FACTORS MODIFYING THE REACTION OF ALPHA STAPHYLOLYSIN TO HEAT*

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The generally accepted view that alpha staphylolysin is thermolabile has recently been questioned by Smith.[21] He further demonstrated that some samples of toxin were rendered inactive by heating at 60° C. for 30 minutes, yet retained considerable lytic activity when heated for the same period at 80°.

The thermostability of staphylolysin during certain stages of its evolution in shake cultures, as well as the phenomenon of greater stability at higher than at lower temperatures, have been fully confirmed. The present report is concerned with an attempt to elucidate further the mechanisms underlying this unusual behavior toward heat.

General Methods

Alpha staphylolysin of the Wood 46 strain was produced by a modification of the method of Casman,[6] consisting in the agitation of the culture throughout its period of incubation. Both a relatively slow, vacuum-driven rocking type of shaking device and a more rapid motor-driven shaker operating in the horizontal plane only were employed. All cultures were grown at 37.5° C., usually on heart infusion broth with physiological saline and 1 per cent neopeptone at an initial pH of 7.2-7.4. The hemolysin tests were carried out in an open water-bath at a temperature of 40°, using thrice-washed rabbit cells suspended in physiological saline. The degree of hemolysis was estimated on a scale of 4+ for the maximum at 2, 5, 10, 15, 30, and 60 minutes, as well as after refrigeration overnight. Whenever numerical representations for hemolysin titers are given, they consist in an expression of the velocity of the reaction during the first hour of observation in the water-bath, and are calculated according to the method of Smith,[21] representing the summation of the reciprocal values of the time by the degree of lysis at the stated intervals. The overnight results are not incorporated in these figures, since their added numerical value was negligible. These later observations were of use as a further check on the interpretation of the degree of lysis

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during the first hour of incubation. The samples of toxin were heated in small volumes in water-baths accurate to 1° C., an error of no significance at the temperatures tested. All filtrations were carried out through Chamberland L_3 or L_5 candles. The hydrogen ion concentrations were determined by the glass electrode method (Beckman pH meter).

Results

The initial studies were carried out with a strain of Wood 46 which had been preserved without passage on an agar slant for three months. The results with respect to the speed of evolution of the lysin were comparable to those reported by Smith.[21] The thermostability of the lysin at 80° was fully established for many of the samples; the 60° fractions likewise exhibited considerable lytic activity (Table 1A). When titrations were carried out with unfiltered material (Table 1B), several of the fractions proved more

TABLE 1A

STAPHYLOLYSIN TITERS OF WOOD 46 AFTER CHAMBERLAND L_3 FILTRATION

Age, hrs.	*Unheated*	60°/30′	80°/30′
18	16,958	13,864	14,030
24	20,024	14,470	13,709
30	21,590	13,180	11,473
48	17,826	7,110	4,167
64	15,994	6,713	3,707
166	10,107	528	264
210	8,878	862	215

TABLE 1B

STAPHYLOLYSIN TITERS OF WOOD 46, UNFILTERED

Age, hrs.	*Unheated*	60°/30′	80°/30′
6	32	0	0
8	9,072	3,900	6,846
12	11,470	4,647	7,207
16	11,519	4,022	7,974
24	20,988	10,134	6,372
48	16,475	6,580	4,680
72	15,339	4,011	81
96	14,057	2,847	81

stable at 80 than at 60°, as noted by Smith for filtered specimens.

In several of the unfiltered toxins, such as the 16-hour sample of Table 2, the difference in the titers of the two heated toxins was

TABLE 2

EFFECT OF PROLONGED HEATING ON A 16-HOUR LYSIN, WOOD 46

Period of heating	*Unheated*	60°	80°
	11,719		
15′		360	5,056
30′		344	4,577
60′		0	3,028
90′		32	1,800
120′		0	692
180′		0	231

marked, and the greater stability of the 80° fraction was maintained during 3 hours of heating. In the course of subsequent titrations, however, the phenomenon of a greater stability of lysin at the higher temperature (80°) than at the lower (60°) was observed with both filtered and unfiltered material, and the discrepancy in the early results, for want of better explanation, might be referred to undetermined technical differences.

Nevertheless, the fact that under certain conditions the greater stability of the 80° samples became manifest in unfiltered preparations suggested the possibility that this phenomenon was, in some manner, influenced by the organisms or their products, since these might not be completely cleared by centrifugation alone, and might therefore exert an effect on the properties of the lysin in such samples. Parallel titrations were carried out on a number of filtered and unfiltered toxins, as well as on unfiltered fractions to which heavy suspensions of organisms were added.

