THE PHAGOCYTOSIS AND INACTIVATION OF STAPHYLOCOCCI BY MACROPHAGES OF NORMAL RABBITS*

By G. B. MACKANESS‡, M.B., D.PHIL.

(From The Rockefeller Institute)

(Received for publication, February 23, 1960)

The attempts which have been made to study the fate of staphylococci in phagocytic cells have led to conflicting reports. Cohn and Morse (1) studied the fate of staphylococci in almost pure suspensions of rabbit polymorphonuclear leucocytes. Their method permitted simultaneous measurement of both extracellular and intracellular bacterial populations, and showed that the fate of staphylococci was critically dependent upon the efficiency of phagocytosis. Whereas coagulase-negative staphylococci were readily ingested and rapidly killed in normal serum, coagulase-positive staphylococci survived because of their resistance to phagocytosis. In immune serum, in which comparable rates of phagocytosis prevailed, coagulase-positive and coagulase-negative staphylococci were both effectively inactivated by rabbit polymorphonuclear leucocytes. The surface component of a strain of *S. aureus* which confers resistance to phagocytosis has since been purified by Morse (2) and shown to be a mucopeptide.

In contrast with these findings, other workers have emphasized the apparent survival of coagulase-positive staphylococci in suspensions of human polymorphonuclear leucocytes (3–5). The cells for these studies were obtained from whole blood, and were contaminated with other cell types. Since it has been reported that coagulase-positive staphylococci survive, and perhaps multiply, within rabbit mononuclear phagocytes (5–7), it was thought that a real difference between the fate of staphylococci in mononuclear and polymorphonuclear phagocytes might afford an explanation for the contradictory reports of those working with pure and mixed cell populations.

The methods used to study mononuclear cells have been ingenious cultural procedures, but they have not been strictly quantitative. In the present work, the quantitative method of Cohn and Morse was used. One objective was to learn the importance of separating the intracellular from the extracellular phase of the interaction of cells and bacteria; the other was to determine

* The work was done during the tenure of a National Institutes of Health Postdoctoral Fellowship.

‡ Present address: Department of Experimental Pathology, John Curtin School of Medical Research, Australian National University, Canberra.

whether a real difference exists between the fate of staphylococci in polymorphonuclear and mononuclear phagocytes.

Materials and Methods

Macrophage Suspensions.—Mononuclear cell exudates were produced in the peritoneal cavity of rabbits by the injection of 50 ml. of sterile heavy mineral oil. The content of granulocytes in exudates produced in this way varies with the time of harvest and the irritant properties of the oil. Some exudates contained as many as 10 per cent granulocytes. For most experiments, however, the oil used was almost devoid of irritant properties,[1] and often the exudates produced with it contained less than 1 per cent granulocytes. The cells were recovered after 4 days by injecting 250 ml. of saline, and then aspirating the contents of the peritoneal cavity to a separatory funnel. The cell suspension was then delivered through gauze into siliconed centrifuge tubes. The cells were recovered by centrifuging (75 *g*) and resuspending the deposit in sterile Gey's solution at pH 7.4. Total and differential cell counts were made.

Bacteria.—Two strains of staphylococci were used: *Staph. aureus* (Smith), a coagulase-positive strain isolated from osteomyelitis in man; and *Staph. albus* (Mendita), a coagulase-negative strain isolated from a nasal swabbing.

Both strains have been maintained in the laboratories of Dr. René J. Dubos. The organisms were grown in penassay broth (Difco Laboratories, Detroit). The age at which the culture was used is given for each experiment. The bacteria were washed twice by centrifuging (2000 *g*) for 10 minutes, and resuspending the deposit in an equal volume of Gey's solution containing 0.01 per cent bovine albumin (Armour & Co., Chicago). The final suspension contained approximately 5×10^8 viable bacteria per ml.

Serum.—Normal serum was obtained from the animals used to produce exudates. The animal was bled from the heart before recovery of an exudate. After its separation, the serum was stored at −20°C. for use in subsequent experiments. *Immune serum*[2] was obtained from two rabbits which had received multiple injections of a heat-killed suspension of *S. aureus* (Smith) (1). The agglutinin titers of these sera were 1:640 and 1:1280. They were stored at −20°C., and at the time of use were diluted 1:2 with fresh serum to provide a source of complement.

Suspension of Cells and Bacteria.—A suspension containing 5×10^7 macrophages per ml. was delivered into one or more 15 × 120 mm. paraffin-lined test tubes. Additions of normal or immune serum and of bacterial suspensions were then made in proportions which gave a final serum concentration of 10 per cent, and approximately equal numbers of cells and bacteria at concentrations of 3 to 4×10^7 per ml. In some experiments, when insufficient cells were available to achieve this density, the same ratio of bacteria to cells was preserved by appropriate dilution of the bacterial suspension. The total volume in each tube did not exceed 4.0 ml. The mixtures were incubated in a reciprocating water bath shaker at 37.5°C.

Bacterial Enumeration.—Samples were removed from the tubes at prescribed intervals for estimation of the total number of viable organisms present in the mixture, the proportion of these remaining in the extracellular phase, and the proportion associated with or contained within cells (1).

Total Number of Viable Bacteria.—A sample of 0.2 ml. was delivered into a tube containing 1.8 ml. of penassay broth. After two cycles of freezing and thawing in CO_2 and alcohol, the suspension was homogenized for 1 minute in a high-speed teflon homogenizer. The homogenate was serially diluted in penassay broth, and 0.1 ml. aliquots of these were spread on the dried

[1] Nujol, Plough, Inc., New York.

[2] Immune serum was kindly supplied by Dr. S. I. Morse

surface of penassay agar in quadrant plates. Colony counts were made after incubation for 16 to 18 hours.

Viable Extracellular Bacteria.—At the same time a sample of 0.2 ml. was delivered into a tube containing 3.8 ml. of a 0.01 per cent solution of bovine albumin in Gey's solution. After thorough mixing the cells were deposited by centrifuging lightly (50 *g*) for 4 to 5 minutes, leaving uningested bacteria in suspension. The upper half of the supernatant fluid was withdrawn, homogenized, and plated as before.

