A COMPARATIVE METHOD FOR TESTING THE ENZYMES OF LIVING HEMOLYTIC STREPTOCOCCI.

I. LIPASE.

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INTRODUCTION.

During the study of a series of cases of acute extensive superficial gangrene in which a hemolytic streptococcus was constantly present and seemed to be the causative agent, it was observed that the organism had a very great affinity for the subcutaneous fat. The infection invariably spread rapidly in this tissue and caused an extensive necrosis and solution of the fatty tissue. This observation led to an investigation of the enzymes of the streptococci recovered from these cases.

It is generally believed that bacteria carry on their metabolic processes by the aid of ferments which act upon the surrounding medium and prepare it in a suitable manner for ingestion. These enzymes may be liberated during the life of the bacteria or upon their death and dissolution. It has been frequently shown that these enzymes are capable of digesting carbohydrates, proteins, and fats (10). The methods which have been described, however, are not adapted to strict quantitative estimations. The recent methods which Stevens and West (9) used in their investigation of the enzymes of hemolytic streptococci are not available for a quantitative study because of the difficulty of completely extracting the enzyme by the grinding process.

Because of the complete solubility of the pneumococcus in bile, Avery and Cullen (1) were probably able to get all of the endoenzymes of that organism into solution. So far, this has not been possible with the streptococcus. We have tried various chemical substances and physical forces in an attempt to find a method that would completely liberate the enzymes without destroying them. All of these efforts were unsuccessful. We therefore discarded the methods of extraction of enzymes from dead organisms and turned to the more logical study of living organisms. It was observed that living streptococci suspended in

water showed a marked lipolytic activity in the presence of ethyl butyrate. It was then found that streptococci, in common with other organisms, die off rapidly when suspended in water. This led to the study of suspending fluids which would maintain the streptococcus for a considerable length of time without increase or diminution of numbers. One of us with Zau (8) found that the hemolytic streptococcus will survive for a week or more in dilutions as high as 100 organisms per cc. in three solutions; namely, Locke's solution, 0.2 per cent sodium citrate, and a balanced solution containing 1.0 per cent sodium chloride and 0.05 per cent calcium chloride, if to each of these solutions 0.1 per cent gelatin be added. Certain other solutions containing 0.1 per cent gelatin, including distilled water, will maintain the streptococci for several days. These gelatin solutions were therefore considered to be available for quantitative determinations. Gates (6) has devised a fairly accurate method of obtaining standard suspensions of bacteria so that in the gelatin solutions different organisms may be quantitatively compared.

The usual method of determining lipolytic activity by other workers has been to titrate the acid produced in the enzyme substrate mixtures with sodium hydroxide. Dietz (3) has shown, however, that enzyme preparations of different activity, as measured by their velocity constants, reach the same equilibrium point if allowed to continue to completion. Therefore, if sufficient time be allowed, the amount of titrable acidity produced will be the same, even if the concentration of the enzyme is varied. Within certain limits, when there is an excess of substrate present in an enzyme-substrate system, the velocity of the reaction is in direct linear proportion to the quantity of active enzyme present. We have found this to be true of preparations of pancreatic lipase and ethyl butyrate. These facts, together with the difficulty of titrating the minute quantities of acid produced by small quantities of bacteria, suggested the possibility of measuring the velocity of the reaction between certain definite hydrogen ion concentrations.

EXPERIMENTAL.

The organism used in these experiments was obtained from a case of acute gangrene of the leg, one of a series of cases previously reported (7). It was a typical *Streptococcus pyogenes* in its cultural characteristics. It was kept as a stock culture in horse blood broth in which it would survive for 2 months without transfer. During

these experiments it was kept actively growing by frequent transplantation. 0.1 cc. of this stock culture was planted in 25 cc. of 1 per cent dextrose liver digest broth in a 50 cc. centrifuge bottle and incubated for 12 to 14 hours. After incubation the culture was centrifuged, washed twice, and then taken up in the suspending fluid to be used for the test. The concentration was then determined by the method of Gates (6) and adjusted so that 1 cc. contained approximately 5 billion cocci. Three standard color tubes were prepared by boiling 6 cc. of the standard suspension for 1 minute, dividing this into three parts, centrifuging, and taking up the sedimented bacteria from each part in 2 cc. of Clark's (2) standard buffer mixtures at pH levels of 8.0, 7.6, and 7.2. These suspensions were placed in 5 cc. test-tubes and 0.05 cc. of phenol red was added to each tube as indicator. Thus each standard color tube had the same relative turbidity as the suspension of living organisms to be tested. With the suspended organisms, the colors of the standard color tubes were slightly paler than the buffer solutions alone but they remained constant for several days. 2 cc. of the living suspension to be tested were placed in a dry sterilized 5 cc. test-tube. Corks were used instead of cotton plugs in order to prevent gaseous exchange. 0.05 cc. of phenol red was added, then 0.2 cc. of ethyl butyrate and enough $\frac{N}{50}$ sodium hydroxide to bring the mixture, after shaking, to a color slightly more alkaline than the pH 8.0 standard color tube. This usually required 0.2 cc. of the alkali. In later tests the alkali was added first and the ethyl butyrate afterward in order to have the ethyl butyrate the last addition before the actual test began. Control tubes were set up of the standard suspension of organisms without ethyl butyrate and the suspending fluid alone with ethyl butyrate. All of the tubes were incubated in the water bath at 37.5° and shaken every minute to maintain contact between the bacteria and the substrate which otherwise promptly floated to the top of the fluid. After each shaking, the color of the fluid was compared with the standard color tubes and a time reading taken when the color was exactly the same. It was found that the colors could be much more accurately compared if the tubes were held in front of a frosted electric bulb covered by wet filter paper than if held up to the daylight. As acid formed in the "active tubes," the color passed through

