LIMITATION OF BACTERIAL GROWTH AT HIGHER TEMPERATURES[1]

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INTRODUCTION

The position of the thermophilic bacteria[2] in any adopted system of classification has long been a subject of considerable controversy. Some writers have considered the thermophiles as a distinct group while, according to others, they may be regarded as varieties of the common spore-forming soil and water organisms. The reports of previous investigators and our own study suggest a close relationship between them and the so-called subtilis group.

The known thermophilic bacteria are rods which are usually Gram-positive and vary considerably in size. They may occur singly, in pairs or in chains. Long filamentous forms are seen frequently. Their oxygen requirements extend over a wide range, some being aerobic, others facultative, and others anaerobic.

They are, with very few exceptions, non-pathogenic and vary to a considerable degree with respect to carbohydrate fermentation, gelatin liquefaction, nitrate reduction, action in milk, indol and hydrogen sulphide production, pigmentation, and growth in the various culture media. Gentian violet inhibits their development (Cameron, 1930). Shaw (1928) reported inhibition of supposedly Gram-negative thermophiles by crystal violet.

[1] This paper covers in part the thesis submitted to the Graduate School of Yale University by the senior author as part requirement for the degree of Doctor of Philosophy.

[2] The terms "thermophilic bacteria" and "thermophiles" are applied in this paper to bacteria having an optimum growth temperature of 55° or above. Ordinary thermoduric organisms as, for example, certain coccus forms occurring in milk, are not included here.

The literature on thermophiles is concerned mainly with four questions, namely (1) classification and taxonomy, (2) the demonstration of growth at high temperatures (55° and above) of known organisms, (3) acclimatization of non-thermophiles to higher growth temperatures, and *vice versa*, and (4) environmental and other conditions which favor resistance to heat.

An observation which is cited quite often is that of Dallinger (1887), who by gradual exposure to increasing temperatures over a period of seven years acclimated a certain flagellate to 70°C., which at the beginning of the investigation did not withstand a temperature of 23°C. Davenport and Castle (1896) succeeded in accustoming tadpoles to higher temperatures. Several apparently successful attempts at acclimatization have been made with known members of the subtilis group.

The influence of increased concentration of medium on resistance of bacteria to heat has received some attention. It has been suggested that such treatment may be instrumental in producing a cell with a low moisture content and consequently a higher thermal resistance.

Several theories have been advanced to explain the ability of protoplasm to resist temperatures generally considered as lethal, but, in so far as the present writers are aware, few extended efforts have been made to gain an actual insight into the mechanisms permitting continued functioning at these temperatures. No definite information concerning the nature of thermal resistance with respect to maximum temperature of active growth has come to the authors' attention.

Space does not permit of the inclusion here of an historical review of publications on the limitation of bacterial growth at higher temperatures. For such a review the reader is referred to the Ph.D. dissertation of the senior author deposited in the Yale University Library.

At the beginning of the present work chief interest was centered in the distribution of obligate thermophiles in nature, and in the conversion of non-thermophiles into strictly thermophilic forms. The procedures adopted were at first purely bacteriological but, as the investigation continued, interest in the mechanisms which

permit growth at higher temperatures became more and more intense. The apparently close relationship observed between thermophiles and the common spore-forming bacteria, the possible rôle of desiccation in the maintenance of life at high temperatures, and the suggestive recent literature bearing on oxidation-reduction aspects, led to an attack on the problem of growth at high temperatures by (1) continued acclimatization attempts with members of the subtilis group, (2) desiccation procedures, and (3) heat lability studies of certain enzymatic oxidation-reduction processes and their possible relation to high temperature tolerance and growth.

EXPERIMENTAL

Attempts were made to raise the maximum growth temperatures of certain well-known members of the subtilis group, to confirm, if possible the successful acclimatizations reported by other investigators.

Beef infusion broth and agar (2 per cent) adjusted to pH 7.0 were employed throughout this study. All cultures were transferred daily in the broth and on the slanted agar, and during the incubation were kept in light test tube racks, to permit of uniform heating. Temperature determinations were made with thermometers held in the racks. Thelco electric incubators were used for temperatures ranging from 37 to 60°C., and Freas electric and Leitz gas-heated for temperatures above 60°C. With all three types of incubators the thermometers kept inside registered a temperature approximately one degree lower than the regular thermometer projecting through the roof of the incubator. By keeping the inside thermometers in test tubes containing water, temporary fluctuations in the temperature readings were reduced to a minimum.

After establishing the maximum growth temperature for each organism, transfers were made daily at this temperature, and from time to time the temperature was raised 0.25 to 0.5 degree. Cultures died frequently during this treatment, necessitating a return to the original stock culture. This procedure was followed for about one year, the acclimatization process being controlled

occasionally by determining the maximum temperatures of continued growth (on two or more transfers) of second twenty-four-hour agar cultures grown at 37°C.

The strains that were employed in this study appear to have had fixed maximum growth temperatures; at least, they did not lend themselves for this type of experimentation in the period of time they were studied, as will be seen in table 1.

An almost negligible degree of acclimatization to higher temperatures was attained with *B. mycoides*, *B. prausnitzii*, *B. megatherium* D, and *B. megatherium* N. *B. subtilis* F, *B. mesentericus* F, *B. mesentericus* A, and *B. cereus* showed practically no ability to adapt themselves to temperatures of growth above their already established maximum.

TABLE 1

Showing maximum temperatures of growth of acclimatization and control cultures

ORGANISMS	ACCLIMATIZATION CULTURES		CONTROL CULTURES (ON AGAR)
	In broth	On agar	
B. subtilis F	56–59	59–60	59–60
B. vulgatus F	56	56	56
B. mesentericus F	51–52	53–54	53
B. mesentericus A	51–52	53–54	52–53
B. subtilis T	49–50	49–50	49–50
B. cereus	47–48	49	47–49
B. megatherium D	46	47	43–44
B. megatherium N	46	47	43–44
B. prausnitzii	45	45	43–44
B. mycoides	43–44	43–44	41–42

Note: These temperatures were approximately one degree lower than those registered by the regular incubator (top) thermometers.

Several of the strains revealed lower maximum temperatures of growth in nutrient broth than on agar medium. *B. subtilis* F varied considerably with respect to its highest growth temperature in broth. *B. subtilis* F and *B. vulgatus* F grew at 55°C., and *B. mesentericus* F and *B. mesentericus* A tolerated a maximum temperature only slightly less.

The relatively short period (one year) in which the adaptation

experiments were conducted, and the presumably harmful effects of temporary fluctuations in the temperatures of the incubators, do not permit of a definite conclusion of inadaptability of the organisms studied. The experiments indicate, however, that acclimatization of bacteria to higher growth temperatures is more difficult than one would be led to expect from the reports of other investigators.

The viscid nature of the growth produced on agar by one organism (*B. megatherium* N), which acquired the ability to grow from two to three degrees above the original maximum, and certain changes in the oxygen requirements accompanying rise in temperature, as indicated by the inferiority of broth tubes in the support of growth at high temperatures, stimulated the following studies on partial desiccation and on oxidation-reduction mechanisms as possible factors determining maximum growth temperatures.

DESICCATION EXPERIMENTS

The possibility of the viscid substance produced by *B. megatherium* N acting as a hygroscopic protector against excessive moisture suggested itself. Although *B. megatherium* D, which was acclimated to the same temperature as N, did not appear viscid on the same medium, the absence of a similar, but less extensive, mechanism or "hygroscopic mantle" need not be assumed. Buchanan (1909) cites references and presents data supporting the theory that bacterial slimes and gums are a result of swelling or solution of the cell wall or capsule.

Numerous attempts of the writers to raise the maximum growth temperatures of members of the subtilis group by cultivation in media of high osmotic pressure proved unsuccessful. For increasing the osmotic pressure, or rather decreasing the relative water content of the nutrient broth, 20 to 40 per cent peptone, 10 to 15 per cent sodium chloride, and 30 to 35 per cent lactose were added separately in the highest concentrations which supported growth. After continued cultivation in these different media the maximum temperatures of growth were determined in the media of lowered water content and in ordinary nutrient broth. Furthermore, cultures in media of high concentration

were allowed to evaporate almost to dryness at different temperatures, and their maximum growth temperatures determined.

The partially desiccated media not only failed to increase the heat tolerance, but were inferior to ordinary meat extract and beef infusion broths in their support of growth at the higher temperatures.

Finally, cells of *B. subtilis* F were also desiccated in washed, ignited sand, in a vacuum and in an atmosphere of carbon dioxide gas over sulphuric acid, for a period of six months. When inoculated into beef infusion broth, the cells failed to grow at temperatures above the original maximum.

OXIDATION-REDUCTION STUDIES

The unsuccessful attempts to raise the maximum growth temperatures appreciably by subjecting the organisms to gradually increasing temperatures and to desiccation led to a search for catalytic and fermentative mechanisms which may play an active rôle in the determination of the upper thermal range limits. The inferiority of broth to sloped agar in the support of bacterial growth at the higher temperatures indicated a dependence of growth at these temperatures upon a definite oxidation-reduction potential, and suggested the following study of bacterial growth in deep agar at various temperatures.