The results of such experiments varied considerably. For the most part, the unheated toxins did not differ significantly from each other, while the heated samples showed a greater range of variation, with an unpredictably greater or lesser stability at 60°. When, however, heavy suspensions of organisms were added to the toxins, striking changes in the effect of heat were frequently observed. In the evaluation of the titers of such samples, chief reliance was placed on the overnight reading after refrigeration, since the lysin was masked during the period of evolution by the turbid cell suspensions. A further source of error was encountered in the rapid appearance

of a violet-colored hemoglobin derivative when suspensions of young staphylococci were used, and in the greater suspension stability of the 60° fraction as compared with the one heated at 80°. The final, overnight results of one such experiment are presented in Table

TABLE 3

EFFECT OF FILTRATION, NON-FILTRATION, AND ADDED SUSPENSION OF STAPHYLOCOCCI ON THE REACTION OF LYSIN TO HEAT

		A Immediate titration			B Retitration of A (18 hrs. contact with staph.)		
	Toxin dilution	*Filtered*	*Unfiltered*	*Added suspension*	*Filtered*	*Unfiltered*	*Added suspension*
Unheated	1:10	++++	++++	++++	++++	++++	++++
	1:20	++++	++++	++++	++++	++++	++++
	1:40	++++	++++	++++	++++	++++	++++
	1:80	++++	++++	++++	++++	++++	++++
	1:160	++++	++++	++++	++++	++++	++++
	1:320	+++	++++	++++	++++	+++	++++
	1:640	++	+++	++	++	++	+++
	1:1280	−	+	+	+	−	++
	1:2560	−	−	−	−	−	+
	1:5120				−	−	−
60°/30′	1:10	++++	++++	+++	−	−	−
	1:20	++++	++++	++	−	−	−
	1:40	++++	++++	+			
	1:80	++++	+++	−			
	1:160	+++	+	−			
	1:320	++	+	−			
	1:640	−	−	−			
80°/30′	1:10	++++	++++	++++	++++	++++	++++
	1:20	++++	++++	++++	++++	++++	+++
	1:40	++++	++++	++++	+++	+++	+
	1:80	++++	++++	+++	++	++	−
	1:160	+++	+++	+	+	−	−
	1:320	++	++	−			
	1:640	−	+	−			

3(A), and were obtained with an 11-hour rapid shake-culture. It will be noted that the titers of the native, unheated fractions were not significantly different. At 60°, there was a progressive loss of lysin from the filtered to the unfiltered to the "suspension" frac-

tion, the most striking fall occurring in the latter. At 80°, the filtered and unfiltered samples differed only slightly, while a considerable loss of potency was evident in the "suspension" preparation. It should be emphasized that such results were by no means always obtainable with fresh cell suspensions, but were encountered with sufficient frequency to lead to the tentative conclusion that they might not be without significance as factors modifying the reaction of the lysin to heat.

If, then, agents might be elaborated by the staphylococci which influence the thermostability of the lysin, the time required for the

TABLE 4

CHANGES IN THERMOSTABILITY OF LYSIN IN CONTACT WITH STAPHYLOCOCCI AT 37.5°

Hrs. after rapid shaking for 14 hrs.	*Heat*	*Standing*	*Further shaking*
0	None	15,809	
	60	6,324	
	80	6,921	
½	None	14,562	15,442
	60	7,924	7,143
	80	10,851	11,533
1	None	15,400	17,026
	60	3,450	10,171
	80	11,070	14,748
2	None	16,659	16,739
	60	3,035	7,043
	80	8,122	9,521
6	None	13,739	14,283
	60	1,604	2,801
	80	8,653	8,735
7½	None	17,087	16,755
	60	1,786	3,250
	80	5,256	5,084
11	None	15,989	15,223
	60	559	2,316
	80	5,837	4,201
24	None	14,586	
	60	163	
	80	3,747	

appearance of such products assumed importance. Accordingly, titrations were carried out immediately after incubation, and after allowing the toxin to stay in contact with the cells at room temperature or in the incubator at 37.5° for variable lengths of time. The results are illustrated in Table 3(B). The same toxin was used as in 3(A), except that the lysin was allowed to stay in contact with the organisms at room temperature, without further agitation, for 18 hours. While the titers of the unheated fractions had risen slightly, the 60° lysin had completely disappeared, and the potency of the 80° samples had been reduced considerably. In a subsequent experiment (Table 4), a 14-hour rapid shake-culture was prepared, half of it left in the incubator without further agitation, while the rest was shaken for a further period of 11 hours. Samples were withdrawn at intervals in order to determine the rapidity with which, in a given instance, the reactions of the lysin to heat undergo change. It will be noted that the unheated fractions did not vary significantly. The 60° sample lost half its lytic activity after standing in the incubator one hour, and underwent further considerable loss in the period from 6 to 11 hours. The 80° fraction showed no consistent trend during the first 6 hours, but dropped significantly at the end of 24 hours. In the toxin subjected to further agitation, the native samples did not differ significantly from each other, while the fall in the titer of the 60° sample was slower and less marked than in the unshaken fractions, with the fall in the titer of the 80° fraction even less marked. It must be pointed out again that not all toxin samples undergo these changes when left in contact with the organisms; indeed, in some instances, no significant changes were observed within similar periods of observation.

