INHIBITORY ACTION OF PHAGE K ON STAPHYLOCOCCAL DEHYDROGENASES

I. EFFECT ON VARIOUS STRAINS OF STAPHYLOCOCCUS AUREUS, INCLUDING MEMBERS OF THE PHAGE-TYPING SERIES

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Received for publication 22 April 1963

ABSTRACT

RALSTON, D. J. (University of California, Berkeley) and B. S. BAER. Inhibitory action of phage K on staphylococcal dehydrogenases. I. Effect on various strains of Staphylococcus aureus, including members of the phage-typing series. J. Bacteriol. 86:666-672. 1963.—The polyvalent phage K was found to depress the dehydrogenase activities of a large number of Staphylococcus aureus strains, as measured by the reduction of triphenyl tetrazolium chloride to the insoluble red formazan. The inhibition occurred immediately after the adsorption of a multiplicity of phage particles, and was independent of the infectibility of the strains and of the killing ability of the phage. It appeared to be closely associated with the phenomenon of sensitization—a change in the cell surface which increased the susceptibility of the wall to digestion by soluble staphylococcal lysins and simultaneously abolished the capacity to synthesize phage. The inhibitory effect occurred both in nutrient media and in a nongrowth glucose-phosphate buffer supplemented with cysteine.

The virulent staphylococcal phage K infects a large number of Staphylococcus aureus strains. Previous studies showed that purified phage preparations, adsorbed in sufficient numbers to living cells of strain K1, brought about a surface change allowing staphylococcal extracellular lysins to digest the wall, resulting in an immediate lysis-from-without (Ralston et al., 1955, 1957a). This phenomenon was referred to as “sensitization.” Sensitizing quantities of phage also blocked active phage infection. Even when adsorbed maximally with particles, the sensitized cells retained their coccal appearance, formed no phage, and exhibited no lysis for long periods.

This phenomenon may be related to the recent effects (also termed “sensitization”) noted by Lanni (1961) with the coliphage T1 at and its host Escherichia coli B, in which phage-bacterium complexes rapidly lost their plaque-forming abilities when they were placed in calcium-deficient challenge buffers.

It was found that the number of particles required to bring about sensitization varied with the cell age; cells reached a peak of sensitivity within 3 to 4 hr after inoculation on Tryptose Phosphate (TP) agar (Difco), at which time they could be sensitized by low multiplicities of phage. Infected cells were shown to remain uniformly resistant to virolysin until the end of the latent period, and evidence was presented to show that infection-lysis was in part mediated by intracellular amount of newly synthesized phage-induced virolysin (Ralston et al., 1961).

In other studies (Ralston, 1963), it was reported that the damage to the cell surface was specific in that it served to expose mainly those regions of the muropeptide substrate(s) affected by the normal cell autolysin and the phage-induced enzyme, virolysin, and only secondarily allowed egg-white lysozyme to act in combination with these lysins.

Mild heat in saline at 44 C also converted the cells to lysin sensitivity. We suggested that a heat-labile structure or mechanism must be intact for the cell to maintain its resistance to wall lysins. This damage was reversible by incubation in broth at 37 C, and it was noted that cells sensitized by phage at 4 C could also regain their lysin resistance and plaque-forming ability upon incubation at 37 C (Ralston et al., 1957a).

In the present studies, we have investigated the possibility that sensitization of the cell surface by phage K is accompanied by a damage to systems involved in energy supply to the cell.
The tests were conducted with triphenyl tetrazolium chloride (TTC) as a general indicator for the dehydrogenase capacity of the cells. It was found that the adsorption of phage in large multiplicity to living cells lowers the dehydrogenase activity of a large number of *S. aureus* strains, all strains capable of undergoing phage sensitization and lysis with virolysin being affected. The inhibitory property seems independent of the infectivity of the strain or of the killing action of the virus. The reaction is perhaps a critical step in the phenomenon of phage sensitization. It appears to represent a new pathological potential of the K phage.