Deep infusion agar containing 1 per cent glucose was used throughout these experiments. The inoculations were made after cooling the melted agar to about 45°C. The cultures were incubated through wide ranges of temperature. In order to detect the effects of acclimatization to higher temperatures the experiments were controlled by the simultaneous use of second or third twenty-four-hour transfer cultures (37°C.) of the corresponding stock cultures of the respective organisms.

The results revealed differences in the temperature influence on the different species, and even strains. For example, *B. cereus*, *B. prausnitzii* and *B. subtilis* F showed an inability to grow in the depth of the agar tubes at the higher temperatures. *B. mycoides*, on the other hand, when subjected to its ordinary maximum growth temperature until on successive transfers

at this temperature no growth could be observed at or near the surface of the agar, retained its original property of growing in the depth of the tubes. *B. vulgatus* grew micro-aerophilically after continued cultivation in deep agar at its maximum growth temperature. *B. megatherium* N, after acclimatization to growth on the surface of agar at 47°C., exhibited microaerophilic properties when incubated at 37°C. in glucose infusion agar. The control (incubated at 37°C.) gave both surface and subsurface growths.

The group of thermophilic bacteria studied contained aerobic, facultative anaerobic and microaerophilic members. The microaerophiles produced surface growth when the culture tubes were kept in jars containing a solution of copper sulphate and covered with close-fitting glass plates to prevent excessive desiccation. After one hundred forty-four hours of incubation in this more or less closed system visible microaerophilic growth of the following organisms was observed at the temperatures indicated.

ORGANISMS	TEMPERATURES AT WHICH MICROAEROPHILIC GROWTH OCCURRED
	°C.
S I, S II, S III	44–45
S IV	47–50
1492 gp 100	37
1503 gp 100	44–45
29 and H37	44–45

Four of these thermophiles (S I, S II, S III and S IV) were obtained from Wisconsin University, 2 (1492 group 100, and 1503 group 100) from the National Canners Association Laboratory, and 2 (29 and H 37) were isolated by the writers, the former from human feces, and the latter from soil.

In interpreting the above observations, the influence of temperature upon the medium as well as upon the organism must be considered. By adding methylene blue to the tubes of beef extract agar in a final dilution of 1:100,000 and holding the tubes at 37°, 44° and 55°C., a difference in oxidation-reduction potential could be detected at the different temperatures. After twenty-

four hours the tubes placed at 37° and 44° remained colored throughout, whereas the one which was subjected to 55° was colored only for a distance of 3 cm. from the surface.

While this influence of temperature on the oxidation-reduction potential of the medium may be responsible for the lessened ability of the organisms to grow in the depths of the tubes at higher temperatures, the inability to grow aerobically or the taking on of a microaerophilic character on subjection to high temperatures indicates changes in the properties of the bacterium. The researches of McLeod and Gordon (1923) suggested that bacterial catalase may be involved in such changes. These investigators based their classification of bacteria on the formation of catalase and hydrogen peroxide, and on their sensitiveness to the peroxide. They postulated that anaerobes are incapable of aerobic growth on account of their extreme susceptibility to hydrogen peroxide, and because they are deficient in catalase.

The inhibition of aerobic growth of *B. mycoides*, and the microaerophilic growth induced by higher temperatures upon other organisms, pointed to a possible inactivation of catalase by temperatures above their maximum temperatures of growth.

The general acceptance of cellular oxidation as a catalyzing process, and the apparent provision of the various bacterial species with different respiratory mechanisms, suggested the advisability of studying certain heat-labile catalytic oxidation-reduction processes and their possible relation to the limitation of bacterial growth at higher temperatures.

Keilin (1928–29) demonstrated the important rôle of the so-called "indophenol"- or "paraphenylenediamine oxidase" in the cellular respiration of yeast. He found that all of the factors which inhibit the activity of paraphenylenediamine oxidase or destroy it completely affect the oxygen uptake of the cells in the same way. The oxidase was responsible for the oxidation of cytochrome, particularly its non-autoxidisable components, while dehydrogenases in the living cell activated organic substances, with the resultant reduction of the cytochrome. All factors which inhibited the activity of the dehydrogenase system of the cell, such as narcotics, heating at 52°C., and exposure to low tem-

peratures, delayed the reduction of oxidized cytochrome. Keilin therefore concluded that cytochrome acts as a carrier between two types of activating mechanisms of the cell: (1) the dehydrogenases which activate the hydrogen of organic molecules, and (2) the indophenol (or paraphenylenediamine) oxidase which activates oxygen.

The studies of Quastel and his co-workers on the activation of different substrates by resting bacteria tend to justify a search for growth-limiting factors among heat-labile catalytic oxidation-reduction systems. Quastel, Stephenson and Whetham (1925) were able to show that the capacity of certain bacterial species to grow under anaerobic conditions is closely related to their ability to activate hydrogen acceptors in the presence of hydrogen donators. They set up the three following conditions which to them seem necessary before growth may occur.

(1) The organism must be able to secure energy, either by anaerobic decomposition of the substrate (for example, glucose into lactic acid) or by the oxidation of the substrate.

(2) The organism must be able to activate the substrate so that the latter is capable of reaction (for example, oxidation or reduction which it does not necessarily undergo in the absence of bacteria).

(3) The products of oxidation (or decomposition) of the substrate, or the substrate itself, must be capable of entering into the synthetic operations of the organism.

Cook and Stephenson (1928) studied dehydrogenase activity under conditions in which molecular oxygen served as the hydrogen acceptor. They found *Bact. coli* capable of oxidizing a number of substrates. *Cl. sporogenes*, under the same conditions, was unable to activate any of the tested substances. These oxidation reactions were found not to depend on the presence of living cells.

Dehydrogenase activity in the presence of oxygen, with the possible formation of hydrogen peroxide, and the formation of this substance during autoxidative processes, suggest a possible functioning of peroxidase in cellular oxidation. Callow (1926) demonstrated the heat stability of bacterial peroxidase. Harrison (1929) concludes that the indophenol (or paraphenylenedi-

amine) reaction is no criterion for the presence of an activator of oxygen. He does not disprove the existence of an indophenol oxidase, but shows that the indophenol oxidation can be brought about without the agency of a specific oxidase. In the presence of peroxidase, paraphenylenediamine was oxidized to the di-imino compound by hydrogen peroxide formed during the aerobic oxidation of hypoxanthin by xanthine oxidase.

These researches pointed to important rôles played by oxidase, peroxidase, catalase and dehydrogenase in catalytic oxidation-reduction, and stimulated the carrying out of the following heat-lability studies of these enzymes with respect to the relation between such labilities and the maximum temperatures of growth.

The incompleteness of our knowledge of the rôle played by these heat-labile catalytic systems and the possible importance of these and perhaps other oxidative-reductive mechanisms is evident. By simulating as far as possible the conditions prevailing in the acclimatization attempts, and by employing organisms having widely different maximum temperatures of growth, the writers hoped to gain at least a partial insight into the mechanisms which are responsible for the inhibitory effects of higher temperatures on bacterial development. Their attempts have met with only partial success, as will be seen.

The heat lability studies were made with the members of the subtilis group which were used in the acclimatization experiments, and with certain obligate thermophiles. The lowest maximum temperature of growth of the organisms included in the list was that of *B. mycoides*, namely 41 to 42°C.; the highest was that of the thermophiles, 69 to 71°C.

METHODS

The bacterial suspensions used in the heat-lability tests were prepared by washing off twenty-four-hour (37°C.) growths of the organisms from beef-extract agar in Kolle flasks with 0.85 per cent saline solution. The routine of this procedure was always made as nearly uniform as possible.

Portions of the suspensions were heated for twenty to twenty-four hours through wide ranges of temperature and examined

for the presence of the following catalytic or enzymatic agents: "paraphenylenediamine-oxidase," peroxidase, catalase and "succino dehydrogenase." Tests were made also for hydrogen peroxide. The importance of making the different enzyme tests at as nearly the same time as possible became apparent quite soon. Also the necessity of detecting traces of hydrogen peroxide in the suspensions.

The test with paraphenylenediamine proved useful not only for the detection of oxidase, but also as an indicator of dehydrogenation activities; the secondary reduction of the oxidized paraphenylenediamine proved to be an adequate criterion of hydrogen activation, with most of the organisms. The inter-relationship of the activities of the different systems will be made evident in the experiments described below.

Oxidase

Paraphenylenediamine gives a red to brown color on oxidation in an alkaline medium. It has been found efficacious as a substitute for the "Nadi" reagent in the study of bacterial oxidative processes by Loele (1929), who used it in saline solution or distilled water without adjusting the H-ion concentration. Keilin (1928–29) found a neutralized 1 per cent solution of paraphenylenediaminehydrochloride, when added to a suspension of yeast buffered at pH 7.3, one of the best reagents for revealing the presence of a thermolabile oxidase in yeast. In preliminary experiments in which known oxidase-positive and oxidase-negative organisms were used, an acceleration of the autoxidation of the indicator to the purple compound in acid media, and the adequacy of a pH of 7.3 were demonstrated by the writers.

