BACTERIAL CALORIMETRY

I. GENERAL CONSIDERATIONS. DESCRIPTION OF DIFFERENTIAL MICROCALORIMETER

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The production of heat by living bacteria is one of the transformations of energy brought about by these organisms. Although the rise in temperature of fermenting liquids, decomposing manure and other organic substances has been studied on account of its economic effects, and although, periodically, investigations of heat production in relation to the energetics of microörganisms have been made, it is not possible at this time to state definitely the significance of this liberation of heat or to correlate it with the various phases of the growth and activity of bacteria. In fact, the most recent and valuable compilation of this knowledge by Buchanan and Fulmer (1928) contains a paragraph on the utilization of energy in the development of heat which concludes with this sentence: "More complete studies on the utilization and partition of energy in the growth of microorganisms are needed."

The rise in temperature of organic substances undergoing bacterial decomposition has been called "thermogenesis." Its importance is connected chiefly with so-called "spontaneous heating," which is useful in some agricultural processes, but harmful in others, particularly those in which destructive combustions occur. Within the past year, James (1927–1928) has investigated this type of thermogenesis and has published a comprehensive review of the literature on the subject. These studies, however, have been concerned primarily with increase in tem-

perature and do not afford data on heat production of the kind which can be used in the investigation of energy exchange.

In considering the energy transformations of microörganisms the assumption is made that, when light is excluded, these organisms derive from the aerobic or anaerobic oxidation of foodstuffs all the energy required for their growth, structure formation, maintenance, repair, reproduction, motility, physical states and all the work associated with their vital processes. There is no evidence that they receive energy from other sources. Absorption of heat from the environment affects their chemical and physical reactions, but this heat is not a source of energy to the minute isothermal cell. Hill (1912) has clearly demonstrated the fallacy of regarding bacteria as heat engines, as was suggested by Simonson (1910).

On this assumption, attempts have been made to strike the energy balance of bacterial processes on a calorimetric basis. Rubner (1902, et seq.), as a part of his epochal investigations of the energy transformations of living organisms, measured the heat produced by bacteria. Rubner's bacterial calorimeter was a vacuum flask, carefully insulated and fitted with a delicate thermometer. In order to arrive at an energy balance he determined the heat liberated by the bacteria, the combustion caloric values of the uninoculated medium, the residue medium after growth and the total growth or "crop" of bacteria. In spite of the difficulties and uncertainties of corrections which must be used to compensate for effects produced by changes in the environmental temperature of this type of calorimeter, Rubner obtained important results, which will be discussed later. In a somewhat similar manner, omitting the direct measurement of heat produced, Tangl (1903) and Terroine and his associates (1922, et seq.) have attempted to discover how much energy microörganisms require for the formation of their structure. It does not seem to be probable that combustion values of foodstuffs, residue and bacteria can furnish the information required for the solution of these problems. Among various factors which are not taken into account by this method is the free energy of the substances utilized. The free energy of foodstuffs may differ

considerably from the energy value derived from measurements of the heat produced on combustion. Oppenheimer (1925) has shown that the combustion values of foodstuffs are "practically" equal to their free energies. On the other hand, Baron and Pólányi (1913) and Linhart (1920), and others, have so clearly pointed out that the free energy of these substances must be taken into consideration that it would be unwise to rest modern studies of bacterial calorimetry upon the older values of energy obtained by combustion methods.

The most recent and valuable studies of bacterial calorimetry are those of Meyerhof, A. V. Hill and Shearer. By combined measurements of oxygen consumed and carbon dioxide liberated, together with measurements of heat produced, Meyerhof (1912) has determined the caloric quotient of some phases of bacterial growth. This quotient is expressed as the ratio: heat produced in calories/milligram of oxygen consumed. Through study of this quotient, Meyerhof discovered evidence of the extreme complexity of the factors involved in the production of heat in a culture of bacteria, showing that not all of the heat found in the cultures can be attributed to the oxidation processes of the bacteria. The contribution made by Hill (1911–1912) to the advance of this work has been most direct in the form of the useful differential microcalorimeter devised by him. The great value of his general investigations of the energetics of cellular processes will appear in all phases of this work. Shearer (1921), using Hill's calorimeter, discovered that bacteria liberate much less heat when utilizing free amino acids than when they use more complex protein split products.