Viable Intracellular Bacteria.—After careful removal of the remaining supernatant fluid the cell deposit was resuspended in 2.0 ml. of penassay broth. The cells were then disrupted, diluted, and plated as before.

All bacterial counts were expressed as bacteria per milliliter of the original suspension. The procedure for disrupting macrophages was shown to have no effect upon the viability of staphylococci.

At each sampling a smear stained with Wright's stain and a slide-coverslip preparation were made. These were used to study the morphology and the viability of bacteria and cells respectively.

Variations of procedure are described in appropriate parts of the text.

RESULTS

The Phagocytosis and Survival of Coagulase-Positive Staphylococci in the Presence of Macrophages and Normal or Immune Serum.—

The cell suspension used contained 94 per cent macrophages, 5 per cent granulocytes, and 1 per cent lymphocytes. The bacterial suspension was a washed stationary phase culture (16 hour) of *S. aureus* (Smith). Equal volumes of a mixture of cells and bacteria were dispensed in two paraffin-lined tubes. Normal serum was added to one and immune serum to the other. After mixing, initial samples were taken and the tubes were incubated with shaking at 37.5°. Further samples were taken at prescribed intervals.

The rate of phagocytosis in a mixture of macrophages and bacteria can be inferred from the numbers of extracellular bacteria which disappear from the cell suspension. The relative rates of phagocytosis in normal and immune serum can therefore be compared on the basis of the rates of fall of the supernatant counts in Table I. In immune serum, 90 per cent of bacteria had disappeared from the extracellular phase within 30 minutes; this degree of phagocytosis was not achieved in normal serum at the end of 180 minutes. In Fig. 1 the results of an identical experiment have been plotted. In this experiment, in which polymorphonuclear leucocytes accounted for 3 per cent of the cells present, an even greater difference was found between the rates of phagocytosis in normal and immune serum.

In keeping with these differing rates of phagocytosis, the rates of inactivation of bacteria in normal and immune serum were also different. This can be seen from the total viable counts of Table I and Fig. 1. It should be noted in both experiments that when immune serum was present almost all of the viable bacteria were found in the cell deposit within 1 hour of the commencement of incubation.

TABLE I

Comparison of the Phagocytosis and Killing of S. aureus by Normal Rabbit Macrophages in the Presence of Normal and Immune Serum

Serum	Viable counts	Bacteria/ml. suspension				
		Zero	30 min.	60 min.	120 min.	180 min.
Normal	Total	7.3×10^7	6.2×10^7	4.4×10^7	2.6×10^7	1.6×10^7
	Supernatant	7.9×10^7	3.9×10^7	1.9×10^7	1.4×10^7	9.0×10^6
	Deposit	2.1×10^4	2.1×10^7	2.5×10^7	1.0×10^7	5.0×10^6
Immune	Total	6.0×10^7	1.9×10^7	8.8×10^6	6.1×10^6	5.9×10^6
	Supernatant	6.0×10^7	4.2×10^6	9.0×10^5	2.7×10^5	2.2×10^5
	Deposit	1.2×10^4	1.2×10^7	8.0×10^6	5.9×10^6	5.9×10^6

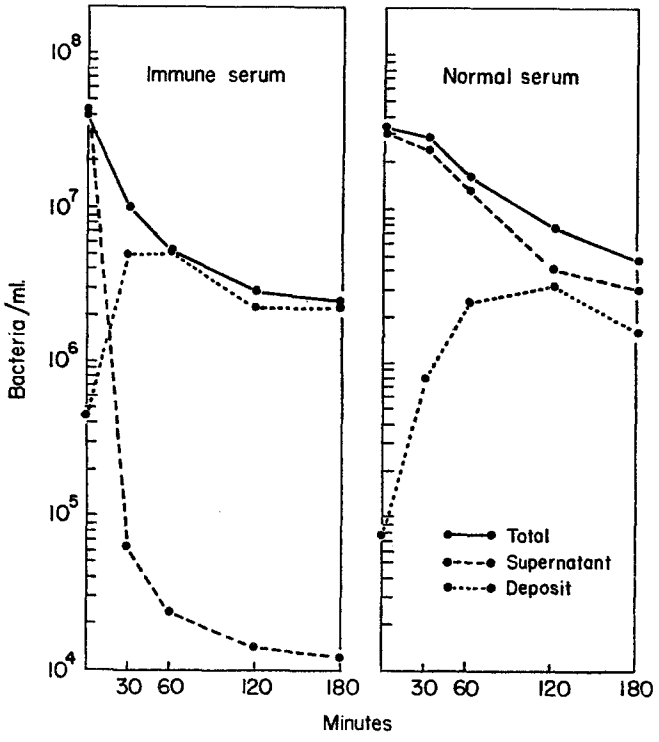

FIG. 1. Viable bacterial counts showing the distribution and survival of *S. aureus* (Smith) during phagocytosis by normal rabbit macrophages in the presence of normal or immune serum.

The foregoing results with macrophages showed an opsonizing effect of immune serum which was identical with that found by Cohn and Morse (1) using polymorphonuclear leucocytes. Moreover, the absolute rates of ingestion of *S. aureus* (Smith) by granulocytes and macrophages in the presence of immune serum appeared to be comparable. However, the subsequent fate of the bacteria in the two cell types was not identical. Whereas with polymorphonuclear leucocytes the total viable count, at least during the first 2 hours, consisted almost exclusively of extracellular bacteria (1), this was not so with macrophages (Fig. 1). Apparently staphylococci are inactivated very rapidly within the former, but much more slowly within macrophages. Furthermore, the number of bacteria surviving in association with the phagocytes is much higher with macrophages than with granulocytes.

A further conspicuous difference between the two types of phagocyte was revealed in stained smears. Whereas almost all ingested bacteria remained morphologically intact for several hours within macrophages, they soon disappeared from the cytoplasm of polymorphonuclear leucocytes. The slower rate of inactivation and the prolonged morphological integrity of staphylococci within macrophages suggest that the mechanism of the antibacterial action of this cell differs from that of the granulocyte.

