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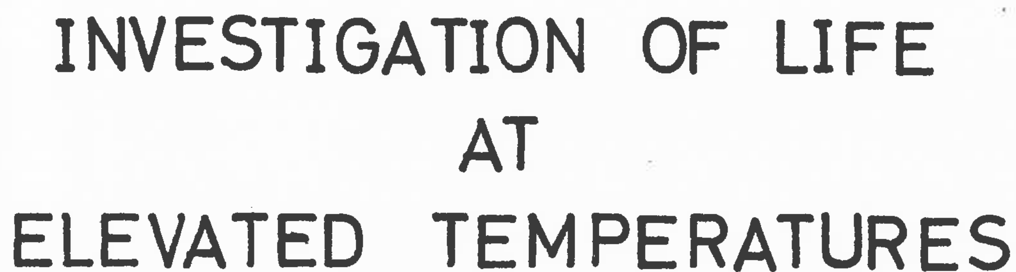

THE STUDY OF ENZYMES OF THERMOPHILIC BACTERIA

Thesis presented in part-fulfilment of the requirements for admission to the degree of Doctor of Philosophy of the University of Adelaide.

by

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SUMMARY

A series of growth curves was done to determine the importance of oxygen in the growth of Bacillus stearothermophilis at 63.5°C, and the effect of change in temperature on growth under anaerobic conditions.

During growth at 63.5° in a glucose medium a high oxygen tension only caused a two-fold increase in cell yield over that produced anaerobically. Addition of nitrate to the medium did not increase the cell yield. Lactate was the major end product of both aerobic and anaerobic growth in a glucose medium. Examination of the end products after resting cell suspensions were incubated with $C_{(14)}$ labelled glucose, and degradation of the $C_{(14)}$ lactate, formed during incubation, showed that B. stearothermophilis catabolised glucose via the glycolytic pathway.

The organism could use amino acids or acetate as energy sources for growth in the absence of glucose, but only if oxygen were present. The tricarboxylic acid cycle did not function in resting cell suspensions grown aerobically with glucose as the energy source, but did function if they had been grown in its absence. Since glucose did not inhibit the tricarboxylic acid cycle directly, it was postulated that there was a defect in synthesis of an oxidative enzyme, due to an oxygen lack caused by the very rapid growth in the presence of glucose.

The mean generation time at 63.5° in a complete glucose medium was 4.5 min.

Changing the temperature of growth from 40° to 68° did not influence the cell yield, which was 21 gm dry weight of cells per 1 gm Mole glucose. But glycolysis and growth were uncoupled at 37° and above 68°, resulting in a smaller yield of cells. The $\log_{10}$ (growth rate) was plotted against the reciprocal of absolute temperature, and the heat of activation for growth, calculated from the Arrhenius equation, was 23,000 cal between 50° and 57°.

The rate of glycolysis (or formation of $C_{(14)}$ lactate from $C_{(14)}$ glucose) was measured between 0 and 60°. An Arrhenius plot, drawn as for growth, revealed that the change in slope when $\log_{10}$ (rate of glycolysis) was plotted against the reciprocal of absolute temperature was less below 30° than above. This was not directly due to lack of glucose transport at low temperatures, for repetition of the experiment with cell free extracts gave the same result.

This change in slope was not reflected when enzymes of the glycolytic pathway were assayed over a range of temperatures, and plotted in the same way. It was suggested that below a minimum temperature growth and glycolysis ceased because the balance of complex reactions necessary for both to proceed was disturbed.

At elevated temperatures glycolysis in resting cell suspensions was unstable unless a rich nitrogen source was supplied. Some

enzymes investigated were heat labile, and others heat stable in crude extracts. Glutamic dehydrogenase and lactic dehydrogenase were apparently heat stable because they were being protected by a cell fragment.

Thermobiosis appears to depend on the ability to maintain the activity of labile proteins, and on the stability of others.

FOREWORD

Scope and General Plan of Thesis

The work reported in this thesis is concerned with the growth and energy metabolism of a thermophilic micro-organism *Bacillus stearothermophilis*, and with the influence of change in temperature on the metabolism of the organism. The first chapter is an attempt to order the published facts and theories pertaining to the biochemistry of life at elevated temperatures. The remainder of the thesis is divided into five chapters dealing with experimental methods, experimental results, and a discussion of these results.