It is not intended to review all of this literature here. It will be more advantageous to take up the consideration of appropriate papers in connection with experimental measurements of the heat produced by bacteria at various stages of their growth and activity. In general the literature shows that the physiologists have for a long time perceived the advantage of using bacteria as subjects for the investigation of the energetics of cells. Rubner's belief that the metabolism of bacteria follows the same laws as that of the cells of higher organisms has been justified by the discovery from the study of bacterial processes of laws govern-

ing the metabolism of the cells of higher organisms. For example, recent advances in the knowledge of the mechanism of the contraction of muscle and of the respiration of cancer cells (Warburg, 1927) owe a part of their success to the results of the investigations of the energy exchanges of bacteria.

From the point of view of the bacteriologist, however, some of the investigations of these physiologists seem to suffer from lack of attention to the strictly bacteriological aspects of the subject. Precautions to keep cultures from contamination have not always been adequate, and in some instances have not even been attempted. It is apparent also that, through ignorance or neglect of the phases of growth of microörganisms, some of the most interesting periods of metabolism have not been studied.

It is evident that one of the first requisites for the investigation of these problems is an apparatus by which heat production by bacteria may be measured accurately.

DESCRIPTION OF DIFFERENTIAL MICROCALORIMETER

A calorimeter for use in the measurement of heat production by bacteria should be sensitive to small differences in temperature in the apparatus and as independent as possible of temperature changes in its external environment. It should be so constructed as to permit sterilization of all parts in contact with the culture, should allow stirring of the culture without causing unequal changes in temperature and should be provided with suitable openings, protected against temperature disturbances due to evaporation, for the introduction of the inoculum and for the withdrawal of small samples of fluid and gases. The differential microcalorimeter described by A. V. Hill (1911–1912) is an apparatus suitable for the measurements of small amounts of heat and automatically eliminates errors due to small changes in the environmental temperature. Since my use of this apparatus for the measurement of the heat liberated by reactions of antigens with antibodies (Bayne-Jones, 1925) I have made some changes in it which permit sterilization of the culture flasks and secure protection against contamination. These modifications, together with the methods used in calibrating the apparatus will be described in the following paragraphs.