Since the inhibition of lysin at 60° by fresh, washed suspensions of staphylococci was not uniformly demonstrable, procedures were undertaken favoring the damage, disruption, or lysis of the organisms. These, among others, included lysis by bacteriophage, prolonged mechanical grinding in the lyophiled state, or simply keeping washed suspensions of organisms in distilled water for various periods of time. The results obtainable with such preparations are illustrated in Table 5; 9- and 15-hour samples of toxin were used. The phaged sample was prepared by adding small increments of staphylococci from an agar slant to a 5 ml. tube of phage Mx* at 8

* Obtained through the courtesy of Dr. M. L. Rakieten.

hourly intervals to the point where no further clearing occurred. The slightly turbid suspensions were then cleared by centrifugation,

TABLE 5

THE EFFECT OF SUPERNATANTS OF DAMAGED STAPHYLOCOCCI ON REACTION OF LYSIN TO HEAT

	Heat	*9-hr. toxin*	*15-hr. toxin*
1. Lysis by bacteriophage.....	None	8,206	8,970
	60	16	81
	80	81	592
2. Mechanical disruption.....	None	8,074	9,018
	60	0	0
	80	0	0
3. Lysin control 1:1 with saline....	None	10,270	9,386
	60	1,492	677
	80	6,550	6,646

and the supernatant was tested for inhibitory properties. The mechanically disrupted fraction was prepared by washing a 12-hour shake-culture in distilled water, lyophiling the sediment, and grinding it with metal ball bearings for from 5 to 6 hours. The toxin was added in equal volumes to the test preparations, and the pH of all samples was adjusted to within 0.1 of each other at about 7.3. Toxin diluted with an equal volume of saline served as control, and all samples were incubated for 30 minutes at 37.5° before heating at 60 and 80° for 30 minutes. Total inhibition of the lysin was accomplished by the mechanically disrupted fraction at 60 and 80°, while the inhibition exerted by the phaged preparation was less marked, and might well be attributed to the lower concentration of the staphylococci available in this preparation.

It appears, then, that substances may be liberated by the organisms which exert a considerable influence on the reactions of alpha lysin to heat. Such effects are not restricted to lysin-producing strains, but have been obtained with strains fundamentally devoid of ability to form toxin. Indeed, similar results have been obtained with properly prepared autolysates of such heterologous strains as pneumococci, beta hemolytic streptococci, and coli. The non-specificity of the inhibitory phenomenon is further made evident by the fact that it may be produced by such diverse agents as ammonium sulfate

precipitates of tetanus and botulism toxins, human and rabbit sera possessing a negligible antilysin titer, and suitable concentrations of pepsin, trypsin, and lecithin. These results are illustrated in Table 6. It may be mentioned that both the undiluted human

TABLE 6

INHIBITION OF STAPHYLOLYSIN BY AGENTS NOT DERIVED FROM STAPHYLOCOCCI

I. Human serum

Heat	*Undil.*	1:10	1:100	*Control*
None	6,570	8,534	8,802	8,254
60°	0	0	810	1,388
80°	coag.	2,353	2,353	4,514

II. Pepsin, 2 mg. per ml.

	Pepsin	*Control*
None	6,522	7,835
60°	0	1,854
80°	342	4,948

III. Lecithin, 0.05 mg. per ml.

Lecithin	*Control*
4,374	4,112
0	1,456
2,852	3,252

serum and pepsin appeared to exert some inhibitory action on the unheated samples, although the maximal effect occurred at 60°. The action of the pepsin cannot be attributed to its enzymatic properties, since the tests were conducted at a pH of 7.5, and there is evidence that the pepsin did not destroy the lysin, since heating at 80° led to some reactivation. Finally, the results obtained with lecithin should more properly be attributed to some breakdown products formed upon heating than to the original compound.

Further procedures were undertaken to determine the nature of the inhibiting agents liberated in the supernatants of suspensions of staphylococci. Accordingly, large quantities of staphylococci were grown, either in Blake bottles on extract agar or in liter flasks as shake-cultures on heart infusion broth. After washing the organisms in saline, they were resuspended in from 200 to 300 ml. of distilled water, and 1 ml. of chloroform was added. After incubation at 37.5° for from 5 to 7 days the suspensions were centrifuged, and the supernatant was filtered through Chamberland L_5 candles. These solutions were then treated by a variety of procedures, including precipitation with saturated and half-saturated ammonium sulfate, with trichloracetic acid, normal HCl, and 4 volumes of acetone

or 10 to 20 volumes of 3 parts of ethyl alcohol and one part ether in the cold.