the ranges covered by the standard color tubes, the rapidity of acid formation being considered indicative of the amount of lipase present, providing the controls were negative. A direct quantitative comparison was thus possible for the same organism under different conditions and for different organisms under identical conditions. The comparison could be made between the pH 8.0 and 7.6 levels or between the 7.6 and 7.2 levels or both together.

A preliminary experiment determined the fact that 0.2 cc. was an excess of substrate for the amount of lipase present. Tubes were set up with the same quantity of standard living suspensions but with increasing amounts of ethyl butyrate, 0.1, 0.2, 0.3, and 0.4 cc.

TABLE I.

Relative Speed of the Reaction in Different Suspending Media.

Tube No.	Suspending fluids.	Time required between pH 8.0 and 7.6.	Time required between pH 7.6 and 7.2.	Total time required between pH 8.0 and 7.2.
		min.	*min.*	*min.*
1	0.2 per cent sodium citrate with 0.1 per cent gelatin.	17	38	55
2	Locke's solution with 0.1 per cent gelatin.	18	40	58
3	1.0 per cent sodium chloride and 0.05 per cent calcium chloride with 0.1 per cent gelatin.	6	18	24
4	Twice distilled water.	8	22	30

Controls: Standard suspension without ethyl butyrate and suspending fluids alone with ethyl butyrate, all negative.

being used. In this series the passage time from pH 8.0 to 7.2 was 22, 22, 22, and 19 minutes respectively. For the subsequent tests 0.2 cc. was used. Tests were then performed to determine (1) the best suspending fluid for the reaction; (2) the effect of diluting the standard suspension; (3) the effect of varying the incubation time for the culture of the organism; (4) the optimum temperature for the reaction; (5) the effect of previous contact with higher temperatures; (6) the optimum hydrogen ion concentration; (7) the action of a strain when relatively avirulent in comparison with the same strain made relatively virulent for rabbits by repeated animal passages; and (8) the action of different strains of hemolytic streptococci from different clinical sources.

The Relative Activity in Various Suspending Fluids.

Since it had been found that the hemolytic streptococcus is well preserved in Locke's solution, in 0.2 per cent sodium citrate, 1.0 per cent sodium chloride with 0.05 per cent calcium chloride ("20:1" mixture), and even in water if 0.1 per cent gelatin be added to each (7), these four solutions were tested. It was found that with "20:1" mixture the results were attained more quickly and the color changes were more clear-cut than with the other solutions. The result is shown in Table I. The "20:1" mixture was used in all of the subsequent tests.

The Effect of Dilution of the Organisms.

The test was set up with five tubes, the first of which contained the standard suspension with approximately 5 billion organisms per cc. The others contained 4, 3, 2, and 1 billion respectively. The dilutions were made with the "20:1"-gelatin suspending fluid. This made the higher dilutions less turbid than the standard color tubes. When the dilution was made with a suspension of heat-killed organisms to make the turbidity the same, the relative result was the same.

The results are given in Table II. The curve in Text-fig. 1 indicates that the rapidity of the reaction is approximately in linear proportion to the concentration of active substance, at least for the three highest concentrations.

The Relative Action of Young and Old Cultures.

0.1 cc. of the stock culture was incubated overnight in 25 cc. of 1 per cent dextrose liver digest broth. In the morning after 14 hours incubation, 2 cc. of this culture were transplanted to another tube containing 25 cc. of media. Incubation was continued until the afternoon when the test was made for lipase activity. At that time the first culture had been incubated for 21 hours and the second for 7 hours.

The 7 hour subculture made the color change in 19 minutes while the 21 hour culture took 66 minutes. This very important finding will be further elaborated below.

The Optimum Temperature of the Lipolytic Reaction.

The test was set up with five tubes containing the standard suspension of living organisms, each one having two control tubes. The lipase activity was then tested at ice box temperature, 15° C.; room temperature, 27°; and water bath at 37.5°, 50°, and 62°.

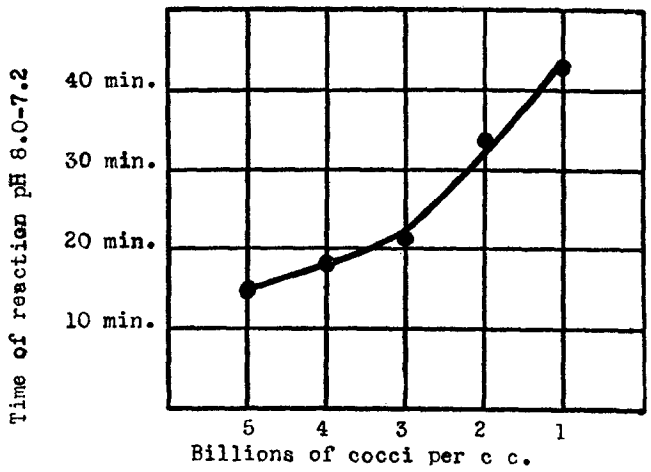
TABLE II.

