OPTIMUM AND LIMITING TEMPERATURES FOR THE GROWTH OF THE PLAGUE BACILLUS IN BROTH

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It has become customary to grow pathogenic organisms in the laboratory at 37°C. This practice seems to be based on the belief that those organisms "which have adapted themselves to a saprophytic or parasitic life in relation with warm-blooded animals have an optimal temperature round 37°C. to 38°C." (Andrewes, 1930). This belief does not appear to be based on any exact measurement of optimum growth temperatures for the various organisms concerned. In the case of *Pasteurella pestis*, at least, it has long been recognized by most workers that it grows "poorly above 35°C." though there is still a wide difference of opinion as to the temperature at which optimum growth results. One of the present authors (Sokhey 1939b) had found the temperature for optimum growth in broth to be about 27°C. More recently Spicer (1940) has reported that, "the optimum temperature for the growth of a Type III pneumococcus strain was found to be 27°C."

In the case of *P. pestis* early workers, quoted by Albrecht and Gohn (1900), found the optimum growth to result at 37°C.; later workers, as reported in standard textbooks, put the optimum growth temperature at 25°C. to 30°C., though Topley and Wilson (1936) state, "growth, however, at both 24°C. and 37°C. is often as good as at 30°C." Recently Tumanksy *et al.* (1935) made a comparative study of the growth of *P. pestis* and *Pasteurella pseudotuberculosis* Pfeiffer, at nine different temperatures beginning with 0°C. and ending with 43°C. They found the optimum growth temperature of *P. pestis* to be 28°C. to 30°C. and the upper limit of growth at about 43°C. Regarding the lower limit of the growth they found that the organisms showed some growth even at 0°C.

It would appear that at least some of the differences in the reported observations are due to different media, solid or liquid, employed, and the number of organisms used as the inoculum by different workers. In a previous paper (Sokhey 1939b), it was shown that on nutrient agar plates, if 5,000 organisms or more were seeded per square centimeter of the surface, more profuse growth resulted at 27°C. than at 37.5°C., but if a smaller inoculum was used, i.e., 500 organisms per sq. cm. of the surface, no growth resulted at all at 27°C. while some growth still appeared at 37.5°C. after incubation for 48 hours. If blood agar was substituted for nutrient agar even with the smaller inoculum, 500 organisms per sq. cm. of surface, the number of colonies resulting both at 27°C. and 37.5°C., were equal in number, though the size of the colonies at 37.5°C. was smaller.

In the present paper results are given of an investigation carried out to de-

termine more precisely the optimum and limiting temperatures for the growth of *P. pestis* in nutrient broth.

TECHNIQUE

The optimum temperature for the growth of an organism depends on a variety of factors. The most important of these are: (1) composition of the medium, (2) number of organisms used as the inoculum, (3) the time chosen for observation, and (4) the criterion used for determining the optimum growth. Therefore, for a comparative study like this the conditions under which the experiment is conducted must be specified and kept constant.

Medium and its pH

The present study refers exclusively to the growth of the organism in nutrient broth. Basal Infusion Broth, Medium No. 748, Committee of A.P.H.A., was used (Levine and Schoenlein, 1930). Two different pH values of broth, 6.4 and 7.2, were used for reasons explained below. Broth for each determination was placed in 10 ml. quantities in test tubes with an internal diameter of 1.7 cm. Keeping the internal diameter of the tubes constant is important since it was found, as reported in a previous paper (Sokhey 1939c), that "a 48-hour growth of the plague bacillus in a liquid medium bore no relation to the total quantity as such of the medium nor to its surface area, but was directly proportional to the circumference of its surface area." Though subsequent work, which will be reported in another paper on the rate of growth of the organism, has shown that this statement is subject to a modification, the observation still remains true in its broad aspect and applies to the present study.

For the experiments reported in this paper the same batch of broth was used throughout for each of the two series of determinations. Large quantities of broth were made and kept in cold storage at 4°C. For the preparation of the inoculum, however, odd batches of broth with pH 6.8 were used.