In each routine test for paraphenylenediamine-oxidase five different tubes of buffered bacterial suspension[3] were employed. The five tubes were treated as follows:

After each heating and cooling
Tube 1 remained untreated.
Tube 2 was heated for fifteen minutes in a boiling bath, and cooled.

[3] Two cubic centimeters of bacteria-KH_2PO_4-NaOH mixture to each tube.

Tube 3 received 0.1 cc. of N/50 KCN.
Tube 4 received 1 drop of 1:100 H_2O_2 solution.
Tube 5 received 3 drops of horse-radish peroxidase.[4]

To each tube was added 0.5 cc. of a freshly prepared 1.0 per cent solution of paraphenylenediamine in KH_2PO_4-NaOH buffer (pH 7.3). After shaking for seven or eight minutes color readings were made immediately and at convenient intervals. The shaking was not repeated after the first reading, in order to permit observations on dehydrogenase activities which were indicated by a secondary decoloration in the depth of the tubes. All of the heated portions of a given bacterial suspension were tested simultaneously; hence, for each organism subjected to heating at eight different temperatures a set of 40 tubes was required.

Tube 2 (heated at 100°C. for fifteen minutes) served as a control on auto-oxidation. The potassium cyanide in tube 3 inhibits oxidative processes, as comparison with no. 2 should reveal. Inhibition of oxidation by the cyanide is indicative of the presence of oxidase in the KCN-free suspensions. Tube 4 (receiving the H_2O_2) serves as a test for peroxidase; and tube 5 (containing the "horse-radish peroxidase") is included in the series to detect the presence of H_2O_2. The added peroxidase supplements any bacterial peroxidase that may already be present in the suspension.

Thus, a positive color test (red to brown) in tube 1 indicates the presence of the oxidase, providing that tube 2 reveals thermolability (negative color test) due to the fifteen minutes heating, and tube 3 reveals an inhibition (no color reaction) of the active principle by the potassium cyanide. Tubes 4 and 5 are employed for the detection of peroxidase and peroxide, respectively, and supplement the other tests made for these agents, as shown below.

Peroxidase

Peroxidase may be detected by mixing the bacterial suspension with a watery suspension of benzidine and various amounts of hydrogen peroxide. Benzidine blue is formed in the presence of peroxidase. A H-ion concentration of pH 5.0 appears to be most satisfactory.

[4] Water extract of horse-radish.

The tests for peroxidase were carried out by mixing five drops of the bacterial suspension on a white procelain plate,[5] with two drops of phthalate buffer (pH 5.0), one drop of a 0.5 per cent finely divided benzidine suspension (adjusted to pH 5.0) and one drop of hydrogen peroxide of varying dilutions. Six tests were made with each buffered mixture of benzidine and bacterial suspension. One received no H_2O_2, and the other five one drop of 1:50, 1:100, 1:1000, 1:10,000 and 1:100,000 dilutions of H_2O_2, respectively. Readings were usually made after the preparations had been allowed to dry overnight.

Catalase

This was detected by mixing two drops of the bacterial suspension with one drop of phosphate buffer (pH 7.3) and one drop of a 1:50 solution of H_2O_2, on a glass slide. Examination for the evolution of gas bubbles was made with the unaided eye and, when necessary, with the aid of the microscope.

Hydrogen peroxide

Three different tests were made for peroxide, as follows:

(1) Particles of titanium sulphate were added to several drops of the bacterial suspension. A yellow color indicates the presence of peroxide.

(2) A drop of a fresh mixture of dilute solutions of ferric chloride and potassium ferricyanide was added to a few drops of the bacterial suspension. In the presence of peroxide the ferric is reduced to ferrous iron, with the characteristic green to blue color production (Schönbein, 1860). The possible action of oxidizing and reducing agents other than peroxide detracts considerably from the reliability of this test in complex biological systems.

(3) This is the same as that employed in the peroxidase test. Four or five drops of bacterial suspension are mixed with two drops of buffer (pH 5.0), a drop of benzidine suspension and one or two drops of horse-radish extract (peroxidase). The formation of benzidine blue indicates the presence of peroxide. The reaction is retarded by reducing mechanisms.

[5] A glass plate resting on a white background may also be used.

The detection of peroxide in the presence of catalase is difficult. With inhibition of the catalase, and consequent accumulation of peroxide, an oxidation in the presence of heat-stable peroxidase may be effected. Such oxidation may mask the thermolability of the "oxidase."

"Succinodehydrogenase"

Methylene-blue reduction was studied under anaerobic conditions at pH 7.3, and in the presence of sodium succinate. Since the bacterial suspensions were not washed, hydrogen donators other than the succinate may have been activated by the bacterial dehydrogenases.

To 1.0 cc. of bacterial suspension 1.0 cc. of KH_2PO_4-NaOH buffer (pH 7.3), 0.2 cc. of a 5 per cent solution of sodium succinate, and 0.3 cc. of a 1:10,000 solution of methylene-blue were added. The buffer solution, succinate and methylene-blue were sterilized separately by autoclaving. Aseptic technique was employed throughout the experiments.

The tubes (12 by 100 mm.) in which the tests were made were fastened around the outside of a glass beaker by means of a rubber band and, after filling the beaker with cotton (white background) the whole was placed in a Novy jar, which was then evacuated with a Cenco hyvac pump and placed in the 37°C. incubator. Readings were made after fifteen minutes and at convenient intervals thereafter.

RESULTS

Proper interpretation of variations in the heat labilities of the "systems" under study, and of the interdependence of their activities, requires consideration and discussion of results obtained with each organism individually. Only those results are presented in full detail which were obtained with organisms that showed at least a reasonable constancy in the thermolabilities of their "systems." They serve to illustrate certain correlations between the thermolabilities of the "systems" and the growth-inhibiting temperatures of organisms which had widely different maximum temperatures of growth.

Experiment I

Heat lability studies were carried out simultaneously with the three following organisms: No. 1608, group 80 (a facultative thermophile which had a temperature limit of 65°C.), and *B. subtilis* F and *B. mycoides*, ordinary subtilis group members (the former having a maximum growth temperature of 59 to 60°C., and the latter of 41 to 42°C.).

The three different suspensions were prepared from agar cultures grown at 37°, and heated twenty to twenty-four hours at the following temperatures:

No. 1608 group 80, 37, 45, 49, 55, 59, 65–66 and 70°C.
B. subtilis F, 37, 45, 49, 55, 59, 62, 65–66 and 70°C.
B. mycoides, 37, 42, 45, 49, 55, 59, 65–66 and 70°C.

After subjection to these different temperatures, the various suspensions were tested for the presence of oxidase, peroxidase, catalase and peroxide, according to the methods described above.[6] The succinodehydrogenase lability tests were made in a separate experiment, but the results are included in tables 1A, 1B and 1C.

Table 1A presents the results obtained with organism No. 1608 group 80. It reveals some interesting relationships between the labilities of the enzymes and the maximum growth temperature.

At 65 to 66°C. (maximum growth temperature) the paraphenylenediamine oxidase begins to weaken, while at 70° C. it is almost completely destroyed. At 70° tube 4 in the "oxidase" series (receiving 1 drop of 1:100 H_2O_2) reveals a sudden increase in the intensity of the color produced, thereby indicating a destruction at 65 to 70° of the mechanisms inhibiting oxidation (catalase or other reducing systems). Tube 5 fails to show the presence of free H_2O_2 at 70°C.

The benzidine tests for peroxidase also reveal thermolability of an oxidase. The control tube (receiving no H_2O_2) exhibited good benzidine-blue production in all of the seven heated tubes but the last (70°C.), in which only a trace was observed. The higher

[6] In the oxidase test, the KCN control (tube 3) received two drops of M/1.000, instead of 0.1 cc. of M/50 KCN.

TABLE 1A

Organism No. 1608 group 80. Maximum temperature of growth, 65°C.

	HEATING TEMPERATURE																														
	37°C.					45°C.					49°C.					55°C.					59°C.					65-66°C.					70°C.				
Oxidase																																	
Tube number	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Reaction time:																																			
25 minutes	2	−	1	2	2	2	−	1	2	2	2	−	1	2	2	1	−	1	2	2	1+	−	1	1	1+	1	−	1	1	1	±	−	±	2	±
50 minutes	3	−	2	3	3	3	−	2	3	3	3	−	2	3	2	3	−	2	3	3	2+	−	2	2	2+	1	−	1	1	1	±	−	±	3	±
2½ hours	4	2	4	4	4	4	1	3	4	4	4	2	4	4	4	3	2	3	4	4	4		1	4	4	4	3	1	3	4	4	2	1	2	4	2
Peroxidase																																			
1:50 H_2O_2	2					2					2					2					2					2					1				
1:100 H_2O_2	2					2					2					2					2					2					1				
1:1,000 H_2O_2	3					3					3					3					3					2					2				
1:10T H_2O_2	3					3					3					3					3					3					1				
1:100T H_2O_2	3					3					3					3					3					3					1				
No H_2O_2	3					3					3					3					3					3					1				
Catalase																																			
	2					2					2					2					2					1					1				
Peroxide																																			
$Ti_2(SO_4)_3$	−					−					−					−					−					−					−				
$FeCl_3 + K_3FE(CN)_6$	−					−					−					−					−					−					−				

			Succinodehydrogenase				
Reaction time:							
20 minutes	4		4	3	—	—	—
40 minutes	4		4	4	—	—	—
2 hours	4		4	4	4	—	—
6 hours	4		4	4	4	—	—
18 hours	4		4	4	4	—	—

Key: For oxidase, peroxidase, catalase and peroxide: 4, very good; 3, good; 2, distinct; 1, trace; ±, faint trace; —, negative. For succinodehydrogenase: 4, complete decoloration; 3, almost complete decoloration; 2, distinct decoloration; —, no decoloration.

