THE SORPTION OF BACTERIOPHAGE BY LIVING AND DEAD SUSCEPTIBLE BACTERIA

I. Equilibrium Conditions

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The first step in the process of bacteriophagy, or lysis of bacteria by bacteriophage, is the combination of phage with susceptible organisms. D'Herelle in his early work (1, 2) noted a rapid and specific attachment of phage to susceptible bacteria under suitable conditions, and von Angerer (3), working with dead organisms, found that the process could be expressed in the form of the usual adsorption isotherm equation with $1/n = 0.75 - 0.80$. Prausnitz and Firle (4) further observed that heating susceptible bacteria to temperatures above 110°C resulted in destruction of the ability to bind phage. It is also known that as a general rule homologous or heterologous resistant bacteria fail to combine with phage to any appreciable extent (4, 5, 6); although Flu (7) and Goih and Jacobsohn (8) have reported cases which are definite exceptions to the rule.

The general trend of the above work has led to the assumption that live bacteria take up phage by adsorption, although there exists no adequate quantitative data in support of this idea. Obviously the question of how phage and bacteria combine is a fundamental one and the writer has felt that a closer study of the process would be in order, particularly since the observation of Krueger and Northrop (9) that extracellular phage per milliliter constitutes a constant low percentage of total phage per milliliter during the logarithmic phase of phage production in a phage-bacteria mixture, suggests the possibility of phage distribution being of a simple type.

The present paper deals with observations on the distribution of phage between bacteria (both living and dead) and the broth in which
they are suspended. The phage used throughout is a highly active antistaphylococcus race and the bacterium is a single phage-susceptible strain of *S. aureus*. Abbreviations and terms employed are defined as follows:

- **P**: Bacteriophage.
- **B**: Bacterium.
- **[P]**: Phage/ml.
- **[B]**: Bacteria/ml.
- **P.U.**: Phage unit. An arbitrary activity unit (11).
- **[P]₀**: Unbound phage/ml at equilibrium.
- **Pₖ**: Phage bound by bacteria.
- **Broth**: Veal infusion, 1 per cent peptone, 0.5 per cent NaCl, pH 7.6.

**Distribution of Phage between Living, Susceptible Bacteria and Broth in the Absence of Growth**

**Methods:** 16 hour cultures of *S. aureus* grown in Blake flasks were taken up in saline, filtered through a Schleicher and Schüll Faltenfilter No. 588, washed twice in saline and the cell content determined by the centrifuged-sediment method (10). The saline suspension was diluted with broth to give a proper **[B]** and the **B**-broth mixture was held at 10°C for ½ hour before being used. Mixtures of **P** and **B** (already cooled to 10°C.) were placed in test tubes and the latter were shaken at 10°C for 2 hours. At the end of this time the tubes were placed in iced holders and were centrifuged at high speed for 6 minutes. The supernatants were at once pipetted off, diluted with broth, and kept at 0°C until they could be titrated.

For determining free **P** at equilibrium 4 ml. aliquots of various dilutions were employed, the titration procedure being that described by Krueger (11, 12). Phage combined with **B** was determined by difference. Controls of **B** in broth without **P** and of known **[P]**'s mixed with **B** (diluted for titration without centrifuging, for total **P/ml.**) were run with each series to detect any bacterial reproduction or formation of **P**.

Since the relationship being studied was that existing between **P** and **B** in the absence of **B** growth, it was essential to prevent bacterial reproduction. Rather than complicate the experiments by introducing chemical inhibitors, it seemed best to work at low temperatures. It soon developed that an additional factor of safety was introduced if the broth suspension of **B** was kept at 10°C. for ½ hour before being mixed with **P**. The controls of **B** alone in broth were sufficient to detect cases of moderate **B** growth, but a much more satisfactory growth criterion lay in the control mixtures of known **[P]**'s with **B**.
The value of such controls rests upon a fact elicited in previous work on the kinetics of the $B$-$P$ reaction (9), namely, that the rate of $P$ production in a $P$-$B$ mixture is a power of the rate of $B$ reproduction. Consequently, even slight increases in $[B]$ give rise to very appreciable and easily detectable increments in total $[P]$. $P$ production therefore serves as a sensitive indicator of $B$ growth.

Control tests run at intervals of 15 minutes on several distribution series indicated that 1½ hours sufficed for the establishment of equilibrium between intracellular and extracellular $P$. This point
was also confirmed by experiments in which equilibrium was approached from both sides (see reversal experiments). The use of periods longer than 3 to 4 hours for contact between \( P \) and live, non-growing \( B \) introduced complications due to the death of considerable numbers of \( B \). It is shown elsewhere (Distribution of phage between heat-killed susceptible bacteria and broth) that the processes by which dead \( B \) and live \( B \) take up \( P \) from solution differ. Also, control experiments indicate that under the experimental conditions obtaining no significant drop in viable survivors occurs during the first 3½ to 4 hours. After this time, however, the proportion of dead

![Graph](image)

**Fig. 1.** Phage distribution between live, resting, susceptible bacteria and broth. Data plotted as adsorption isotherm. Contact period 2 hours at 10°C. The points are seen to lie along a line of slope \((1/n) = 1\).

cells in the mixture becomes considerable and their presence accounts for the inconstant and not altogether predictable results secured when long contact periods are employed.