While all of the above procedures yielded products possessing the ability to mask the lysin when heated with it at the appropriate temperatures, acetone and alcohol-ether extraction was most commonly resorted to, the extraction with the larger volumes of alcohol-ether probably removing any lipoids which might have been present.[4] After the reagents were added to the solutions under test, they were acidified to the point of maximum cloudiness, and placed in the ice-box for some 12 hours. The supernatants were removed by centrifugation and by decanting, the precipitates were washed, and then dried in vacuuo. While the yields varied considerably, as much as 500 mg. was obtained by the acetone precipitation from the supernatant of a 24-hour growth of staphylococci harvested from 20 Blake bottles. The precipitates varied in appearance from a fine white powder to gray or yellowish material, moderately soluble in water, but readily soluble in dilute alkali. The points of minimum solubility of several of the yields were found to be at approximately 2.75, although in some instances precipitation occurred at as high as pH 3.5 to 4.5. Repeated solution of an acetone precipitate in dilute alkali and reprecipitation at the isoelectric point yielded precipitates which were increasingly less soluble in water, suggesting some denaturation of the proteins present. However, the inhibitory properties were concentrated in such preparations, and lost, or greatly reduced in the acetone precipitates of the supernatants. These products gave positive Biuret, Millon, xanthoproteic, and tryptophane tests (Rosenheim, Ehrlich, and Adamkiewitz). The Molisch and Seliwanoff reactions were positive, but the Benedict test was negative. Tests for pentoses (Bial, phloroglucinol, and aniline hydrochloride reactions) were not elicited, and the special thymonucleic acid reactions described by Dische[7] (indol, carbazole, and diphenylamine tests) were not obtained. When solutions of the precipitates were subjected to ultrafiltration through cellophane membranes,* the inhibiting agents were held back by such membranes, and the ultrafiltrates lacked inhibitory properties. The total nitrogen* of one sample obtained by acetone precipitation was 7.7 per cent of dry weight, while the nitrogen* and organic phosphorus* content of an alcohol-ether precipitate were 9.94 and 3.1 per cent

* These determinations were kindly performed by Dr. P. Lavietes and Dr. K. A. Klinghoffer, and by Miss P. M. Hald of the Department of Internal Medicine.

respectively. Since definitive chemical identification of the products obtained has not been carried out, the possibility has not been excluded that other products elaborated by the staphylococci exert similar effects when heated with the lysin.

Observations were made on the concentration of the inhibitors required to produce a demonstrable effect. Accordingly, suitable dilutions of an acetone precipitate were prepared, mixed with an equal volume of lysin, and tested unheated and after heating at 60° and 80° for 30 minutes (Table 7). With the higher concentrations

TABLE 7

EFFECT OF CONCENTRATION OF ACETONE-PRECIPITATED INHIBITOR ON THERMOSTABILITY OF LYSIN

Toxin dil.	*Dilutions of inhibitor, mg./ml.* 5.0	0.5	0.25	0.125	0.0625	0.031	0.015	0 (*contr. tox* 1:1)
A) Unheated								
1:10	++++	++++	++++	++++	++++	++++	++++	++++
1:20	++++	++++	++++	++++	++++	++++	++++	++++
1:40	++++	++++	++++	++++	++++	++++	++++	++++
1:80	+++	+++	+++	+++	+++	+++	++++	+++
1:160	++	++	++	++	++	++	+++	++
1:320	+	+	+	+	+	+	++	+
1:640	−	−	−	−	−	−	+	−
1:1280	−	−	−	−	−	−	−	−
B) Heated (60°/30′)								
1:10	−	−	−	−	++	+++	+++	++++
1:20					+	++	+++	+++
1:40					−	+	++	++
1:80						−	+	+
1:160							−	−
1:320								
C) Heated (80°/30′)								
1:10	−	++	++++	++++	++++	++++	++++	++++
1:20		+	+++	+++	+++	+++	+++	+++
1:40		−	++	++	++	+++	+++	+++
1:80			+	+	+	++	++	++
1:160			−	+	−	+	+	+
1:320				−		−	−	−
1:640								

of 5 and 0.5 mg. per ml., there was marked inhibition at both 60° and at 80°. As the inhibitor was diluted, a zone appeared in which inhibition remained complete at 60°, but was diminished or lost at 80°. Further dilution of the inhibitor resulted in progressive further loss of activity at both temperatures, and finally both heated fractions approached the control in stability. No significant inhibition was noted in the unheated samples. This titration demonstrated the manner in which the inhibitors, when present in the proper concentration, might operate to produce the effect described by Smith—the preservation of the lysin at the higher temperature and its apparent inactivation at a lower range.