TABLE 1B

B. subtilis F. Maximum temperature of growth, 59–60°C.

	HEATING TEMPERATURE																																					
	37°C.					45°C.					49°C.					55°C.					59°C.					62°C.					65–66°C.					70°C.				
Oxidase																																						
Tube number	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Reaction time:																																						
12 minutes	±	−	+	1	±	±	−	−	1	±	−	−	−	±	−	−	−	−	1	±	−	−	−	1	−	−	−	−	1	−	−	−	−	1	−	−	−	−	1	−
40 minutes	2	−	±	2	1	1	−	±	2	1	1	−	−	1	±	1	−	−	2	1	−	−	−	3	−	−	−	−	3	−	−	−	−	3	−	−	−	−	3	−
2½ hours	4	±	3	4	3	3	±	2	3	2	2	±	1	3	2	2	±	1	4	3	1	±	±	5	1	1	±	±	5	1	±	±	±	5	±	±	±	±	5	±
Peroxidase																																						
1:50 H_2O_2	±					±					±					2					2					2					2					2				
1:100 H_2O_2	±					±					±					2					2					2					2					2				
1:1,000 H_2O_2	1					1					1					2					2					2					2					2				
1:10T H_2O_2	1					1					1					±					±					−					−					−				
1:100T H_2O_2	1					±					±					±					−					−					−					−				
No H_2O_2	1					±					±					±					−					−					−					−				
Catalase																																						
	3					3					3					2					2					±					±					±				
Peroxide																																						
$Ti_2(SO_4)_3$	1					−					−					−					−					−					−					−				
Succinodehydrogenase																																						
Reaction time:																																						
15 minutes	4										3					−					4										4					4				
40 minutes	4										4					3					4										4					4				
2 hours	4										4					4					4										4					4				

TABLE 1C

B. mycoides. Maximum temperature of growth, 41–42°C.

	HEATING TEMPERATURE																																					
	37°C.					42°C.					45°C.					49°C.					55°C.					59°C.					65–66°C.					70°C.				
Oxidase																																						
Tube number.....	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Reaction time:																																						
15 minutes....	3	–	1	2	3	2	–	1	2	3	2	–	1	2	2	1	–	–	2	1	–	–	–	1	±	–	–	–	1	±	–	–	–	1	±	–	–	–	1	±
40 minutes....	3	–	2	3	3	3	–	2	3	3	3	–	1	2	2	1	–	–	2	1	–	–	–	2	±	–	–	–	2	±	–	–	–	2	±	–	–	–	2	±
2½ hours.....	3	±	3	3	3	(2)	±	3	3	3	3	±	3	3	4	3	±	1	3	3	±	±	±	3	1	±	±	±	3	1	±	±	±	3	1	±	±	±	3	1
Peroxidase																																						
1:50 H_2O_2.....	2					2					2					2					1					1					1					1				
1:100 H_2O_2.....	2					2					2					2					1					1					1					1				
1:1,000 H_2O_2.....	1					1					1					1					1					1					1					1				
1:10T H_2O_2.....	–					–					–					–					–					–					–					–				
1:100T H_2O_2.....	–					–					–					–					–					–					–					–				
No H_2O_2.....	–					–					–					–					–					–					–					–				
Catalase																																						
	2					2					–					–					–					–					–					–				
Peroxide																																						
$Ti_2(SO_4)_3$.....	±					–					–					–					–					–					–					–				
Succinodehydrogenase																																							
Reaction time:																																						
15 minutes.....	1										–					–					–																			
1 hour.....	4										–					–					–																			
5 hours.....	4										1					–					–																			

Parentheses indicate decoloration in the depths.

concentrations of peroxide tended to inhibit the oxidation. Although the activity and nature of the peroxidase are not revealed in this test, it was unquestionably present as a heat-stable catalyzer, as was evidenced by the higher degree of oxidation of the paraphenylenediamine at 70°C. in tube 4 of the "oxidase" set-up.

Catalase activity is distinctly lessened within the 59 to 65° range, while in the same range the succinodehydrogenase is apparently destroyed.

The presence of peroxide was not revealed in the suspensions by the titanium sulphate or the $FeCl_3 - K_3Fe(CN)_6$. However, minute traces of H_2O_2 may not have been detected by these reagents, and the presence of an active catalase may also account for the negative results.

We may sum up the results presented in table 1A by saying that there is apparently a correlation between the temperature which inhibits the activities of oxidase, catalase and succinodehydrogenase, on the one hand, and that marking the maximum growth temperature, on the other.

The results obtained with *B. subtilis* F are presented in table 1B. Here the relationships between the maximum temperature of growth and thermolabilities of the enzymes studied are not all as clear-cut as those obtained with No. 1608 group 80.

The oxidase, which was apparently weak, was detected in the forty minutes reading, which reveals an inhibition of this enzyme between 55° and 59°C. At the end of two and one-half hours a total inactivation was observed between 62° and 65°C.

Tube 4 in the oxidase set-up indicated, by the greater degree of oxidation beginning at 55 to 59°, an inhibition of catalase or other reducing mechanism within this range. Tube 5 indicated the absence of peroxide at temperatures above 55°. The positive test for peroxide with titanium sulphate in the suspension heated at 37° indicates that the peroxide may have played a rôle in the oxidase tests. Harrison (1929) has shown that the paraphenylenediamine oxidation is not necessarily proof of "oxidase" activity. Furthermore, *B. subtilis* F was found to be an active producer of peroxide.

The peroxidase tests demonstrated the thermostability of this

catalytic agent, and when considered together with the catalase tests, reveal the rôle of catalase in the inhibition of the peroxidase reaction. With partial inhibition of the catalase at 49 to 55°C., peroxidase activity becomes evident. The control tube (without added H_2O_2) indicates the functioning of an oxidase having a thermolability close to the maximum temperature of growth. This heat lability and the negative results obtained with the higher dilutions of H_2O_2 with suspensions heated above 59° indicate that we are dealing with a direct aerobic oxidase, and not a combination of heat-stable peroxidase and H_2O_2. However, the possibility of the functioning of a very active thermolabile peroxidase must be considered.

The catalase of *B. subtilis* F was almost completely inactivated between 59 and 62°.

The succinodehydrogenase of this organism is apparently heat-stable. However, this test was made apart from the others, a bacterial suspension considerably heavier than the others being used. Whether attempts to correlate under anaerobic conditions such activation with maximum growth temperature of an organism which grows aerobically are justified may be questioned. However, if such active dehydrogenation is obtained with oxygen as the hydrogen acceptor, the consequent formation of peroxide and dependence upon the functioning of the catalase becomes evident. The presence of active oxygen may be necessary for the oxidation of the succinate under aerobic conditions. If it is, the oxidase assumes an important rôle as the activator of this oxygen. A dependence of ability to grow on the presence of a suitable hydrogen acceptor is indicated in the results obtained on growing *B. subtilis* F in deep agar at various temperatures. It was incapable of growth in the depth of the medium at the higher temperatures. This may have been due to decreased oxygen potential in the depth when the medium was subjected to the higher temperatures.

The inhibiting action of higher temperatures upon growth in deep agar, and the inhibiting or inactivating effects upon the oxidase and catalase of this organism indicate a dependence of growth upon the completion of the respiratory processes, and tend to

justify the search for growth-limiting factors in the field of heat-labile catalytic oxidation-reduction systems.

In suspensions of *B. mycoides* (table 1C) catalase and succinodehydrogenase were apparently inactivated at the maximum growth temperature. Oxidase and peroxidase appeared to be heat-labile, but the lability of the latter was not complete and both were inhibited by temperatures above the maximum temperature of growth. The oxidase is inhibited between 45° and 49°, and inactivated between 49 and 55°C. However, upon repeating the experiment, an inhibition of the oxidase was evident at temperatures nearer the maximum growth temperature (41 to 42°C.)

The peroxidase was inhibited between 49° and 55°, but was not completely inactivated at any of the temperatures. This partial lability of the peroxidase was demonstrated in both the paraphenylenediamine oxidase test (tube 4) and the benzidine test.

The possible presence of peroxide, as indicated by tube 5 of the oxidase set-up and by the titanium sulphate test, suggests that the apparent stability of the oxidase at temperatures above the maximum temperature of growth is due to oxidation of the paraphenylenediamine by the peroxidase-H_2O_2 system at these temperatures.