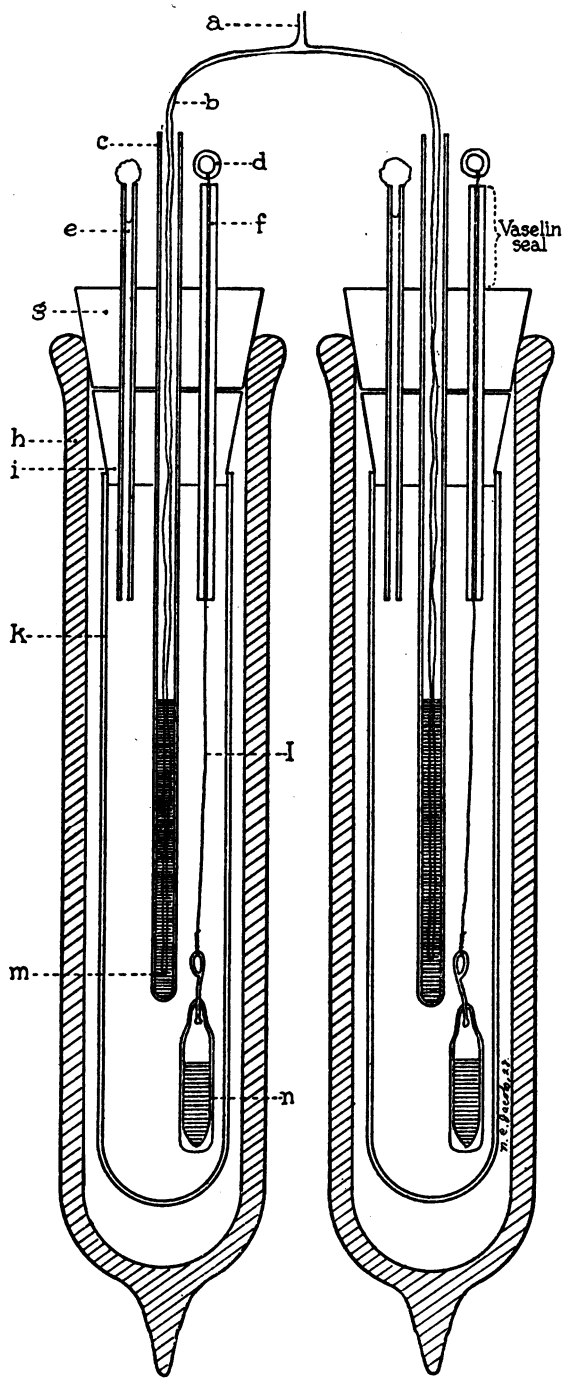

FIG. 1. SECTIONAL VIEW OF DIFFERENTIAL MICROCALORIMETER

The basic parts of the calorimeter are two cylindrical silvered vacuum Dewar flasks, 30 cm. long, 4.5 cm. wide, 380 cc. volume. Figure 1 is a sectional drawing showing these flasks and their contents. The Dewar flasks cannot be sterilized by heat without altering their thermal properties. Therefore, a vessel which can be filled with the requisite amount of medium and sterilized in the autoclave is fitted inside the Dewar flask. This vessel, *K*, is a rounded bottom, thin-walled pyrex glass tube measuring 20 by 4 cm. Its upper end is closed with a rubber stopper, *I*, or may be made of glass with suitable tubes fused into it. A second rubber stopper, *G*, is used to close the Dewar flask. Three glass tubes are passed through these stoppers. The tube marked *E* has an inside diameter of 3 mm. It is open at both ends, passes into the inner vessel, reaching almost the level of the fluid and has its outer end, 8 cm. above the outside stopper, plugged with cotton. Inoculation of the medium is made through this tube, and through it small samples are withdrawn by capillary pipette. Tube *C* is a thin-walled glass tube, 4 mm. in diameter, closed at the lower end to serve as a receptacle for the thermocouple. As it is impracticable to sterilize the thermocouples, *A* and *B*, they are placed inside this tube after the sterilization of the culture vessel. A small amount of fluid is placed in this closed tube to aid in the conduction of heat to the junctions of the thermocouple. This arrangement makes the temperature measuring system slightly less sensitive, introducing a short lag. These slight errors, however, having values which can be determined, are much less serious than the uncertainties produced by thermocouples contaminated with bacteria. The third tube, *F*, is a thick-walled capillary tube to provide a sleeve for the thread or fine wire, *L*, to which the stirring bulb, *N*, is attached. This bulb is composed of glass partly filled with mercury and is of such a size as to allow it to be moved up and down in the vessel without striking the wall of the thermocouple tube. After sterilization, the upper part of this capillary tube is filled with vaseline to shut out bacteria and prevent evaporation. The ring, *D*, is attached to the arm of an automobile wind-shield wiper, the oscillations of which raise and lower the bulb, *N*, stir-

ring the contents of the culture vessel. Bulbs of this type are more satisfactory than air bubbles or rotating blades for stirring the cultures. Air causes frothing of the culture medium, evaporation, temperature disturbances which cannot be controlled and irregular distribution of the bacteria. Rotating blades stir adequately. But it is very difficult to revolve the blades in each flask at the same rate and the mechanism for rotating the blades takes up more room than that used to raise and lower the bulbs.

Two Dewar flasks with sterilizable inner vessels compose the differential calorimeter. One vessel, into which is placed one junction of the thermocouple, contains the culture. The second vessel, serving as a control, contains the other junction of the thermocouple. The pair of vessels is so adjusted that temperature differences between them due to any other causes than the changes occurring in the culture are automatically almost completely balanced out. The temperature changes indicated by deflections of a galvanometer attached to the terminals of the thermocouples are, therefore, directly referable to changes in the culture. In order to protect the calorimeter against temperature disturbances from the outside, as an additional precaution, the flasks are submerged in a water bath, the temperature of which rarely varies more than 0.01°C., and often remains constant for many hours.