Effect of Dilution of the Organisms on the Rapidity of Lipase Action.

Suspending fluid "20:1"-gelatin mixture (1.0 per cent sodium chloride and 0.05 per cent calcium chloride with 0.1 per cent gelatin).

Tube No.	Estimated No. of cocci per cc.	Time required between pH 8.0 and 7.6.	Time required between pH 7.6 and 7.2.	Total time required between pH 8.0 and 7.2.
		min.	*min.*	*min.*
1	5 billion.	4	11	15
2	4 "	5	13	18
3	3 "	6	15	21
4	2 "	9	25	34
5	1 "	14	29	43

Controls: Standard suspension (approximately 5 billion cocci per cc.) without ethyl butyrate and "20:1"-gelatin solution with ethyl butyrate both negative.



TEXT-FIG. 1. Curve representing the effect of dilution of the organisms on the rapidity of lipase action.

The optimum temperature was found to be 37.5°. At 50° the control containing the suspending fluid and ethyl butyrate changed slightly, while at 62° this control tube passed through the whole zone from pH 8.0 to 7.2 faster than the suspension of organisms with ethyl butyrate. The results are shown in Table III and the curve for the reaction in Text-fig. 2.

TABLE III.

Optimum Temperature for the Lipase Action.

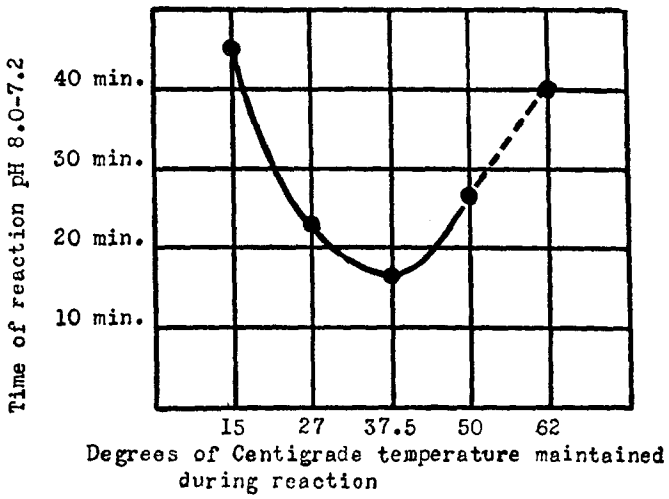
Suspending fluid "20:1"-gelatin mixture.

Tube No.	Temperature.	Contents of test-tubes.	Time required between pH 8.0 and 7.6.	Time required between pH 7.6 and 7.2.	Total time required between pH 8.0 and 7.2.
	°C.		*min.*	*min.*	*min.*
1	15	Standard suspension and substrate.	19	26	45
2	15	" " alone.	—	—	—
3	15	"20:1"-gelatin solution and substrate.	—	—	—
4	27	Same as No. 1.	11	12	23
5	27	" " " 2.	—	—	—
6	27	" " " 3.	—	—	—
7	37.5	" " " 1.	7	10	17
8	37.5	" " " 2.	—	—	—
9	37.5	" " " 3.	—	—	—
10	50	" " " 1.	14	13	27*
11	50	" " " 2.	—	—	—
12	50	" " " 3.	26	—†	—
13	62	" " " 1.	14	26	40‡
14	62	" " " 2.	—	—	—
15	62	" " " 3.	8	27	35

* Cannot be said to be entirely enzyme action because of slight change in control tube.

† Did not reach pH 7.2 by end of experiment (1 hour).

‡ Cannot be said to be enzyme action because of more rapid change in the control tube with ethyl butyrate.



TEXT-FIG. 2. Curve representing the optimum temperature for lipase action. Dotted line represents uncertainty of enzyme action because of changes in control tubes.

The Effect of Previous Contact with Higher Temperatures.

The test was set up with five tubes containing the standard suspension of organisms. The tubes were then subjected to temperatures of 37.5°, 40°, 60°, 80°, and 100°C. respectively for 10 minutes. They were then cooled and the test for lipase activity carried out with the proper controls in water bath at 37.5°. It was found that the tubes previously heated to 60°, 80°, and 100° made the change from pH 8.0 to 7.6 very slowly but never reached 7.2, while the other two tubes promptly passed on to the acid end of the color range. The test was then repeated between the active and inactive levels. Five portions of the standard suspension were heated for 10 minutes at 40°, 45°, 50°, 55°, and 60°C. respectively. After heating, 0.1 cc. from each tube was cultured in plain broth to determine the viability of the organisms. The lipolytic activity of the heated suspensions was then tested.

TABLE IV.

Effect of Previous Contact for 10 Minutes with Higher Temperatures.

Suspending fluid "20:1"-gelatin solution.

