MECHANISM OF ACTION OF STAPHYLOCOCCAL ALPHA-HEMOLYSIN

II. Analysis of the Kinetic Curve and Inhibition by Specific Antibody

AMERICO A. MARUCCI

Department of Microbiology, Upstate Medical Center, State University of New York, Syracuse, New York

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Abstract

Marucci, Americo A. (Upstate Medical Center, Syracuse, N.Y.). Mechanism of action of staphylococcal alpha-hemolysin. II. Analysis of the kinetic curve and inhibition by specific antibody. J. Bacteriol. **86**:1189–1195. 1963.—At least two steps are necessary before the rabbit erythrocyte is lysed by staphylococcal alpha-hemolysin. The first step involves the reaction of alpha-hemolysin with the red cell. The second step, leading to the release of hemoglobin, is an intrinsic reaction of the damaged red cell and takes place without further participation of hemolysin. The speed of this intrinsic reaction is temperature-dependent. Erythrocytes taken from the same rabbit do not vary in their susceptibility to the alpha-hemolysin. From the results of the experiments described herein, a preliminary hypothesis on the mechanism of action of staphylococcal alpha-hemolysin is given.

The concept that staphylococcal alpha-hemolysin is an enzyme has received consideration from several quarters. Van Heyningen (1950), in discussing gram-positive toxins, noted their similarity to enzymes in being capable of acting in extremely low concentrations, but he cautioned that it does not follow that all toxins are enzymes. Robinson et al. (1960) reported that their highly purified alpha-hemolysin preparation could hydrolyze casein. This was the first report of alpha-hemolysin acting on an isolated substrate. However, Bernheimer and Schwartz (1963), using casein, clotted milk, or gelatin as substrate, could not demonstrate enzymatic activity. They concluded that the alpha-hemolysin is probably not a proteolytic enzyme. The results of Robinson et al. (1960) have not yet been confirmed by other workers. Lominski and Arbuthnott (1962), on the basis of their kinetic results, also advanced the enzymatic hypothesis.

By use of the methods described in the accompanying paper (Marucci, 1963), experiments were done to elucidate the mechanism of action of the staphylococcal alpha-hemolysin. This has been approached through two main avenues of experimentation: (i) by analyzing conditions and varying reagents which affect the different portions of the kinetic curve, and (ii) by studying the inhibition of alpha-hemolysin by specific antibody added at various times during the reaction. On the basis of the results of these experiments, a preliminary hypothesis on the sequence of steps leading to lysis of the erythrocyte by alpha-hemolysin is proposed.

Materials and Methods

All the reagents and conditions for performing the analyses described in this report are given in detail in the accompanying paper (Marucci, 1963). New variations will be described with the experiments in which they are introduced. Each experiment has been repeated at least once, but most often they have been rerun three to four times with the same results.

Results

Demonstration of excess hemolysin activity. To show that there is no demonstrable diminution in alpha-hemolysin activity as the kinetic analyses are usually performed, the experiment shown in Table 1 was done. Flask A represents a typical kinetic analysis. In the remaining flasks, the cells were removed by centrifugation after exposure for the indicated times. To 14 ml of each supernatant fluid, 1.0 ml of new intact cells was added, and the kinetic course of hemolysis was followed in the usual fashion (Fig. 1).

Despite the varying degrees of lysis obtained during the first exposure of the hemolysin to the rabbit erythrocytes, the lysis of new cells by the supernatant fluids from these reactions was identical. This would indicate that, under these condi-

tions, the hemolysin is not used up or fixed in its reaction with rabbit erythrocytes.

Further demonstration of undiminished hemolysin activity after erythrocyte lysis. Four flasks were set up with identical amounts of hemolysin. At the starting time, 2.0 ml (5×10^7) of cells were added to flasks A, B, and C, and samples were removed from flask A. At 32 min, when the hemolysis in all flasks had gone to completion, a second portion of cells was added to flasks B and C, and samples were removed from flask B. After 35 min, 2.0 ml of the same cell suspension were added to flasks C and D. This represented the third addition of cells to flask C and the first to flask D, which served as the control for the nonspecific inactivation of the alpha-hemolysin held at 20 C for 1 hr and 8 min (Fig. 2).