To determine more precisely the temperature range of maximal activity, two concentrations of an inhibiting agent were heated with the lysin at 50, 53, 56, 60, 65, 70, 80, and 100° (Table 8). The

TABLE 8

THE EFFECT OF INHIBITOR ON STAPHYLOLYSIN AT DIFFERENT TEMPERATURES

*Heat**	*Undil. inhib.*	*Inhib.* 1:5	*Toxin-saline control*
None	7,640	7,770	7,574
50°	8,422	8,735	8,714
53°	16	1,225	5,214
56°	16	81	859
60°	0	0	1,593
65°	16	164	3,347
70°	16	595	4,347
80°	65	2,156	4,543
100°	213	1,140	1,920

* Heating for 30′; at 100° for 15′ only.

inhibitor was prepared by growing 4 liters of staphylococci (Wood 46) for 48 hours in the shaking machine, washing the organisms, resuspending them in 300 ml. of distilled water, and after 5 days at 37.5° using the supernatant filtered through Chamberland L_5 candles. With the undiluted solution, the zone of marked inhibition extended from 53° to 100°, with the maximal effect at 60, and some loss at 80 and 100. With the inhibitor diluted 1:5, the zone of marked inhibition was limited to from 56 to 70°, although not insignificant inhibition of the lysin was present over the wider range noted for the undiluted agent. It therefore became apparent that it was not possible to dissociate the optimal range of temperature

from the concentration of the inhibitors present. However, the maximal effect is certainly exerted between 56 and 65°. It is especially noteworthy that neither the unheated nor the 50° fractions showed any significant inhibition.

The effect of heat on the inhibitors themselves was then investigated (Table 9). The inhibitors were derived from strain Wood 46

TABLE 9

EFFECT OF HEAT ON THE INHIBITING AGENTS

Heat of mixture Inhib. toxin	A) Inhib. not preheated *Wood 46 Inhib.*	*Reddish Inhib.*	B) Inhib. heated 60° *Wood 46 Inhib.*	*Reddish Inhib.*	*9-hr. toxin control-saline 1:1*
None	7,506	9,302	9,202	9,334	9,966
60°	0	0	0	0	1,392
80°	0	16	0	824	4,448
	C) Inhib. heated 80°		D) Inhib. heated 100°		
None	9,138	9,202	10,064	10,766	
60°	261	0	261	0	
80°	5,110	2,688	5,100	4,084	

and strain Reddish by allowing the supernatant of washed staphylococci to stay in contact with the cells at 37.5° for 7 days. The inhibitors were used unheated and after heating at 60, 80, and 100° for 30 minutes before being added in equal amounts to the toxin. After the usual preliminary incubation, the samples were again left unheated, and heated at 60 and 80° for 30 minutes, and at 100° for 15 minutes. In the case of the Wood 46 inhibitor, there was complete inhibition of lysin in the unheated sample and the one in which the lysin was preheated at 60° when the lysin-inhibitor mixtures were heated at 60 and 80°. When the inhibitor was preheated at 80 and 100°, partial return of lysin occurred at 60°, while complete return took place at 80. With the inhibitor derived from strain Reddish no return of lysin took place at the 60° level, while progressively increasing return of lysin occurred at 80, the maximal lysin titer being demonstrable when the inhibitor was preheated at 100. In subsequent titrations, the concentration of the inhibitors appeared to play a vital rôle in the extent to which the lysin was re-liberated when the inhibitors were preheated. In general, there was a far greater lag in the return of lysin on reheating at 60 than

at the higher temperatures, although, with sufficient dilution of the inhibitors, the lysin might become as manifest at 60 as at 80°. Although preheating the inhibitors did not produce an all-or-none effect on its subsequent action on the lysin, the evidence pointed to some attenuation by heat, and the fact that this was more marked at 80 and 100° than at 60° explained, in part, its diminished ability to inhibit the lysin at the higher temperatures.

Titrations were then carried out to determine whether the inhibitor combined with the lysin stoichiometrically. Appropriate volumes were prepared and all samples heated at 60° for 30 minutes (Table 10). Within the narrow limits within which it was possible to test this relationship the combination followed the laws of multiple proportions.

TABLE 10

COMBINATION OF LYSIN WITH INHIBITOR IN MULTIPLE PROPORTIONS AT 60° C.