The catalase lability at a temperature between 42 and 45°C. is in accord with the results obtained in the "deep agar" experiments with acclimated *B. mycoides*, in which subjection of the organism to temperatures slightly above the normal maximum temperature of growth resulted in an inability to grow aerobically, but with retention of ability to grow in the depths. The loss of ability to grow aerobically may have been due to the inactivation of catalase and consequent accumulation of peroxide. While this inactivation may account for the failure to grow aerobically, inability to grow anaerobically may possibly be due to the inactivation of the dehydrogenase or of the activators of certain hydrogen acceptors at the higher temperatures.

In view of the variation in the temperatures inhibiting or inactivating the catalase of *B. mycoides*, a definite conclusion regarding the rôle of this enzyme is not yet possible. The experiments were

carried out with quite turbid suspensions. However, in another experiment a considerably heavier suspension was used, and this showed a distinct catalase activity at 50°C. That this variation may have been due to the difference in turbidity of the suspensions employed, and perhaps to the presence of small clumps of bacteria, is indicated by similar results obtained with other organisms.

Experiment II

In this experiment three different organisms were employed, *B. mesentericus* A, *B. subtilis* T, and *B. cereus* H. The results are summarized in tables 2A, 2B and 2C, respectively.

Bacillus mesentericus A (2A). This organism, like the *B. vulgatus* strain studied, showed very little general correlation between the temperature at which growth is inhibited and the temperatures retarding the activity of the oxidation-reduction systems investigated.

Strong direct oxidase action was demonstrated in both the p-phenylenediamine and benzidine tests. In both instances inhibition begins near the temperature above which the organism is unable to grow (52 to 53°C). The correlation is more nearly complete with the benzidine than with p-phenylenediamine. The latter reagent showed inactivation to be incomplete even at 70°C. Secondary reduction of the p-phenylenediamine is evident in all suspensions except those which were heated at 65 and 70°C.

The benzidine oxidase test and the $FeCl_3 + K_3Fe(CN)_6$ test for peroxide are both more pronounced below than above 52 to 53°C. The titanium sulphate tests were negative. It is possible that minute traces of peroxide formed as a result of continued respiratory activity in the suspensions heated at or below 52 to 53° may account for the stronger oxidase activity at or below this temperature. Addition of dilutions of H_2O_2 stronger than 1:100,000 seemed to inhibit the oxidation of benzidine.

Except for the partial inhibition of the succinodehydrogenase and oxidase of *B. mesentericus* A, the thermolabilities of the systems studied do not coincide with the maximum temperature of growth (52 to 53°C.).

TABLE 2A

B. mesentericus A. Maximum temperature of growth, 52–53°C.

	HEATING TEMPERATURE																																			
	37°C.					45°C.					49°C.					52–53°C.					55°C.					59°C.					65°C.					70°C.				
Oxidase																																						
Tube number.....	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
10 minutes.....	3	−	1	3	2	3	−	1	3	2	3	−	+	3	3	3	−	±	2	2	2	−	±	2	1	2	−	±	2	1	1	−	−	1	±	±	−	−	±	−
30 minutes.....	2	−	2	2	2	2	−	1	2	2	2	−	1	2	2	2	−	1	2	2	2	−	1	2	2	2	−	1	2	2	2	−	±	2	1	1	−	−	1	±
$4\frac{1}{2}$ hours.....	(2)	3	(2)	2	(2)	(2)	3	(2)	(2)	(1)	(2)	3	(3)	2	(1)	(2)	2+	2	(2)	(1)	(2)	3	2	2	(1)	(1)	3	(2)	2	(1)	2	2	3	2	3	3	1	1	3	2
Peroxidase																																								
1:50 H_2O_2.....	2					2					2					2					2					2					2					2				
1:100 H_2O_2.....	2					2					2					2					2					2					1					1				
1:1,000 H_2O_2....	2					3					3					2					2					2					1					1				
1:10T H_2O_2.....	3					3					3					2					2					2					1					±				
1:100T H_2O_2....	3					3					3					3					3					2					1					−				
No H_2O_2.....	3					3					3					2–3					2					1					1					−				
Catalase																																								
	2					2					2					2					2					2					2					2				
Peroxide																																								
$Ti_2(SO_4)_3$.....	−					−					−					−					−					−					−					−				
$FeCl_3$ + $K_3Fe(CN)_6$.....	2					2					2					1					1					1					1					1				

Succinodehydrogenase

15 minutes.....	3		4	—	—	—	—	—
40 minutes	3		4	3	—	—	—	—
2 hours.....	4		4	4	3	3	—	—
4 hours.....	4		4	4	3	4	—	—
7½ hours.....	4		4	4	4	4	3	—
20 hours.....	4		4	4	4	4	3	2

Parentheses indicate decoloration in the depths—secondary reduction.

TABLE 2B

B. subtilis Tp. Maximum temperature of growth, 49–50°C.

	HEATING TEMPERATURE																																			
	37°C.					45°C.					49°C.					52 5°C.					55°C.					59°C.					65°C.					70°C.				
Oxidase																																						
Tube number	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
20–35 minutes	–	–	–	1	–	–	–	–	1	–	–	–	–	1	–	–	–	–	1	–	–	–	–	1	–	–	–	–	1	–	–	–	–	1	–	–	–	–	1	–
4½ hours	1	1	1	3	2	1	1	1	3	2	1	1	1	3	2	1	1	1	3	2	1	1	1	3	2	1	1	1	3	2	1	1	1	3	2	1	1	1	3	2
Peroxidase																																								
1:50 H_2O_2	1					1					1					1					1					1					1					1				
1:100 H_2O_2	±					±					±					±					±					±					±					±				
1:1,000 H_2O_2	–					–					–					–					–					–					–					–				
1:10T H_2O_2	–					–					–					–					–					–					–					–				
1:100T H_2O_2	–					–					–					–					–					–					–					–				
No H_2O_2	–					–					–					–					–					–					–					–				
Catalase																																								
	3					2					1					1					–					–					–					–				
Peroxide																																								
$Ti_2(SO_4)_3$	–					–					–					–					–					–					–					–				
Benzidine + HR	–					–					–					–					–					–					–					–				
$FeCl_3 + K_3Fe(CN)_6$	–					–					–					–					–					–					–					–				
Succinodehydrogenase																																								
15 minutes	4					4					–					–					–					–					–					–				
2 hours	4					4					–					–					–					–					–					–				
4 hours	4					4					3					–					–					–					–					–				
7½ hours	4					4					4					–					–					–					–					–				
20 hours	4					4					4					–					–					–					–					–				

B. subtilis T (2B). This organism exhibited no oxidase, and a peroxidase action so weak that thermolability of these agents could not be demonstrated.

Aside from a slightly positive reaction in tube 5 of the oxidase set-up (four and one-half hours reading), the tests for peroxide were negative.

Catalase and succinodehydrogenase were readily demonstrable. Both were inactivated at temperatures approximating the maximum growth-temperature (49 to 50°C.).

Bacillus cereus H (2C). This organism exhibited no clear-cut relation between the temperatures limiting its growth and those inhibiting the activity of oxidase and peroxidase. This lack of correlation seems to have been due, however, to the concomitant variation, with rise of temperature, in the activities of the catalase and reducing mechanisms, and to the presence of peroxide. Thus, failure to demonstrate lability of the oxidase at 49°C. may be due to the increase in peroxide concentration at the higher temperatures (as indicated by the $FeCl_3$ + $K_3Fe(CN)_6$ test) and to the inhibition or inactivation of the catalase and reducing mechanisms. The peroxidase tests with benzidine tended to confirm this supposition, in that they exhibited a stronger reaction at 55° and 59° than at 49°C. Tube 5 of the oxidase set gave a stronger reaction than tube 1, indicating the presence of H_2O_2 at these temperatures. The results obtained with tube 4 (receiving one drop of 1:100 H_2O_2) indicate an inhibition of either oxidase or peroxidase. The tube exhibited a weaker reaction at 52.5°C. than below this point.