In order to balance out temperature changes due to fluctuations in the environmental temperature, the two flasks are given the same coefficient of thermal conductivity by placing an appropriate amount of fluid in each.

The conductivity constant for loss of temperature by a vacuum flask may be calculated from the following expression:

$$K \log e = \frac{1}{t} \log \left(\frac{A - T_0}{T - T_0} \right)$$

where K = conductivity coefficient for temperature loss.
t = time, usually in hours.
A = initial temperature.
T = temperature of fluid in flask at time t.
T_0 = temperature of environment (e.g., water bath).
$\log e$ = 0.434.

This coefficient K, varies with the amount of fluid in the flask. Hence K can be made to have any desired value within certain limits by increasing or decreasing the amount of fluid in the flask. After determining K for a standard volume of fluid in one flask, the other flask can be given an identical value of K by putting into it more or less than the standard amount of fluid. This amount of fluid can be determined from tables prepared when the calibrations are made.

Corrections for heat loss, which are used in the final computation of the total heat produced, are based upon the value of this coefficient of temperature loss, K. In order to calculate the total amount of heat produced during an experiment it is necessary to add to the observed heat production an amount equal to that which has been lost by conduction to the outside. The formula for this is given by Hill as follows:

Total heat liberated = (heat capacity of the flask and fluid) $(T - T') - (A - A')$ [K (area of the curve)]

where $T - T'$ = the observed difference in temperature between the two flasks;

$A - A'$ = the initial value of $T - T'$.

K = the coefficient of conductivity for loss of temperature. The value used is taken proportional to the interval of time for which the calculation is to be made.

These values are known from the observations.

The expression K (area of the curve) refers to the curve relating the difference in temperature between the two flasks to the time during which the observations are made. The area of the curve can be obtained with sufficient accuracy for any interval by multiplying the observed value $(T - T')$ in the middle of that interval by the duration of the interval in hours or fractions of hours. Therefore, K (area of the curve) = K [value of $(T - T')$] in the middle of the interval. This expression gives the temperature lost during the particular interval of time.

The loss of heat calculated thus for each interval, added to the loss calculated to the end of the previous interval and finally

added to the observed heat production, gives the total heat liberated.

The temperature changes are measured by means of thermocouples composed of junctions of copper and constantan wires. The constantan wire, B & S. No. 30 is 0.25 mm. in diameter, the copper wire, B. & S. No. 32 measures 0.202 mm. in diameter. These fine wires are silk-covered and are coated with shellac after completion of the junctions. The junctions are made by winding the copper around the constantan wire and fixing it with a small piece of solder. Elements composed of one to 10 junctions have been used. A single pair, or at most three pairs of junctions give sufficient electromotive force for the measurements to be made in this work. As an additional insulation and protection against wetting, the wires and junctions are enclosed in very thin capillary tubes, sealed at the bottom.

The copper wires leading from the thermocouple junctions are covered with rubber tubing and passed through glass tubes, well covered with rubber, to two pools of mercury in glass cups set into a block of wood. These pools of mercury are placed close together and protected as much as possible against unequal heating or cooling, to avoid thermoelectric effects at this point. Copper wires connect the mercury pools to the terminals of the galvanometer. Numerous tests have demonstrated that this system of wiring is free from parasitic currents arising from bimetallic junctions.

The galvanometer is a Type HS reflecting instrument made by the Leeds and Northrup Company. The total resistance of the galvanometer is 17.5 Ω. Its microvolt sensitivity is 4.5 mm. per microvolt at 1 meter and its external critical damping resistance is 50 Ω. The resistance of the galvanometer is approximately equal to the resistance of the thermocouples and circuit.

The readings indicative of temperature changes at the junctions of the thermocouple are taken directly from the scale divisions of the galvanometer deflection at 1 meter. No potentiometer is used. The calibration of the scale divisions in terms of temperature is made by numerous comparisons with Beckmann thermometers in the flasks with the thermocouple junctions.

Most of the thermocouples used give deflections in which 1 scale division = 0.003°C.

