STREPTOLYSIN PRODUCTION IN CARBOHYDRATE MEDIA.*

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The inhibition of the hemolytic power of streptococci in media to which fermentable sugars have been added has been commented on by Ruediger (1), Sachs (2), Kuhn (3), Lyall (4), Davis (5), and Sekiguchi (6). After extended experiments Sachs concluded that in dextrose media the growth of the streptococcus was inhibited by the acid produced and the production of the hemolysin was correspondingly diminished. Kuhn, however, explained the effect of glucose by the theory that in carbohydrate media which were fermented by streptococci the metabolism of the organism changed so that the red cells were spared the direct lytic action of the bacteria. Although this in no way accounts for the diminished amount of hemolysin in filtrates of dextrose cultures, it was the first intimation that the protein-sparing action of carbohydrate might be applied to this phenomenon. Kendall and Farmer (7), studying the nitrogen metabolism, found that this principle was true in bacterial as well as animal physiology. Brown (8) has inferred that as there is undoubted evidence that streptolysin is a product of protein metabolism (it is thermolabile, can be filtered only through the coarsest filters, and is non-dialyzable), "in the presence of fermentable sugar the protein metabolism of the streptococci is reduced to a minimum and for this reason the formation of streptolysin is reduced or inhibited altogether." He suggested also that the acids formed might prevent the formation of streptolysin by acting on the growing streptococci, that the acids might inactivate the streptolysin during its formation, and, furthermore, might render the blood corpuscles insusceptible to hemolysis. It is possible on this basis to explain the action of sterile filtrates.

* From a report to the Surgeon General of the Army by a board of officers convened to study respiratory diseases.

In 1903 Schottmüller (9) noted that pneumococci and certain streptococci constantly produced green colonies on blood agar plates. Ruediger (1) attempted to show that this discoloration was due to acid production, and since the ferric chloride test on glucose broth cultures showed a reaction similar to that of lactic acid which he found would discolor blood media, he concluded that the green color was due to the action of lactic acid on red cells. He further states that not only did the addition of glucose increase the ability of pneumococci and streptococci to produce green colonies but that hemolytic cocci failed to hemolyze and after 36 to 48 hours incubation were surrounded by a distinct green halo. Cole (10) and Blake (11), working respectively with pneumococcus and *Streptococcus viridans*, discredited the action of acid in this reaction and showed that it was probably due to some inherent oxidative process acting on the cells immediately surrounding the colonies. Davis (5) found that after the addition of calcium carbonate to dextrose blood plates the average size of the zone of hemolysis was as large as on plain blood agar. By studying the solution of the carbonate particles and red cells simultaneously he concluded that the production of hemolysin and acid was coincident. Sekiguchi (6) stated that the hemolysin production was hindered by the glucose and not checked or destroyed by the acid. Finally, Brown (8) has observed that greenish or brownish discoloration similar to the alpha appearance is not produced by beta or gamma type of streptococcus in dextrose blood agar, but if there is sufficient fermentation the entire plate may be browned, first in the neighborhood of the colonies. The most noticeable effect was the inhibition of hemolysis by the actively hemolytic type. He further showed that acids produced in dextrose might account for some of the hemolysis of the gamma type streptococcus in poorly buffered media.

The reaction of the culture media for the production of streptolysin was described by Lyall (4) as optimum at 0.3 per cent acid to phenolphthalein, but he found inhibition if he varied the reaction much on either side of this point. M'Leod (12) obtained the greatest production in media distinctly alkaline to litmus. Since cultures become excessively acid in sugars which are fermented, calcium carbonate was added by Sachs (2) and Lyall (4), and the hemolysin determined. Lyall found that there was still marked inhibition. Sachs' tables show but little variation from the ordinary culture media except that the lysin was active slightly longer; by the addition of lactic acid to media before inoculation he shortened the life of the hemolysin by half. Braun (13) added acid and alkali to filtrates and after 6 hours incubation the alkaline preparation was most hemolytic; a filtrate obtained from a serum broth culture was made alkaline and incubated with a quantity of the original filtrate as a control. Both results were similar. Braun (13) and Sachs (2) concluded that the ability of the streptococcus to form acid was not to be identified with its hemolysin-producing power. That sufficient acid is formed in dextrose media to hemolyze red cells was shown by Sekiguchi (6); a 24 hour culture heated at 60°C. was not hemolytic after neutralization.