TABLE 1. *Protocol for experiment to demonstrate constant hemolysin activity in supernatants after exposure of alpha-hemolysin to erythrocytes for varying periods*

Flask	Hemolysin (1:100)	Buffer	Cells*	Time reaction took place before centrifuging	Per cent lysis in supernatants
	ml	*ml*	*ml*	*min*	
A	5.0	23.0	2.0	—	—
B	5.0	23.0	2.0	2	3
C	5.0	23.0	2.0	15	57
D	5.0	23.0	2.0	60	99
E	—	28.0	2.0	—	—

* Optical density = 0.600.

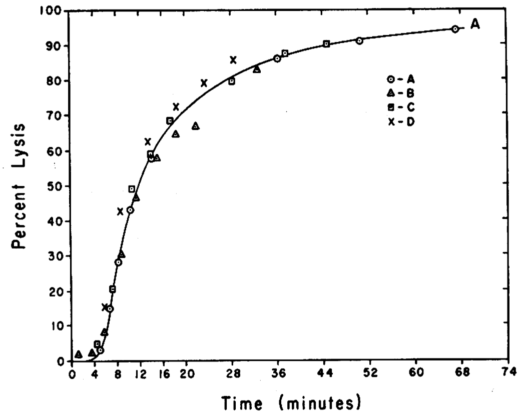

FIG. 1. *Demonstration of constant alpha-hemolysin activity in supernatant fluids removed from reactions in which varying amounts of lysis had taken place.*

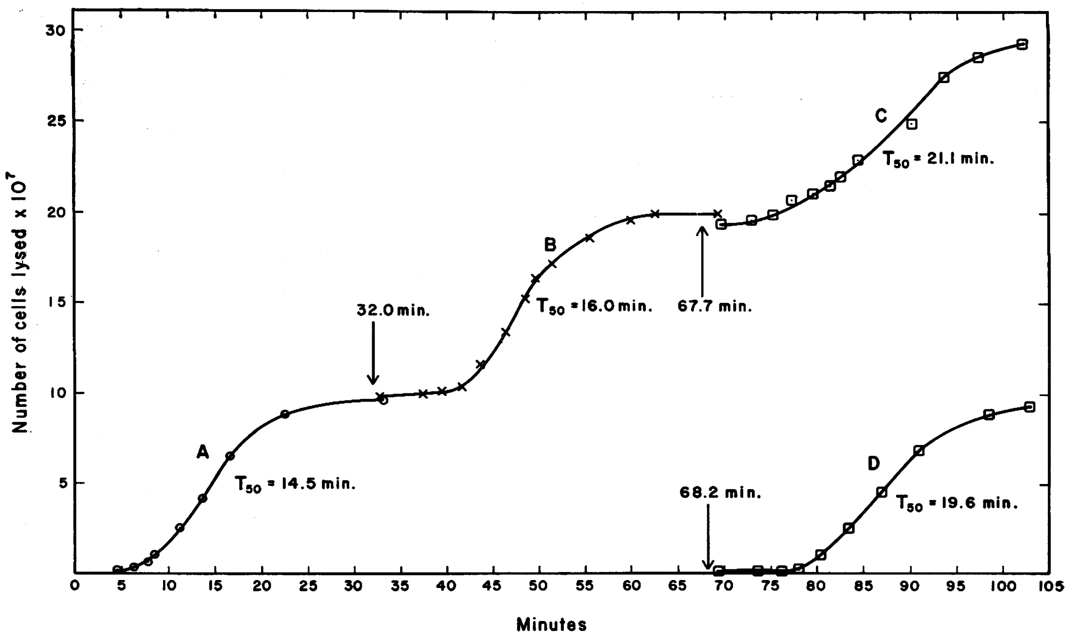

FIG. 2. *Kinetic study of hemolysis of a new batch of cells added to a reaction mixture in which the previous cells had been lysed by staphylococcal alpha-hemolysin.*

The T_{50} values for flasks A, B, C, and D were 14.5, 16.0, 21, and 19.6 min, respectively. When the nonspecific inactivation of the alpha-hemolysin is taken into consideration (*see* Table 1, Marucci, 1963), the T_{50} for each of these reactions can be considered as being identical. This means that there is an excess of hemolysin activity under the conditions of these experiments.

Effect of antibody addition before and at varying times after addition of the standardized erythrocyte suspension at 20 C. This kind of experiment was performed to learn what, if any, difference in inhibition could be demonstrated by addition of antibody at various times during the course of the kinetic analysis. Seven flasks, labeled A to G, each containing identical amounts of hemolysin and buffer, were set up. Flask G was the control for kinetic analysis without added antiserum. The times for addition of the antiserum are indicated in reference to the time of addition of the standardized erythrocyte suspension (Fig. 3).