In the foregoing experiments, the cell suspensions which were used contained 3 and 5 per cent polymorphonuclear leucocytes. It seemed possible that these cells could have contributed disproportionately to the observed inactivation of bacteria. However, in ten experiments in which the fate of coagulase-positive staphylococci was followed from zero time, a similar pattern emerged. Although the granulocyte content of the cell suspensions ranged from less than 0.1 to 15 per cent, the percentage of bacteria surviving at the end of 180 minutes varied only between 3 and 10 per cent. It happened that the most effective inactivation occurred with a cell suspension containing less than 0.1 per cent granulocytes.

The Interaction of S. aureus and S. albus with Rabbit Macrophages.—Cohn and Morse (1) found that *S. albus* (Mendita) was readily ingested by granulocytes in the presence of normal serum, but that *S. aureus* (Smith) was not. It was found with macrophages that a similar situation exists. Since a comparison of the intracellular fates of these two strains in macrophages could only be made if comparable rates of phagocytosis prevailed, it was necessary to add immune serum to the suspension containing *S. aureus*.

Sixteen-hour cultures of *S. aureus* (Smith) and *S. albus* (Mendita) were prepared in the usual way. They were added in approximately equal numbers to a suspension of cells containing 1.5 per cent granulocytes and 97 per cent macrophages. Immune serum was added to the suspension containing *S. aureus* and normal serum to the other. Bacterial counts were made in the usual way.

A comparison of the rates of phagocytosis and survival of *S. aureus* and *S. albus* in the presence of rabbit macrophages is shown in Fig. 2. It will be seen that the ingestion and inactivation of the two strains were similar. In each case, the bacteria surviving after 180 minutes were comparable in number, and located in association with the cells; they comprised between 2 and 3 per cent of the original populations. In stained smears the ingested bacteria of

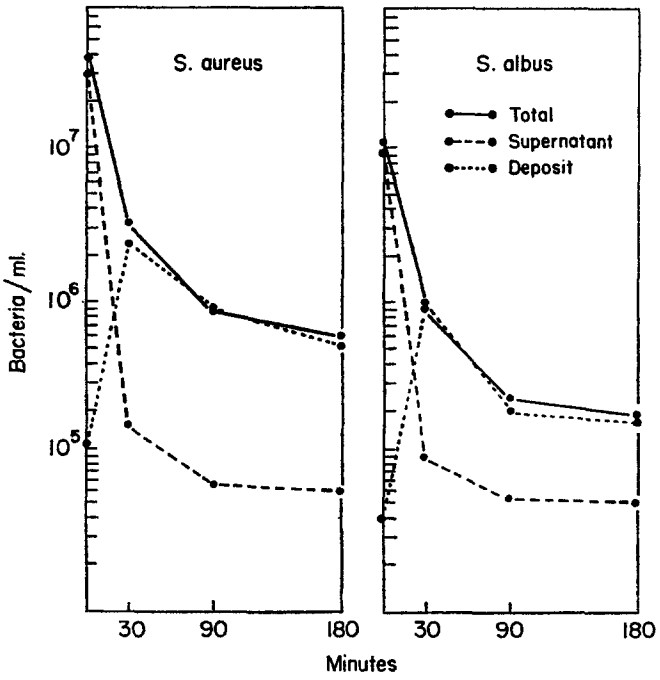

FIG. 2. Viable bacterial counts showing the phagocytosis and survival of *S. albus* and *S. aureus* in the presence of normal rabbit macrophages, and normal and immune serum respectively.

both strains were still morphologically intact at the end of 180 minutes of incubation.

Penicillin and Streptomycin Sensitivity of S. aureus (Smith).—In the experiments which follow, use was made of streptomycin or penicillin to kill or inhibit extracellular bacteria. Although these substances are known to be relatively ineffective against intracellular organisms, the drug concentrations used for this purpose must be carefully appraised for any effect they may have on intracellular bacteria.

The respective minimal bacteriostatic concentrations of penicillin and streptomycin were determined for an inoculum of 10^6 *S. aureus* (Smith) grown in penassay broth. They were

0.1 and 2.5 μg./ml. when read after 4 hours' incubation; and 0.1 and 20 μg./ml. after 16 hours' incubation. The bactericidal activities of the 4 hour minimal bacteriostatic concentrations of the two drugs were then determined in a medium of 10 per cent rabbit serum in Gey's solution, using an inoculum of 10^6 washed bacteria from a 4 hour culture in penassay broth. The suspension was shaken at 37.5°, and samples were taken at intervals for dilution and plating.

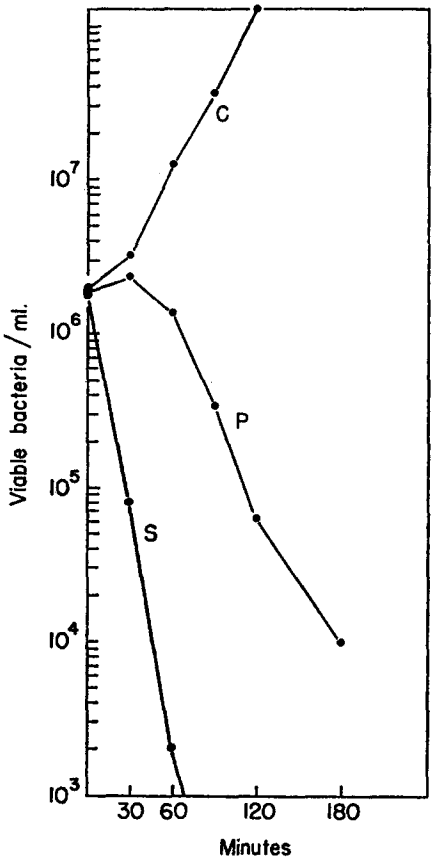

FIG. 3. Viable counts showing the bactericidal activities of streptomycin (*S*) (2.5 μg./ml.), and penicillin (*P*) (0.1 μg./ml.), upon *S. aureus* (Smith) in a medium of 10 per cent rabbit serum in Gey's solution. *C* = untreated control.

These conditions were chosen because they corresponded to the conditions under which the drugs were to be used experimentally. The results are given in the survival curves of Fig. 3.

Any potential effect of the minimal bacteriostatic concentrations of penicillin or streptomycin on intracellular staphylococci was then assessed by comparing the survival of staphylococci in suspensions of parasitized macrophages during exposure to varying concentrations of either drug.