Methods.

The most important factor in the production of strong hemolysin is the media employed for the growth of the streptococcus. After testing various sera and broths M'Leod (12) considered beef infusion peptone broth with the addition of 20 per cent inactivated horse serum the most satisfactory, since he was able to obtain filtrates which were strongly hemolytic and fairly constant. In the following experiments a similar medium was used with 2 per cent peptone. No difficulty was experienced at any time in obtaining hemolysins and the filtrates were very active if obtained through new Mandler filters after 10 to 14 hours incubation. Since the object of the study was to observe the effect of dextrose and other fermentable sugars on the lysin production, it seemed necessary to measure the acid in the cultures at stated intervals. This was accomplished by means of a series of phosphate mixtures prepared according to Sörensen (14). To overcome the interference of the protein in the heavy serum media, 0.25 cc. was removed and diluted to 10 cc. before the indicators were added. The facts concerning the growth of the streptococcus were established on plate counts usually taken every 4 hours. It is realized that colony counts are open to much criticism, especially when an organism is counted which occurs in chain formation; films were made, a large number of groups of cocci was counted, and the number of individuals in each specimen was averaged. This factor usually varied from 4 to 7. In view of these facts, since the purpose was to obtain an idea of the relative growth of the organisms, the counts obtained by multiplying this number by the calculated colonies per cubic centimeter of the culture were sufficiently accurate. Hemolysis was determined by a series of tubes set up with increasing amounts of sterile filtrate or, as in the determination of hemolysin production, with the supernatant fluid obtained after centrifuging a portion of the culture at high speed for 10 minutes. 1 cc. of a 5 per cent suspension of washed human cells was added to each tube, and after an hour at 37°C. all were made up to 5 cc. volume and the undissolved cells were counted in a Levy chamber; the degree of red cell destruction was calculated in percentages by comparison with a standard control. Such counts are accurate since the occurrence

of shadow cells observed in hypotonic salt solution is rare; a perfect curve can be constructed from the degrees of hemolysis in the increasing amounts of hemolytic filtrate.

EXPERIMENTAL.

The effects of sugars were first observed on a series of plates made with 5 per cent defibrinated human blood and 1 per cent carbohydrate. The streptococci were obtained in pure culture from acute empyema fluids; five strains were chosen which gave a beta type hemolysis and had a final hydrogen ion concentration of pH 5.2 to 4.9 in dextrose broth. In liquid media they fermented dextrose, maltose, saccharose, lactose, and salicin, but did not so utilize mannite, inulin, glycerol, or raffinose. The first three sugars constantly inhibited hemolysis with discoloration of the media after 24 to 48 hours incubation, but lactose and salicin were more inconstant in action and some degree of cell destruction was observed at times without apparent cause. The typical green colony attributed to the pneumococcus or *Streptococcus viridans* was not observed. The remaining four substances had little effect on the size of the hemolyzed zone. Rarely the edges of the decolorized areas were not so sharp as on plain blood agar plates. It was apparent that the sugars which were easily fermented inhibited hemolysin formation most (Lyall (4)), but the resistant carbohydrates occasionally gave uncertain reactions. It is among this last group that the majority of sugars employed for the differentiation of various strains occurs; hence the appearance of the colonies on sugar blood agar plates does not seem accurate as a test of fermentation (Davis (5)). This inhibition of lysin production occurs in liquid as well as solid media. Sekiguchi (6) noted that distinct hemolysin was found only in plain broth, seldom in a weak content of glucose, and rarely in 3 per cent. The degree of inhibition is necessarily dependent to a large extent on the enrichment of the media with serum, since in simple media which are not suitable for the development of a strong hemolysin no trace is evident when an easily fermented sugar has been added previous to inoculation. In the following tests active hemolysin could always be obtained in the presence of carbohydrates.