Table I lists the pertinent data obtained in one distribution experiment run at 10°C. \([P] \)'s varied from \( 1 \times 10^9 \) p.f.u./ml. to \( 5 \times 10^7 \) p.f.u./ml. and over this range three different \([B] \)'s were employed: \( 4 \times 10^8 \)/ml, \( 2 \times 10^9 \)/ml. and \( 1 \times 10^9 \)/ml. respectively. A period of 2 hours was allowed for the system to come to equilibrium before samples were taken, and it is apparent that the observed values give a reasonably constant partition coefficient. In calculating \( K \) the ratio \( P_{obs}/B \) is multiplied by \( 25 \times 10^6 \) in order to obtain the concentration.
of \( P/\text{ml.} \) of \( B \) (there are approximately \( 25 \times 10^8 \) bacterial cells in 1 ml. of packed bacteria). Fig. 1 presents the same data plotted in the log form of the adsorption isotherm equation. The points are seen to lie on the line of slope 1 (i.e., \( 1/n = 1 \)).

Any single \( B \) suspension gives satisfactorily constant values for the partition coefficient. Different preparations of \( B \), however, produce moderate fluctuations in the coefficient over and above the variations due to experimental errors in estimating \( B \) and determining \( P \). [Determinations of \( B \) can be made with an accuracy of \( \pm 2 \) per cent (10) while \( [P] \) can be determined within \( \pm 3 \) per cent (12).]

It would appear then that the distribution of \( P \) between broth and live, non-growing \( B \) may be expressed in the form of the common Normal Distribution Equation. If the distribution is strictly normal it not only should furnish data which fit the equation but equilibrium should be attainable from both sides. Experimentally this proves to be true as is demonstrated in the following experiment:

\[ 4 \times 10^9 \text{ p.u.} \text{ and } 1.6 \times 10^9 \text{ } B \text{ were mixed in a total volume of } 4 \text{ ml. of broth. The mixture was shaken for } 1\frac{1}{2} \text{ hours at } 10^\circ\text{C, to permit establishment of equilibrium and then was diluted } 1/10 \text{ with broth. After an additional } 1\frac{1}{2} \text{ hours shaking at } 10^\circ\text{C, } [P]_e \text{ of the diluted mixture was determined. For comparison } [P]_e \text{ was also ascertained in mixtures having the } B \text{ and } P \text{ concentrations of the undiluted and diluted original set-up. For this purpose two tubes were prepared: in both of which } [P] = 1 \times 10^9 \text{ and } [B] = 4 \times 10^9. \text{ To be sure that equilibrium had been reached free } P/\text{ml.} \text{ was determined in one tube at } 1\frac{1}{2} \text{ hours and in the other at } 2 \text{ hours. To check } [P]_e \text{ in the diluted suspension a flask containing } 4 \times 10^9 \text{ p.u. and } 1.6 \times 10^9 \text{ } B \text{ in } 40 \text{ ml. of broth was included. Samples for determinations of free } P/\text{ml.} \text{ were taken at } 1\frac{1}{2} \text{ and } 2 \text{ hours.}

As shown in Table II the partition coefficients agree quite well. Moreover, the total free \( P \) after dilution is nearly ten times the amount present before dilution, so there is no doubt that equilibrium in the dilute suspension was reached by diffusion of phage from the bacteria instead of diffusion into them, as is the case in the previous experiments. Consequently it follows that the removal of \( P \) from solution into living, susceptible \( B \) is a reversible process, thus further substantiating the idea that \( P \) distribution between \( B \) and broth in the absence of growth is a case of true Normal Distribution. Repetition of this experiment at \( 0^\circ\text{C.} \) and \( 10^\circ\text{C.} \) with other concentrations of \( B \) and \( P \) has given like results.
### TABLE II

**Reversibility of Phage Distribution Between Broth and Living, Resting Bacteria. 10°C.**

<table>
<thead>
<tr>
<th></th>
<th>Total P.U.</th>
<th>Total B</th>
<th>Volume</th>
<th>([P])</th>
<th>([B])</th>
<th>([P]_4)</th>
<th>Total extracellular (P)</th>
<th>(K = \frac{(P_{2}\text{/}P_4)(25 \times 10^9)}{[P]_4})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentrated P-B mixture after 1½ hrs.</td>
<td>4 × 10⁶</td>
<td>1.6 × 10⁶</td>
<td>4 ml.</td>
<td>1 × 10⁶</td>
<td>4 × 10⁶</td>
<td>5 × 10⁶</td>
<td>2 × 10⁶</td>
<td>11.9 × 10⁹</td>
</tr>
<tr>
<td>“ 2 “ “ “</td>
<td>“</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Identical with A except diluted 1/10 after 1½ hrs. ([P]_4) determined at: 1½ hrs. after dilution</td>
<td>“</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>“ 2 “ “ “</td>
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<tr>
<td><strong>C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control suspension made up directly to same condition as B after dilution. ([P]_4) determined at: 1½ hrs.</td>
<td>“</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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<td>“ 2 “ “ “</td>
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<tr>
<td><strong>D</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control to detect B growth by determining initial and final total ([P]) of mixture after 1 hr.</td>
<td>“</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total \([P]\): 3.9 × 10⁹