Catalase activity, although retarded at 45 to 49°, is completely inactivated only at 55 to 59°C. However, when the test was repeated with more homogeneous suspensions (fewer and smaller clumps of bacteria) the following results as to catalase lability were obtained:

Temperature, °C	37	45	47	49	55	59	65
Catalase activity	2	2	—	—	—	—	—

Titanium sulphate gave a faintly positive test for peroxide in all suspensions. The benzidine-horseradish-peroxidase test failed

TABLE 2C

B. cereus H. Maximum temperature of growth, 47–49°C.

	HEATING TEMPERATURE																																					
	37°C.					45°C.					49°C.					52.5°C.					55°C.					59°C.					65°C.					70°C.				
Oxidase																																						
Tube number	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
25 minutes	3	–	–	3	3	2	–	–	2	2	2	–	–	2	2	2	–	–	1	3	2	–	–	1	3	1	–	–	1	2	–	–	–	1	±	–	–	–	1	±
35 minutes	1	–	–	2	(1)	1	–	–	1	(1)	(1)	–	–	1	(1)	(1)	–	–	1	1	1	–	–	2	2	2	–	–	1	2	–	–	–	1	±	–	–	–	1	±
4½ hours	(2)	2	2	3	(2)	(2)	2	2	3	2	(2)	2	2	3	2	(1)	2	2	3	2	(2)	2	2	3	2	2	2	2	3	2	2	2	2	2	2	2	2	2	3	3
Peroxidase																																						
1:50 H_2O_2	±					1					1					1					2					2					2					2				
1:100 H_2O_2	±					±					±					±					1					1					1					1				
1:1,000 H_2O_2	–					–					–					–					–					±					±					±				
1:10T H_2O_2	–					–					–					–					–					–					–					–				
1:100T H_2O_2	–					–					–					–					–					–					–					–				
No H_2O_2	–					–					–					–					–					–					–					–				
Catalase																																						
	2					2					1					1					1					±					–					–				
Peroxide																																						
$Ti_2(SO_4)_3$	±					±					±					±					±					±					±					±				
Benzidine + HR	–					–					–					–					–					–					–					–				
$FeCl_3 + K_3Fe(CN)_6$	3					2					2					3					3					3					3					1				

Succinodehydrogenase

15 minutes	4	—	—	—	—	—	—	
40 minutes	4	3	—	—	—	—	—	
2 hours	4	4	—	—	—	—	—	
4 hours	4	4	3	3	—	—	—	
7½ hours	4	4	4	4	—	—	—	
20 hours	4	4	4	4	—	—	—	

Parentheses indicate decoloration in the depth—secondary reduction.

to reveal the presence of peroxide, while the $FeCl_3 - K_3Fe(CN)_6$ test gave a distinct reaction with all but the 70° suspension.

Succinodehydrogenase was apparently inactivated between 49° and 55°C. Slight reduction in the depths of the paraphenylenediamine tube was evident in the 52.5° suspension, and distinct reduction in the 55° suspension. The ability to dehydrogenate at temperatures above 49° indicates that the peroxide responsible for the positive oxidase tests at these temperatures was produced as a result of continued respiration while the organism was subjected to these temperatures. The consequent accumulation of hydrogen peroxide in the absence of active catalase at these temperatures may possibly be the mechanism responsible for the inhibition of growth by the higher temperatures. However, the degree of secondary reduction of the oxidized p-phenylenediamine was less above 49°C. than below.

Thus, the thermolabilities of the catalase and succinodehydrogenase of *B. cereus* distinctly approximate its maximum temperature of growth. The results obtained with oxidase, although indicating a similar relationship, are complicated by other factors. The presence of a thermostable peroxidase is demonstrable.

It should be interesting to note that, while *B. mesentericus* A gave the stronger tests for peroxide at temperatures below its maximum growth temperature, *B. cereus* was less active in this respect at 45° and 49°, than at 52.5°, 59° and 65°C. This difference was due apparently to a difference in the thermolabilities of their respective catalases. The peroxide already present when the suspensions were prepared could be destroyed by the catalase of *B. mesentericus* at all of the temperatures, whereas that of *B. cereus* was considerably inhibited above 49°C. At temperatures below its maximum temperature of growth *B. mesentericus*, continuing to respire, was able to replace some of this peroxide. That such respiratory activity may account for the presence of the peroxide is indicated in the stronger test for this agent obtained with the 37°C. suspension of *B. cereus* than with the 45° suspension.

Experiment III. Thermolability study of suspensions of an obligate thermophile grown at its minimum and maximum temperatures

Two suspensions of an obligate thermophile (No. 59) were prepared, one (*a*) from an agar culture grown at 45° (its minimum growth temperature) and the other (*b*) from a culture incubated at 65°C. (its maximum). These suspensions were heated and their enzymatic activities studied simultaneously. The results are summarized in table 3.

The p-phenylenediamine oxidase of suspension B, although not as strong as A, seemed slightly more resistant to heat. This difference may be due to the greater activity of the peroxidase of B, rather than to any differences in peroxide content or in reducing activities. A comparison of the results obtained in the benzidine test with and without horse-radish peroxidase makes evident the importance of the rôle of peroxidase in such oxidations. In fact, the weakness of the oxidase in suspension B, together with the gradual decrease in its activity with increased temperature and a concomitant decrease in peroxide content, indicates a peroxidase and not an oxidase activity. Suspension A, furthermore, reveals a destruction of the oxidase between 55° and 65°C.

Tube 5 (containing horse-radish peroxidase) failed to reveal peroxide, although this substance was present in amounts detectable by the titanium sulphate and benzidine-horseradish-extract tests.

The peroxidase of suspension B is evidently more resistant to heat than that of A. Slight discrepancies obtained with the higher dilutions of H_2O_2 are probably due to the peroxide present in the tubes of suspension A that were heated at 65°, 70° and 75°C.

The catalase of both A and B suspensions was inactivated at approximately 65°. However, distinct catalase action in B could be demonstrated only when the suspension was considerably heavier than that of A. Aside from indicating a weakening of catalase activity as a result of cultivation at the maximum temperature of growth, this observation makes evident the importance of the rôle played by the turbidity (number of cells) of the bacterial suspensions in the catalase determinations.

TABLE 3

No. 59. Maximum temperature of growth, 65°C.

	SUSPENSION A (GROWN AT 45°C.)																					SUSPENSION B (GROWN AT 63–65°C.)																					
	Temperature																																											
	37°C.					55°C.					65°C.					70°C.					75°C.		37°C.					55°C.					65°C.					70°C.					75°C.			
Oxidase																																												
Tube Number..	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
15 minutes.....	3	–	–	3	2	2	–	–	2	2	–	–	–	±	–	–	–	–	±	–	–	–	–	±	–	1	–	–	±	1	1	–	–	±	1	±	–	–	±	±	±	–	–	±	±	–	–	–	–
25 minutes.....	2	–	–	3	1	1	–	–	1	1	–	–	–	1	–	–	–	–	1	–	–	–	–	±	–	1	–	–	1	1	1	–	–	1	1	1	±	–	–	±	±	–	–	–	–	–
65–70 minutes...	(2)	–	–	(2)	(2)	(1)	–	–	(2)	(1)	1	–	–	2	1	±	–	–	3	±	–	–	–	3	–	(±)	–	–	1	±	±	–	–	1	(±)	1	–	–	2	1	1	–	–	1	1	–	–	1	–
2 hours.....	2	±	±	3	3	1	±	±	3	1	2	±	±	3	2	1	±	±	3	1	±	±	±	4	±	1	–	–	2	1	1	–	–	2	1	1	–	–	3	1	2	–	–	3	2	–	–	–	3	±
Peroxidase																																												
1:50 H_2O_2.....	2					2					1					1					1		2					2					2					2					2			
1:100 H_2O_2.....	2					2					1					1					1		2					2					2					2					2			
1:1,000 H_2O_2.....	2					1					1					1					1		2					2					2					2					2			
1:10T H_2O_2.....	±					±					1					1					1		2					1					1					1					–			
1:100T H_2O_2.....	±					1					±					±					1		1					1					–					–					–			
No H_2O_2.....	±					±					–					±					±		±					±					–					–					–			
Catalase																																												
	2					2					±					±					–		–					–					–					–					–			
																							*2					*2					*±					*–					*–			
Peroxide																																												
$Ti_2(SO_4)_3$.....	–					–					1					1					1		1					1					±					±					–			
Benzidine + HR.....	±					±					3					3					3		2					1					±					–					–			

Succinodehydrogenase

35 minutes.....	4	—	—	—	—	—	—	—	—	—	—	—
65 minutes.....	4	4	—	—	—	—	—	—	—	—	—	—
1½ hours.....	4	4	—	—	—	—	4	—	—	—	—	—
2½ hours.....	4	4	—	—	—	—	4	4	—	—	—	—
3½ hours.....	4	4	3	—	—	—	4	4	—	3	—	—
4½ hours.....	4	4	3	3	—	—	4	4	3		—	—

* Results obtained when a much heavier suspension was used.
Parentheses indicate decoloration in the depths.

The tests for peroxide are worthy of special note. The tubes of suspension A heated at and above 65° gave distinct reactions for peroxide, while the tubes exposed to temperatures below 65° gave practically no indication of its presence, when tested with titanium sulphate and with the benzidine-horseradish extract. Suspension B, on the other hand, revealed a decrease in the amount of peroxide in tubes heated at and above 65°. Here again must be taken into consideration the activity of the catalase and the continued respiration of the bacterial cells. The catalase of suspension A was much more active than that of B. The possibility of respiration with production of peroxide at temperatures below 65° is supported by the evidence of dehydrogenase activity at these temperatures. It seems, therefore, that at temperatures below 65° the differences in the reactions obtained for peroxide are due primarily to differences in catalase activity. The results obtained at temperatures above 65° may be attributed to differences in the amount of peroxide present before the heating. The inhibition of dehydrogenase activity at 65° (as shown by the secondary decolorization of p-phenylenediamine and by the tests with succinate) indicates a smaller amount of peroxide in suspension B than in A.