Experiment 1.—Two flasks of media of 275 cc. volume, identical in every respect except that 1 per cent dextrose had been added to one, were inoculated with equal amounts of a 20 hour culture of Strain A. Bacterial counts, hemolysin determinations, and the hydrogen ion concentration were done at once and at intervals during a 48 hour period. The results are shown in Table I and further illustrated in Text-fig. 1.

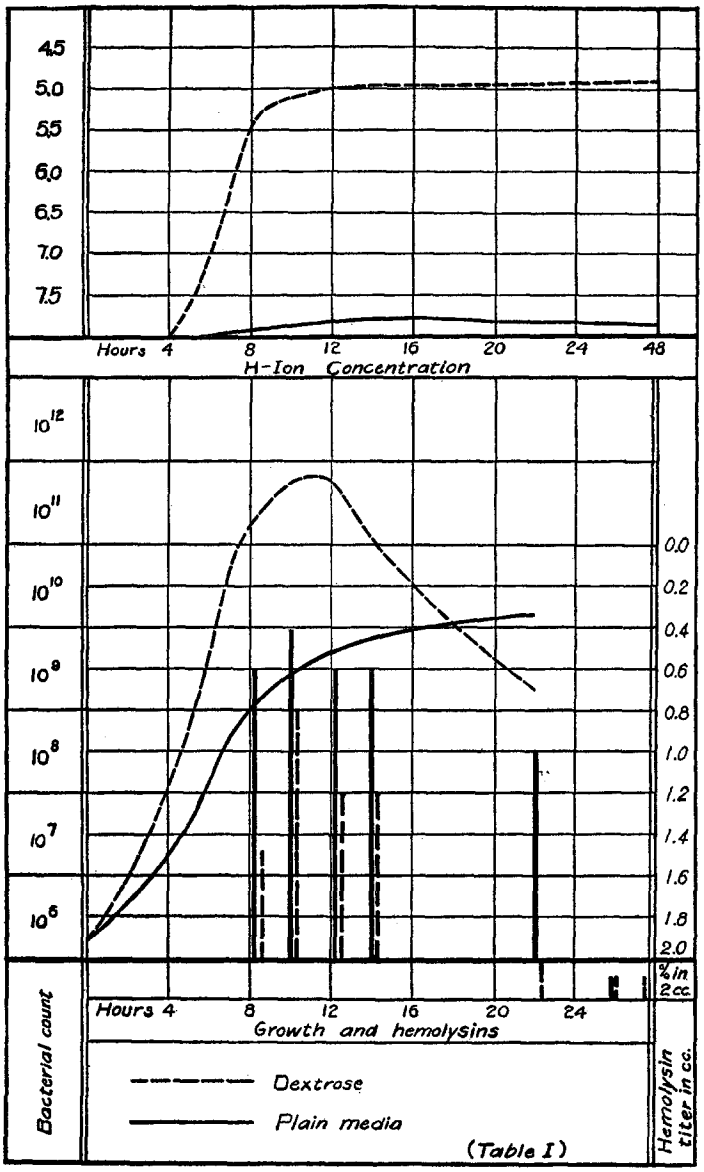
TABLE I.