**Materials and Methods**

**Bacterial strains.** *S. aureus* K1 was isolated in 1931 by A. P. Krueger from a case of multiple furunculosis. It has been maintained continuously in the laboratory, the last 10 years being on TP agar. Strains PS 29, 77, 83, 80, 81, 44A, 3A, 71, 6, 53, 42E, 73, 42D, 187, and 51, members of the staphylococcal phage-typing series, were obtained from J. E. Blair, New York Hospital for Joint Diseases, and were maintained on Tryptic Soy (TS) Agar (BBL). The propagating strain for phage 51 has been referred to as 51 in former publications but is here redesignated 51, in conformity with current nomenclature practice for these hosts.

For most tests, the strains were grown for 4 hr at 37 C on TP agar. The inoculum was an 18- to 24-hr slant of the same medium, suspended in 20 ml, and dispensed in 1-ml portions on the surface of standard agar plates. The cells were harvested in 0.55% (w/v) saline, washed in saline, and suspended in buffer to approximately 3.5 × 10⁶ cocci/ml.

**Phage.** The polyclonal, virulent staphylooccal K1 phage was used throughout. This has been referred to as P1, and is the parent phage from which a host-controllable mutant, P1a, now redesignated K1, was isolated (Ralston and Krueger, 1952). It was produced on host K1 in a double-strength TP broth. Usually, 500-ml Erlemeyer flasks containing 250 ml of medium were inoculated with 2 × 10⁶ cocci/ml, and were aerated by shaking at 37 C until the cells had reached 10⁶/ml (generally 3 hr). Then phage was added to give a phage-cell ratio (P/B) of 1:50, and the mixtures were incubated until lysed, about 4 to 5 hr. The lysates were stored at 4 C overnight, adjusted to pH 7.5 with 1 N NaOH, treated at room temperature with 0.5 μg/ml of ribonuclease and deoxyribonuclease (crystallized; Worthington Biochemical Corp., Freehold, N.J.) until the viscosity was reduced (visual estimation). Then 0.5 μg/ml of trypsin was added, and the flasks were incubated for another 2 hr at room temperature (sometimes at 37 C). Ethylenediaminetetraacetic acid (EDTA) was added to 0.02%, and the bacterial debris was centrifuged and sedimented at 2500 × g. The lysates were then subjected to several cycles of low- and high-speed centrifugation; the phage pellet was resuspended in EDTA-saline until the final cycle, when the EDTA was eliminated. The phage was generally concentrated about 30- to 50-fold by this procedure, to a titer of 3 to 5 × 10¹⁵/ml.

**Enzyme.** The lysin, virolysin, induced by staphylooccal phage K1 was obtained from the phage lysates, with the exception that trypsin was not used in the treatment of the crude lysates. The lysin was present in the high-speed supernatant after removal of the phage. In some experiments, this material was used directly; in others, the virolysin was purified as described previously (Ralston et al., 1957b), or by a procedure involving its adsorption on Super-Cel and elution at 4 C in 50% saturated salt solution, containing 0.1 M K3H2/KH2PO4 phosphate buffer, at pH 6.3. The eluate was concentrated by dialysis against dry Carbowax 4000 (Soller, 1961). The enzyme was labile and did not store well at 4 C or −20 C, so that during the course of these studies several separate preparations were used. There did not appear to be any major differences in these preparations relative to the subjects under investigation.

**Detection of sensitized cells, uninfected cells, and infected cells.** The methods for assaying phage, estimating lysin, and conducting lysis tests have all been described previously (Jones and Krueger, 1951; Ralston et al., 1957a, b; Ralston and Baer, 1960). In brief, cells were sensitized by addition of known multiplicities of purified phage in broth, in phosphate-saline buffer, or in the cystine-glucose-phosphate-saline (CGPS) buffer described below. Generally, with 4-hr cells of K1, a P/B of 10:1 was sufficient to sensitize more than 50% of the cells and thus provide sufficient lysable cells for a satisfactory test. The exact ratios are reported in Results. Where essential, the numbers of uninfected cells were estimated by
plate count on TP agar. Infective centers (cells capable of initiating plaque formation) were determined by plaque assay, the free phage having first been inactivated by exposure to 1:50 specific antiphage K serum for 10 min. The total numbers of "sensitized" cells (those susceptible to lysin) were estimated either by direct count or by turbidimetric determination of their lysis at 37 C in the presence of a strong virolysin preparation. A Klett photoelectric colorimeter, equipped with a 660-mu (red) filter, was used. The absolute numbers of cells were obtained from a standard curve relating Klett units (optical density = Klett units x 0.002) to direct microscopic counts. Lysis was followed by turbidimetric reading until it had come to a constant reading, and the fraction sensitized was calculated from the known number of cells lysed/initial cells. It had been shown in numerous tests that the debris from the lysed cells contributed no light-scattering effects in the concentrations used for these studies.