Antibody present at zero time can completely inhibit the action of alpha-hemolysin on rabbit red blood cells (flask A). In this experiment, the hemolysin was also completely inhibited when antihemolysin was added 0.3 min after the cells (flask B). For this to occur, the hemolysin must be immediately bound and rendered ineffective by the antihemolysin. This result was expected and it was further expected that the addition of antihemolysin at later times in the kinetic curve would immediately arrest the hemolytic process and no further release of hemoglobin could take place because all of the hemolysin would be bound. However, in flasks C, D, E, and F, the hemolysis progresses despite the addition and continued presence of sufficient antibody to inhibit efficiently this amount of hemolysin. Since the inhibition of hemolysin by antihemolysin is instantaneous (flask B), this must mean that those cells which continue to lyse no longer need hemolysin. They have already been damaged by hemolysin early in the reaction and are doomed to progress to lysis.

Effect of antibody addition before and at varying times after addition of the standardized erythrocyte suspension at 37 C. This experiment was identical to the preceding experiment, with the exception that the reaction was run at 37 C instead of 20 C.

At 37 C the kinetic curves describing the lysis after addition of antihemolysin approach a plateau (Fig. 4). At 20 C the cells continue lysing

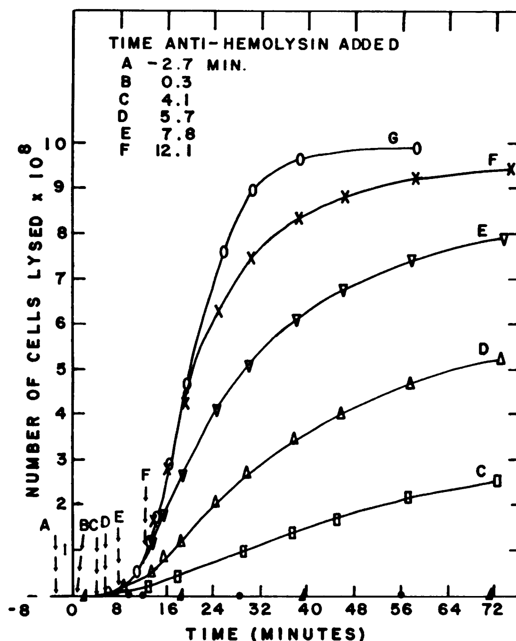

FIG. 3. *Inhibition of alpha-hemolysin by antihemolysin added at varying times during the kinetic reaction at 20 C. Each flask contained the following: hemolysin (1:50), 1.5 ml; rabbit erythrocytes (5 × 10^8 cells per ml), 2.0 ml; buffer, 25.0 ml. Flasks A–F received 1.5 ml of antihemolysin (1:200) at the indicated times. To flask G, the control without antihemolysin, 26.5 ml of buffer were added so that the total volume would be 30.0 ml.*

with no apparent leveling off, at least for the time in which the reactions were followed.

Postantibody sampling. The results from the experiments on antibody inhibition raised the question of whether the sampling procedure employed was in fact measuring all of the cells which had been acted upon by the alpha-hemolysin. The hemoglobin in the supernatant fluids collected in the usual sampling technique represents the reaction from only those cells which have progressed to lysis at the instant that the supernatant fluid is poured off. It is obvious from the preceding experiments that there is a number of cells which have already been damaged by the hemolysin, but have not yet released their hemoglobin because the intrinsic reaction has not yet had time to go to completion. This experiment was set up to study simultaneously the two types of sampling, viz., (i) the regular method in which the sample removed from the reaction mixture was immediately centrifuged and the supernatant