Interval.	pH	Quantity of fluid.	Hemolysis.*	Bacterial count.†
		Plain media.		
hrs.		*cc.*	*per cent*	
0	8.0	2.0	0	$1.6(10^6)$
4	8.0	2.0	0	$1.9(10^7)$
6	8.0	2.0	0	$2.0(10^8)$
8	7.9	0.6	100	
10	7.9	0.4	100	
12	7.8	0.6	100	$6.5(10^9)$
14	7.8	0.6	100	
22	7.8	1.0	100	$1.1(10^{10})$
28		2.0	50	
48	7.9	2.0	0	
		Dextrose media.		
0	8.0	2.0	0	$1.6(10^6)$
4	8.0	2.0	0	$9.9(10^7)$
6	7.1	2.0	0	$3.8(10^9)$
7	6.3			
8	5.5	1.5	100	$2.1(10^{11})$
10	5.1	0.8	100	
12	5.0	1.2	100	$7.8(10^{11})$
14	5.0	1.2	100	$1.0(10^{11})$
22	4.9	2.0	100	$2.6(10^9)$
28	4.9	2.0	50	
48	4.9	2.0	50	

* Hemolysins are indicated by the amount of supernatant fluid required to hemolyze 1 cc. of the red cell suspension or in the percentage of hemolysis if incomplete.

† The counts are given in powers of 10.

Experiment 2.—The previous experiment was repeated with three flasks, one of plain horse serum beef infusion media, one with 1 per cent dextrose, and one with dextrose and 1 per cent calcium carbonate. These were inoculated with



TEXT-FIG. 1. Comparison of the effects of plain media and dextrose media on lysin production.

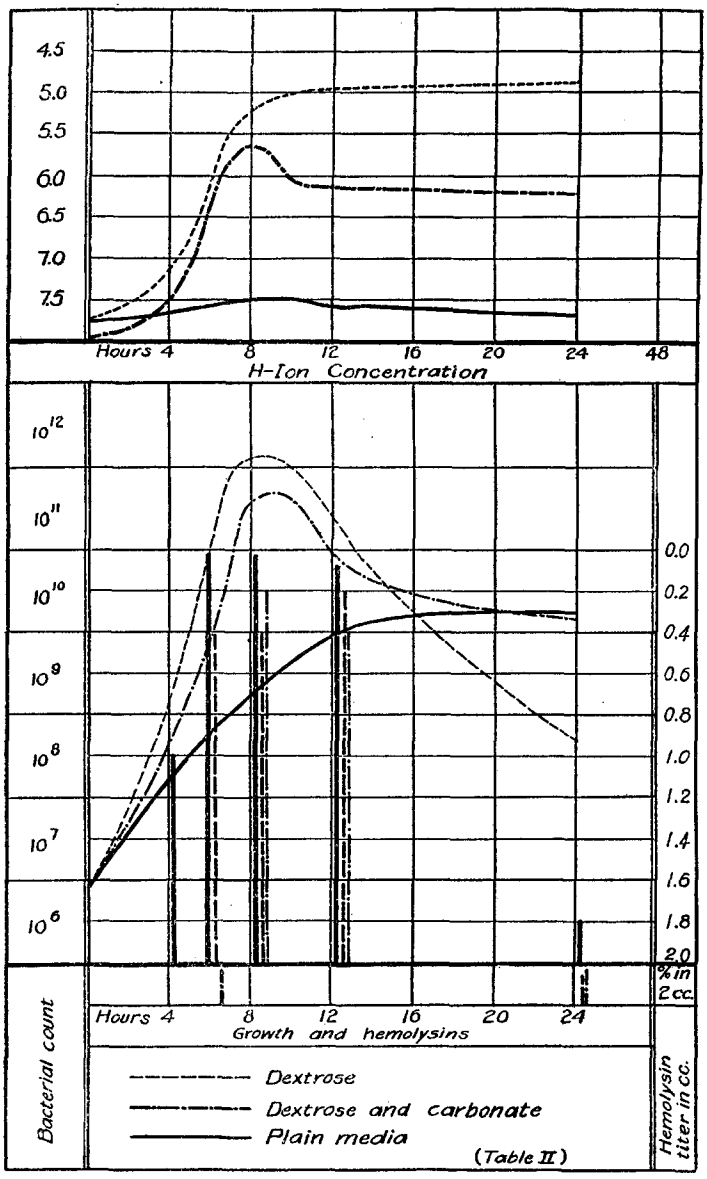
equal amounts of a 20 hour culture of Strain B. The results are illustrated in Table II and Text-fig. 2.