Assay of dehydrogenase activity. Cells of known ages were washed in saline and suspended in TP broth or CGPS buffer, generally to concentrations of 3 or 4 x 10^8/ml. After equilibration at 37 C for 5 to 20 min, TTC was added to final concentrations of 0.005 to 0.02% (w/v), and the relative amount of formazan produced was measured by reading the increases in optical density at 660 mu. (Some experiments were performed with a blue filter.) Calculations of the relative dehydrogenase activity were based upon the formazan (expressed as Klett units) produced per unit time by a constant number of cells.

Generally, after an initial period before the appearance of visible red formazan, the curves assumed an almost linear course, the slope of which varied with the cell concentration. The rate of reduction was not directly proportional to the initial concentration of cells (dehydrogenase). These effects appeared to resemble the observations of Slater (1959), who reported a nonlinearity of reduction of neotetrazolium by varying concentrations of rat liver mitochondria, and thought the response might be due to the removal of a heat-stable cofactor from the tissue preparations. We do not know whether this nonlinear relationship also applied to our measurements under conditions where the dehydrogenases of constant amounts of cells were depressed by phage. At best, the values for the dehydrogenase activities were relative, and were valid only for comparisons made on individual preparations within a single experiment.

Chemicals and buffers. The 2,3,5-2H-triphenyl tetrazolium chloride was obtained from Eastman Chemical Products, Inc. It was dissolved in distilled water and stored away from light. In some experiments, autoclaved preparations were used.

CGPS buffer contained: K2HPO4, plus KH2PO4, 0.066 M; MgCl2, 0.0002 M; cysteine-HCl, 0.013 M; glucose, 0.011 M; and NaCl, 0.105 M; pH 7.0.

Results

Characteristics of TTC reduction by S. aureus. Cells of S. aureus K1 caused a rapid reduction of TTC to red formazan in TP broth (Fig. 1). In this measurement, resting (18 hr) cells (2.3 x 10^8/ml) were equilibrated for 20 min at 37 C under static and shaken conditions. Then TTC was added to 0.01%, and growth and dehydrogenase activity were measured. In aerated flasks, cell growth was more rapid, but TTC reduction was poor. Therefore, all our tests were performed in stationary tubes, where the reduction was optimal.

To make comparisons of the dehydrogenase activity, the formazan formed was converted to an aqueous solution by the addition of 5 ml of 6% trichloroacetic acid. This solution was then filtered to remove cell debris and other insoluble material, and 0.4 ml of filtrate was added to 0.4 ml of 1% phenol in 0.011% HCl. The absorbancy was read at 540 mμ. With standard solutions of formazan, a straight line was obtained from 0 to 0.2 absorbancy.
activities of different *S. aureus* strains, it was necessary to correct the data for any cell growth which occurred during the incubation periods. Of course, when phage K was added to the cells, no correction was made for cell growth, for above one particle per cell division was promptly stopped. In general at 0.005% TTC, in TP broth, most strains grew equal to broth controls, but some strains seemed to be inhibited at higher concentrations. When cultured on the surface of TP agar, all strains were inhibited at 0.01%, were partially inhibited at 0.005%, but grew well at 0.001%. Members of phage group III appeared to be slightly more sensitive. The amount which was lethal (MLD) for *S. aureus* has been reported to be greater than 0.05% in Heart Infusion Broth at 37 C (May et al., 1960).

*S. aureus* has been shown to reduce TTC in glucose-phosphate buffers (Forbes and Sevag, 1951), especially in media supplemented with amino acids. We have found that the K1 strain required the presence of cysteine, and it differed from the strain 1A of these authors in that it could not reduce TTC in the presence of glycine and glutamic acid or use succinate as a substrate, even in the presence of cysteine (Table 1).