" 3 “ “ "
Distribution of Phage Between Growing Susceptible Bacteria and Broth

In an earlier paper (9) Krueger and Northrop reported a simple relationship found to exist between intracellular and extracellular P in a P-B mixture during the log phases of B growth and P formation. The log plots of total P/ml. and extracellular P/ml. against time in several of our kinetic experiments were practically parallel lines, indicating that extracellular P/ml. was a constant low percentage (ca. twenty per cent) of total P/ml., while the intracellular P fraction constituted a constant major percentage of total P/ml. at any particular time.

TABLE III
Calculation of Partition Coefficients for Phage Distribution in Phage-Growing B Mixture at 36°C. Data from a Previously Reported Experiment (9)

<table>
<thead>
<tr>
<th>t (sampling)</th>
<th>B/ml.</th>
<th>Total P/ml.</th>
<th>Free P/ml.</th>
<th>K = (P_B/25 × 10^9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.5 × 10^7</td>
<td>2.5 × 10^4</td>
<td>2.5 × 10^4</td>
<td>—</td>
</tr>
<tr>
<td>.50</td>
<td>4 &quot;</td>
<td>2.5 × 10^5</td>
<td>1.6 &quot;</td>
<td>90 × 10^3</td>
</tr>
<tr>
<td>1.00</td>
<td>6.3 &quot;</td>
<td>2 × 10^8</td>
<td>6.3 &quot;</td>
<td>123 &quot;</td>
</tr>
<tr>
<td>1.50</td>
<td>1.6 × 10^8</td>
<td>2 × 10^7</td>
<td>4 × 10^6</td>
<td>76 &quot;</td>
</tr>
<tr>
<td>2.00</td>
<td>3.2 &quot;</td>
<td>1.6 × 10^8</td>
<td>4 × 10^8</td>
<td>31 &quot;</td>
</tr>
<tr>
<td>2.50</td>
<td>6.3 &quot;</td>
<td>1.6 × 10^9</td>
<td>4 × 10^7</td>
<td>16 &quot;</td>
</tr>
<tr>
<td>3.00</td>
<td>1 × 10^9</td>
<td>1.6 × 10^11</td>
<td>5 × 10^4</td>
<td>7.7 &quot;</td>
</tr>
<tr>
<td>3.50</td>
<td>&quot;</td>
<td>1.4 × 10^11</td>
<td>1.6 × 10^9</td>
<td>21.6 &quot;</td>
</tr>
</tbody>
</table>

With [B] constant this relationship would signify that a true normal distribution obtained with reference to P in the bacterial and broth phases. However, the system is not static, for [B] is increasing logarithmically with time and a priori the data will not yield a constant partition coefficient. Table III, an analysis of such an experiment, shows this to be true, K decreasing markedly during the period studied.

Since the distribution of P between living susceptible B and broth in the absence of growth is so definitely of normal type it seemed advisable to reconsider our earlier kinetic data. In the first place, there was the possibility that with such a rapid rate of P production there might exist a lag in diffusion of P through the cell membrane,
samples being taken too fast for attainment of equilibrium. To influence the data in the direction of maintaining a constant partition coefficient we should have to assume then that \( P \) is formed extracellularly and that a progressively greater lag arose in the diffusion of \( P \) into the cell. While there was no special reason to suppose that this represented the true situation the possibility could be experimentally checked. The test was carried out by allowing a \( P \)-growing \( B \) mixture to reach a point well within the log phase of \( B \) growth, stopping the reaction by rapidly cooling the reactants to 0°C. and titrating the free and total \( P/\text{ml.} \) at intervals. Table IV gives the essential data of the experiment and shows that no lag in diffusion of \( P \) either in or out of the cell is detectable.

The next desirable bit of information was the exact relative slopes of the log plots of extracellular \( P/\text{ml.} \) and intracellular \( P/\text{ml.} \) against \( t \). In the experiments previously reported (9) samples taken for total \( P/\text{ml.} \) determinations were diluted at once and were mixed with the test \( B \) suspension. The time period occupied by the procedure was short and the titration data furnished very uniform slopes for the rate of total \( P \) formation. However, this was not so in the case of free \( P/\text{ml.} \) determinations. These samples had to be centrifuged for 0.1 hour during which time the \( P-B \) reaction continued. They were then diluted and titrated immediately.