Tube No.	Previously subjected temperature for 10 min.	Time required between pH 8.0 and 7.6.	Time required between pH 7.6 and 7.2.	Total time required between pH 8.0 and 7.2.
	°C.	*min.*	*min.*	*min.*
1	37.5	14	18	32
2	40	18	22	40
3	60	75	*	
4	80	68	*	
5	100	60	*	

Controls: Standard suspension (5 billion cocci per cc.) without ethyl butyrate negative; "20:1"-gelatin solution with ethyl butyrate negative.

* Did not reach pH 7.2 by end of experiment (3 hours). Initial change manifestly not due to enzyme action.

It was found that the suspensions that had been heated to 40°, 45°, and 50° were about equally active, while the action of the suspension heated to 55° was markedly delayed and the suspension heated to 60° was practically inactive. Correspondingly the cultures of the heated organisms showed profuse growth from the suspensions heated to 40°, 45°, and 50°, while from the suspension heated to 55° out of approximately 500 million organisms planted, only six to eight colonies were found clinging to the sides of the test-tube. When shaken up and incubated longer they grew out profusely. The suspension heated to 60° was sterile. The results of these tests are shown in Tables IV and V and the curve in Text-fig. 3.

TABLE V.

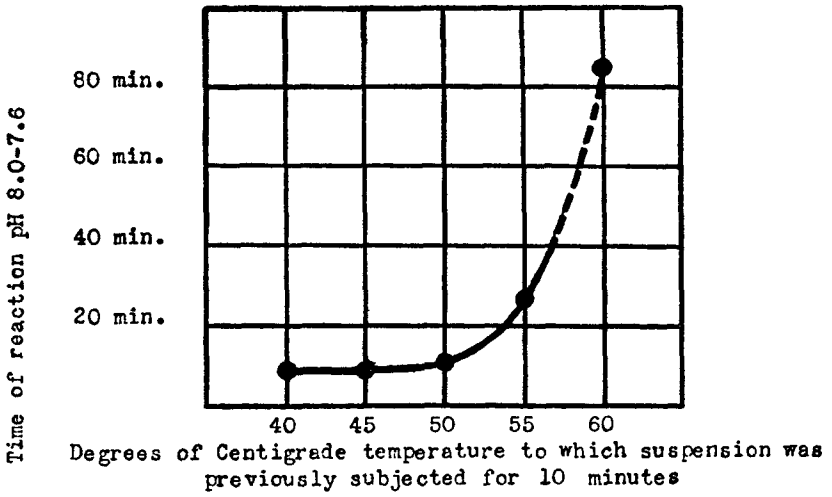
Effect of Previous Contact with Temperatures between 40° and 60° C. for 10 Minutes on the Lipolytic Activity and Viability of the Organisms.

Suspending fluid "20:1"-gelatin mixture.

Tube No.	Previously subjected temperature for 10 min.	Time required between pH 8.0 and 7.6.	Time required between pH 7.6 and 7.2.	Total time required between pH 8.0 and 7.2.	Culture after heating
	°C.	*min.*	*min.*	*min.*	
1	40	9	15	24	Profuse.
2	45	9	15	24	"
3	50	11	15	26	"
4	55	27	37	64	6 to 8 colonies.
5	60	85	*		No growth.

Controls: Heated standard suspension without ethyl butyrate and suspending fluid alone with ethyl butyrate at each temperature. The controls at 60° gradually changed color, thus making enzyme action at this temperature questionable.

* Did not reach 7.2 by end of the experiment (3 hours).



TEXT-FIG. 3. Curve representing the effect of previous contact with heat on the rapidity of lipase action. Dotted line represents uncertainty of enzyme action because of slight changes in control tubes.

When the experiment was repeated and the heating process continued for 30 minutes at 55°C., the organisms were completely destroyed and there was no lipolytic activity, while at the three lower temperatures the viability of the organisms and the lipolytic action were not affected.

The Optimum Hydrogen Ion Concentration.

For this test, the speed of color change could not be used as an indicat on of acid production because a given amount of acid has more effect on the color changes in the higher numerical pH levels than in the lower pH levels. It was found necessary to start the reaction at standard color levels and after a period of time, titrate back to the standard color. The standard colors were made by suspending heat-killed organisms, in the standard concentration of approximately 5 billion cocci per cc., in Clark's (2) buffer solutions at the pH levels 5.4, 6.0, 6.6, 7.2, 7.8, 8.4, 9.0, and 9.6 and using the corresponding indicators. The standard suspension of living organisms was centrifuged in 4 cc. quantities, washed once, and then taken up in the same concentration with "20:1"-gelatin solution previously titrated to the levels indicated above. 2 cc. of the live suspension at each hydrogen ion level were used for the test. After adding the ethyl butyrate, the reactions were again adjusted to the levels indicated. Controls were set up for each hydrogen ion level containing standard suspension alone, suspending fluid alone, and suspending fluid plus ethyl butyrate. After incubation for 45 minutes, enough $\frac{N}{100}$ sodium hydroxide was added to each tube to bring the color back to the standard. No change occurred in the tubes at pH 5.4 and 6.0. At pH 9.0 and 9.6 the controls changed to such an extent that the actual lipolytic effect was uncertain. Between pH 6.6 and 8.4 the slight changes in the control tubes could be adjusted by subtraction so that the relative amount of lipolytic action could be fairly accurately determined.