Microscopic examinations of wet mounts of untreated cells were made under oil immersion. Formalin was added to a final concentration of 1.3% (v/v) to stop all reaction (Lester and Smith, 1961). In our suspension, the dye was retained by the cocci for long periods without complications from autolysis. As early as 1 min after the dye was added, reduction had begun in over 95% of the cocci. In confirmation of the studies of Diena, Wallace, and Greenberg (1962), the formazan appeared to be located around the cocci, often seeming to bulge out from the cell. It increased in intensity with time, but we could not distinguish between an intracellular and an extracellular location.

**Effect of phage K on dehydrogenases of S. aureus K1.** In growth media and in the CGPS buffer, phage K caused a decrease in the reduction of TTC to formazan (Fig. 1). The inhibition occurred immediately after adsorption. It could be demonstrated both when phage was preadsorbed in the cold and TTC then added, and when phage was added at intervals to cells already in the process of reducing the dye. The inhibition required large amounts of phage. Its extent was increased with increasing phage-cell ratios. The conditions under which phage produced dehydrogenase inhibi-

### Table 1. Effect of amino acids on tetrazolium reduction by logarithmic-phase *Staphylococcus aureus* K1 in glucose-phosphate buffer

<table>
<thead>
<tr>
<th>Component tested*</th>
<th>Relative amount of formazan (Klett units/90 min at 37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell control in buffer†</td>
<td>3</td>
</tr>
<tr>
<td>Glucose</td>
<td>0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4</td>
</tr>
<tr>
<td>Glycine</td>
<td>1</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>3</td>
</tr>
<tr>
<td>Glucose + glutamic acid</td>
<td>13</td>
</tr>
<tr>
<td>Glucose + glycine</td>
<td>13</td>
</tr>
<tr>
<td>Glucose + cysteine</td>
<td>211</td>
</tr>
<tr>
<td>Succinic acid + glutamic acid</td>
<td>0</td>
</tr>
<tr>
<td>Succinic acid + glycine</td>
<td>4</td>
</tr>
<tr>
<td>Succinic acid + cysteine</td>
<td>4</td>
</tr>
</tbody>
</table>

* Concentration of glucose and succinic acid = 1 mg/ml; cysteine, glutamic acid, and glycine = 100 μg/ml.
† Initial turbidity = 30 Klett units.
‡ Buffer = 0.02 M phosphate (pH 7.0), containing 0.02 M Ca++, Mg++, and Mn++ ions.

Dehydrogenase inhibition by phage K, a general occurrence with *S. aureus* strains. Our previous studies had shown that a large number of *S. aureus* strains (all those capable of adsorbing phage K) were susceptible to sensitization by sufficient amounts of phage (Ralston et al., 1957b). In the present study, a test was made of the effect of sensitizing quantities of phage on the dehydrogenases of several of these *S. aureus* strains. The degree of inhibition of their dehydrogenases was compared with the total lysis produced by virolysin. Purified K phage was added to cells in TP broth. After adsorption of the phage, TTC was added to a final concentration of 0.02%, for measurement of the residual
dehydrogenases. Comparable phage-treated samples were exposed to strong amounts of virolysin, and the fractions of cells sensitized were calculated from the known numbers of cells lysed at 37 C. Every strain susceptible to sensitization exhibited a susceptibility to dehydrogenase inhibition, but the degree of lysis was not directly related to the inhibition of TTC-reducing capacity (Table 2). The quantities of walls which the phage underwent lysis, whereas 3A, exposed to a P/B of 14 particles, lysed 64%. This was not related to the percentage of dehydrogenase depression. Some of these differences may have depended upon the structural configuration of the walls which might have affected their rate of lysis. The data show that dehydrogenase inhibition is a rather general property of phage K.