A suspension of macrophages in 10 per cent immune serum was incubated for 25 minutes with a washed suspension of *S. aureus*. The cells were then washed 3 times to remove ingested bacteria, and were resuspended in 10 per cent serum in Gey's solution. Aliquots of the parasitized cell suspension were then added to paraffin-lined tubes containing varying concentrations of penicillin and streptomycin. The final drug concentrations ranged upwards from the minimal bacteriostatic concentrations. Total bacterial counts were made in the usual way at zero time and after 1 and 2 hours of incubation.

Table II shows the numbers of intracellular bacteria which remained viable within macrophages during exposure to varying concentrations of penicillin and streptomycin. Since viability was unaffected by either drug, at any of the concentrations tested, it was assumed that the minimal bacteriostatic concentrations (the lowest of the concentrations tested) could safely be used to

TABLE II

Two Experiments Showing the Effect of Penicillin and Streptomycin on the Survival of Intracellular S. aureus (Smith) in Normal Rabbit Macrophages

Time	Bacteria/ml. suspension							
	Penicillin, μg./ml.				Streptomycin, μg./ml.			
	0	0.1	0.5	2.5	0	2.5	5	10
min.								
Zero	2.4×10^5	2.4×10^5	2.6×10^5	2.5×10^5	7.8×10^5	6.9×10^5	7.2×10^5	6.8×10^5
60	2.1×10^5	2.1×10^5	2.2×10^5	2.2×10^5	6.9×10^5	6.4×10^5	7.1×10^5	7.4×10^5
120	2.0×10^5	1.7×10^5	1.9×10^5	2.1×10^5	7.8×10^5	6.5×10^5	6.4×10^5	6.1×10^5

inhibit extracellular bacteria without affecting the viability of bacteria within cells.

The Effect of Immune Serum on the Survival of Intracellular Staphylococci.—In a previous section it was shown that coagulase-positive and coagulase-negative staphylococci were inactivated to a similar extent by normal rabbit macrophages. In order to show this, however, the coagulase-positive staphylococci were sensitized with immune serum in order to promote an adequate rate of phagocytosis. Conceivably, the treatment of bacteria with antibody might also have made them more susceptible to inactivation within macrophages. An effect of antibody upon the fate of bacteria after phagocytosis has neither been demonstrated nor excluded as a possibility. Advantage was taken of the relatively slow inactivation of staphylococci within macrophages to determine whether sensitization with antibody does in fact influence their intracellular survival.

Equal volumes of a suspension of macrophages containing 3 per cent granulocytes were added to two paraffin-lined tubes. Normal serum was added to one, and immune serum to the other. A suspension of *S. aureus* (Smith), prepared in the usual way, was added to the

tube containing immune serum. A 10-fold concentration of the same bacterial suspension was added to the tube containing the normal serum. The mixtures were incubated with mixing for 20 minutes at 37.5°C. The tubes were then chilled and the cells were washed 3 times with 5.0 ml. of Gey's solution containing 5 per cent serum. The parasitized cells were finally suspended in 10 per cent serum containing 2.5 μg. of streptomycin per ml. The streptomycin was added because it was not known whether uningested bacteria would be effectively removed by washing. The tubes were now incubated, and samples were taken at intervals for estimation of the total numbers of viable bacteria present.

The uptake of *S. aureus* proceeds very slowly in the absence of immune serum. To compensate for this a higher multiplicity of organisms was used

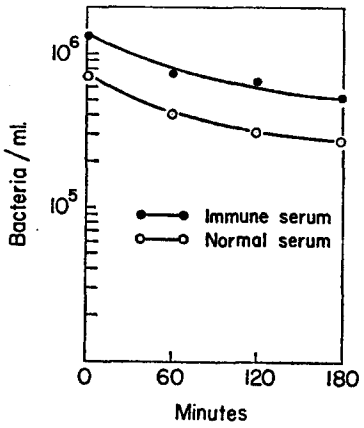

FIG. 4. Viable counts showing the survival of two predominantly intracellular populations of *S. aureus* ingested by normal macrophages in the presence of normal or immune rabbit serum.

during phagocytosis in normal than in immune serum. It was hoped that the two macrophage suspensions obtained in this way would be comparable with respect to the numbers of sensitized or unsensitized staphylococci present within cells. However, the data plotted in Fig. 4 show that, despite the 10-fold higher multiplicity, fewer organisms were ingested in normal than in immune serum. This was confirmed in the smears made at zero time. These showed approximately twice as many bacteria in cells containing sensitized organisms. This difference does not alter the significance of the survival curves shown in Fig. 4, which indicate that the sensitized bacteria were no more susceptible than the unsensitized bacteria to inactivation by macrophages.

The Influence of the Age of Bacteria on Their Survival in Macrophages.—Extracellular organisms are potentially capable of multiplication during the course of an experiment, and of contributing unknown numbers of organisms to the total population. Cohn and Morse (1) used organisms from a stationary phase culture and conducted their experiments within the period of the lag

phase of growth. This measure, which was also adopted in most of the present experiments, was intended to minimize the error due to extracellular multiplication. However, there may be biologically important differences between the intracellular survival of lag phase organisms, and of those ingested in an active phase of their growth cycle.

A subculture was made from a 13 hour penassay broth culture of *S. aureus* (Smith). Four hours later the subculture and the original culture were washed in the usual way and resuspended to equivalent optical densities. Equal volumes of a cell suspension, containing 95 per cent macrophages and 3 per cent granulocytes, were dispensed in four paraffin-lined tubes. Bacteria from the 4 hour culture were added to two of them, and from the 17 hour culture to the other pair. Following the addition of immune serum, the cell suspensions were incubated as usual. After 55 minutes, streptomycin (2.5 μg./ml.) was added to one tube of each pair. Samples were taken at prescribed intervals and treated in the usual way for estimation of the total number of viable organisms present.