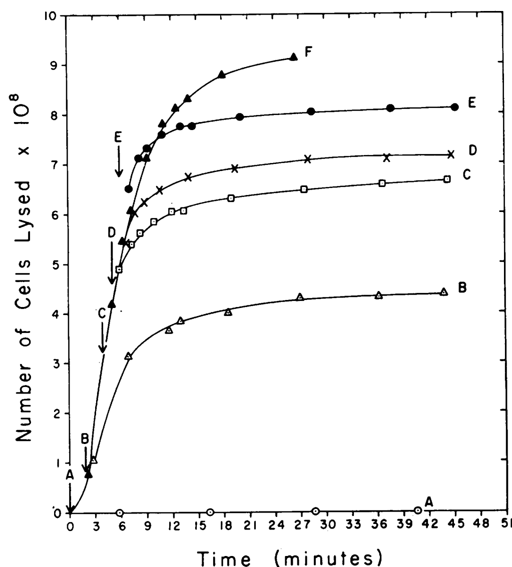

FIG. 4. *Inhibition of alpha-hemolysin by antihemolysin added at varying times during the kinetic reaction at 37 C. Each flask contained the following: hemolysin (1:50), 1.5 ml; rabbit erythrocytes (5 × 10⁸ cells per ml), 2.0 ml; buffer, 25.0 ml. Antihemolysin (1:200), 1.5 ml, was added to flask A at 0 min, flask B at 2.04 min, flask C at 4.00 min, flask D at 5.04 min, and flask E at 5.99 min. No antihemolysin was added to flask F; 26.5 ml of buffer were added to bring the total volume to 30.0 ml.*

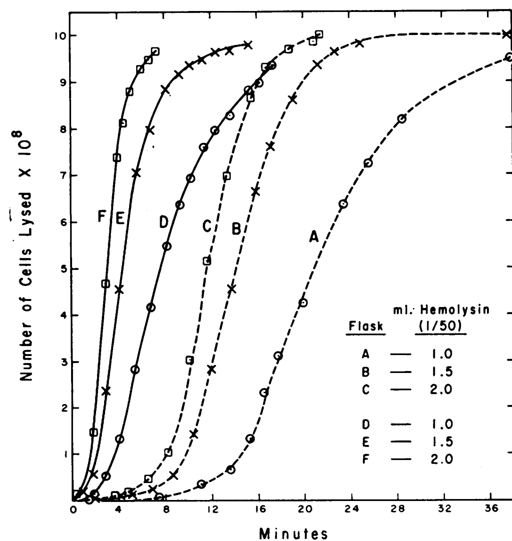

FIG. 5. *Comparison of the kinetic curves obtained by the regular sampling technique (flasks A, B, and C) and by postantibody sampling (flasks D, E, and F) with three different levels of hemolysin.*

fluid was removed for optical density determinations, and (ii) a new sampling method (postantibody) in which an accurately measured sample of the reaction mixture was pipetted into an equal volume of antihemolysin, mixed, and placed at 37 C for 1 hr. The hemolysin is immediately bound and rendered ineffective, but all of the cells which have been damaged will progress to lysis. The supernatant fluid from these tubes will represent all of the cells which had been acted upon by the alpha-hemolysin at the instant of sampling. The results of one such experiment, using varying amounts of hemolysin, are shown in Fig. 5.

This postantibody sampling procedure results in a displacement of the kinetic curves to the left. It was felt that this sampling technique would eliminate the lag period, but, though the lag is greatly reduced, it is still present.

Uniformity of susceptibility to alpha-hemolysin. A common explanation for the decreasing rate of lysis in the terminal portion of the kinetic curve is that the cells differ with respect to their susceptibility to lysis by alpha-hemolysin. It has been postulated that those cells most resistant are destroyed more slowly (Lominski and Arbuthnott, 1962). Heretofore, it has not been possible to test this explanation, and it has generally been assumed to apply. With the results of our studies on the inhibition of hemolysin by antibody, the way was cleared for the experimental examination of this postulate. The experimental design was to have alpha-hemolysin act on an erythrocyte suspension and then, at the proper time, to add suffi-

TABLE 2. *Experimental protocol for selection of cells to demonstrate the homogeneity of rabbit erythrocytes with respect to susceptibility to lysis by alpha-hemolysin*

Flask*	Hemolysin (1:50)	Rabbit erythrocytes†	Buffer	Antihemolysin (1:200)	Time of addition of antihemolysin	Per cent lysis after 1 hr at 37 C
		ml	*ml*	*ml*	*min*	
I	3.0	30.0	7.5	4.5	4.2	85.0
II	—	10.0	3.5	1.5	3.2	0
III	—	10.0	5.0	—	—	0

* Cells from these flasks were washed five times with buffer at 0 C and were restandardized to contain 5 × 10⁸ cells per ml.

† Optical density = 0.600.

TABLE 3. *Kinetic analyses demonstrating the homogeneity of rabbit erythrocytes with respect to susceptibility to lysis by alpha-hemolysin*

Flask	Flask from which cells were taken	Cells*	Buffer	Hemolysin (1:100)
		ml	*ml*	*ml*
A	I	2.0	26.5	1.5
B	II	2.0	26.5	1.5
C	III	2.0	26.5	1.5
D	—†	2.0	26.5	1.5
E	I	1.0	14.0	—
F	II	1.0	14.0	—
G	III	1.0	14.0	—
H	—†	1.0	14.0	—

* Optical density = 0.600.