TABLE II.

Interval.	pH	Quantity of fluid.	Hemolysis.	Bacterial count.
		Plain media.		
hrs.		*cc.*	*per cent*	
0	7.8	2.0	0	$8.7(10^6)$
4	7.7	1.0	100	$2.8(10^8)$
6	7.6	0.05	100	
8	7.5	0.05	100	$2.9(10^9)$
10	7.5			
12	7.6	0.1	100	$1.0(10^{10})$
24	7.7	1.8	100	$1.8(10^{10})$
		Dextrose media.		
0	7.8	2.0	0	$8.7(10^6)$
4	7.3	2.0	0	$1.3(10^9)$
6	6.1	0.4	100	
8	5.3	0.4	100	$1.2(10^{12})$
10	5.0			
12	5.0	0.2	100	$4.1(10^{11})$
24	4.9	2.0	50	$5.7(10^8)$
		Dextrose carbonate media.		
0	8.0	2.0	0	$8.7(10^6)$
4	7.5	2.0	0	$5.7(10^8)$
6	6.3	2.0	75	$1.5(10^{10})$
8	5.6	0.2	100	$7.0(10^{11})$
10	6.2			
12	6.2	0.4	100	$6.2(10^{10})$
24	6.1	2.0	75	$1.0(10^{10})$

Experiment 3.—Experiment 2 was repeated, beginning with media titrated to pH 7.6. The flasks were inoculated with equal quantities of a 20 hour broth culture of Strain B (Table III).

The growth of streptococci in dextrose media is much more rapid and profuse than in plain broth, and concomitant with this growth there is a rapid change in hydrogen ion concentration. In cultures which are not so heavily seeded as were those in Tables I to III the maximum acidity is reached within 24 hours (Avery and Cullen (15)).



TEXT-FIG. 2. Comparison of the effects of plain media, dextrose media, and dextrose carbonate media on lysin production.

TABLE III.

Interval.	pH	Quantity of fluid.	Hemolysis.	Bacterial count.
		Plain media.		
hrs.		*cc.*	*per cent*	
0	7.6	2.0	0	$9.2(10^6)$
3½	7.6			
4				$3.7(10^8)$
5	7.5	1.2	100	
6	7.2	0.2	100	
8	7.3	0.1	100	$3.6(10^{10})$
11	7.3	0.05	100	
12				$4.6(10^{10})$
24	7.3	1.0	100	$6.8(10^{9})$
		Dextrose media.		
0	7.6	2.0	0	$9.2(10^6)$
3½	7.6			
4				$2.4(10^8)$
5	6.6	2.0	0	
6	5.5	1.5	100	
8	5.0	0.4	100	$5.0(10^{11})$
11	4.9	0.3	100	
12				$1.9(10^{12})$
24	4.9	2.0	75	$3.2(10^9)$
		Dextrose carbonate media.		
0	8.0	2.0	0	$9.2(10^6)$
3½	8.0			
4				$9.5(10^8)$
5	7.0	2.0	0	
6	6.1	2.0	50	
8	5.5	0.6	100	$5.8(10^{13})$
11	5.5	0.2	100	
12				$2.7(10^{10})$
24	6.4	2.0	50	$5.1(10^{10})$