TABLE III

Total Viable Counts of Bacteria from 4-Hour and 17-Hour Cultures of S. aureus during Phagocytosis by Rabbit Macrophages. Streptomycin (2.5 μg./ml.) Was Added after 55 Minutes of Incubation to One of Each Pair of Cell Suspensions

Bacterial culture	Bacteria/ml.			
	Zero	60 min.	120 min.	180 min.
4 hr.	3.0×10^7	2.4×10^6	1.9×10^6	2.0×10^6
4 hr. + STM	2.8×10^7	1.8×10^6	1.1×10^6	7.8×10^5
17 hr.	4.0×10^7	2.8×10^6	1.4×10^6	1.2×10^6
17 hr. + STM	3.7×10^7	1.8×10^6	1.1×10^6	6.0×10^5

The results of intracellular survival of bacteria which had been ingested either in the log phase or the lag phase of their growth cycle, appear in Table III and Fig. 5. They show no difference between the respective rates of inactivation, either during the 1st hour in the absence of streptomycin, or during the subsequent 2 hours in its presence. Without streptomycin, the numbers of bacteria present in the suspension inoculated with actively growing bacteria did not fall significantly beyond the first hour. This suggests that with the shorter latency sufficient multiplication occurred to balance the rate of ingestion and inactivation by macrophages. From this it will be appreciated that if a higher multiplicity of actively growing bacteria had been used, the growth of organisms in the extracellular phase could have obscured the evidence that *S. aureus* was inactivated following its ingestion by macrophages.

Since the behavior of log phase and lag phase organisms was alike so far as inactivation by macrophages is concerned, the use of lag phase organisms seems justified as a means of minimizing extracellular multiplication.

The Long Term Survival of Staphylococci in Macrophages.—Some 3 to 10 per cent of coagulase-positive staphylococci have been found to survive in the presence or normal rabbit macrophages. The ultimate fate and the nature of these surviving organisms were studied in more detail in three experiments which will be considered together.

For all three of the following experiments, suspensions of macrophages were parasitized in in the usual way, using immune serum and washed suspensions of bacteria from 16-hour

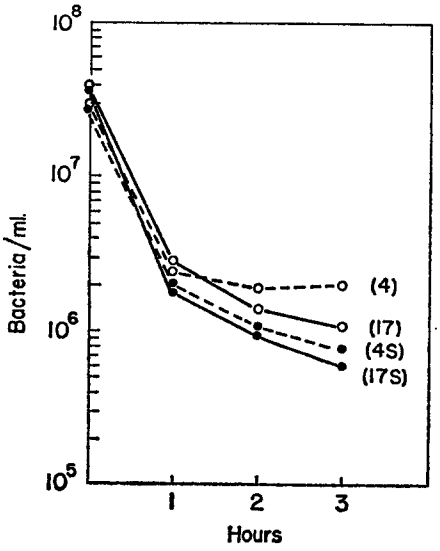

FIG. 5. Total viable counts showing the survival in the presence of rabbit macrophages of bacteria from a 4 hour log phase culture (4) and a 17 hour stationary phase culture (17) of *S. aureus*. At the end of 1 hour streptomycin (*S*) (2.5 μg./ml.) was added to one of each pair of macrophage suspensions.

cultures of *S. aureus* (Smith). After incubation for 25 minutes the cell suspensions were centrifuged (50 *g*) and the cell deposit was washed 3 times with 5 per cent rabbit serum in Gey's solution in order to remove most of the extracellular bacteria. In the first experiment equal numbers of parasitized cells were added to two paraffin-lined tubes; one was left untreated and to the other streptomycin (2.5 μg./ml.) was added. Normal serum was then added to a final concentration of 10 per cent, and the tubes were incubated with shaking. At intervals samples were taken and treated as usual to give estimates of the total number of viable organisms present. The second experiment was performed in the same way except that penicillin (0.1 ug./ml.) was used in place of streptomycin.

The procedure used in the third experiment was more complex and the bacteria used were from a 4 hour culture. Parasitization was done as before. After washing, the cells were resuspended in Gey's solution to a density of 2.0×10^6 macrophages per ml. Ice cold rabbit plasma (without anticoagulant) was added to the cell suspension to give a concentration of 25 per cent. After thorough mixing, 0.5 ml. samples were distributed in Leighton tissue culture tubes

and were left to clot on a horizontal surface. At this stage, the macrophages in four tubes were killed by freezing in a mixture of CO_2 and alcohol. Two ml. of a nutrient medium, consisting of 10 per cent rabbit plasma in Gey's solution at pH 7.4, was then added to each tube. In the medium of some tubes streptomycin was incorporated at a concentration of 2.5 μg./ml. The tubes were then incubated in a rotating drum at 37.5°. At the end of 1 hour the medium in all tubes (except those containing dead macrophages) was replaced by medium free from streptomycin; but to half the tubes previously containing streptomycin, the medium added contained penicillin (0.1 μg./ml.). Initially, and at prescribed intervals, the medium was removed from representative cultures, the tubes were irrigated with Gey's solution to remove excess antibiotic, and were frozen and thawed 3 times. The fibrin clot was then digested by adding to each tube 4.5 ml. of 0.25 per cent trypsin in Gey's solution, followed by incubation for 10 minutes at 37.5°C. The dispersed contents of each tube were then removed, homogenized, and plated as usual. In summary, the plasma clot cultures comprised four categories: (*a*) cultures incubated throughout in the absence of antibacterial substances; (*b*) cultures treated for 1 hour with streptomycin to kill virtually all extracellular bacteria and incubated thereafter in the complete absence of antibacterial substances; (*c*) cultures treated for 1 hour with streptomycin and thereafter with penicillin; and (*d*) cultures containing dead macrophages which were treated throughout with streptomycin to show the efficiency of its bactericidal action under the conditions of test.

The numbers of staphylococci found in fluid and solid cultures of parasitized macrophages are compared in Fig. 6. In the absence of antibacterial substances, the behavior of the bacterial population was quite different in fluid and solid media. In fluid medium the bacterial count remained almost constant for 4 hours (Fig. 6, *A* and *B*). In the solid medium, in which contact between cells and bacteria was almost prevented by immobilization in a plasma gel, continued phagocytosis could no longer prevent an increase in the extracellular population. As a result, the bacterial numbers increased logarithmically during the first 8 hours of incubation (Fig. 6 *C*).