Inoculum

Preliminary observations showed that as small an inoculum as 40 to 60 organisms sown in 10 ml. of nutrient broth, in our test tubes, was enough to give growth, but with this inoculum appreciable amount of growth resulted only after a very long period of incubation, about 8 days. A larger inoculum, say 3 to 4 million organisms, gave a good growth in 36 hours and could be relied upon to minimize the stationary (lag) period of growth, or rather the stationary periods of growth of *P. pestis*. In a subsequent paper we shall show that the plague bacillus when grown in a liquid medium has two stationary periods of growth. For our inocula 48-hour growths of the organism in nutrient broth were used. They were prepared in the following manner. A 2 mm. loopful of a culture on blood agar was inoculated into 10 ml. of nutrient broth in a test tube. The growth on the loop was carefully mixed with broth to obtain a tolerably uniform suspension and was incubated at 27°C. for 48 hours. A second

subculture was made by sowing 0.5 ml. of the first subculture into 9.5 ml. of broth in a test tube (1.7 cm. internal diameter). This was incubated in a vertical position for 48 hours at 27°C. The second subculture contained approximately 400 million organisms per ml. It was diluted to 1 in 10, and 0.1 ml. of this dilution was used as the inoculum for inoculating 9.9 ml. of the broth for each determination. Actual counts showed that the inocula on an average contained 3.46 million organisms.

Criterion of optimum growth

Crop yield after exactly 36 hours of incubation was used as the criterion of optimum growth. In our hands crop yield proved to be quite an effective criterion and its measurement was less laborious than the measurement of the rate of growth. The broth cultures were incubated in a vertical position and were specially protected against mechanical jars. The crop yield was estimated by the method of counting the number of viable plague organisms in broth cultures, described in a previous paper (Sokhey 1939c).

Strains

Two strains of *P. pestis*, 55/H and 145/Bit, were used, one for each of the two series of experiments reported. These strains were the first subcultures on blood agar made from primary culture of venous blood from severe septicaemic human cases. The virulence of the subcultures had been measured by the method described in a previous paper (Sokhey 1939e), and both strains were highly virulent, i.e., 5 to 10 organisms killed 100 per cent of the white mice infected.

RESULTS

Two lots of nutrient broth were employed. The pH of one was adjusted at 6.4 and the pH of the other at 7.2. Though the optimum pH for the growth of *P. pestis*, as will be described in a subsequent paper, is 7.2 to 7.6, for reason to be explained elsewhere we grow cultures for making Haffkine plague vaccine in broth with pH 6.4. Therefore, broths with both these hydrogen ion concentrations were used in these experiments. For experiments with the broth of pH 6.4 the temperatures of incubation employed were 23°C., 25°C., 27°C., 28°C., 29°C., 30°C. and 31°C., and the strain used was 145/Bit. Four to six sets of determinations were made and 8 parallel plates were used for counting the number of organisms in each test tube, giving 32 to 48 counts for each temperature. The results of these counts are given in table 1. For experiments with the broth of pH 7.2, the temperatures of incubation employed were 25°C., 27°C., 28°C., 29°C., 30°C. and 32°C., and the strain used was 55/H. Four sets of experiments were made, and again 8 parallel plates were used for counting the number of organisms in each test tube, giving 32 counts for each temperature. The mean numbers of colonies per plate are given in table 3.

To determine the limiting temperatures of the growth of the organism incubation temperatures of −2°C., 0°C., 2°C., 4°C., 43°C. and 45°C. were

TABLE 1

Colony counts of 36-hour growths of Pasteurella pestis in broth, pH 6.4, in 10 ml. quantities placed in test tubes of 1.7 cm. internal diameter