The optimum hydrogen ion concentration was found to be pH 7.8. The results are shown in Table VI and Text-fig. 4.

The Relation of the Virulence of the Organism to the Lipolytic Activity.

The strain used in all of the preceding experiments had been passed through three rabbits and had a minimal lethal power, on intravenous injection, of 1 cc. of a standard suspension estimated to contain 1 billion organisms per cc. The same strain was passed through thirteen rabbits, after which it killed repeatedly at 0.001 cc. of the standard suspension.

TABLE VI.

Optimum Hydrogen Ion Concentration for Lipolytic Action.

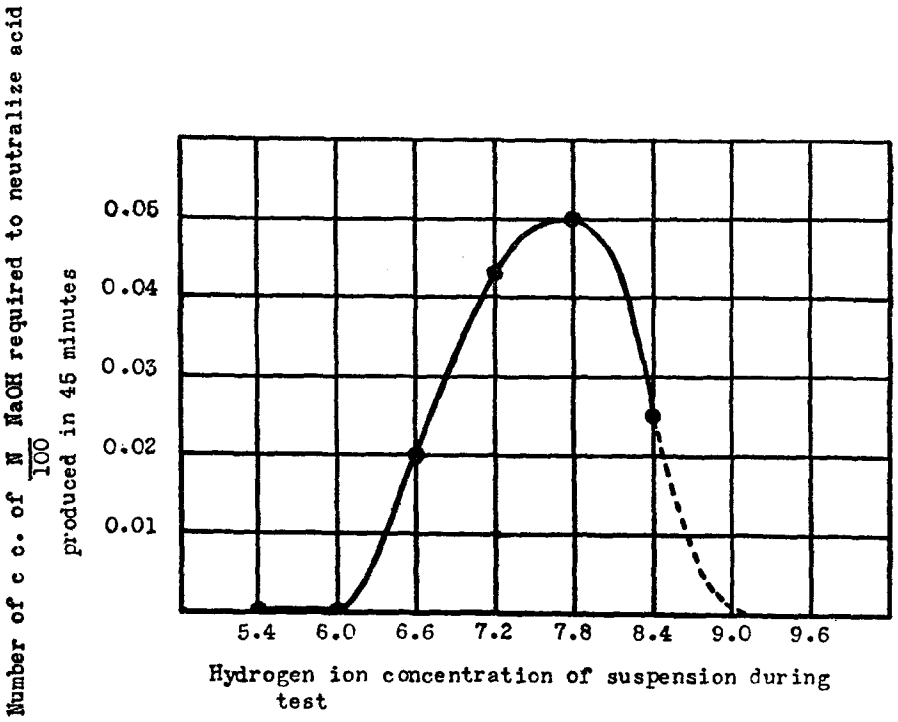
Suspending fluid "20:1"-gelatin mixture.

Tube No.		Levels of hydrogen ion concentration.							
		5.4	6.0	6.6	7.2	7.8	8.4	9.0	9.6
1	Standard suspension with ethyl butyrate.	0	0	0.25	0.7	0.95	0.9	+++	++++
2, 3, and 4	Summation of controls.	0	0	0.05	0.275	0.45	0.65	+++	++++
Difference (representing enzyme action).....		0	0	0.2	0.425	0.5	0.25	–	–

The tubes were set up with the colors exactly conforming to standard color tubes for each hydrogen ion level.

The figures represent the amount of $\frac{N}{100}$ NaOH required to bring the color of the mixtures back to the standard colors after 45 minutes incubation at 37.5°C. in the water bath.

Controls: Standard suspension without ethyl butyrate, suspending fluid with ethyl butyrate, suspending fluid alone.



TEXT-FIG. 4. Curve representing the optimum hydrogen ion concentration for lipase action. Dotted line represents uncertainty of enzyme action because of marked changes in the control tubes.

The lipolytic test was set up for standard suspensions of the virulent and the relatively avirulent organism in the usual way. The speed of the reaction of the two suspensions proved to be almost the same for both.

There was no proportionate increase of lipolytic activity with the increase in virulence and no indication that virulence in any way depends upon the lipolytic property. The figures are given in Table VII.

TABLE VII.

Comparison in Lipolytic Action between an Organism of Low Virulence for Rabbits and the Same Strain When in a Condition of Relatively High Virulence.

Suspending fluid "20:1"-gelatin mixture.

Tube No.	Virulence.	No. of rabbit passages.	Time required between pH 8.0 and 7.6.	Time required between pH 7.6 and 7.2.	Total time required between pH 8.0 and 7.2.
			min.	*min.*	*min.*
1	Low.	3	7	18	25
2	High.	13	9	20	29

Controls for each tube: Standard suspension of organisms without ethyl butyrate and the suspending fluid with ethyl butyrate all negative.

The Action of Different Strains of Hemolytic Streptococci Obtained from Various Clinical Sources (with Particular Reference to Strains Recovered from a Series of Cases of Acute Superficial Gangrene.)