Inhib.	*Tox.*	*Saline*	1:10	1:20	1:40	1:80	1:160
ml.	ml.						
0.2	0.4		+	0	0	0	0
0.2	0.8	to 2 ml.	++	++	+	0	0
0.2	1.6		++++	+++	++	+	0
0.4	0.4	to 2 ml.	0	0	0	0	0
0.4	0.8		++	+	0	0	0
0.4	1.6		+++	++	+	0	0
0.5	0.5	to 4.5 ml.	0	0	0	0	0
0.5	1.5		++	+	0	0	0
0.5	3.0		+++	++	+	0	0
1.0	0.5	to 4.5 ml.	0	0	0	0	0
1.0	1.5		+	0	0	0	0
1.0	3.0		++	+	0	0	0
1.5	0.5	to 4.5 ml.	0	0	0	0	0
1.5	1.5		0	0	0	0	0
1:5	3.0		+	+	0	0	0

Some effects of pH on the reaction of alpha staphylolysin to heat

Since the hydrogen ion concentration had been extensively studied in its relation to the production and thermostability of staphylolysin,[22] the present observations were largely limited to the questions of whether changes in the pH might reactivate lysin or

operate to produce greater stability at the higher than at the lower temperatures.

Preliminary studies had shown that altering the initial pH of the medium within the range of 6.6 and 8.2 failed to influence significantly the ratio of the titers at 60 and 80°, although some diminution of lysin production was evident at the extremes.

The changes in the pH of a culture on unbuffered heart infusion broth with 1 per cent neopeptone and saline were observed during the period of incubation. As had been well established, the initial

TABLE 11

THE EFFECT OF PH ON THE THERMOSTABILITY OF FORMED TOXIN

Age	*pH*	*Unheated*	60°	80°	100°
hrs.					
8	6.10	9,866	3,187	6,306	7,970
	6.65	11,162	2,550	7,306	7,870
	7.55*	11,227	3,810	6,490	5,442
	8.40	11,293	4,282	5,058	3,184
	9.50	11,052	523	0	16
11	6.55	8,173	1,854	5,110	7,878
	6.95	9,134	1,770	6,510	6,610
	7.73*	12,130	1,770	4,878	5,712
	8.45	12,130	1,604	2,966	3,852
	9.05	13,926	261	1,257	213
16	6.50	11,866	16	3,535	6,809
	7.10	12,062	16	5,374	6,446
	7.50	10,530	81	3,266	6,546
	7.85*	11,930	213	2,853	4,880
	8.35	16,470	48	3,026	3,784
	8.75	14,410	16	493	531
	9.65	14,290	65	0	0
21	6.05	11,866	1,956	7,306	7,606
	8.10*	14,266	1,388	4,846	2,586
	9.55	13,866	16	16	16
40	6.75	11,988	3,188	6,909	6,746
	7.25	11,294	4,384	6,646	5,248
	7.65	12,626	4,614	4,630	4,052
	8.65*	14,590	2,482	2,466	247
	9.15	17,087	959	163	65

* Native samples.

60 and 80° samples heated for 30′; 100° samples for 15′.

acid phase was followed by a rising pH, with the rate and degree of change greatly influenced by the speed of agitation and the resulting acceleration of growth. The pH of heated samples was also determined, and while the difference in the hydrogen ion concentrations of the heated and unheated samples was usually insignificant, the corresponding lysin titers often varied markedly. Therefore, the pH cannot be regarded as the determining factor of the lysin titers of such fractions. However, the possibility still remained that changing the hydrogen ion concentration of various prepared samples of toxin before subjecting them to heat might modify the subsequent activity of the lysin. Accordingly, the hydrogen ion concentrations of various prepared samples of toxin were changed with 0.2*N* NaOH or HCl, and these fractions were then titrated with and without heating. The results obtained with 5 samples of different ages are illustrated in Table 11.

In general, the variations in the unheated fractions were not striking, although there was a trend toward some increase with rise in pH. When highly alkaline samples were heated a marked fall in the lysin titer might take place, even to the point of complete disappearance. Thus, in the 8- and 16-hour fractions heated at 80° the lysin varied from 0 to a maximum of 7306. The high alkalinity of the older shake-cultures might therefore account, in part, for the greater lability of such samples, as noted by Smith[21] and in the present study.

Closer inspection of the data presented in Table 11 revealed considerable individual variation in the response of different fractions. Thus, the 60° fractions of the 16-hour toxin varied slightly over the pH range of 9.65 to 6.5, while the 21-hour culture changed from 16 at 9.55 to 1388 at 8.1. Again, changing the pH of the 80° fraction of the 40-hour toxin from 9.15 to 7.65 led to a 28-fold rise in the titer, while a comparable change in the pH of the 11-hour toxin, from 9.05 to 7.73, produced only a four-fold increase.