Plots of the titration readings, \( i.e., \) log extracellular \( P/\text{ml.} \) against
t, gave slopes varying somewhat in different experiments and the points of any one plot were not as regularly aligned as in the total P/ml. plots. To obtain better data the same experiments were repeated with this alteration in technic: Samples for total P/ml. and for extracellular P/ml. were removed from the reaction mixture at intervals, chilled at once to 0°C. and the total P aliquots were diluted in 0°C. broth. Meanwhile, the chilled extracellular P samples were packed in ice, centrifuged 0.1 hour and the supernatants then diluted with chilled broth. All samples were held at 0°C. after dilution until the end of the experiment and were then titrated together. Control experiments showed that immediate reduction in temperature could be relied upon to stop B growth and P production and that the dilution of samples with subsequent storage at 0°C. prevented any significant change in the P content.
Following this procedure, time of sampling, particularly for extracellular \( P \), could be more accurately fixed and the data so obtained furnished curves of the type shown in Fig. 2. In the plot of log extracellular \( P/\text{ml} \) against time the slope is clearly not parallel to the log total \( P/\text{ml} \) plot. The difference is slight but definite, and makes for considerable improvement in calculations of the partition coefficient. Thus in Table V the data from the experiment of Fig. 2 are listed and it is evident that the partition coefficients agree very well.

It seems reasonable to conclude therefore, that our earlier data for extracellular \( P/\text{ml} \) were not entirely accurate due to the mechanical difficulty of fixing \( t_{\text{sampling}} \) in a rapidly reacting mixture. With an improved technic, this source of error has been eliminated and the data fit the Normal Distribution Equation satisfactorily.

The distribution of \( P \) between broth and live, susceptible, growing \( B \), i.e., in a reacting mixture of \( P \) and \( B \), is then of the same simple type found to hold for \( P \) distribution in the case of live, non-growing \( B \).

### Distribution of Phage between Heat-Killed Susceptible Bacteria and Broth

**Methods.**—Saline suspensions of staphylococci were prepared as described under *Distribution of phage between living bacteria and broth in the absence of growth*. After heating at 80\(^\circ\)C. for 3 hours the suspensions were washed several times with...
saline and samples for sterility tests were taken. Various concentrations of $P$ and $B$ in broth were placed in the mechanical shaker at 0°, 10° or 36°C. At the end of 16 to 18 hours the tubes were centrifuged at high speed and the $[P]$'s of the supernatants determined. Controls of known $[P]$'s with known $[B]$'s (for total $[P]$ determinations) were included in each set-up. The long period of contact between $B$ and $P$ in these experiments was used merely as a matter of convenience and not because the attainment of equilibrium conditions required such prolonged interaction (see section on velocity, page 508).

The adsorption isotherm equation states that $a = k c^{1/n}$, where $a = $ quantity of sorbate per unit of adsorbent—here P.U. adsorbed per $B$, $c = $ concentration of sorbate at equilibrium and $k$ and $1/n$ are constants. Table VI presents the data from an experiment typical of many performed with dead $B$. The constants $\log k$ and $1/n$ are calculated from the experimental observations and are seen to give values for $\log a$ when substituted in the logarithmic form of the adsorption equation ($\log a = \log k + 1/n \log c$) which agree well with the observed figures. In Fig. 3 the same data are plotted for comparison with the calculated curve.

Table VII includes the data from two other adsorption experiments run at 10°C. with different $B$ preparations. In the plot of the data, Fig. 4, observed points of Fig. 2 are reproduced to bring out the degree of variation between results obtained with different lots of organisms. The two sets of data in Table VII include regions of high $P/B$ ratios and show a distinct flattening in their upper segments due to satura-
tion of B with P. Such behavior is not predicted by the Freundlich equation although frequently observed experimentally. However, the general adsorption theory developed by Langmuir (13) predicts this

![Graph showing phage distribution between heat-killed, susceptible bacteria and broth. Data plotted as adsorption isotherm. Contact period 16 hours at 10°C. 1/\(m\) = 0.84.](image)

**TABLE VII**

Adsorption of Phage by Heat-Killed Susceptible Bacteria at 10°C.

*Two Different B Preparations.* Total B: \(2.4 \times 10^9\). Volume 8 Ml. Period of Contact 16 Hours. B Killed at 80°C.

