used in serological studies rather than those usually employed in ingestion tests. The heated suspensions and the surrounding medium were tested for M content. Parts of the suspensions were used in parallel ingestion tests to show that the living and heat-killed cocci reacted typically in the light of other experience with phagocytosis of this strain.

Table X gives the experimental details and shows that although measurable amounts of M appeared in the surrounding fluid on heating at 56°C., much more was released at 100°C. However, there still remained large amounts of M on the coccal bodies, removable by extraction with hot acid.

Studies similar to those described immediately above have been done with three other strains (Types 1/119, D58/47, and AD240) with essentially similar results. In an additional experiment the supernate from a suspension of AD242 that had been immersed in a water bath at 56°C. for 3 minutes, (the exposure that had been used in many of the ingestion experiments with heat-

killed cocci), was tested with Type 14 antiserum and gave a ± reading after standing overnight. Supernate from a control portion of the same suspension that had stood at room temperature for 3 minutes formed no precipitate with the same serum.

Any study of the streptococcal M substance is hampered by two technical difficulties: first, the difficulty of removing all the M from the coccal bodies

TABLE X

*Effect of Heat on M Protein and Ingestion Resistance*

| Preparation | Per cent neutrophils containing cocci | | Capillary precipitin tests* | |
|---|---|---|---|---|
| | 15 min. rotation | 30 min. rotation | Anti-14 | Anti-36 |
| Living cocci from 2 hr. culture used for heat-treated preparations | 5 | 6 | | |
| Acid extract of unheated cocci | | | ++++ | − |
| Cocci exposed to 56°C. 10 min. | 8 | 14 | | |
| Supernate from cocci exposed to 56°C. 10 min. | | | + | − |
| Acid extract of cocci exposed to 56°C. 10 min. | | | ++++ | − |
| Cocci exposed to 100°C. 30 min. | 18 | 33 | | |
| Supernate from cocci exposed to 100°C. 30 min. | | | ++++ | − |
| Acid extract of cocci exposed to 100°C. 30 min. | | | ++++ | − |

Donor: G.G.W.

*Method.*—80 ml. of autoclaved beef heart–infusion broth was inoculated with 8 ml. of 18 hour blood broth culture of strain AD242 and incubated 2 hours at 37°C. The growth was centrifuged and the sediment resuspended in 0.5 ml. of fresh broth. This suspension was exposed to heat as indicated in the table, centrifuged and the supernates were neutralized and tested for serological activity. Crude acid extracts were prepared from heated and unheated cocci. Uninoculated broth was carried in parallel with the heated coccal suspensions and tested with the antisera. No reactions were observed. The living and heated suspensions were diluted with autoclaved broth to a chain count of 2.0 million for use in the ingestion tests.

* 24 hour readings of the capillary tests are reported.

and second, the lack of a method for determining quantitatively the M content of extracts. Nevertheless, the qualitative studies described above and the common experience of using heat-treated suspensions for serological studies of M and its antibodies make it clear that M is a rather highly heat-stable material. Although some M may leave the coccal bodies during heating, much remains associated with the cell and can exercise whatever function it possesses in preventing phagocytosis after heat treatment of the cocci.

*Effect of Heat on the Capsule.*—All the strains in this study, whether highly resistant or highly susceptible to phagocytosis, produced good capsules when grown in serum broth, as shown by wet india ink preparations. In cultures

of the type used in the ingestion test the capsule appears early, reaches maximal size at 2 to 3 hours, and declines in size thereafter, being mostly gone by 5 hours. Our interest centered in the capsule as an anatomical structure and no quantitative chemical analyses have been made of the hyaluronic acid attached to the coccal bodies or released into the medium during growth or heat treatment.

Short exposures to 56°C. did not cause the cocci to lose their capsules if they had been grown and resuspended for heat treatment in serum broth. This is illustrated in Figs. 1 to 8 which show wet india ink preparations made from a 2 hour culture of strain D58/48, before and after being heated at 56°C. for 3 minutes. The capsule is seen to persist as a well defined anatomical structure. When a portion of the heated culture was kept at room temperature overnight the cocci became denuded. However, when the heated suspension was stored in the refrigerator at 2–4°C., the capsule persisted, diminishing somewhat in size, for at least 6 days, but was lost from most of the cocci after 14 days' storage. These photographs were made from the same suspensions that furnished the data given in Table VIII.