The test has not been applied to a large series of cases but a direct comparison has been made between eighteen strains recovered from a definite clinical disease entity which has been studied by one of us and called hemolytic streptococcus gangrene (7) and strains from seven other sources including two from the throats of scarlet fever cases. Inasmuch as there seemed to be a great affinity between the organism and the subcutaneous fat in the gangrene cases, it was thought that this might be explained by an increase in lipolytic ferment. This theory is hardly tenable after studying the results of this test. Although there is more active lipase in the organisms of the gangrene group as a whole than in the group of controls, two of the latter are regularly more active than several of the former and there is no clear-cut difference based upon this property. The freshness of the strain is immaterial. Some strains which were kept in horse blood broth for over a year were just as active in the production of lipase

as those strains recently recovered from patients. The tests were done at three different times with certain of the controls used in all three tests. The organisms were planted, first in 5 cc. of plain broth, and after 10 to 12 hours incubation 2 cc. were transferred to 1 per cent dextrose broth. The periods of incubation and standardization of suspension were kept as uniform as possible. It may be that in a large series of cases it will be possible to make some biologic grouping on this basis.

For the present all that can be said is that the activity of lipolytic ferment does not appear to correspond to any specific disease-producing function.

DISCUSSION.

The delay in color change from pH 8.0 to 7.2 in the gelatin-citrate and gelatin-Locke's solutions employed in the first set of tests (Table I) is probably due to the fact that these solutions have more buffer action than the "20:1"-gelatin mixture. The citrate anion is a buffer and the Locke's solution contains a small amount of sodium bicarbonate. The slower action of gelatin-water may be due to the poorer preserving action of that fluid. It was thought possible that in the gelatin solutions some of the acid formed was due to the action of a gelatinase. On the other hand, it was recognized that gelatin itself has a buffer action. When the test was repeated with the final suspension of the organisms in one tube in "20:1" mixture *with* gelatin and in the other *without*, the color change was definitely more rapid in the solution without gelatin. This shows that if there is any acid formed from the gelatin it is not sufficient to counterbalance the buffer action of the gelatin itself.

The effect of the dilution of the organisms on the lipase action is about what would be expected. The margin of experimental error may be enough to explain why the curve is not a straight line. Or it may be that the prolonged contact with the products of the reaction, in spite of their extreme dilution, delays the reaction. This point is considered below.

The most significant finding in the whole series of experiments has been the great activity of young cultures. This led to a more thorough investigation of the factor of the length of incubation.

Was the fat-splitting a function performed only by young, rapidly multiplying, metabolizing bacteria? In what phase of growth was the lipase action most rapid?

At 8 p.m., 0.1 cc. of the stock culture was planted in two centrifuge bottles each containing 25 cc. of 1 per cent dextrose liver digest broth pH 7.6. At 4 a.m., 2 cc. of this 8 hour culture were transferred to each of twenty similar bottles of media. At 4 hour intervals for a period of 28 hours certain of these cultures were taken and the following tests carried out on them.

(*a*) Hydrogen ion concentration of the supernatant fluid.

(*b*) Concentration of organisms in the media. The total number was determined by the Gates (6) turbidimeter method and that number divided by the number of cubic centimeters of media used. This estimation was only approximate because there were not exactly 25 cc. of media in each bottle.

(*c*) The viability of the organisms. A standard suspension estimated to contain 1 billion cocci per cc. was made by means of the Gates (6) turbidimeter in the "20:1"-gelatin mixture and a series of seven dilutions made, each one containing a tenth of the number in the previous dilution. The final dilution was, therefore, estimated to contain 100 organisms per cc. 5 cc. of broth medium were then added to 1 cc. of each dilution and the whole series incubated. The results were read 24 hours after the 28 hour test was completed.

(*d*) The lipolytic action of the organisms suspended in the "20:1"-gelatin solution.

The various steps in the tests were done at precisely similar periods after incubation ceased. For the 4, 8, and 12 hour tests, six, four, and three bottles of culture were respectively used. For the next three tests two bottles were used. For the 28 hour test one was used.

The results of these tests are shown in Table VIII and Text-fig. 5. The significant points brought out by these tests were the following.

1. In glucose broth the hydrogen ion concentration reached a point of equilibrium at pH 5.1 in 16 hours.

2. Multiplication was rapid up to 8 hours but gradually slowed up and after 16 hours entirely ceased.

3. After 12 hours the organisms began to die off rapidly and at 24 hours none survived in a suspension containing 100 million organisms per cc.

4. The lipase action was rapid and constant as long as the organisms were actively multiplying but became slower as multiplication became slower, although the organisms were still viable. When the organisms were all dead the lipase action reached another constant five or six times as slow as that of the actively multiplying organisms.

The slow action may represent the action of an endoenzyme while the rapid action appears to be a function of the living organism. It may be an ectoenzyme. It is evidently not enough that the organisms be alive but they must be in an actively multiplying growth phase. Foster (5) has shown that the acid curve for hemolytic streptococci in a dextrose broth medium depends upon a number of factors; namely, the initial hydrogen ion concentration of the medium, the age of the parent culture, and the amount of the inoculum. He

TABLE VIII.

Effects of a Varying Duration of Incubation on the Hydrogen Ion Concentration of the Medium and Concentration and Viability of Organisms and the Lipase Activity.

Suspension fluid for the viability tests and lipase reaction "20:1"-gelatin mixture.