† In these cases, the original cell suspension was used.

cient antihemolysin to combine with and neutralize the action of the hemolysin. The mixture was left for 1 hr at 37 C to permit all of the damaged cells to progress to lysis. The remaining cells represent those which are supposed to be most resistant to lysis. These cells were washed and restandardized. A kinetic analysis was then performed to compare the susceptibility of these selected "resistant" cells with (i) cells to which the same amount of antibody had been added and which were washed and restandardized as were the test cells; (ii) control cells to which no antihemolysin was added, but which were carried along at 37 C for 1 hr followed by washing and restandardization; and (iii) the original cell suspension not carried through these procedures. The protocol for this experiment is given in Table 2, and the results are shown in Table 3 and Fig. 6.

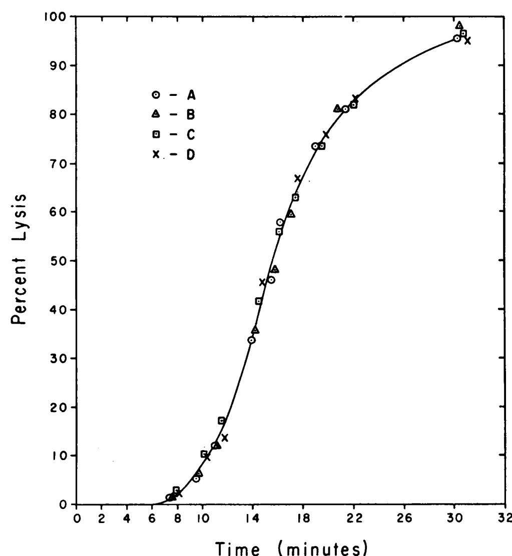

FIG. 6. *Comparison of kinetic analyses of a standardized selected cell suspension (flask A); cells with antihemolysin, kept at 37 C for 1 hr, washed, and restandardized (flask B); cells kept at 37 C for 1 hr, washed, and restandardized (flask C); and the original standardized cell suspension kept at 0 C (flask D).*

There was no difference in the susceptibility of erythrocytes remaining after 85% of the population had gone on to lysis, as compared with the original erythrocyte suspension. The kinetic curves describing the lysis of each batch of cells are superimposable. Another experiment, in which the final 5% of the cell population was isolated, yielded the same result.

DISCUSSION

From the results of the experiments reported herein, it appears that the lysis of rabbit erythrocytes by alpha-hemolysin progresses in at least two steps. The first reason for this conclusion is based on the presence of the lag period in the kinetic curves. The lag can be due to the progress of several sequential steps leading to lysis or to the necessity for the accumulation of a critical number of hits before the erythrocyte is destroyed. The second reason stems from our finding that lysis continues after the addition of antihemolysin. This occurs despite the fact that the hemolysin is immediately bound and rendered ineffective by antibody. Therefore, those cells which progress to lysis, after antihemolysin addition (post-antibody), represent cells which have already been damaged by the hemolysin; i.e., they have been critically damaged and are doomed to eventual destruction without any further contribution from the medium. We have called these cells "Er*." This is analogous to the situation found by Mayer (1961) in his studies of the lysis of sensitized sheep erythrocytes by guinea pig complement. He called this step the terminal transformation reaction. It takes place independently of any of the components of complement. As was shown with the rabbit erythrocytes used here, the speed of the terminal transformation of sheep erythrocytes is temperature-dependent. I have

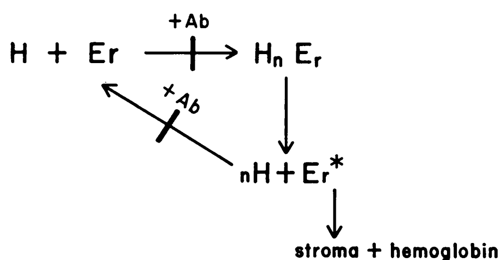

FIG. 7. *Schematic presentation of the steps leading to lysis of rabbit erythrocytes by staphylococcal alpha-hemolysin.*

not yet determined whether the final degree of lysis reached in my system is independent of temperature.