No experiment is begun until the pair of flasks composing the calorimeter are in temperature equilibrium with each other and with the water bath in which they are submerged. The Dewar flasks are rarely lifted out of the water bath, but are raised only high enough to allow removal of the inner vessels. After the inner vessels, containing sterilized culture medium, are brought from the autoclave they are placed in a pot of water until their temperature has cooled down to approximately that of the water bath. They are then placed in the Dewar flasks, submerged below the surface of the water bath, and at this time the thermocouples are let down into their appropriate tubes. The stirrers are connected and started. The whole system is left in this condition for ten to eighteen hours, which is the time required for the establishment of temperature equilibrium. At the end of this period, and after at least thirty minutes observation showing zero deflection of the galvanometer, the experiment is begun by the introduction of the inoculum into one of the flasks. With proper precautions, 1 ml. of a fluid culture may be introduced through tube *E* (fig. 1) by means of a capillary pipette without causing more than a slight change in temperature. Compensation for this is made by a slight shift of the zero of the galvanometer. Aseptic precautions at this time, and at subsequent removals of small amounts of the culture through tube *E* avoid contamination with undesirable bacteria.

CALIBRATION

Calibration of flasks

The determination of the value of the coefficient of temperature loss, *K*, is made by placing in a flask warm water, 1° to 2°C. higher than that of the surrounding temperature and allowing this to cool. Observations of the temperature at regular intervals of time provide data for the calculation of *K*. A number of such observations are made and an average value of *K* is taken from these results. The temperature may be measured with a

Beckmann thermometer or with the thermocouples. When the temperature in the flask is within 1°C. of the temperature of the water bath the cooling curve is in a region in which errors in measurement of the temperature have a larger effect than they

TABLE 1

Determination of K for flask No. 1, containing 100 cc. water

Values of A and T are expressed as the degree of temperature above that of the water bath.

t TIME	T_0 WATER BATH	A INITIAL TEMPERATURE	T TEMPERATURE AFTER INTERVAL	K
hours	°C.	°C.	°C.	
0	37.5	0.6405		
1	37.5	0.6405	0.528	0.1932
2	37.5	0.6405	0.453	0.1729
3	37.5	0.6405		
4	37.5	0.6405	0.333	0.1633
5	37.5	0.6405	0.285	0.1620
6	37.5	0.6405	0.242	0.1626

The value of K for each hour is calculated from the equation:

$$K \log e = \frac{1}{t} \log \left(\frac{A - T_0}{T - T_0}\right)$$

The average value of K per hour from these 5 determinations is 0.1708. Calculations of temperature corrected for loss by cooling are made as follows, using 0.1708 as the value of K per hour. The values for the temperature at the half-hour intervals are taken from the graph.

TIME	TEMPERATURE OBSERVED	$K \times$ VALUE OF MID. ORDINATE	TOTAL TEMPERATURE LOSS	CORRECTED TEMPERATURE
hours	°C.			°C.
0	0.6405	0	0	0.6405
1	0.528	0.099	0.099	0.627
2	0.453	0.083	0.182	0.635
3	0.395	0.071	0.253	0.648
4	0.333	0.061	0.314	0.647
5	0.285	0.053	0.367	0.652
6	0.242	0.045	0.412	0.654

do in cases in which the temperature difference is greater. Nevertheless, the equation for the calculation of the coefficient of temperature loss holds. An illustration of the method of determining K and of the calculations for corrections for loss of temperature is

given in table 1 and the accompanying graph, figure 2. The values of the corrected temperature lie on an approximately straight line parallel to the time axis at the level of the value of the initial temperature.