**Relationship of the antidehydrogenase effect to other biological functions of K phage.** It is already known that sensitized cells cannot support phage synthesis. In fact, sensitization has been found to block phage replication and also to prevent the appearance of the induced enzyme, virolysin (Ralston et al., 1961). In other words, sensitization and infection have been shown to be mutually exclusive events. From this, it seems possible that if the antidehydrogenase activity of phage were to apply mainly to sensitized cells the dehydrogenases of infected cells would be expected to remain diminished. Unfortunately, studies of infected cells have been difficult to perform with strain K, because, even at low phage multiplicities, the population has been found to be mixed with a large fraction of sensitized cells. The possibility that dehydrogenase changes pertained to these cells could not be easily eliminated. However, by using older cells of strain PS 51, we have been able to infect larger percentages of cells and thus obtain sufficient proportions of infected cells to measure their TTC-reducing capacities. In this study, resting cells were suspended in TP broth to 2.4 × 10^8/ml; phage K was added from P/B = 0.23 to 4.36. After 20 min at 28 C, the uninfected cells were

| Table 2: Effect of K phage on dehydrogenase activities of Staphylococcus aureus strains |
|-----------------|--------|----------------|-----------------|-----------------|-----------------|-----------------|
| Phage type | Strain | Phage-cell ratio | Per cent lysis (phage + virolysin) | Relative dehydrogenase (Klett units per 20 min per cell × 10^{-4}) | Per cent inhibition of dehydrogenase | Per cent killing |
| I | 80 | 16 | 53 | 1.1 | 0.5 | 56 | NT* |
| I | 44A | 11 | 26 | 1.1 | 0.0 | 100 | NT |
| II | 3A | 14 | 64 | 2.5 | 0.3 | 88 | NT |
| II | 71 | 14 | 38 | 2.0 | 0.7 | 67 | NT |
| III | 6 | 14 | 44 | 2.3 | 1.4 | 39 | NT |
| III | 7 | 26 | 50 | 1.2 | 0.9 | 27 | NT |
| III | 42E | 22 | 61 | 3.4 | 0.2 | 95 | NT |
| III | 53 | 16 | 54 | 0.7 | 0.2 | 66 | NT |
| IV | 42D | 21 | 50 | 1.4 | 0.4 | 74 | NT |
| Misc. | 81 | 13 | 42 | 1.7 | 1.0 | 41 | NT |
| Misc. | 73 | 27 | 21 | 1.6 | 0.3 | 85 | NT |
| Misc. | 187 | 51 | 18 | 1.5 | 0.5 | 66 | NT |
| I | 29† | 3 | 63 | 2.4 | 1.4 | 40 | 99 |
| I | 9 | 70 | 2.4 | 0.5 | 78 | NT |
| I | 27 | 96 | 2.4 | 0.2 | 90 | NT |
| III | 83† | 5 | 95 | 5.0 | 3.0 | 20 | NT |
| III | 10 | 105 | 5.0 | 2.8 | 44 | NT |
| III | 20 | 100 | 5.0 | 2.4 | 52 | NT |
| III | 77† | 3 | 95 | 5.0 | 0.4 | 92 | 91 |
| III | 4 | 100 | 5.0 | 0.2 | 96 | 96 |
| III | 11 | 100 | 5.0 | 0.1 | 99 | 99 |

* Not tested.
† Plaque-nonforming strains. Strain 83 tested in CGPS buffer instead of TP broth.
determined by plating on TP agar. The numbers of infected cells were assayed by plaque count (after exposure to specific antiserum), and the dehydrogenase levels were assayed in the presence of 0.01% TTC. At P/B = 1.66, an estimated 87% of the cells had received at least one particle, and at least 51% of the cells formed plaques, i.e., were actively infected, whereas there was only an insignificant decrease (9%) in the dehydrogenase activity (Table 3). At P/B = 4.36, the population of cells could be accounted for as composed of 1.9% uninfected cells plus 41.5% infected (plaque-forming) plus 50% sensitized (plaque-nonforming, virolysin-lysable), a total of 93.4%. At the lesser P/B, no estimate was made of the number of sensitized cells, and therefore the entire population was not accounted for. However, since the main interest in this experiment was to obtain a large fraction of infected cells and to measure the attendant dehydrogenase activity, it was not considered essential to assay all the sensitized fractions.

The antidehydrogenase effect of phage K was also shown to occur with strains which could not support phage formation (plaque formation), e.g., PS 83, 77, and 29 (Table 2). The antidehydrogenase activity thus seems to be unrelated either to the processes involved in the initiation of infection—at least at low P/B—or to the potential capacity of a strain to support phage synthesis.