It is clear that, as in the case of the M susbstance, the capsule survives an amount of heat which kills the cocci but which fails to reduce their resistance to phagocytosis.

The constitution of the medium in which streptococci are grown has an effect on the development of the capsule. Some strains produce little or no demonstrable capsule when grown in autoclaved beef infusion broth, but produce a well developed capsule in serum broth. Other strains produce a good capsule no matter what the medium. Serum also has a definite protective effect on the susceptibility of the capsule to injury during heat treatment since, if it were omitted, total or marked loss of the capsule was commonly observed. A point of technical importance is that when streptococci were grown in serum broth, centrifuged, re-suspended in fresh serum broth and then immediately placed in the 56°C. water bath, considerable loss of capsule occurred. If, however, the suspensions were allowed to stand at room temperature for 15 minutes or so after being resuspended and before the usual 3 or 4 minute heat treatment, the capsule suffered little or no change.

When, for one reason or another, the capsule is lost, the cocci usually agglutinate into clumps and have a tendency to adhere to platelet masses if they are then mixed with blood. Although we do not have sufficient pertinent data to allow an analysis of the effect this has on the number of leukocytes that accomplish phagocytosis, it was considered an undesirable occurrence and the material reported here has, whenever possible, been done with encapsulated, unagglutinated suspensions. Exceptions are found in the studies in which serum-free media were used and in which more than the minimum exposure to heat was employed.

## DISCUSSION

The use of killed streptococci makes it possible to study phagocytosis of non-proliferating organisms, thereby eliminating the influence of increasing bacterial numbers during the test, and eliminating the effect of any diffusible bacterial toxins and metabolic products normally released by proliferating organisms. It also makes possible the evaluation of the role in resistance or susceptibility to phagocytosis of stable elements of the cell which survive a lethal agent, and this role can be studied in the absence of the active metabolic processes going on during proliferation. The work presented here demonstrates that resistance to ingestion does not depend on the cocci being in an actively dividing state, but persists after the bacteria are killed by heat or mercury arc irradiation. Some exploratory experiments have been performed in which cocci killed with free chlorine were found to lose, partially at least, their resistance to phagocytosis, and it may be supposed that the chlorine caused an alteration in the substances responsible for ingestion resistance.

Some comment may be appropriate here about the state of the killed cocci. The criterion we have used for death of the cells has been that they are incapable of proliferating when transferred to an adequate medium known to support excellent growth of living streptococci from small inocula. Work of Heinmetz (9, 10) and others has shown that bacteria thought to be killed by heat or other agents may be revived under special conditions, specifically when certain members of the Krebs cycle are added to the medium. It is quite probable that several enzymic functions of the streptococcus may survive a brief exposure to 56°C. but there does not seem to have been any work done on such persisting functions. We know that the heat- and irradiation-killed cocci used in these studies do not revive or proliferate in the course of the test as it is run, because terminal subcultures have invariably been sterile. In the experiments in which the cocci were stored for several weeks after lethal exposure to heat, it is reasonable to suppose that there may have been further decline in any undefined persisting metabolic capacities; but if so, this was not reflected in an increased susceptibility to phagocytosis. Thus, although it cannot be said that residual metabolic activities could not be playing a role in the persisting resistance of the cocci to ingestion by phagocytes, it seems more reasonable to attribute this persistence to surviving structural elements of the cell. This view is supported by some preliminary evidence showing that a brief treatment of the heat-killed cocci with active trypsin results in a dramatic and almost complete loss of phagocytosis resistance.

Reque (11) in 1906 and Rosenow (12) in the same year showed that the capacity of diphtheria bacilli and pneumococci to resist phagocytosis persisted

after lethal exposures to heat. Since this is also true of the streptococci, it is quite possibly a general phenomenon among bacteria.