<table>
<thead>
<tr>
<th>Total initial p.c.</th>
<th>(\log [P]_a)</th>
<th>(\log P_b/B)</th>
<th>Total initial p.c.</th>
<th>(\log [P]_a)</th>
<th>(\log P_b/B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(6 \times 10^9)</td>
<td>9.4</td>
<td>2.22</td>
<td>(7 \times 10^9)</td>
<td>9.52</td>
<td>2.26</td>
</tr>
<tr>
<td>4 &quot;</td>
<td>9.0</td>
<td>2.12</td>
<td>5 &quot;</td>
<td>9.06</td>
<td>2.23</td>
</tr>
<tr>
<td>2 &quot;</td>
<td>7.9</td>
<td>1.91</td>
<td>3 &quot;</td>
<td>8.35</td>
<td>2.07</td>
</tr>
<tr>
<td>1 &quot;</td>
<td>7.0</td>
<td>1.61</td>
<td>8 \times 10^9</td>
<td>6.95</td>
<td>1.52</td>
</tr>
<tr>
<td>4 (\times 10^8)</td>
<td>6.40</td>
<td>1.22</td>
<td>(4 \times 10^9)</td>
<td>6.60</td>
<td>1.22</td>
</tr>
<tr>
<td>2 &quot;</td>
<td>6.15</td>
<td>0.92</td>
<td>2 &quot;</td>
<td>6.24</td>
<td>0.92</td>
</tr>
<tr>
<td>1 &quot;</td>
<td>5.70</td>
<td>0.62</td>
<td>1 &quot;</td>
<td>5.80</td>
<td>0.62</td>
</tr>
<tr>
<td>(4 \times 10^4)</td>
<td>5.18</td>
<td>0.21</td>
<td>(4 \times 10^4)</td>
<td>5.24</td>
<td>0.22</td>
</tr>
<tr>
<td>2 &quot;</td>
<td>4.80</td>
<td>-0.08</td>
<td>2 &quot;</td>
<td>4.90</td>
<td>-0.08</td>
</tr>
<tr>
<td>1 &quot;</td>
<td>4.42</td>
<td>-0.39</td>
<td>1 &quot;</td>
<td>4.56</td>
<td>-0.38</td>
</tr>
</tbody>
</table>
type of curve with high ratios of sorbate to adsorbent and lends to the present data adequate theoretical support.¹

It should be mentioned that several early adsorption experiments run at 10°C, as already described gave isotherms for which \(1/n\) in the log plot = 1.25. This suggested the possibility of \(P\) distribution in dead \(B\) being of the associative type. However, in associative distribution \(n\), in the equation \(C_2 = k_1 C_1^n\), is ordinarily a whole number greater than one. Further, associative distribution is commonly

![Graph](image)

**FIG. 4.** Phage distribution between heat-killed, susceptible bacteria and broth. Three different \(B\) preparations. Data plotted as adsorption isotherm. Contact period 16 hours at 10°C.

- \(\triangle\) Data of Table VII
- \(\square\) Data of Table VI
- \(\circ\) Data of Table VI

reversible while no evidence of reversal could be obtained in the present instance. It was finally found that peculiar results of this sort, giving distribution plots midway between those of normal and associative distribution, depended upon some substance present in unwashed cultures. This material, derived from the medium

¹ In order to apply Langmuir's equation quantitatively it is necessary to make arbitrary assumptions as to the constants owing to lack of experimental data, so that no definite significance would attach to the calculations.
upon which the bacteria were grown and amboceptor-like in action, furthered the binding of $P$ by dead $B$ and was shown in control experiments with ultrafiltrates not to exert any inactivating action upon phage. Its presence in $P$-live $B$ mixtures used for distribution experiments resulted in the $B$ taking up considerably more $P$ than is normally the case and was responsible for several apparently anomalous series until the cause was discovered. In subsequent adsorption and distribution experiments, the $B$ were washed several times with broth or saline to remove the disturbing factor. When new lots of agar (of the same formula) were used for growing the bacteria no differences were found between washed and unwashed $B$, both giving strictly regular results.

It is quite possible occasionally to obtain what appear to be fairly good adsorption isotherms when the ratio $P/B$ of the entire series is

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Total initial P.U.} & \text{[P]$_e$} & \text{Total extracellular P.U.} & K = \frac{(P/B)(25 \times 10^9)}{[P]$_e$} \\
\hline
(1) P-B mixture for establishing [P]$_e$ after 16 hours & $4 \times 10^4$ & $3 \times 10^4$ & $2.4 \times 10^3$ & $1,380 \times 10^6$ \\
(2) Reversal experiment & $2.1 \times 10^4$ & $1.7 \times 10^3$ & $19,800 \times 10^3$ & \\
At equilibrium (16 hours) diluted to 80 ml. with broth and shaken 24 hours at 10°C. Final volume $10 \times$ initial volume & \\
\hline
\end{array}
\]

high and if lower $P/B$ ratios are not included in the test the results may be misleading. However, the plotted points do not give a smooth curve, the slope is very low and other experiments including lower $P/B$ ranges indicate that the region is one of $B$ saturation with $P$.

Apparently, then, the combination of $P$ with dead $B$ may be expressed in the form of the usual adsorption isotherm equation except in cases of very high $P/B$ ratios. If the process is a true adsorption and proceeds to $> 99$ per cent completion as in the present instance, it should be very difficult to demonstrate any reversibility. This
point was tested experimentally by running at the same time as the reversal experiment with live B, a similar series employing dead B. Table VIII gives the results obtained and clearly demonstrates that no detectable reversal has taken place. Other experiments performed at 10°C. and 0°C. with various [P]'s and [B]'s lead to the same general conclusion.