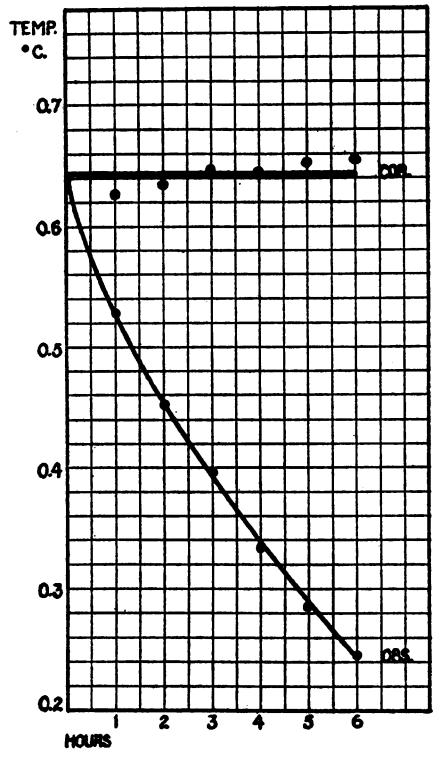

FIG. 2. GRAPH OF COOLING CURVE OF CYLINDER AND INNER VESSEL

Determination of sensitivity and accuracy

By liberating a small known amount of heat from a resistance coil in the fluid in one of the flasks it is possible to determine the sensitivity and lag of the system and to evaluate the errors in the measurements. A small coil of nichrome wire is placed in the fluid in one of the pair of differential flasks and a measured electric current is passed through it for a definite period of time.

A short lag occurs, but measurable changes in temperature are

detectable within a minute after the start of the flow of current. This interval is of no significance in the kind of work for which the apparatus is intended.

The heat produced by the coil is calculated from the formula:

$$H = \frac{RI^2}{4.18} \text{ gram cal. per sec.}$$

where H = heat produced.
R = resistance in ohms.
I = current in amperes.
4.18 = factor for converting joules into gram calories.

In one experiment the resistance of the coil was 11 Ω and the current 0.025 amperes. This produced 5.91 gram calories per hour. The corrected temperature at the end of five hours was 0.249°C. The heat capacity of the flask and fluid was equivalent to that of 125 cc. of water. Hence the observed heat production was 6.02 gram calories per hour. The error in this case was +1.88 per cent. In another experiment, lasting six hours, the observed heat production with the same coil and a current of 0.05 amperes was 22.89 gram calories per hour. The calculated heat production amounted to 23.68 gram calories. The error in this case was 3.33 per cent. These experiments demonstrate that the calorimeter and measuring devices are sufficiently sensitive and accurate to measure the production of a small amount of heat, of the order of magnitude of that produced by cultures of microörganisms, with an error of approximately ±3.0 per cent.

Experiments on the measurement of the heat produced by mixtures of definite volumes of absolute ethyl alcohol and water in the calorimeter gave results which differed only within this range of error from the heat production expected by calculation.

The heat capacity of the flask and its contents can be approximated from computations based upon the amount and kind of fluid in the flask, the specific heat of this, and the glass and other materials composing the vessels and apparatus in them. This estimate can be verified and corrected by calibrations in which a known amount of heat is liberated in the flasks. In general, the

heat capacity of the flasks and fluids used has been in the neighborhood of 125, e.g. equivalent to the heat capacity of 125 cc. of water.

As the methods of observation and computation which have been used in the experiments upon the heat produced by bacteria are applied in the case in which a known amount of heat is

TABLE 2

Calibration experiment

Dewar cylinder and vessel No. 1.
100 cc. water in vessel. Heat capacity of flask and contents = 125.
Coefficient of loss of temperature, K, = 0.1780 per hour.
Coil, resistance = 11 Ω.
Current 0.050 amperes. Gram. cal. per hour (calc.) = 23.68.
Thermocouple No. 3 gives 1 scale div. = 0.003°C.
Water bath temperature 37.5° ± 0.01°C.

HOURS	GALV. S.D.	°C. OBSERVED	K × VAL. MID. ORD.	TOTAL TEMPERATURE LOSS	°C. CORRECTED	HEAT GRAM CAL. OBS.	HEAT GRAM CAL. CALC.	DIF. GRAM CAL.	ERROR PER CENT
0	0	0	0	0	0	0	0	0	0
0.5	32	0.096							
1	58.8	0.1764	0.017	0.017	0.1934	24.22	23.68	0.54	+2.28
1.5	82	0.246							
2	105	0.315	0.0438	0.0608	0.3758	46.97	47.36	0.39	−0.82
2.5	127	0.381							
3	144.5	0.4335	0.0678	0.1286	0.5621	70.26	71.04	0.78	−1.09
3.5	163	0.489							
4	180	0.540	0.087	0.2156	0.7556	94.44	94.72	0.28	−0.29
4.5	194	0.582							
5	209	0.627	0.1035	0.3191	0.9461	117.26	118.40	1.14	−0.96
5.5	225	0.675							
6	235	0.705	0.120	0.4391	1.441	143.01	142.08	0.93	+0.65

liberated in the calorimeter, one of the calibration experiments is given in detail in table 2.