The work presented here showing that with minimal exposure of cocci to heat there are slight if any changes in the M substance and in the capsule as an anatomical structure, and at the same time little or no change in the capacity of the cocci to resist phagocytosis, is compatible with the idea that these substances are in part if not entirely responsible for that resistance.

Evidence of such a role for these substances consists of the demonstration that mouse-virulent strains almost always contain M (13); that mice may be protected from infection by virulent strains with anti-M serum, but not with antibodies for other cellular components (14); that in bactericidal tests strains containing M are capable of growing in whole human blood whereas strains without M are destroyed by the phagocytes (15); that strains that have acquired increased virulence for mice through serial intraperitoneal passage also, if their M content was low to begin with, have an increase in M content (13); that some strains grown on artificial media show a reduction in M production and mouse virulence and that a recovery from this degraded state by growth in serum-containing medium or animal passage is associated with increased M production and mouse virulence (2, 13, 16); that removal of the capsule by hyaluronidase results in a moderate decrease in virulence which is associated with a moderate increase in susceptibility to phagocytosis, Rothbard (1), Kass and Seastone (17); and that digestion of living cocci by active trypsin, which is known to digest M without affecting viability of cocci, leads to an increased susceptibility to phagocytosis, Morris and Seastone (18). Much of this work rests on the assumption that virulence and the ability to resist phagocytosis can be equated, as suggested early by Marchand (2), Denys and LeClef (19) and other workers.

There seems to be little doubt of a close relationship between M production and the virulence and phagocytosis resistance of a strain. To a lesser extent the capsule is similarly involved. That there may be other structural elements concerned in virulence aside from M and the capsule is entirely possible, but the nature, or indeed, even the existence of such substances has not been demonstrated. Although it has often been shown that strains that are virulent for a particular host are able to resist phagocytosis by the leukocytes of that host, it has not been shown on an adequate basis that all the phenomena of streptococcal virulence can be explained simply by resistance or susceptibility of the cocci to the host's phagocytes. The studies by Wood (20) of the phenomenon of surface phagocytosis raise questions concerning the adequacy of customary *in vitro* methods, of which the method we have employed is one, to give a complete and accurate picture of phagocyte-bacterium interactions as they may occur in host tissues. Much more work needs to be done along these lines.

The work presented here indicates that the elements responsible for phago-

cytosis resistance of group A streptococci are cellular components that remain effective after the cocci have been rendered non-proliferative. Investigation of the antiphagocytic capacities of the cocci may profitably be directed towards the nature of these substances, the conditions under which they are synthesized by the cell, and the mechanism by which they prevent ingestion by the phagocytes.

## CONCLUSION

Living group A streptococci which resist ingestion by human neutrophils in an *in vitro* test still do so after they have been killed by exposure to heat or to mercury arc irradiation.

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# PLATE

## EXPLANATION OF PLATE 1

Photographs of wet India ink preparations of a streptococcal suspension (strain D58/48) which was grown for 2 hours in serum broth, centrifuged, resuspended in fresh serum broth, and immersed in the 56°C. water bath for 3 minutes. Subcultures demonstrated that the culture had been sterilized. One portion of the suspension was stored at room temperature for 24 hours. Other portions were stored at 2–4°C. for 23 days, and were tested periodically for persistence of capsule and resistance to phagocytosis.

The photographs were taken with American Optical Company 1.8 mm. dark medium phase contrast objective and condenser which had been adapted to the Leitz panphot. A Strobinar IV electronic flashlight (Heiland Research Corp., Denver) giving an exposure time of approximately $\frac{1}{1000}$ second served as light source. Magnification on the film was 1100 and on the photographs 2600.

FIG. 1. Fresh, living resuspended cocci.

FIG. 2. Shortly after heating at 56°C. for 3 minutes. The cocci have been killed but the capsule persists.