EXPERIMENT NO.	NUMBER OF COLONIES PER BLOOD AGAR PLATE, 40 SQ. CM., SEEDED WITH 0.05 ML. OF 10^{-6} DILUTION OF THE GROWTHS AT DIFFERENT TEMPERATURES. 8 PARALLEL PLATES USED FOR COUNTING COLONIES FROM EACH GROWTH						
	23°C.	25°C.	27°C.	28°C.	29°C.	30°C.	31°C.
1	8	4	8	8	14	9	11
	6	8	6	15	14	17	10
	6	14	15	17	14	13	4
	9	9	18	20	15	24	10
	7	11	11	11	25	9	6
	9	9	13	8	14	5	9
	6	6	16	8	12	10	8
	10	6	14	11	18	8	4
2	8	18	13	9	18	7	12
	6	20	6	7	21	7	11
	4	13	18	12	17	6	5
	16	6	18	15	17	8	2
	11	7	13	13	10	6	7
	12	9	11	20	12		9
	6	6	16	14	16	13	14
	5		18	12	14	6	6
3	12	9	11	11	14	10	
	8	13	12	12	8	12	
	8	7	17	21	11	7	
	12	7	10	20		8	
	12	15	9	10		11	
	11	17	10	11		5	
	7	7	11	16	15	10	
	8	14	11	13	11	17	
4	14	5	13	10	14	16	10
	4	6	9	22	12	12	9
	12	11	15	7	9	11	11
	12	5	15	14	11	13	9
	15	13	12	16	16	15	9
	12	19	15	20	12	18	9
	10	19	10	15	14	12	8
	10	10	8	9		12	9
5		12	9	6	15	11	9
		14	11	10	13	4	9
		12	7	9	8	5	8
		12	13	10	12	10	16
		4	10	9	17	12	13
		11	8	12	10	15	7
		7	10	8		16	6
		9	9	7		6	10

TABLE 1—*Continued*

EXPERIMENT NO.	NUMBER OF COLONIES PER BLOOD AGAR PLATE, 40 SQ. CM., SEEDED WITH 0.05 ML. OF 10^{-6} DILUTION OF THE GROWTHS AT DIFFERENT TEMPERATURES. 8 PARALLEL PLATES USED FOR COUNTING COLONIES FROM EACH GROWTH						
	23°C.	25°C.	27°C.	28°C.	29°C.	30°C.	31°C.
6		5	11		5	9	15
		5	6		14	8	10
		3	12		11	7	6
		13	16		8	6	8
		8	12		19	9	10
		12	10			11	9
		5	15		15	6	10
		7	12		3	9	6
Mean number of colonies per plate ($\bar{x}$).	9.2500	9.8298	11.9375	12.4500	13.3658	10.2340	8.8500
Mean number of organisms per ml. (millions).....	185	197	239	249	267	204	177
Sum of squares of individual colony counts (Sx^2).....	3044	5436	7359	6954	7998	5715	3458
Square of the mean x number of readings ($n\bar{x}^2$).....	2738.00	4541.37	6840.19	6200.10	7324.43	4922.53	3132.90
Sum of squares of deviations from the mean ($S(x - \bar{x})^2$).....	306.00	894.63	518.81	753.90	673.57	792.47	325.10

TABLE 2

Analysis of data in table 1 to determine whether the observed differences in the growth at different temperatures are statistically significant

TEMPERATURES BETWEEN WHICH THE SIGNIFICANCE OF THE DIFFERENCES IN THE GROWTH IS TESTED	DIFFERENCES BETWEEN THE MEANS $\bar{x}_1 - \bar{x}_2$	SUM OF THE SQUARED DEVIATIONS FROM MEANS AT THE TWO TEMPERATURES $S(x - \bar{x})^2 + S(x' - \bar{x}')^2$	s	t	REMARKS
°C.					
29–30	3.1318	1466.04	4.1287	3.5495	Highly significant
29–28	0.9158	1427.47	4.2507	0.9694	Not significant
29–27	1.4283	1192.38	3.7020	1.8142	Not significant
27–25	2.1077	1413.44	3.8984	2.6347	Highly significant

employed. Broth with pH 7.2 was used for the determinations. The results are given in table 5. Table 5 also includes the mean values of growths at 25°C. to 37°C. based on counts for table 3.

TABLE 3

Colony counts per plate of 36-hour growths of Pasteurella pestis cultivated in broth, pH 7.2, in 10 ml. quantities placed in test tubes of 1.7 cm. internal diameter, according to temperature

TEMPERATURE	MEAN NUMBER OF COLONIES PER PLATE*
°C.	
25	13.8064
27	26.0323
28	24.8750
29	23.0937
30	23.1875
32	20.2812

* 4 sets of determinations were made for each temperature; 8 parallel plates were used for counting the organisms in each test tube, making 32 counts for each temperature.