The curve of temperature changes which occurred in this experiment is shown in the accompanying graph, figure 3, in which temperature is plotted against time. The temperature, corrected for heat loss, falls on a straight line, ascending regularly, which is to be expected as the result of the continuous liberation of a constant amount of heat in the calorimeter. It is additional

evidence of the validity of Hill's formula for the calculation of the coefficient of temperature loss and shows the correctness of the value calculated for this case.

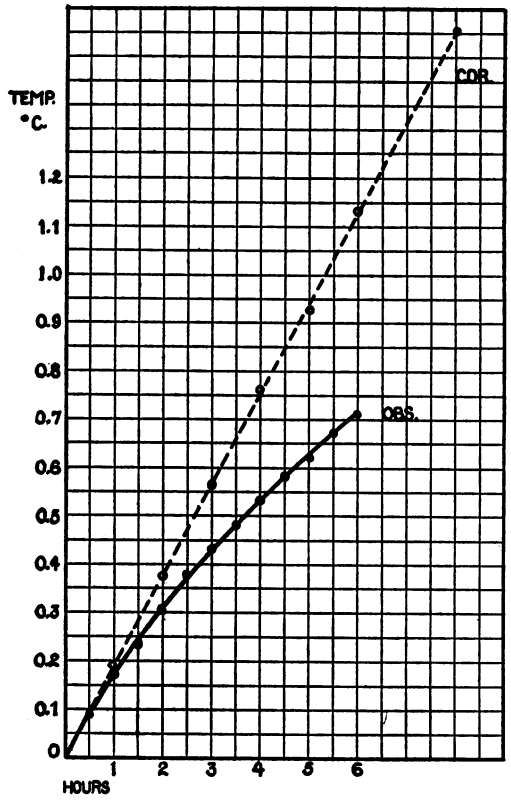

FIG. 3. GRAPH OF OBSERVED TEMPERATURE CHANGES PRODUCED BY HEATING COIL IN ONE VESSEL OF CALORIMETER

The corrected curve is a straight line, showing liberation of a constant amount of heat.

As an additional check on the ability of the calorimeter to eliminate automatically or balance out temperature changes due to warming or cooling the environment of both flasks, a pair of flasks, adjusted to have the same value of the coefficient of temperature loss, K, have been placed close together and left in posi-

tion for forty-eight and ninety-six hours. During this time, the temperature of the room or water bath rose and fell during intervals of two to ten hours through a range of about 2°C. The temperature difference within the flasks during this period, as measured by the thermocouple, varied between 0 and 0.012°C. These changes took place very slowly. Small rapid fluctuations produce no detectable temperature differences between a pair of balanced flasks.

This calorimeter has been used to measure the heat production of bacteria in several types of culture media. Most of these cultures have produced a rise in temperature of 0.2 to 0.5°C. in eight to ten hours, liberating an amount of heat easily measured by the calorimeter, and the recording system. As the curves of heat production require detailed analyses, they will be described and discussed in the subsequent papers of this series.

SUMMARY

This paper describes modifications of the differential microcalorimeter of A. V. Hill which adapts it to the study of heat produced by growing bacteria. The chief changes are the addition of an inner vessel which can be filled with the required amount of culture fluid and sterilized. Other minor changes permit the insertion of the thermocouples without contaminating the media, mechanical stirring in place of stirring by air bubbles, and a tube for the introduction of the inoculum and the withdrawal of small samples.

Data obtained from calibrations are presented showing that the apparatus is sufficiently sensitive and accurate to measure the production of approximately 6 gram calories per hour with an error of ±1.88 per cent and the liberation of 23.68 gram calories per hour with an error of ±3.33 per cent in a vessel and fluid having a heat capacity equivalent to 125 cc. of water. Heat production by cultures of bacteria is within these limits in most of the media employed and it is estimated that the error in the measurements within this range is approximately ±3 per cent.

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