It may be further noted that changing the pH before heating the toxin, might, in certain instances, result in the appearance of greater stability of the lysin at the higher temperature. Thus, in the native 40-hour sample of pH 8.65, the titers of the 60 and 80° samples were essentially the same; changing the pH to 7.25 and 6.75 produced a relatively greater return of lysin in the 80° preparation than in the one heated at 60°, so that at pH 6.75 the ratio of the lysins became 2:1 in favor of the 80° fraction.

While some of the highly alkaline, apparently inactive older cultures might be reactivated by acidification or, at least, by rendering the reaction less alkaline, an attempt was made to determine whether an alkaline sample already heated at 60° for 30′ might also be reactivated by acidification and reheating. In this experiment (Table 12), a 48-hour rapid shake-culture of pH 9.35 was

TABLE 12

THE REACTIVATION OF A 60° LYSIN BY ACIDIFICATION

pH	*Unheated*	60°/30′	80°/30′	100°/15′	60°/30′ & 100°/15′
9.35	15,655	129*	97	81	145
6.45	15,623	48	7,145	6,610	6,598
6.45*	195	0	1,624	2,884	2,058

* Retitration of the 60° sample of pH 9.35 after adjusting the pH to 6.45.

quite inactive at all heat levels tested. When the pH was adjusted to 6.45, although the toxin continued to be quite inactive at 60°, a striking return of lysin took place at the higher temperatures, as well as in the sample which was first heated at 60° for 30 minutes, and then at 100 for 15 minutes. The sample of toxin of pH 9.35, heated at 60°, and giving a low lysin titer of 129, was then acidified, and its titer was now found to be essentially the same, 195. Reheating this sample showed no lysin at 60°, although the higher temperatures released a not inconsiderable quantity of toxin.

The mechanism of the hydrogen ion reactivation of staphylolysin is not readily explainable. Such studies as those of Ponder,[17] Gordon,[13] and others on the influence of pH on relatively simpler hemolytic systems have only served to emphasize their extreme complexity, with effects on lysin and red cells contributing to the final result. The extent to which the inhibitors might play a rôle in these reactions has not been determined, though it is not entirely beyond the realm of possibility that they might be involved. Thus, the failure of the lysin titers to change materially at 60° in the 16-hour sample of Table 11 might, perhaps, have been conditioned by an excess of inhibitors acting at that temperature, although the alternative explanation of inherent qualitative differences might be invoked.

The question might be raised as to the identity of the reactivated lysin—whether effected by heat or changes in the hydrogen ion

concentration—and the native lysin. Forssman,[11] Smith,[20] and Morgan and Graydon[15] have advanced evidence suggesting that alpha lysin is not a single pure antigen. The observations of Burnet[5] on the changes in neutralization and flocculation with specific antisera of heated toxins, when compared to the unheated lysins, point to definite modification in some of the properties of the heated toxins. That the reactivated lysin is not fundamentally different from the native lysin has been shown by the demonstration of lethal toxin in such preparations and the ability to neutralize them with specific antitoxin developed against the unheated lysin. It must be conceded, however, that other and more delicate tests might disclose some differences in the native and reactivated toxins.

Discussion

The present studies fully confirm the observations of Smith[21] on the relative thermostability of many samples of alpha staphylolysin Wood 46 when produced on heart infusion neopeptone broth by a modification of the Casman continuous shake technic. The unanimity with which alpha lysin has come to be regarded as heat labile, i.e., more or less completely inactivated by heating at from 56 to 60° for 30 minutes, has been discussed elsewhere.[21] In recent years, Burnet,[5] Smith,[21] and Dolman and Kitching[8] have been virtually alone in drawing attention to the possible thermostability of alpha lysin, and a recent brief report by Singer and Hagan[19] likewise takes this view. The early work which claimed that the lysin was not labile has fallen into complete obscurity. Thus, in 1905, Fraenkel and Baumann[12] reported that some lysins resist 80 and 100°. The observations of Dreyer[9] and of Atkin[2] on megatheriolysin and those of Arrhenius[1] and of Landsteiner and Rauchenbichler[14] on staphylolysin bear the closest relation to the present study.

Dreyer observed that when megatheriolysin was heated at 60° for 30 minutes, it was completely inactivated, while subsequent heating at 100° for 10 minutes restored half its original strength. No explanation was advanced for this phenomenon, which was subsequently confirmed by Atkin. Arrhenius carried these observations over to staphylolysin: "The staphylolysin behaves in a very peculiar manner. If it is heated to 70, it loses a great deal of its hemolytic power, which curiously enough, returns almost completely after heating for 5 minutes at 100." Landsteiner and Rauchenbichler undertook to explain this phenomenon. The clue to its nature was

obtained by noting that dilution with saline or broth eliminated the phenomenon of inactivation at lower temperatures (65°) and reactivation at the higher level (100°). This immediately suggested that something extraneous to the lysin was involved, since, the authors reasoned, if it were a property of the lysin *per se*, dilution should not eliminate it. After the constituents of the medium were ruled out, autolysates of staphylococci were found to confer on lysin the property of inactivation at 65 and restoration at 100°. Landsteiner therefore postulated that the inactivating products unite at the lower temperature with the lysin, but that the union is dissolved at the higher temperature. These authors concluded that staphylolysin is heat stable and that true inactivation may not occur until boiling for one hour.