These observations are suggestive, in view of the theory of McLeod and Gordon according to which ability to grow aerobically is a function of catalase and peroxide production, and degree of susceptibility of the organism to peroxide. Eckford (1927) found obligate thermophiles to be incapable of catalase activity until they were set in a water bath at 45°C. The present experiments with thermophile No. 59 grown at 63 to 65°C. indicate a weakening of the catalase activity in organisms grown at higher temperatures when this activity is tested at a lower temperature. They indicate also a continued production of peroxide at the lower temperature. May not such peroxide production, together with retarded catalase activity, play an important rôle in the inhibition of the growth of obligate thermophiles at the lower temperatures? Such a possibility is supported further by the microaerophilic growth obtained with nine obligate thermophiles upon incubating them for 144 hours in deep glucose infusion agar at the lower temperatures (see previous statements).

The tests for succinodehydrogenase indicated an inhibition or partial inactivation of this enzyme at 65°C.

We may conclude from the results obtained with suspension A (cells grown at 45°), that organism No. 59 exhibits a clear-cut inhibition of three of the four studied systems at the temperature marking its upper limit of growth (65°C.). The partial inhibition of peroxidase seems to be due to the partial inactivation of the oxidase between 55° and 65°. This is indicated by the results obtained in the control suspension with the benzidine test. Here a faint oxidase reaction occurred in the control tubes heated at 37°, 55°, 70° and 75°C., but none in the tube heated at 65°, although at the last-named temperature distinct amounts of peroxide remained active. The reappearance of the positive test at 70° and 75° is not due to an oxidase, but to inactivation of the reducing mechanisms and to the action of the peroxidase upon traces of peroxide present. The inferiority of suspension B in oxidase activity also indicates such inhibition of the oxidase at 65°C.

Experiment IV. Thermolability study of an obligate thermophile cultivated at a temperature slightly above its minimum growth temperature

This organism (SIV) had a maximum temperature of growth of about 70°C. Although it grew faintly at 45°C. (minimum temperature), it was grown at 49° in order to obtain sufficient material during twenty-four hours' incubation for the different tests. The results are presented in table 4.

The p-phenylenediamine oxidase set-up revealed an inhibition of the oxidase between 55° and 65°, and complete inactivation between 70 and 75°. The same material showed inactivation of the reducing mechanisms between 70° and 75°, and the presence of a heat-stable peroxidase (tube 4 of 75° suspension). Tube 5 gave negative tests for free peroxide.

Heat-stable "benzidine-peroxidase" was demonstrated in the suspensions prepared from cultures that were heated at 65 to 75°C. By the same test the 37° and 55° tubes gave a negative reaction. This indicated the presence of heat-labile mechanisms which inhibited the oxidation.

TABLE 4

SIV. Maximum temperature of growth, 70°C.

	HEATING TEMPERATURE																								
	37°C.					55°C.					65°C.					70°C.					75°C.				
Oxidase																									
Tube number	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
15 minutes	3	−	−	3	3	2	−	−	2	2	1	−	−	1	1	1	−	−	1	1	−	−	−	±	−
30 minutes	(±)	−	−	(±)	(±)	(±)	−	−	(±)	(±)	1	−	−	1	1	1	−	−	1	1	−	−	−	±	−
75 minutes	(2)	−	−	(2)	(1)	(2)	−	−	(2)	(1)	(2)	−	−	(1)	±	(1)	−	−	(1)	(±)	−	−	−	3	−
2 hours	1	±	±	1	2	1	±	±	1	1	1	±	±	2	1	1	±	±	1	1	±	±	±	3	±
Peroxidase																									
1:50 H_2O_2			−					−					2					2					2		
1:100 H_2O_2			−					−					2					1					2		
1:1,000 H_2O_2			−					−					−					−					1		
1:10T H_2O_2			−					−					−					−					−		
1:100T H_2O_2			−					−					−					−					−		
No H_2O_2			−					−					−					−					−		
Catalase																									
			4					4					2					2					−		
Peroxide																									
$Ti_2(SO_4)_3$			−					−					−					−					−		
Benzidine + HR			−					−					−					−					−		

Succinodehydrogenase

35 minutes	4	4	–	–	–
65 minutes	4	4	–	–	–
1½ hours	4	4	–	–	–
2½ hours	4	4	4	4	–
3½ hours	4	4	4	4	–

Parentheses indicate decoloration in the depths.

The inhibiting mechanisms appear to have been catalase and dehydrogenases. The activities of both of these are decidedly inhibited between 55° and 65°C. Complete inactivation occurs only between 70° and 75°, however.

The tests for peroxide gave negative results. However, in the presence of very active catalase the detection of free peroxide is hardly to be expected.

We have, then, with this organism an inhibition of oxidase, catalase and dehydrogenase activities at 55 to 65°, and complete inactivation of these systems at 70 to 75°C.

Experiment V. Thermolability determinations of B. mesentericus F

In this study an almost complete correlation was established between the oxidase, catalase and dehydrogenase labilities, on the one hand, and the maximum growth temperature, on the other (53°C.).

With inhibition of the catalase and reducing mechanisms, the peroxidase activity becomes more and more evident up to 59 to 65°, when it is partially inhibited. A positive test for peroxide was not obtained. The beginning of inhibition of dehydrogenating mechanisms at 52 to 53° was indicated in the succinodehydrogenase tests and by the appearance of autoxidation in tube 3 at the two and one-half hours reading of the p-phenylenediamine set-up.

A detailed presentation of results obtained with *B. prausnitzii*, *B. megatherium* D and *B. megatherium* N is not possible as yet.

Considerable variation in the labilities of the enzymes of *B. prausnitzii* was observed. In several experiments the catalase activity was found to have as its upper limit the temperature of maximum growth (45°). In others, however, a distinct catalase activity was evident in suspensions heated at 49°C., and a trace of activity in 55° suspensions. The variations do not permit definite conclusions regarding the relations between the labilities of the "systems" and the maximum temperature of growth of *B. prausnitzii.*

B. megatherium D and *B. megatherium* N also exhibited considerable variation. However, the results obtained with these two

strains permit certain conclusions. The former, having a maximum growth temperature, after limited acclimatization, of 47°, revealed no catalase activity, but did give indications of a trace of peroxide; furthermore, there was only a weak succinodehydrogenase action, which manifested an initial inhibition at 47°C. *B. megatherium* N, whose maximum temperature of growth was 47°C., after acclimatization, also revealed no catalase activity, and no peroxide could be detected. Here, too, a weak succinodehydrogenase exhibited initial inhibition at 47°C. The oxidase activity of both organisms was very weak.

The apparent absence of catalase and the weak oxidase activity of these two *B. megatherium* strains (D and N) are of interest in view of their strictly aerobic properties. The dehydrogenase action, although weak, indicates possible production of peroxide. Traces of peroxide were detected in *B. megatherium* D. A utilization of this peroxide by the peroxidase suggests itself. It is possible, however, that the apparent absence of catalase activity is due to treatment other than heating, since by dropping some H_2O_2 onto agar slant cultures of these organisms grown at 25° traces of catalase activity were observed. By using slant agar cultures developed at 43°C., and employing the same technique, a slight catalase activity was exhibited by *B. megatherium* D, but none by *B. megatherium* N.

In table 6 are presented the observed correlations between the maximum temperatures of growth of 12 different organisms employed in this investigation, and the heat labilities of their oxidation-reduction systems. The correlations are not complete, but in view of the fact that the suspensions were subjected to the higher temperatures for only 20 to 24 hours, whereas the maximum temperatures of growth were those for growth during continued transfer at twenty- to twenty-four-hour intervals, any distinct inhibition of the activities of the systems at or near the maximum growth-temperature was regarded as sufficient for inclusion in the table. Because of less distinct inhibitions and of variations in the results obtained with certain organisms, some of the attempted correlations must be considered as questionable. (See table 6.)

TABLE 5

B. mesentericus F. Maximum temperature of growth, 53°C.

	HEATING TEMPERATURE																																					
	37°C.					45°C.					49°C.					52–53°C.					55°C.					59°C.					65°C.					71–72°C.				
Oxidase																																						
Tube number	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
15 minutes	2	–	–	2	2	2	–	–	2	1	2	–	–	2	1	2	–	–	2	1	2	–	–	1	1	±	–	–	±	±	–	–	–	±	±	–	–	–	–	–
30 minutes	1	–	–	1	1	2	–	–	2	2	2	–	–	2	2	1	–	–	1	1	1	–	–	1	1	±	–	–	±	±	–	–	–	±	±	–	–	–	±	–
70 minutes	(1)	–	–	(1)	(1)	(1)	–	–	(2)	(1)	(1)	–	–	(2)	(1)	(1)	–	–	(1)	(1)	1	–	–	1	1	1	–	–	1	1	–	–	–	1	±	–	–	–	1	±
2½ hours	(1)	±	–	(2)	(1)	(1)	±	–	2	(1)	2	±	–	2	(1)	2	±	±	2	2	1	±	±	2	1	2	±	±	2	2	±	±	±	2	1	±	±	±	2	1
Peroxidase																																						
1:50 H_2O_2	–					–					–					2					2					2					1					1				
1:100 H_2O_2	–					–					–					1					2					2					1					1				
1:1,000 H_2O_2	–					–					–					–					2					2					1					1				
1:10T H_2O_2	–					–					–					–					±					1					±					±				
1:100T H_2O_2	–					–					–					–					–					±					–					–				
No H_2O_2	–					–					–					–					–					–					–					–				
Catalase																																						
	4					4					4					3					2					–					–					–				
Peroxide																																						
$Ti_2(SO_4)_3$	–					–					–					–					–					–					–					–				
Benzidine + HR	–					–					–					–					–					–					–					–				

	Succinodehydrogenase							
15 minutes.....	3		4	—	—	—	—	—
40 minutes.....	4		4	2	—	—	—	—
2 hours.....	4		4	—	—	—	—	—
4 hours.....	4		4	2	—	—	—	—
7½ hours.....	4		4	3	2	—	—	—
20 minutes.....	4		4	4	3	—	—	—

Parentheses indicate decoloration in the depths.