The greatest increase occurs in the first few hours of incubation during the time that the hemolysin is produced. As the limiting acid concentration is approached the streptococci are found to be less viable, and from that time there is a gradual reduction in the count. Cultures in plain serum broth show a stronger hemolysin

titer during this period of active growth, while the production of hemolysin not only begins earlier but the cultures are actively hemolytic for a longer time. In general, the hemolysin curves obtained approximate the figures of Lyall (4), M'Leod (12), and Besredka (16). The addition of calcium carbonate allows a pH intermediate between those of flasks of plain serum bouillon and of flasks to which glucose alone has been added. With this partial neutralization the growth approaches or exceeds that in dextrose, but the production of hemolysin is still further delayed. In several experiments it was found that the rate of growth in the carbonate dextrose media was inverse to the hemolysin production, and, furthermore, in the cultures without sugar, although there were fewest streptococci, the hemolytic property was always greatest. It is apparent from Tables I to III that with the increased utilization of dextrose the hemolysin production is

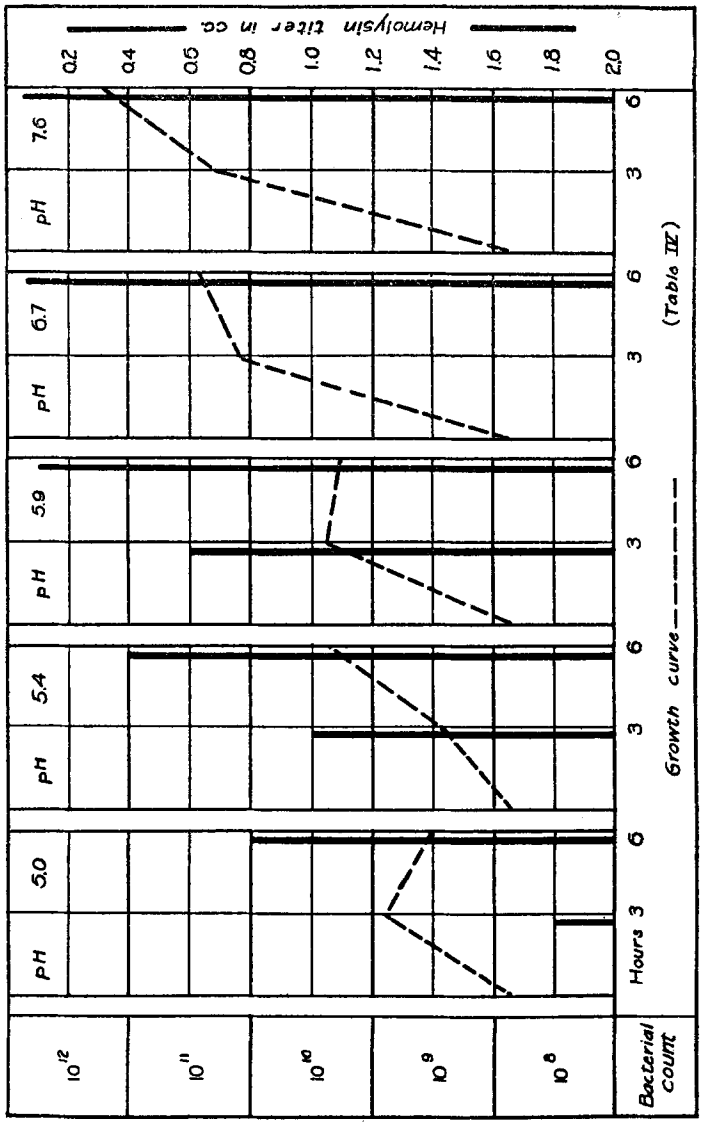
TABLE IV.

pH	3 hrs.		6 hrs.	
	Titer.	Bacterial count.	Titer.	Bacterial count.
	cc.		*cc.*	
7.6		$2.6(10^{11})$	0.05	$1.7(10^{12})$
6.7		$1.1(10^{11})$	0.05	$3.8(10^{11})$
5.9	0.6	$3.0(10^{10})$	0.1	$2.1(10^{10})$
5.4	1.0	$4.0(10^{9})$	0.4	$3.7(10^{10})$
5.0	1.8	$8.8(10^{9})$	0.8	$5.0(10^{9})$

inhibited because of the protein-sparing action of the carbohydrate. The effects of the acids developed in the cultures still further explain these figures.