When phage K was adsorbed in TP broth at values in the order of P/B = 1 to cells of strain 83, it was found that more than 90% of the cells could be killed, as defined by failure to form colonies on agar, without losing their ability to reduce TTC. Similar results have been observed for strains K₁ and 51. Although it is recognized that the antidehydrogenase activity probably includes a killing action, it is evident that killing per se by one particle can occur via a separate pathway, i.e., in the absence of dehydrogenase inhibition.

**DISCUSSION**

The analysis of the antidehydrogenase and sensitizing activities of phage K is quite complex. Our investigations suggest that sensitization by phage is accompanied by a decrease in the cellular dehydrogenase activities. The antidehydrogenase effect does not appear to be related to the ability of the phage to kill; neither does it seem to occur when cells are infected at low multiplicities in the range of one particle per cell, such cells having been shown to be nonsensitized, i.e., resistant to virolysin. Under conditions of high phage multiplicity, the degree to which the antidehydrogenase effect pertains to sensitized and infected (nonsensitized) cells is less clear. At high phage levels the cell populations contain mixtures of sensitized and infected (nonsensitized) cells, and there was no estimate of the relative activities of the individual cells in each category. In some tests, all the TTC-reducing capacity of the sensitized fractions was obliterated by phage. In other tests, there appeared to be a proportion of sensitized cells still capable of reducing the dye; these, of course, could be lysed by virolysin. This indicates that loss of resistance to virolysin is not dependent upon an all-or-none block in the dehydrogenases; therefore, if there is any relationship between the two

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**TABLE 3. Dehydrogenase activity of infected Staphylococcus aureus 51**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phage-cell ratio</th>
<th>Uninfected cells/ml × 10⁹</th>
<th>Per cent uninfected cells</th>
<th>Infected cells/ml × 10⁹</th>
<th>Per cent infected cells</th>
<th>Resident dehydrogenase (Klett units per 60 min per cell × 10⁻²)</th>
<th>Per cent inhibition of dehydrogenase</th>
<th>Per cent lysis with virolysin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>2.40</td>
<td>100</td>
<td>0.00</td>
<td>0.0</td>
<td>2.00</td>
<td>0</td>
<td>NT*</td>
</tr>
<tr>
<td>2</td>
<td>0.23</td>
<td>2.40</td>
<td>100</td>
<td>0.13</td>
<td>5.5</td>
<td>2.10</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>0.38</td>
<td>2.40</td>
<td>100</td>
<td>0.41</td>
<td>17.0</td>
<td>2.00</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>0.48</td>
<td>1.20</td>
<td>50</td>
<td>0.34</td>
<td>14.0</td>
<td>1.85</td>
<td>7</td>
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<tr>
<td>5</td>
<td>0.79</td>
<td>1.00</td>
<td>41.5</td>
<td>0.94</td>
<td>39.0</td>
<td>1.85</td>
<td>7</td>
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</tr>
<tr>
<td>6</td>
<td>1.66</td>
<td>0.42</td>
<td>17.5</td>
<td>1.22</td>
<td>51.0</td>
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<td>7</td>
<td>4.36</td>
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<td>1.9</td>
<td>1.00</td>
<td>41.5</td>
<td>1.30</td>
<td>35</td>
<td>50</td>
</tr>
</tbody>
</table>

* Not tested.
phage properties, it is dependent upon a more subtle, less drastic interference with these cellular enzymes.

In a separate publication (Ralston and Perry, 1963), a more detailed analysis has been presented of the quantitative effects of the antidehydrogenase and sensitizing activities of the phage. In these studies, the accumulated evidence strongly suggests that sensitization is accompanied by a severe decrease in the dehydrogenases to critical threshold levels. Above these levels, the cells appear capable of maintaining resistance to virolysin.

Regardless of its role in cellular resistance to lysis, the antidehydrogenase activity of this phage provides a new basis for studying the pathological potentials of this virus for the staphylococci.

ACKNOWLEDGMENT

This research was supported by grant E-3776 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

LITERATURE CITED