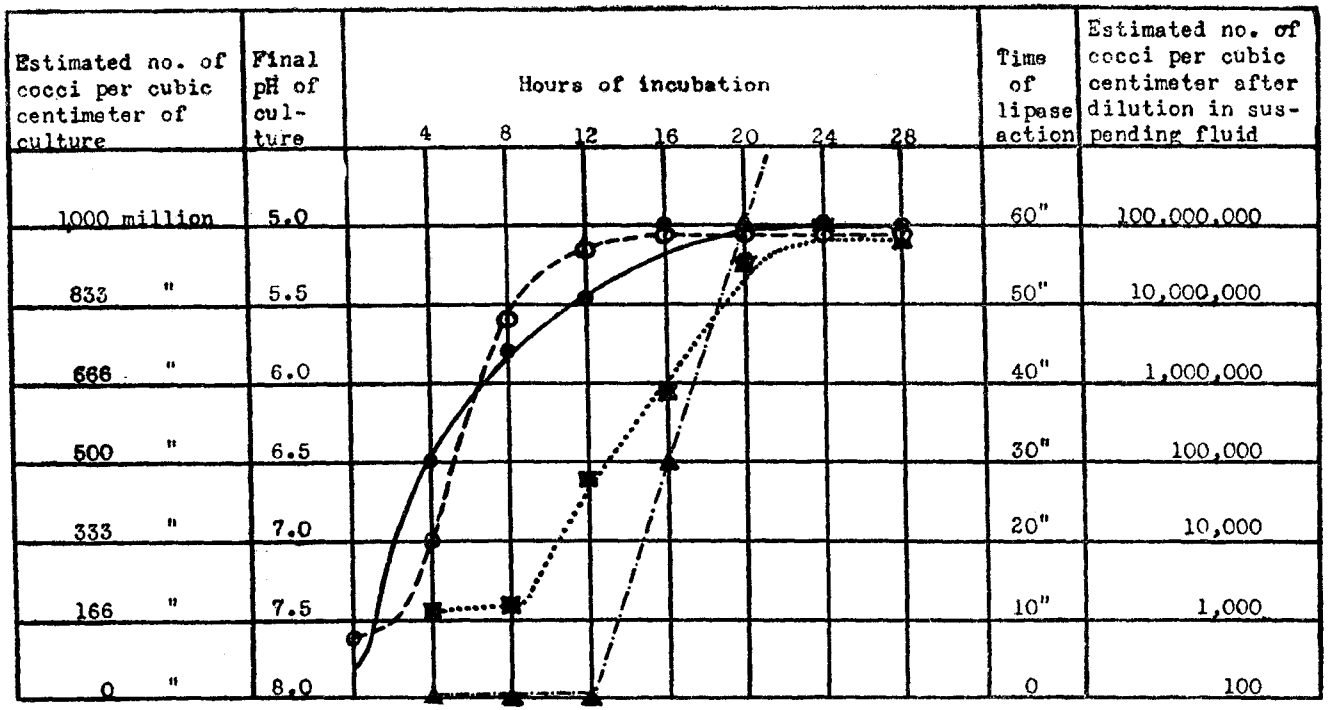
Duration of incubation.	pH level reached.	Concentration of organisms per cc. of culture medium.	No. of cocci per cc. in highest viable dilution.	Total time taken for lipase action pH 8.0–7.2.
hrs.				*min.*
4	7.0	500 million.	100	11
8	5.6	748 "	100	12
12	5.2	848 "	100	28
16	5.1	1,028* "	100,000	39
20	5.1	928* "	100,000,000	56
24	5.1	1,026 "	∞	60
28	5.1	1,028 "	∞	58

Hydrogen ion concentration determined by comparator method.

Lipase action determined in the usual way in "20:1"-gelatin suspending fluid.

* This discrepancy is probably due to the fact that the quantity of media in the tubes was not exactly 25 cc. The Gates method also has slight experimental error.

did not have a suitable method for determining the viability of the organism, for, as he remarks, the plating method is unreliable. Therefore, he could not fully appreciate the factor of viability. If dextrose broth is used, the incubation of the parent culture should not be over 8 hours and the subculture time should be the same, in order that one may be sure that the organisms are all alive and actively growing. It is obvious that these factors must all be constant



TEXT-FIG. 5. Curves representing the effects of varying duration of incubation in 1 per cent dextrose liver digest broth. A few organisms grew out in the highest dilutions of the 16 hour culture when incubated from 36 to 48 hours. Not grossly apparent before 36 hours.

⊙-------⊙ Hydrogen ion concentration of medium after incubation for the hours indicated.

●——— Estimated number of cocci per cubic centimeter of medium after incubation for the hours indicated.

■.....■ Time taken for lipase action color change pH 8.0 to 7.2 after incubation for the hours indicated.