After 7 hours it could be seen microscopically that a large increase had occurred in the bacterial population of untreated cultures; the macrophages seemed unaffected by the numerous bacteria present. It was possible to demonstrate that this increase in bacterial numbers had arisen by extracellular multiplication. Streptomycin (2.5 μg./ml.) was added to an untreated culture at 7 hours. The bacterial counts found in this and another untreated culture at 8 hours were 1.0×10^7 and 1.2×10^9 ml. respectively. Thus, streptomycin produced a fall in the viable count of an untreated culture in the interval between 7 and 8 hours which was comparable with that produced initially at the same drug concentration in cultures of dead macrophages (Fig. 6 *C*). In the latter the bacterial count fell during the first hour from 4.6×10^5 to 6.3×10^3/ml.

The immediate bactericidal action of streptomycin in cultures of dead macrophages contrasts with the absence of any significant drug effect in either fluid or solid cultures containing intact cells (Figs. 6 *A* and *C*). This establishes the fact that most bacteria in the preparations were located initially within cells. The ultimate fate of these bacteria seems to have been a slow inactivation.

This is evidenced by the declining viable counts in both fluid and solid cultures exposed to ineffective concentrations of either penicillin or streptomycin (Figs. 6 *A* to *C*). It is difficult to be sure, however, that this was not partly a drug effect. The prolonged shaking of a fluid culture would be expected to damage macrophages and expose intracellular bacteria to the action of the drugs. The

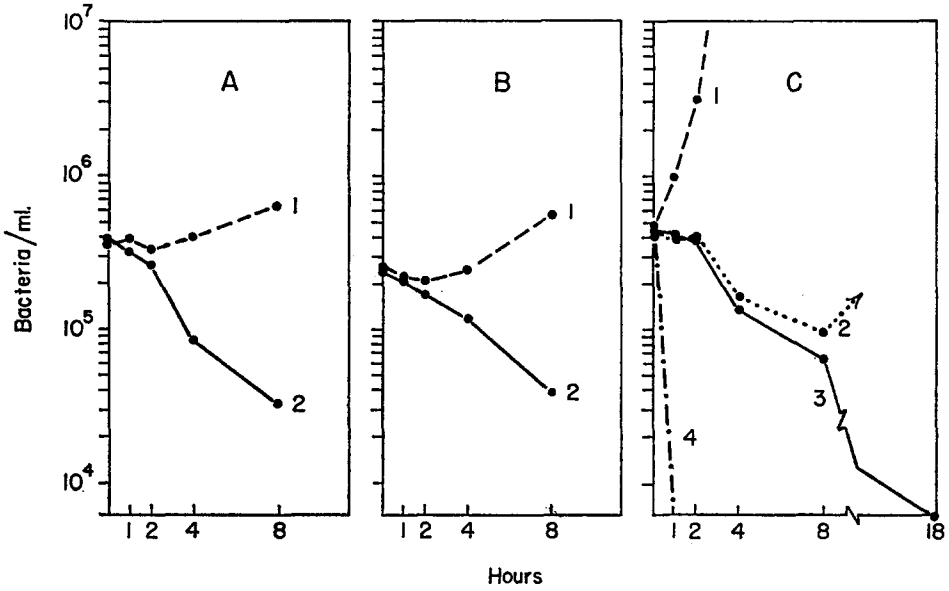

FIG. 6. Three experiments showing the survival of predominantly intracellular populations of *S. aureus* in rabbit macrophages:

A. Macrophages incubated in fluid suspension. Curve 1, untreated; Curve 2, treated with streptomycin (2.5 μg./ml.).

B. Macrophages incubated in fluid suspension. Curve 1, untreated; Curve 2, treated with penicillin (0.1 μg./ml.).

C. Macrophages maintained in a plasma clot bathed with nutrient medium. Curve 1, untreated; Curve 2, treated for 1 hour only with streptomycin (2.5 μg./ml.); Curve 3, treated 1 hour with streptomycin (2.5 μg./ml.); and thereafter with penicillin (0.1 μg./ml.); Curve 4, macrophages killed by freezing, treated with streptomycin (2.5 μg./ml.).

experiment using a solid medium was performed in order to avoid this. In the plasma clot cultures no apparent death of macrophages occurred during 18 hours, yet the fall in viable count in the presence of penicillin was similar to that seen in fluid cultures. Thus, the continuing inactivation of bacteria cannot be attributed to the action of penicillin on organisms released from damaged macrophages.

The use of a solid culture medium had the additional advantage that cultures could be exposed to streptomycin for a period long enough to kill virtually

all extracellular bacteria, and then washed free of drug in order to study the fate of intracellular bacteria in the absence of antibacterial substances. It will be seen in Fig. 6 *C* that the bacterial count continued to fall for 7 hours after the withdrawal of streptomycin. It seems reasonable to conclude, therefore, that those staphylococci which survive early inactivation within macrophages

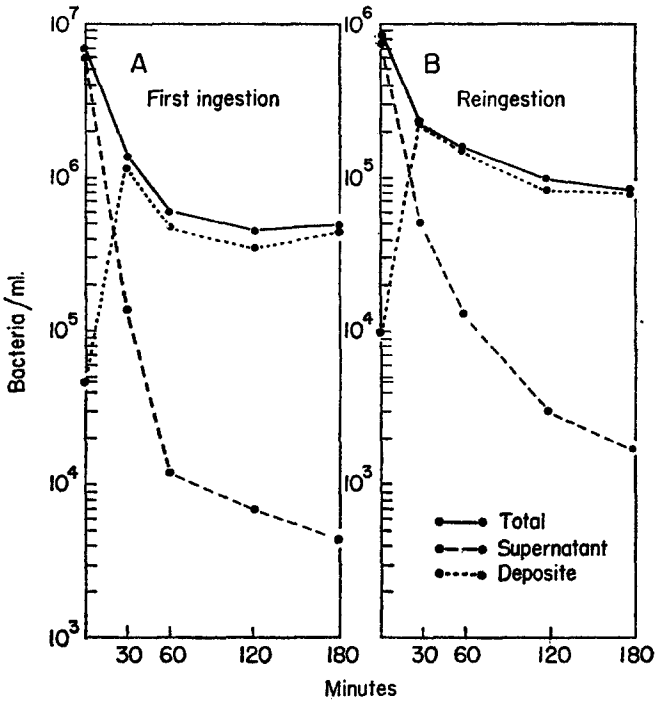

FIG. 7. Viable counts showing the distribution and survival of a population of *S. aureus* ingested for the first time by normal rabbit macrophages (*A*), and of the viable intracellular population recovered from a duplicate suspension at 1 hour and reexposed to fresh macrophages (*B*).

ultimately die in an intracellular environment. It is apparent, however, that this phase of inactivation is very slow, for it was still incomplete at the end of 18 hours.