The control tubes for total [P] in distribution experiments performed with dead B invariably give a [P] value identical with or less than that of the supernatant fluid left after centrifugation of B, although these determinations are made with dilutions of the entire P-B mixture. This apparent paradox is readily explicable on the basis of the foregoing facts concerning P adsorption. P taken up by dead cells is retained by them and consequently does not function in the P titration. Hence the [P] of the P-B mixture ordinarily will be that of the supernatant. However, if low dilutions of the P-B suspension are employed in the titration set-up an appreciable concentration of dead B may be introduced. Now, as the live organisms of the test series grow and produce P the latter will be adsorbed by the dead B in considerable amounts, thereby increasing time of lysis and causing the mixture to have an apparent [P] less than the free [P] at equilibrium.

The fact that the combination of P with dead B is non-reversible brings up the possibility that the phenomenon studied may not one of adsorption but rather an inactivation of P by some hypothetical substance derived from the dead B and set free in the broth. This possibility was tested by filtering heavy suspensions of dead B in broth and saline through acetic-collodion membranes (14) just dense enough to retain all organisms while allowing free passage of substances in solution and adding the filtrates to known [P]'s. The filtrate-P mixtures were shaken at 10°C. and 36°C. for 16 hours and the [P] of the liquid then determined. In no instance was it possible to detect the least loss in P titre, showing that dead B remove P from solution by a process of P-B combination and not by liberating any soluble inactivating substance.

The Effect of Varying Temperatures at Which B Are Killed on B Adsorptive Capacity.—This relationship was investigated by exposing aliquots of the same B preparations for 3 hours to temperatures of 65°C., 80°C. and 100°C. The heat-killed bacteria were then tested in
the usual way for ability to adsorb phage. No significant changes were found over this range of temperatures.

II. Reaction Velocity

In order to obtain some idea of the course of events between the time of mixing phage with susceptible bacteria and the final attainment of equilibrium the reaction velocity of the P-B system was investigated, using both live and dead bacteria.

Methods: The live bacterial reactant was prepared as outlined under Distribution of phage between living susceptible bacteria and broth in the absence of growth. Appropriate concentrations of P and B, previously cooled to 10°C., were mixed and the mixtures were shaken at 10°C. Samples taken at intervals were centrifuged at high speed 0.1 hour in iced cups. The supernatants were pipetted off, diluted with 0°C. broth and kept at 0°C. until they could be titrated.

In the case of the reaction of P with dead B the latter reactant was made up according to the procedure described in Distribution of phage between heat-killed susceptible bacteria and broth. Sampling and titrating were carried out as above.

The removal of P from solution by resting live bacteria or by heat-killed cells proceeds very rapidly and the time of sampling must be accurately fixed to obtain reliable data. Consequently the middle of the centrifuging period was arbitrarily designated as t-sampling in these experiments. The initial \([B]\) used throughout was \(1 \times 10^8 B/ml\) while initial \([P]\) was \(1 \times 10^8 P.v./ml\).

The distribution of P between live, susceptible B and broth in accordance with the Law of Normal Distribution would suggest that the combination of P with B involves diffusion of the lytic substance into the cell. It is not surprising therefore to find that the rate of the process, in common with those found for many other cases of diffusion into living cells, is proportional to the concentration gradient and to the number of cells present.

That is:

\[
\frac{dP}{dt} = ([B]) ([P]_t - [P]_e),
\]

where \([B]\) = concentration of bacteria, \([P]_t = \) free P/ml. at any time \(t\) and \([P]_e\) = concentration of P at equilibrium.

It follows that

\[
\frac{dP}{dt} = k ([B]) ([P]_t - [P]_e),
\]

which on integration gives:

\[
k = \frac{1}{t [B]} \ln \frac{([P]_t - [P]_e)}{([P]_e - [P]_t)}.
\]
TABLE IX

Summary of Reaction Velocity Experiments on: (a) Combination of Live, Resting, Susceptible Bacteria with Phage (b) Combination of Heat-Killed, Susceptible Bacteria with Phage

\[ [P]_0 = 1 \times 10^6 \text{ p.v.}/\text{ml. throughout}. \quad [B]_0 = 1 \times 10^6 \text{ B/ml. throughout}. \quad \text{Temperature} 10^\circ\text{C}.

K's Are Those of Calculated Curves Best Fitting Experimental Data

<table>
<thead>
<tr>
<th>B preparation used: Live, resting, susceptible B</th>
<th>B preparation used: Heat-killed, susceptible B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>K calculated from ( \frac{t}{[B]} \ln \left( \frac{[P]_0 - [P]_t}{[P]_0 - [P]_s} \right) )</td>
</tr>
<tr>
<td>Broth with shaking</td>
<td>0.0139 \times 10^{-5}</td>
</tr>
<tr>
<td>&quot; without shaking Run together</td>
<td>0.0148 &quot;</td>
</tr>
<tr>
<td>Broth with 20 per cent glycerine Run together</td>
<td>0.0138 &quot;</td>
</tr>
<tr>
<td>Broth</td>
<td>0.0125 &quot;</td>
</tr>
<tr>
<td>Average</td>
<td>0.0142 &quot;</td>
</tr>
</tbody>
</table>

The close agreement of the average K's is obviously fortuitous.
Fig. 5 shows graphically a single experiment performed in duplicate with live and dead B. There is no significant difference in the reaction velocities. This in general expresses the results of the series of velocity experiments (cf. Table IX), i.e., live, resting, susceptible B and heat-killed, susceptible B take up P from solution at much the same rate, although it should be noted that equilibrium is rapidly reached with live cells whereas dead bacteria continue reacting with P until much more of the latter is removed from solution.