It was found that when the hydrogen ion concentration of the media was increased before inoculation by the addition of lactic acid, the growth of the streptococci and the hemolysin produced were proportional to the pH. This interferes in no way with the previous findings, since in the curves established the multiplication of the bacteria was not retarded in the first few hours of incubation, yet less lysin was produced. The effect of the acid in Table IV is primarily one of growth inhibition.

Experiment 4.—Five 100 cc. flasks of veal infusion horse serum media were used. The original titer of the media was pH 7.6. Four of the flasks were



TEXT-FIG. 3. The effect of acid on lysin production.

titrated to pH 6.7, 5.9, 5.4, and 5.0 respectively with 0.5 N sterile lactic acid. The volumes were then made equal with sterile 0.85 per cent sodium chloride solution. Each of the flasks was inoculated with the washed streptococci centrifuged from 20 cc. of an 18 hour culture of Strain B. Counts and hemolysin determinations were made after 3 and 6 hours incubation (Table IV, Text-fig. 3). The pH had not changed at the end of the period. Hemolytic titers are designated by the smallest amount of supernatant fluid which would completely dissolve 1 cc. of a 5 per cent suspension of human cells in 1 hour at 37°C. None of the flasks was hemolytic at the beginning. The counts before incubation were 8.5 (10^8).

The effects of acid are still more far reaching. Sterile hemolytic filtrates incubated in various strengths of acid for 6 hours lose a large part of their hemolytic property. That there is destruction of the hemolytic substance is evident, since after titration back to the original pH with sodium hydroxide the solution of the red cells is still less than that of the control tubes. The percentages of hemolysis in the tubes containing the lowest amounts of filtrate serve best as a means of checking this destructive action. In the determinations of hemolysis by the addition of 1 cc. of a 5 per cent suspension of blood cells in salt to small amounts of acid filtrate, the pH is raised to a point which in itself is not destructive to red cells. The acidity in the larger quantities is sufficient to account for the greater hemolysis in the tubes incubated at pH 5.0 for 6 hours. Supernatant fluids acidified and titrated back to neutrality with secondary sodium phosphate after incubation gave similar results. Braun's (13) tables also show this point.

Experiment 5.—An 18 hour culture of Strain B was found actively hemolytic. It was filtered through a new Mandler filter and divided into three equal portions. One was left at its original pH, 7.4. The others were adjusted to a pH of 5.0 and 6.0 by the addition of sterile 25 per cent lactic acid. The hemolytic titer was determined at once and after 3 and 6 hours incubation (Table V). At the end of 6 hours the flasks were neutralized (7.4) and the volumes were made up by the addition of normal salt solution so as to contain an equal concentration of the original filtrate. Cultures of the flasks at the beginning and at the end of the experiment were sterile.

In Table V it was assumed that some of the hemolysis in the strongly acid tubes was due to acid concentration. It was observed that salt solution, to which acid had been added until a pH was reached which

would check the growth of streptococci, had no effect on red cells. If red cells were incubated 1 hour in media made to contain a large quantity of weakly ionized electrolytes and titrated the same way with lactic acid, the solution and discoloration were striking. The destruction of corpuscles begins at about pH 6 and in broth is complete

TABLE V.