The Fate of a Surviving Intracellular Population of Staphylococci upon Reingestion by Fresh Macrophages.—The foregoing studies have shown that although 90 per cent or more of the bacteria in a population of staphylococci die relatively soon after their ingestion by normal rabbit macrophages, a significant number of them survive for a considerable time. Because of their insensitivity to drugs, they are believed to be located within cells. The prolonged survival of some

bacteria in this location may be an indication of the heterogeneous nature of the bacterial population; but it could also be caused by heterogeneity of the host-cell population. An attempt was made to distinguish between these two possibilities.

The phagocytosis and survival of bacteria from a washed 16 hour culture of *S. aureus* in a suspension containing normal rabbit macrophages and immune serum was followed in the usual way. Total, supernatant, and sediment counts were made on samples taken from one of duplicate tubes incubated at 37.5°. After 1 hour the cells in the unsampled tube were washed 3 times to remove most of the extracellular bacteria. The cells were then disrupted by freezing and subsequent digestion with 0.25 per cent trypsin in Gey's solution for 10 minutes at 37.5°. After light centrifugation (75 *g*) to deposit cell debris, the supernatant was removed and the bacteria were deposited at high speed (2,000 *g*). After resuspension in a small volume of 0.01 per cent bovine albumin and a further light centrifugation, the recovered bacteria were added to a fresh suspension of macrophages in 10 per cent immune serum. The fate of these bacteria was followed as before.

The phagocytosis and survival of an untreated population of *S. aureus* is compared in Fig. 7 with that of the survivors of a similar population of bacteria which had been recovered from macrophages after incubation for 1 hour. It was estimated that 70 per cent of the bacteria had been resident within macrophages for more than 30 minutes at the time of recovery. The number recovered was fewer than expected, so that during rephagocytosis the ratio of bacteria to cells was much lower than it had been in the original mixture. The lower multiplicity, since it provided for fewer contacts between cells and bacteria, would account for the somewhat slower rate of phagocytosis and death of recovered bacteria. Allowing for this, it is apparent that bacteria which survived the first ingestion by macrophages were no less susceptible to inactivation than were bacteria ingested for the first time.

DISCUSSION

The phagocytosis of a population of bacteria is neither instantaneous nor complete; it is a rate process which is influenced by many factors. For these reasons it is virtually impossible to obtain a uniform and exclusively intracellular population of bacteria for the purpose of studying their intracellular fate. A definitive conclusion concerning the fate of intracellular bacteria would depend upon a simultaneous measurement of the rate of ingestion, as well as the rates of multiplication or death of both intracellular and extracellular bacteria. Since these cannot all be measured, at least one variable must be minimized or eliminated. To this end, Cohn and Morse (1) used lag phase bacteria to avoid the complication of extracellular multiplication. With this simplification, they were able to measure the bacteria remaining in the extracellular phase during phagocytosis, and so determine the fate of a bacterial population in relation to its rate of ingestion. In normal serum coagulase-positive and coagulase-negative staphylococci were ingested by polymorpho-

nuclear leucocytes at different rates, so that a direct comparison of their behavior in leucocytes was not possible. But in the presence of immune serum, in which the rates of phagocytosis were comparable, the inactivation of coagulase-positive and coagulase-negative staphylococci was found to be equally rapid and efficient.

In the present experiments the same has been found true for rabbit macrophages; but again the comparison could only be made by using immune serum to enhance the uptake of the coagulase-positive staphylococci. Since sensitization with antibody did not appear to render the bacteria any more or less susceptible to inactivation within macrophages, it is concluded that no difference exists between the two types of staphylococci with respect to their inactivation within macrophages. This view conflicts with the reports of Kapral and Shayegani (6), Baker (7), and Tompsett (5), who found that coagulase-positive staphylococci survived within mononuclear phagocytes, whereas coagulase-negative ones did not. The methods used by these workers were so different from those used in the present experiments that the contradiction in results cannot be directly evaluated. It is worth recalling, however, that the present experiments have shown that the multiplication of extracellular bacteria can, in some circumstances, obscure completely the behavior of bacteria within cells. This was clearly demonstrated by the contrasting behavior of untreated fluid and plasma clot cultures of parasitized macrophages. For several hours the bacterial population in a fluid suspension was prevented from increasing by the continued phagocytosis of extracellular bacteria; but in a solid medium, where conditions were unfavorable for efficient phagocytosis, it was seen to increase logarithmically. This comparison serves to illustrate the inhibitory effect of ingestion upon the growth potential of staphylococci; it also emphasizes the impossibility of assessing the fate of intracellular bacteria if extracellular bacteria are free to multiply. It is an extreme example; but it is true of any situation in which *all* the bacteria are not freely accessible for ingestion by the cells.

Previous workers with macrophages have resorted to frequent or continuous washing of their cultures in order to control the number of extracellular bacteria present. In this laboratory it was found impossible to wash all bacteria from a glass surface after they had settled there in the presence of serum proteins. It must be presumed, therefore, that washing is not an effective measure for removing extracellular bacteria. When this method has been used (6, 7), the phagocytes have been restrained from effective contact with *all* extracellular bacteria by being immobilized on a glass surface. Under such conditions, extracellular bacteria would be free to multiply, and would contribute unknown numbers of bacteria, some of which would be available for ingestion by cells. The washing of cultures has the further disadvantage that the results obtained cannot be regarded as truly quantitative.

Another measure adopted by earlier workers for the control of extracellular multiplication has been the use of antibacterial substances. In some circumstances this device seems unobjectionable; but antibiotics cannot be introduced into the test system until after the organisms have been ingested. By this time, as is clear from the present observations, the major part of the bacterial population may already have been killed. Previous workers have not been able to observe this early phase of inactivation of staphylococci by mononuclear phagocytes.