▲—·—·—·▲ Highest dilution at which organisms were viable after incubation for the hours indicated. Readings made 24 hours after 28 hour test.

for any test involving a comparison of different strains. The time variations in all of the tests presented in the first part of this paper, are attributable to a variation in these factors for the fact had not been recognized when they were done that the organisms begin to die off after 12 hours. Most of the tests were carried out 12 to 14 hours after inoculation with 0.1 cc. of the stock culture. With this small amount of inoculum, the period at which death began was probably somewhat later than in the test presented above, in which 2 cc. of an 8 hour culture were used as the inoculum. In the early test which compared a young with an old culture, the inoculum for the 7 hour culture had been incubating for 14 hours and was probably not as active as at an earlier period. However, the facts brought out by the tests still stand, because the preliminary steps in each test were the same and the conditions were changed only after the standard suspensions had been made. Thus the relation of the varying conditions is the same as it would be under more ideal cultural conditions. The time of the reaction is slower, in general ranging between 20 and 30 minutes. The tests with different strains were all repeated with 8 hours incubation of both the parent culture and the subculture. With this short incubation, the virulent and avirulent strains produced their effects in 11 and 10 minutes respectively. The gangrene strains did not show a clear-cut increase of lipolytic activity over that of certain of the control strains.

It was thought that if the enzyme were an ectoenzyme, it might be given off into the medium in an old culture. It was found, however, that the supernatant fluid of an old culture had no demonstrable effect on ethyl butyrate. It may be that the buffer action of the medium concealed a slight action. There was no difference to be found between fresh and supernatant broth. Avery and Cullen (1) were not able to find any lipolytic action in the filtrate from a pneumococcus culture and concluded that that organism did not give off the enzyme. From these tests it is apparent that the living organisms must be in contact with the substrate in order that they may produce the active lipase.

The fact that the optimum temperature for the lipolytic action lies in the neighborhood of the optimum temperature for the growth of streptococcus seems to argue in favor of the lipolytic action being a function of actively growing metabolizing organisms.

The effect of previous contact with heat on the lipolytic action tends to confirm the idea that this action is a function of living organisms, for the thermal death point of the streptococcus coincides quite definitely with the cessation of lipase action whether the heating be continued for 10 or for 30 minutes.

The optimum hydrogen ion concentration for the lipolytic action also conforms to the level of alkalinity favored by the streptococcus for most rapid growth (Fennel and Fisher (4)).

In the course of a number of the lipase tests an important fact was observed which should be presented here; namely, that the streptococcus is killed by the products of the ethyl butyrate digestion. Cultures made at 5 minute intervals during one test were sterile after the test had run between 25 and 30 minutes. At this time, the pH was slightly above 7.2 which was reached in 30 minutes. This is well above the acid death-point of the streptococcus in ordinary media and there was no death and no color change in the control tube containing organisms in suspension without ethyl butyrate. Thus it is seen that the organisms are killed by the products of the reaction rather than by the mere property of acidity. Therefore it is important not to start the test at a pH more than a fraction above pH 8.0 for if it is much higher, the digestive products may kill the organisms and thus delay the reaction. For this reason, the interval between pH 8.0 and 7.6 is probably more representative of the action of the living organisms than the interval between 7.6 and 7.2. It is in the nature of pH values for more acid to be required to accomplish the color change from 7.6 to 7.2 than to bring about that from 8.0 to 7.6 but in certain tests the delay may have been partly due to the death of some of the organisms. The presence of a buffer delays the color change but may not affect the toxic action of the digestive products. For this reason the "20:1" mixture has a decided advantage over the citrate solution and Locke's solution. The facts here presented all tend to confirm the idea that the rapid lipase action is a function of living, actively metabolizing organisms.

Although the organisms from the gangrene series do not show any greater activity in the splitting of ethyl butyrate than the control strains, the fact that a rapid lipase action is a function of rapidly multiplying organisms suggests another possible explanation for the

rapid necrosis and solution of the subcutaneous fat in these cases. It is possible that because of some biological relationship between these organisms and the invaded body, at present not understood, there is in these cases an unusually rapid multiplication of the organisms in the body or else they are not killed off as rapidly in the body fat by the products of their metabolism, as other strains are. Such hypotheses may be extremely difficult to test. We have failed to get a necrosis of human fat outside of the body either by injecting the organisms into the center of a piece of fat and then incubating or by adding a piece of fat to ordinary broth media and then inoculating with the streptococcus.

CONCLUSIONS.

1. A method is presented for the quantitative comparison under various conditions of the activity of the lipolytic enzyme of the hemolytic steptococcus. The speed of acid production as shown in the color change from pH 8.0 to 7.2, when the living streptococcus is suspended in association with ethyl butyrate, is considered to be indicative of the amount of ferment elaborated by the organism.

2. The lipolytic action is a function of living, actively growing organisms such as are present in 4 to 8 hour cultures.

3. The speed of the lipolytic action is approximately in linear proportion to the concentration of the organisms.

4. The lipolytic action is most rapid at 37.5°C., slower at 50°, and absent at 62°.

5. The organisms are partially destroyed and the lipolytic action is markedly delayed by previous heating to 55°C. for 10 minutes. Both the organisms and the lipolytic activity are completely destroyed by continued contact with this temperature for 30 minutes.

6. Increasing the virulence of the organism for rabbits by repeated animal passage does not increase the lipolytic action.

7. The predilection of the hemolytic streptococcus for the subcutaneous fat in local streptococcus infection associated with extensive superficial gangrene cannot be explained on the basis of an increase of lipolytic ferment in the organisms recovered from these cases over that of heterologous strains of streptococci.

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