TABLE 4

Analysis of data summarized in table 3 to determine whether the observed differences in the growth at different temperatures are statistically significant

TEMPERATURES BETWEEN WHICH THE SIGNIFICANCE OF THE DIFFERENCES OF GROWTH IS TESTED	DIFFERENCES BETWEEN THE MEANS $\bar{x}_1 - \bar{x}_2$	SUM OF THE SQUARED DEVIATIONS FROM THE MEANS AT THE TWO TEMPERATURES $S(x - \bar{x})^2 + S(x' - \bar{x}')^2$	s	t	REMARKS
°C.					
27–29	2.9386	1833.70	5.4827	2.1267	Significant
27–28	1.1573	1469.52	5.2656	0.8083	Not significant
27–25	12.2259	1653.78	5.2500	9.1681	Highly significant

TABLE 5

Growth in Pasteurella pestis in broth, pH 7.2, placed in 10 ml. quantities in test tubes of 1.7 cm. internal diameter

TEMPERATURES OF INCUBATION	INOCULUM PER ML. OF BROTH	NUMBER OF ORGANISMS PER ML. AFTER 36 HOURS' GROWTH	NUMBER OF ORGANISMS PER ML. AFTER 48 HOURS' GROWTH	NUMBER OF ORGANISMS PER ML. AFTER 96 HOURS' GROWTH	NUMBER OF ORGANISMS PER ML. AFTER 192 HOURS' GROWTH
°C.					
−2*	375,000		430,000	400,000	350,000
0	497,500		522,500	675,000	970,000
2	497,500		608,000	943,000	3,367,000
4	530,000		1,155,000		
25	544,000	276,000,000			
27	544,000	520,000,000			
28	544,000	497,000,000			
29	544,000	462,000,000			
30	544,000	464,000,000			
32	544,000	406,000,000			
37	544,000	98,050,000			
43	560,000		56,350,000	26,850,000	7,400,000
45	587,000		nil		

* For making colony counts of the growths incubated at −2°C., the cultures were quickly warmed by placing them in water bath at 37°C. It was found that if the cultures were allowed to attain room temperature (25°C.) slowly by mere exposure to room air, some of the organisms died and lower counts were obtained than were expected. For this reason it is possible that the counts we have given for growths at 0°, 2°, and 4°C. are lower than they should be, because we did not quickly warm our cultures.

DISCUSSION

The suitability of the technique and the medium (blood agar) employed for making counts was checked by the χ^2 test suggested by Fisher (1936). Detailed statistical analyses will be given in a subsequent paper. It would suffice for the present to state that the observed distribution of χ^2 values closely agrees with the expected distribution of χ^2 values for true samples of a Poisson series. Thus the counts, given in table 1 and averaged in table 3, show a high degree of accuracy.

To verify whether the apparent differences between the mean counts at different temperatures given in tables 1 and 3 are statistically significant, the method of analysis of variance was employed. Values of z for the two sets of experiments given in table 1 and summarized in table 3 are 1.045 and 1.450, respectively. These values indicate that the variations in the counts at different temperatures are due to the significant effect of temperature and not to random sampling errors. Having satisfied ourselves on this point, it only remained to determine the range of optimum temperature of growth. For this purpose the t test (Fisher, 1936) was employed, and the results of analysis are given in tables 2 and 4. The statistical analysis shows that in the broth with optimum hydrogen ion concentration for the growth of *P. pestis*, pH 7.2, maximal growth took place between 27°C. and 28°C. It is to be noted that the growth at this temperature was about five times the growth at 37°C. In the broth with a comparatively unfavourable hydrogen ion concentration, pH 6.4, the growth was less and the zone of optimum growth was slightly widened out to 27°C. to 29°C.

For determining the limiting temperatures of growth the cultures were incubated at −2°C., 0°C., 2°C., 4°C., 43°C. and 45°C. Since the growth at these temperatures was likely to be slow or none at all, cultures were incubated longer than the 36-hour period employed for the optimum growth determinations, for periods varying from 48 hours to 192 hours. If the observations had been limited to 36-hour periods of growth, 0°C. would have appeared as the lower limiting temperature, since no growth resulted at this temperature in even 48 hours.

CONCLUSION

In nutrient broth the optimum growth temperature for *Pasteurella pestis* was found to be 27°C. to 28°C. The growth at this temperature was about five times the growth at 37°C. The limiting growth temperatures were −2°C. and 45°C.

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