In order to determine whether the period consumed in the reaction represented delay due to diffusion of P to the B cell or time utilized in a reaction between P and B, once contact was established, the following experiments were performed:

1. Duplicate set-ups with P and dead B were made. One series was shaken vigorously at 10°C, while the other was not shaken.
Fig. 6a

Fig. 6b

Fig. 6. Influence of doubling viscosity upon velocity of reactions:
a. Live, resting, susceptible \( B + P \)
b. Heat-killed, susceptible \( B + P \)

\[
[B]_0 = 1 \times 10^8 \quad [P]_0 = 1 \times 10^8 \quad \text{Temperature } 10^\circ C. \quad (a - x) = [P]_{\text{free}}
\]

- \( B + P \) normal viscosity of broth.
- \( B + P \) viscosity of medium doubled with twenty per cent glycerine.

There is no marked difference in the velocities of the reactions attributable to the increased viscosity in either set of experiments.
2. No. 1 was repeated with live B.
3. Viscosity of the P-dead B mixture was doubled by addition of twenty per cent glycerine. The reaction was followed in this medium and in an unaltered control.
4. No. 3 was repeated with live, resting cells. Controls demonstrated that the concentration of glycerine employed affected neither B nor P over the required time interval.

No detectable differences were observed in the reaction rates of shaken and stationary mixtures. As shown in Figs. 6a and 6b, doubling the viscosity of the medium with glycerine likewise produced no change in the rate of reaction. It may be concluded, therefore, that the reaction interval is concerned not with the diffusion of P through the medium to the bacteria but rather with a reaction between B and P. Whether this "reaction" implies diffusion of P into the bacterial cell or some chemical combination of B and P cannot be decided from the present data, although the fact that P distribution between live B and broth proceeds according to the Law of Normal Distribution would favor the view of the process being one of diffusion.

**DISCUSSION**

In the case of the antistaphylococcus phage and susceptible strain of *S. aureus* studied, it is clear from the experiments described that distribution of P between broth and B may be of two types. With live, resting, susceptible B, P is distributed in the same manner as is any substance soluble in both phases of a two phase system; *i.e.*, distribution is of normal type and may be expressed by the equation

\[ \frac{C_b}{C_a} = K \]

where \( C_b \) = extracellular P/ml. and \( C_a \) = intracellular P/ml. of B. The ready reversibility of the combination of P and B, a phenomenon noted as occurring qualitatively in earlier experiments on the kinetics of the P-B reaction (9) and shown in this paper to occur quantitatively, further supports the idea of distribution being strictly normal.

During the logarithmic phases of B growth and P formation (9) in a B-P mixture exactly the same type of distribution of P between growing B and broth is found to take place.

The procedure for determining P used throughout this work as well as in previously reported experiments is based upon the fact that the
t (lysis) of a set number of B contained in a unit volume of broth is quantitatively related to the amount of P initially present. No complications develop as long as the P added to the test B is free, extracellular P. However, when the method is employed for determining Total P/ml., i.e., extracellular and intracellular P, as in experiments on the kinetics of the bacterium-bacteriophage reaction (9) an obvious question arises; namely, whether or not it is justifiable to assume that the intracellular P fraction in a P-live B mixture is active in the titration system. The answer is that the P is not intracellular but extracellular for all samples are diluted somewhere within the limits 1/100-1/1,000,000 in setting up the titration and this factor operates to bring out the intracellular P fraction as free, unbound P. The mechanism leading to this result is found in the readily reversible, normal type distribution of P between live B and broth. Table II indicates the working of this mechanism in an actual case.

For the same strain of B killed at 65°C., 80°C. or 100°C. P distribution is of the adsorptive type and is expressible in the form of the adsorption isotherm equation \[ a = k \frac{C^{1/n}}{1} \] with \[ 1/n = 0.80 \] (average). The adsorption under ordinary conditions proceeds to > 99 per cent completion, is not reversible to any measurable degree, and as the ratio \( P/B \) rises saturation of B with P is approached. B killed at the three temperatures mentioned above show no significant differences in adsorptive ability.