Any hypothesis proposed to explain the mechanism of action of staphylococcal alpha-hemolysin must take into account the decreasing rate of lysis in the terminal portion of the kinetic curve. Five possible mechanisms have been considered to account for this phenomenon, and, on the basis of our experimental results, all have been rejected. They are: (i) the hemolysin might be used up in performing its hemolytic work; (ii) the hemolysin might be diverted to unfruitful reactions with stroma, the proportion of which increases greatly in the later portion of the reaction; (iii) the hemolysin might be inactivated by contents of the erythrocytes which are released into the medium after lysis; (iv) the hemolysin might be thermally inactivated during the period of hemolysis; and (v) the cells might be heterogeneous with respect to susceptibility to lysis by the hemolysin and those cells lysed last represent the most resistant cells. The first four possibilities would all result in a real (i, iii, iv) or only an apparent (ii) decrease in hemolytic activity. However, the experiments reported here demonstrate that there is no decrease in hemolysin activity, and these four propositions can be eliminated as major contributory factors to the shape of the terminal portion of the kinetic curve.

Furthermore, as shown in the accompanying paper (Marucci, 1963), the lysis of rabbit erythrocytes by alpha-hemolysin follows the "percentage law" (Andrewes and Elford, 1933). This further substantiates the conclusion that alpha-hemolysin activity is in excess under the conditions of these experiments. The role of cell heterogeneity has also been tested. Isolation of 15% of the cells which were "most resistant" to lysis, and comparison of the rate of lysis of this selected cell population with the original, supposedly heterogeneous, population, showed no difference in susceptibility to lysis by alpha-hemolysin.

The possibility of the production of an intermediate compound which, in turn, produces hemolysis was also eliminated. This is the mechanism by which snake venom lecithinase lyses red blood cells; i.e., the venom acts by splitting lecithin to produce lysolecithin, which is the compound responsible for hemolysis (van Heyningen, 1950). If this were the mode of action of staphylococcal alpha-hemolysin, the supernatant fluids in Fig. 1 would yield progressively increased rates of lysis, and one would expect faster lysis of the second and third added cell samples in Fig. 2. Neither of these events took place; therefore, it can be concluded that no hemolytic intermediate is liberated.

On the basis of the results thus far available, a postulated sequence of events leading to hemolysis of rabbit erythrocytes is given schematically in Fig. 7.

Alpha-hemolysin, H, reacts with the erythrocyte, Er, to give the intermediate, H_nEr. This, in turn, dissociates into active H and Er*, the damaged erythrocyte. The Er* eventually lyses, releasing its hemoglobin and leaving a ghost. The subscript, n, denotes the number of molecules of hemolysin necessary to alter the intact erythrocyte to Er*. The results (Fig. 5) indicate that n is probably small, because the lag is almost completely eliminated by postantibody sampling. Antihemolysin, Ab, can completely block the formation of H_nEr, by combining with H. However, Ab can play no role in the progress of Er* to stroma and free hemoglobin (Fig. 3 and 4).

The representation given in Fig. 7 attributes catalytic activity to the staphylococcal alpha-hemolysin. However, we have not isolated the intermediate complex, H_nEr. Similarly, we have not yet rigorously proven that n molecules of H are available for further reaction with Er after they have acted to form Er*. The results in Fig. 1 and 2 indicate that H is capable of cycling as shown in Fig. 7. These results appear to substantiate the view that staphylococcal alpha-hemolysin acts like an enzyme. Its molecular weight, ca. 40,000, puts it in the class of proteins (Bernheimer and Schwartz, 1963; Marucci, *unpublished data*). The fact that its activity is completely inhibited by specific antibody is the usual finding with enzymes active on substrates of high molecular weight (Cinader, 1963).

A point difficult to reconcile with the enzyme hypothesis is the diverse origin and nature of alpha-hemolysin substrates. Alpha-hemolysin is active, to varying degrees, on many mammalian erythrocytes (Elek, 1959; Bernheimer and Schwartz, 1963). In addition, if the dermonecrotic, lethal, and leukocidal activities are attributed to this molecule (Kumar and Lindorfer, 1962), its spectrum of cellular substrates is further diversified. This, of course, does not eliminate it from this class, since these various cells may present the same chemical compound or grouping for which the enzyme is active. An example of this is the alpha-toxin of *Clostridium welchii* which is known to be the highly specific enzyme, lecithinase C, yet which hemolyzes red blood cells, is dermonecrotic, and is lethal. The final decision on the enzymatic nature of the staphylococcal alpha-hemolysin must await isolation, identification, and kinetic analyses of its substrates.

ACKNOWLEDGMENTS

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