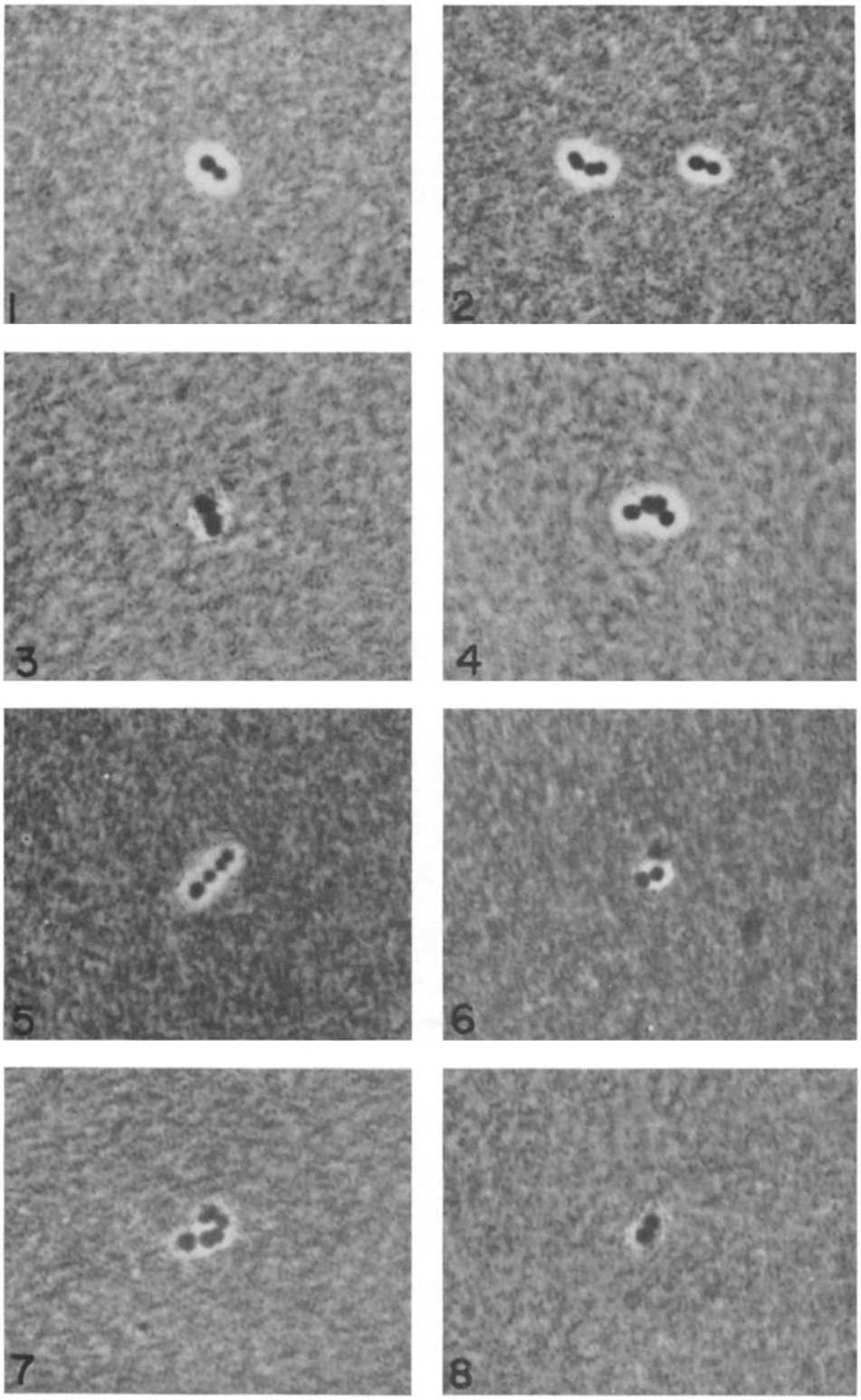
FIG. 3. After standing at room temperature 24 hours. The capsule has disappeared.

FIG. 4. After storage at 2–4°C. for 24 hours. The capsule persists.

FIG. 5. After storage at 2–4°C. for 48 hours. The capsule is still present, but reduced in size.

FIG. 6. After storage at 2–4°C. for 6 days. A diplococcus showing one encapsulated and one unencapsulated coccus.

FIGS. 7 and 8. After storage at 2–4°C. for 14 days. A few cocci are still encapsulated (Fig. 7) but most are denuded (Fig. 8).




(Wiley and Wilson: Ingestion resistance of killed streptococci)