The fate of staphylococci within macrophages has been found in the present studies to be similar to that described for polymorphonuclear leucocytes (1). It is unlikely, therefore, that contamination by mononuclear cells can explain the conflicting results of workers studying the fate of staphylococci in polymorphonuclear leucocytes. However, the results obtained with the two phagocytic cell types have differed sufficiently to suggest that their antibacterial mechanisms may be fundamentally different. In general, the rate of inactivation of staphylococci was slower in macrophages than in polymorphonuclear leucocytes, where it occurred so quickly following ingestion that viable bacteria were largely confined to the extracellular phase (1). It is interesting to recall that Metchnikoff believed that bacteria were destroyed more slowly in macrophages than in granulocytes (8). The morphological finding that staphylococci disappeared rapidly from the cytoplasm of granulocytes, but persisted intact for many hours within macrophages, indicates a second difference between the potentialities of the two cell types. It also explains why Metchnikoff was able to draw his conclusion from morphological studies alone.

A third point of difference between macrophages and granulocytes concerns the percentage of bacteria remaining alive at the end of 180 minutes of incubation. It was significantly different for the two cell types. Less than 0.1 per cent survived in the presence of granulocytes (1) in comparison with 3 to 10 per cent in the case of macrophages. In the latter, the surviving bacteria were shown to be located within cells. When observed over a longer period of time, these bacteria were observed to die slowly; but when recovered from cells and exposed to fresh macrophages, they were as susceptible to ingestion and inactivation as were the bacteria of the original population. On this evidence, it is suggested that the prolonged survival of some bacteria is not due to their insensitivity to the antibacterial environment of the macrophage cytoplasm, but results from the fact that some macrophages are less able than others to destroy ingested staphylococci. This would not be surprising, for the macrophages of a peritoneal exudate are not functionally uniform. In preparations stained with supravital dyes, the appearance of the cells ranges from that of unstimulated monocytes, without vacuoles and with very few granules in the neutral red rosette, to highly stimulated macrophages showing neutral red vacuoles as well as hypertrophy of the neutral red rosette (9). When main-

tained *in vitro* the monocytes of an exudate undergo functional and morphological changes, and assume the characters of the macrophage (10, 11). Exactly the same changes in function and morphology occur *in vivo* (12). It is quite possible, then, that the bacteria with a protracted survival time are those contained in cells whose physiological activities do not provide an efficient antibacterial environment; and that their subsequent gradual death is due to the functional changes which occur in such cells during continued incubation.

Whatever its explanation, the property of prolonged survival of some staphylococci *in vitro* cannot be regarded as a determinant of virulence, for it is shared equally by coagulase-positive and coagulase-negative staphylococci.

SUMMARY

The phagocytosis and survival of staphylococci in the presence of rabbit macrophages has been studied quantitatively. The method permitted an independent measurement to be made of intracellular and extracellular bacteria during the course of phagocytosis. It was found that *S. aureus* was relatively resistant to phagocytosis. In the presence of specific immune serum, however, it was ingested at a rate comparable with that of *S. albus* in normal serum; under these conditions more than 90 per cent of bacteria of either strain were inactivated within 60 minutes. Since immune serum did not alter the susceptibility of *S. aureus* to inactivation within macrophages, it is concluded that *S. aureus* and *S. albus* are similar in respect to their ability to survive within macrophages.

The inactivation of staphylococci occurred more slowly, and a greater percentage survived incubation for 180 minutes, in macrophages than in polymorphonuclear leucocytes. Moreover, they retained their morphology for many hours within the former cell, but disappeared rapidly from the cytoplasm of granulocytes. It is suggested that the antibacterial mechanisms of the two cell types are fundamentally different.

When studied over a prolonged period the staphylococci which survived early inactivation within macrophages were observed to die slowly over many hours. When they were recovered from cells and exposed to fresh macrophages, however, they were ingested and inactivated as readily as bacteria of the original population. It is suggested, therefore, that the prolonged survival *in vitro* of some staphylococci is due to variation in the efficiency of the antibacterial mechanism of exudative mononuclear phagocytes.

BIBLIOGRAPHY

1. Cohn, Z. A., and Morse, S. I., Interactions between rabbit polymorphonuclear leucocytes and staphylococci, *J. Exp. Med.*, 1959, **110,** 419.
2. Morse, S. I., Isolation of a phagocytosis-inhibiting substance from culture filtrates of an encapsulated *Staphylococcus aureus*, *Nature*, 1960, **186,** 102.
3. Rogers, D. E., and Tompsett, R., The survival of staphylococci within human leucocytes, *J. Exp. Med.*, 1952, **95,** 209.

4. Rogers, D. E., Observations on the nature of staphylococcal infections, *Bull. New York Acad. Med.*, 1959, **35,** 25.
5. Tompsett, R., The survival of staphylococci within phagocytic cells, *Bull. New York Acad. Med.*, 1954, **30,** 480.
6. Kapral, F. A., and Shayegani, M. G., Intracellular survival of staphylococci, *J. Exp. Med.*, 1959, **110,** 123.
7. Baker, H. J., Effects of penicillin and streptomycin on staphylococci in cultures of mononuclear phagocytes, *Ann. New York Acad. Sc.*, 1954, **58,** 1232.
8. Hirsch, J. G., Immunity to infectious diseases: Review of some concepts of Metchnikoff, *Bact. Rev.*, 1959, **23,** 48.
9. Cappell, D. F., Intravitam and supravital staining. IV. The cellular reactions following mild irritations of the peritoneum in normal and vitally stained animals, with special reference to the origin and nature of the mononuclear cells, *J. Path. and Bact.*, 1930, **33,** 429.
10. Lewis, M. R., The formation of macrophages, epithelioid cells and giant cells from leucocytes in incubated blood, *Am. J. Path.*, 1925, **1,** 91.
11. Lewis, M. R., and Lewis, W. H., The transformation of white blood cells into clasmatocytes (macrophages), epithelioid cells and giant cells, *J. Am. Med. Assn.*, 1925, **84,** 798.
12. Ebert, R. H., and Florey, H. W., The extravascular development of the monocyte observed *in vivo*, *Brit. J. Exp. Path.*, 1939, **20,** 342.