Studies of the velocities of the reactions (a) Live, resting, susceptible B + P and (b) Heat-killed susceptible B + P show that the rates at which P is removed from solution by live and dead cells do not differ significantly. Experiments with shaken and unshaken P-B mixtures and with mixtures in which viscosity was doubled, lead to the conclusion that the reaction time is concerned with some sort of interaction between P and B and not with diffusion of P through broth to the bacteria. The velocity constant of the process of P-B combination may be calculated from the equation \[ k = \frac{1}{t[B]} \ln \frac{[P]_0 - [P]_t}{[P]_t - [P]_e} \] with good agreement throughout.

Two points not relating to the question of distribution were brought out by the experimental part of this work. First it was found that [P] in a mixture of live B and P is a very sensitive indicator of B growth
and that $[P]$ determinations will detect degrees of $B$ growth otherwise easily overlooked if direct estimation of $[B]$ is relied upon. The value of such an indicator is obvious when experimental requirements necessitate maintenance of live $B$ at a constant $[B]$ level. The sensitivity of $[P]$ to $B$ reproduction rests upon a previously described relationship (9) existing between the rates of $P$ production and $B$ reproduction; namely, that $dP/dt$ is proportional to a power of the rate $dB/dt$. Therefore very small increments in $[B]$ give rise to measurable increases in $[P]$.

The second point of interest relates to $[P]$ determinations in the presence of dead $B$. Total $[P]$ determinations of $P$-dead $B$ mixtures invariably give values identical with or less than the $[P]$'s of the supernatants left after centrifugation of $B$. The obvious conclusion is that $P$ taken up by dead cells is retained by them in an inactive form and consequently does not function in the $P$ titration. The experimental demonstration of the irreversibility of $P$ adsorption by dead $B$ substantiates this view, and with this in mind one would expect the total $[P]$ of a $P$-dead $B$ mixture to be that of the supernatant—as is actually true. If, however, $[B]$ is high or if low dilutions of a $P$-$B$ mixture are used in running the titration a considerable number of dead $B$ may be introduced into the titration set-up. As the test $B$ grow and produce $P$ appreciable amounts of the latter will be adsorbed by the dead cells. Since lysis depends upon the attainment of a particular $P/B$ ratio (9) the time of lysis will be prolonged and the mixture will appear to have a total $[P]$ less than the free $[P]$ at equilibrium.

The mixtures of known $[P]$'s with known concentrations of live $B$ used as controls to detect $B$ growth in distribution experiments with live non-growing $B$ served also as useful indicators of $B$ death. Dead $B$ take up relatively large amounts of $P$ irreversibly and since such $P$ does not function in the titration of $P$-$B$ mixtures for total $[P]$ determinations, it is clear that any large number of deaths among the $B$ population will reduce the apparent $[P]$ of control tubes. Consequently the control series of $P$-live $B$ mixtures served in this work as satisfactory criteria both of $B$ growth and $B$ survival under test conditions.
SUMMARY AND CONCLUSIONS

The above data relating to the antistaphylococcus phage and single strain of *S. aureus* with which previous papers have been concerned (9, 10, 11, 12), bring out the following points.

(a) For live, resting, susceptible *B* suspended in broth as well as for *B* in a *P-B* mixture during the logarithmic phases of *B* growth and *P* formation, *P* is distributed in a manner typical of numerous materials soluble in both phases of a two phase system, *i.e.*, distribution proceeds in accordance with the equation \( C_b/C_a = K \) where \( C_b \) = extracellular *P*/ml. of broth and \( C_a \) = intracellular *P*/ml. of *B*. The combination is quantitatively reversible.

(b) With heat-killed, susceptible *B*, *P* distribution is of the adsorptive type, expressible in the form of the adsorption isotherm equation \( a = kC^{1/n} \). The average value of \( 1/n \) is 0.80 in agreement with the results of von Angerer (2). Under ordinary conditions dead *B* take up much more *P* than do live *B*, the reaction proceeding to > 99 per cent completion. The combination of *P* with dead *B* is not demonstrably reversible and with high initial *P/B* ratios saturation of *B* with *P* is effected. Bacteria killed at 65°C., 80°C. and 100°C. show no differences in adsorptive ability.

(c) The rates at which live, resting, susceptible *B* and heat-killed, susceptible *B* remove *P* from solution do not differ significantly. Velocity constants of the process calculated from \( k = \frac{1}{t[B]} \ln \frac{[P]_0 - [P]_t}{[P]_0 - [P]_e} \) agree satisfactorily. It is shown that the time interval consumed is concerned with an actual reaction between *P* and *B* and not with diffusion of *P* through the broth to *B*.

(d) *P* determinations have been found to serve as satisfactory indicators for *B* growth in *P-B* mixtures where \([B]\) is to be maintained at a constant level. Very small increments in \([B]\) give rise to measurable increases in *P* by virtue of the fact that \( dP/dt \) is proportional to a power of the rate \( dB/dt \) (9).

(e) Similarly \([P]\) estimations will detect death of *B* cells in *P*-live *B* suspensions. Dead *B* take up large amounts of *P* irreversibly; such *P* cannot function in the titration and the result is a sharp drop in \([P]\) of controls.
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BIBLIOGRAPHY