It appears from this table that all of the organisms but *B. vulgatus* and *B. mesentericus* A exhibit a correlation between the temperature limiting growth and that distinctly inhibiting one or more of the "systems." It is possible that the presence of peroxide may have masked the thermolabilities of the oxidases of these two organisms. The dehydrogenase system appears to show with most of the organisms a distinct inhibition at the maximum temperature of growth, while the oxidase and catalase vary considerably in this respect. The authors have tried to make

TABLE 6

Showing the relations between the maximum temperatures of continued growth and the heat labilities of oxidase, catalase and succinodehydrogenase

ORGANISMS	MAXIMUM TEMPERATURE OF GROWTH	OXIDASE	CATALASE	SUCCINODEHYDROGENASE
	°C.			
B. subtilis F	59–60	+	+	−
B. vulgatus F	56	−	±	±
B. mesentericus F	53	±	+	+
B. mesentericus A	52–53	±	−	±
B. subtilis T	49–50	−	+	+
B. cereus	47–49	±	±	+
B. megatherium D	43–44		−	+
B. megatherium N	43–44		−	+
B. mycoides	41–42	±	±	+
No. 1608 group 80	65	+	+	+
No. 59	65	+	+	+
SIV	70	+	+	+

Key: +, distinct correlation; ±, questionable correlation; −, no correlation.

clear, however, that the variation in the thermolability of the oxidase may be due to certain interfering factors, and that the thermolability of the catalase may, in a measure, vary with the numbers, and degree of clumping of, the bacteria tested.

GENERAL DISCUSSION

In living organisms depending upon processes of oxidative decomposition for the energy necessary for the performance of mechanical work, and for chemical and structural syntheses and

other energy transformations, a study of growth limitation due to heat is logically directed toward the effects upon the systems involved in these oxidations. The dependence of growth and metabolism upon oxidative processes, however, does not preclude the possibility that other, but perhaps less, fundamental processes may be involved in the inhibition of growth by heat.

The coincidence between the temperatures limiting bacterial growth and those inhibiting the activities of the oxidative systems selected for study does not necessarily prove that the apparent effects of higher temperatures upon these systems are directly responsible for the growth limitations. The inhibition by heat of mechanisms selected from a complex biological system may be obtained as a result of other changes in this system.

Decreased permeability of the cell wall, as a result of heating, or the coagulation of the cellular protoplasm, may result in a lessened accessibility of the indicators used. Harrison (1929) was able to double the rate of reduction of methylene blue by yeast cells, and to effect a complete and fairly rapid reduction of indigo carmine in both warmed and unwarmed yeast, by inducing plasmolysis by means of toluene. He concluded that the rate of access of methylene blue (or indigo carmine) to the cell-reducing systems is the limiting factor in a suspension of intact yeast cells, and that the time required for the reduction of the dye stuffs gives no measure of the reducing systems present.

However, Dallinger's observations (1887) on increased vacuolization with acclimatization of flagellates to higher temperatures indicated by this more rapid expulsion of waste products an increased rate of metabolism, and also a dehydration. If such desiccations are brought about as a result of increased metabolic activity at higher temperatures, it appears possible that the dehydrations which raise the coagulation temperature of the protoplasm are dependent upon a thermostability of the respiratory mechanisms. That a dehydration may result from increased respiratory activity is suggested by the fact that *B. anthracis* requires the presence of oxygen for sporulation. Schreiber (1896) found oxygen to be necessary for spore formation by aerobic bacteria, and that more oxygen was required for sporulation than

for growth. Matzuschita (1902) observed that under hydrogen and under atmospheric pressures of less than 30 mm., aerobes never produce spores, and that facultative anaerobes and obligate anaerobes form spores very rapidly upon the addition of oxygen.

Assuming then that the dehydration of the cellular protoplasm raises its coagulation temperature, it becomes evident that a decreased accessibility of the indicators due to the coagulation of the protoplasm may be intimately associated with the lessened activity of the respiratory processes.

Aside from the effect of heat on the degree of accessibility of the reagents used for the detection of the oxidation-reduction systems, a coagulation and inactivation of these "systems" may result. Waentig and Gierisch (1916) concluded that liver catalase is a coagulable protein. They found that the inactivation of the catalase was complete at 68°C., while at 48°C., where the catalase extract began to cloud, there was a considerable diminution in activity. Desiccation may thus result in a greater degree of thermostability of the "systems" themselves.

The writers' attempts to demonstrate such dependence of growth at high temperatures upon the degree of dehydration of the bacterial protoplasm by cultivation in media of high osmotic pressure do not support this hypothesis. The methods employed may not have been satisfactory, however, since not only did they tend to desiccate the bacterium, but they also made less available the water and nutrients of the external environment. Since water itself plays a very important rôle in oxidation processes,[7] it is perhaps necessary that an even greater accessibility of water and nutrients prevail at higher temperatures. Thus, while a state of desiccation within the cell would be necessary for resistance to higher temperatures, an abundance of water would have to be available for utilization in the various metabolic processes. The maintenance of such a state of unequal osmotic

[7] "Die langsame Verbrennung des Wasserstoffpalladiums, ist sonach zweifellos an die Mitwirkung von Wasser geknüpft und da es wenige Körper giebt, die sich mit gleicher Energie bei gewöhnlicher Temperatur in Sauerstoffgas oxydieren, so darf man wohl annehmen, *dass kein Körper bei gewöhnlicher Temperatur auf trocknen Sauerstoff zu wirken vermag*" (Traube 1885).

pressure may again depend upon the continued functioning of the oxidative "systems." Hill (1930) has demonstrated the possible rôle of oxidative processes in the maintenance of differences in osmotic pressure.

It has been indicated that certain growth limitations may be due to the inhibition of systems that are responsible for the removal of toxic metabolic products as, for example, hydrogen peroxide.

The failure to demonstrate inactivation of certain systems at the temperatures marking the maximum temperatures of growth may be due to the functioning of other thermostable mechanisms. Thus, the apparent heat stability of the succinodehydrogenase of *B. subtilis* F. (table 1B) may be due to the activity of a heat-stable sulfhydryl system. Abderhalden and Wertheimer (1923) concluded that increased reduction obtained on coagulation of frog muscle is due to an increase of firmly-bound SH groups. Methylene blue is not reduced by these bound SH groups, but cystine is. If free cystine is added to the system (fixed SH groups and methylene blue) the methylene blue is reduced. The cystine acts as an intermediary acceptor and transportor of hydrogen. That the functioning of such a system may prevail with *B. subtilis* F. is indicated by the apparent diminution in activity of the reductive processes when heated at 55°C. and the better reduction of methylene blue after heating at still higher temperatures. Here we may have an inhibition of the heat-labile methylene blue reducing systems, and then above 55°C., coagulation and appearance of the heat-stable SH systems.

It is evident from these considerations that oxidation-reduction thermolability and protoplasm coagulability may be very intimately associated with each other and may play an important rôle in growth inhibition by heat. It has been indicated that, while the inaccessibility of the reagents involved in the respiratory processes and inactivation of the oxidation-reduction mechanisms may be due to a coagulation of the bacterial protoplasm, the coagulability of the protoplasm may be influenced considerably by the activity of the respiratory processes.

SUMMARY

1. Attempts to acclimate members of the "subtilis group" to growth at higher temperatures resulted in only a small degree of success.

2. Media of relatively high osmotic pressure failed to raise the maximum temperatures of growth of members of the "subtilis group," and were inferior to the ordinary beef extract and beef infusion broths, in their support of growth at higher temperatures.

3. Members of the "subtilis group" revealed considerable diversity with respect to the effects of temperature upon oxidation-reduction relations, as indicated by growth in deep media and by the thermolabilities of certain of their catalytic oxidation-reduction systems.

4. Certain correlations between the thermolabilities of catalytic oxidation-reduction systems and the temperatures marking the maximum temperatures of growth of members of the "subtilis group" and of thermophiles have been observed.

CONCLUSION

The evidence presented indicates that the limitation of bacterial growth by temperatures higher than the maximum temperature of growth may be due to the inhibition of the activities of certain catalytic oxidation-reduction mechanisms involved in cellular respiration.

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