Quantity of filtrate.	Hemolysis.		
	pH 5.0	pH 6.0	pH 7.4
Hemolysis before incubation.			
cc.	*per cent*	*per cent*	*per cent*
0.05	100	100	100
Hemolysis after 3 hrs. incubation.			
0.8	100	100	100
0.4	98	100	100
0.2	96	100	100
0.1	78	98	99
0.05	54	96	98
Hemolysis after 6 hrs. incubation.			
2.0	99	87	99
1.0	98	87	98
0.6	84	84	98
0.2	50	68	92
0.1	40	59	86
0.05	30	43	85
Titrated back to pH 7.4 after 6 hrs.			
1.5	28	11	99
1.0	0	22	98
0.8	0	7	97
0.4	0	1	94
0.2	0	0	92

at pH 5. Coincident with this acid hemolysis the hemoglobin is turned brown. Due to the fact that there is so much cell destruction it is impossible to determine the effect of hemolysin on blood cell suspensions which have been previously treated with acid. Lyall (4) has already noted that cells which have been discolored by actively

TABLE VI.

	Control.	0.5 per cent.	1 per cent.	2 per cent.
Filtrate	0.1 cc.	0.1 cc.	0.1 cc.	0.1 cc.
Sterile sugar solution (10 per cent)	0.0 "	0.05 "	0.1 "	0.2 "
Saline solution	0.9 "	0.85 "	0.8 "	0.7 "
Hemolysis before incubation.				
	per cent	*per cent*	*per cent*	*per cent*
Dextrose	99	99	98	98
Mannite	100	97	97	96
Invert sugar	100	99	99	99
Hemolysis after 3 hrs. incubation.				
Dextrose	97	92	81	82
Mannite	96	95	90	82
Invert sugar	98	96	98	94
Hemolysis after 6 hrs. incubation.				
Dextrose	95	84	61	64
Mannite	87	76	70	61
Invert sugar	90	88	88	83

*Series with Large Quantities.**

Quantity of filtrate.	Control tube.	Dextrose.	Mannite.	Invert sugar.
Hemolysis after 3 hrs. incubation.				
cc.	*per cent*	*per cent*	*per cent*	*per cent*
0.8	100	100	100	100
0.6	99	100	99	99
0.2	99	100	97	98
0.05	97	97	95	96
Hemolysis after 8 hrs. incubation.				
2.0	95	96	95	97
1.0	90	93	90	95
0.6	85	87	86	89

* Cultures were sterile after incubation.

growing pneumococci are more resistant to streptolysin, but this action of the pneumococcus, mentioned by Cole (10), probably enters very little into the discoloration of hemoglobin in dextrose cultures of the beta type streptococcus.

The presence of either dextrose, mannite, or invert sugar exerted a slight inhibition on hemolytic filtrates. This cannot account for any of the marked differences in the titer of broth cultures or in the gross appearance of blood agar plates, but it is probably the cause of the hazy edges occasionally observed in the hemolyzed zones on solid media containing unfermented sugars. It is impossible to demonstrate this effect on large quantities of filtrate, but if the minimal hemolytic amount is incubated in 1 per cent of these sugars the solution of the red cells is much retarded after a few hours (Table VI).

Experiment 6.—The effect of sugars on hemolytic filtrates was studied in the following way. It was found that 0.05 cc. of a sterile filtrate obtained from a 12 hour culture of Strain C caused complete hemolysis of 1 cc. of the red cell suspension. A series of tubes was set up containing 0.1 cc. of the filtrate in 1 per cent dextrose, mannite, and invert sugar; the tubes were incubated 3 and 6 hours. The hemolysin tests are indicated in Table VI. To determine the effect of these substances on larger quantities of filtrate, 50 cc. lots were treated in the same manner and the titer was determined after 3 and 8 hours.

SUMMARY.

It is evident that there are numerous interacting factors which affect streptolysin in the presence of fermented sugars. The principal action is the change in the metabolism of the streptococcus by which more carbohydrate and less protein is utilized; although the growth is much increased there is proportionately less hemolysin. The acid developed in these cultures not only lessens the vitality of the growing organism and so lessens proteolysis, but is destructive to hemolysin at incubator temperature. The concentration of the acid produced causes some hemolysis, and a coincident brown discoloration of the hemoglobin. These principles may be applied to both liquid and solid media.

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