Neisser[16] failed to duplicate any of the results of Landsteiner and Rauchenbichler. Similarly Atkin, although working at the Danish State Serum Institute where this phenomenon was demonstrated, failed to find the staphylolysin anything but heat labile, and did not succeed in reactivating the toxin at the higher temperature. The claims of Arrhenius and Landsteiner regarding the thermostability of staphylolysin at higher temperatures were then completely dropped from the literature, until the recent studies of Smith revived them.

The present studies are in complete accord with the observations of Landsteiner, although the same basic conclusions were reached by a different chain of evidence. Once attention was drawn to the rôle of the organisms as distinct from the lysin in modifying the reaction of the toxin to heat, it was possible to obtain agents which exerted maximal inhibition on the lysin at about 60°. It was further shown that these products did not modify unheated lysin titers significantly, nor, indeed, lysin heated with the inhibitors at 50° for 30 minutes. In this respect, these inhibitors differ from the well-known antihemolytic properties of serum[3, 10, 18] when tested against such agents as bile salts or saponin; in which case the action takes place without heat. The distinctive characteristic of the inhibitory agents recovered from the staphylococci and other non-specific sources lies in the fact that they exert a maximal effect at about 53 to 70°, though this range may be considerably modified by the concentration of the inhibitor under test.

The data presented suggest a possible basis for reconciling the contradictory views on the reaction of alpha lysin to heat. It is

possible that the apparent thermolability was merely a masking by excess of inhibitory substances, which might explain both the absence of the lysin at the 60° range as well as at the higher ranges tested by such observers as Neisser and Atkin. Where methods of prolonged cultivation were used to produce the toxin, the factor of excess alkalinity might likewise have contributed to the masking of the lysin.

In discussing the inhibitory hypothesis as a factor in the greater stability of the lysin at 80° than at 60, Smith[21] was inclined to the view that the phenomenon might represent a basic property of the lysin. As not supporting the inhibitory point of view, an experiment was presented in which a native filtrate was diluted in a 1:40 dilution of a non-hemolytic 60° filtrate. This failed to inhibit the lysin, indeed, it led to some increase in the titer, attributed to a subreactive amount of lysin in the diluent. This, however, is not inconsistent with the observations in the present report, since it has been demonstrated that the action of the inhibiting agents requires heating with the lysin to at least 53°, and that no significant effects may be expected without such heat.

The fundamental factors determining the liberation of the inhibiting agents in a given culture are not known. Whether they play any significant rôle in the physiology of the staphylococcus, or are merely autolytic products—as some of the evidence presented suggests—has not been definitely established. It is further quite possible that these agents themselves might be acted upon and destroyed by the enzymes of the staphylococci. Upon such a basis might be explained the finding by Smith that after a period during which the lysin titers at 60° are suppressed, there is a zone in which the lysin reappears.

The fundamental importance of the intrinsic properties of the toxin in determining its reaction to heat cannot be questioned. The present study has sought to emphasize the possible importance of extrinsic factors as well in modifying this reaction and the difficulty of defining such basic properties as reaction to heat when dealing with impure mixtures. Final opinion on the stability or lability of staphylococcal alpha lysin should await isolation of the toxin in pure form.

That such considerations are not without bearing on the heat properties of other toxins is suggested by the similar behavior of megatheriolysin.

Summary

Alpha staphylolysin of Wood 46, produced on heart infusion broth in modified Casman shake-cultures, is relatively heat stable.

Inhibiting agents, liberated by the staphylococci, are described. The inactivation of lysin at lower temperatures (60°) and reactivation at higher levels (80 to 100°) is influenced by these agents. The maximal zone of inactivation is between 53 and 70°; there is no significant effect up to 50°. Higher concentrations of inhibitors may mask the lysin heated at higher temperatures.

The release of lysin at higher temperatures may be due to the partial heat lability of the inhibitors themselves or to a dissociation of the lysin-inhibitor complex.

Many other substances, unrelated to the staphylococci, may exert similar effects on the thermostability of the lysin.

Changes in the hydrogen ion concentration of the lysin, before it is subjected to heat, may modify this reaction in either direction.

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