Obligatory Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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CHAPTER I: SURVEY OF PREVIOUS WORK

A. History of Thermobiosis.

Although few people seem aware of it, thermobiosis is a very wide spread phenomenon. Many forms of life can both survive and multiply at temperatures generally thought to be incompatible with life. The first scientific record of thermophilic life was published by Sonnerat (1774), who described a fish which lived at 69°R. (80°C) in a thermal pool on the Island of Luzon, in the Philippines. Forty-three years later, a mould was reported growing at 50-70°C, and fifty years after that a description of thermophilic algae was published.

Bacteriology was still in its infancy at that time, and the first thermophilic bacterium was not noted until 1879 (by Miquel) which was the time agriculturalists, following a suggestion by Pasteur, were looking for bacteria in soil to account for soil fertility (Topley & Wilson 1958). It is interesting that the first psychrophilic bacterium was also discovered at that time.

Meantime, zoologists and botanists both became interested in this life at elevated temperatures and found fish, molluscs, arthropods, worms, and all classes of algae and moulds growing between 60-98°C (Gaughran 1947). Plants growing in hot arid regions, and those growing at the edges of thermal pools must be considered thermophilic (Thomas 1962). However, bacteria soon became the most commonly encountered forms of thermophilic life, mainly because they constituted a nuisance in the canning industry and in pasteurization processes.

B. Occurrence of Thermophiles.

Thermophilic micro-organisms are ubiquitous, and can be isolated from soils in tropical or temporate regions (Allen 1953) and from any material having any contact with soil: e.g., water (fresh, salt, cold or hot) and from the ocean bottom (Bartholomew 1949), from the faeces of man, animals, birds and fish (Neilson 1957), from slag wool (Rathbaum 1961), from air, snow (Allen 1953) and decaying plant material. Most families of the Schizomycetes are represented among the thermophils (Bergey 1959) but those most frequently isolated are aerobic spore forming bacilli.

C. Origin of Thermophilic Life.

Early reports on thermobiosis were mainly descriptive and it was not until the turn of the century that theories about the origin and nature of these seemingly unique forms of life began to be formulated.

In 1927, Arrhenius (cited by Caughran) considered the natural habitat of thermophilic bacteria to be the planet Venus, which has an average temperature near 48°C. He showed that, theoretically, the spores of the organisms could be propelled by the radiation pressures of the sun and travel from Venus to Earth in a few days. This theory does not account for thermophilic fish. Others suggested that thermophils may be relics of the time when the temperature of the earth was very much higher than it is to-day, and that life as we know it has adapted from life at elevated temperatures. Still others proposed that thermophilic bacteria may have adapted from the mesophilic forms. However, if it is considered that many forms of life survive and grow at elevated temperatures, and that life can be

found at any temperature where water is liquid:- from -8°C (Meaney 1958) to 101°C in oil well brine (Zobell and Johnson 1949), it seems logical to think that the origin of thermophilic life is an evolutionary problem.

D. Definition of Thermophilic Bacteria.

Micro-organisms may be classified as psychrophiles (organisms growing between -8°C and 20°C), mesophiles (growing 20°C to 40°C) or thermophiles (growing above 55°C). This classification is quite arbitrary since the biochemical behaviour of each of these groups is apparently the same; they vary only in the rates of their reactions. Examples of almost every family of bacteria as cited by Bergey (1959) can be found among the psychrophiles, mesophiles and thermophiles.

There are an infinite number of bacteria with an infinite number of temperature parameters, graduating from psychrophilic through mesophilic to thermophilic ranges. This study will investigate whether the thermophiles are only biologically different in degree or have some characteristic feature.

Thermophiles have been divided into two groups:

(1) Facultative thermophiles which grow above 55°C, and will also grow at 37°C, and

(2) Strict thermophiles or stenothermophiles which will not grow below 40°C.

However, Campbell and Williams (1953) and Baker etal (1955) showed that they could increase the maximum temperature of growth of the stenothermophile Bacillus stearothermophilis by enriching

the medium in which they were growing, with selected amino acids. Campbell & Williams (1953), Campbell (1954) and Long & Williams (1959) showed that they could also decrease the minimum temperature of growth of B. stearothermophilis by enriching the medium with amino acids. Allen (1953) isolated 21 strains of aerobic bacilli which grew at 55°C. She showed that their temperature range varied with propagation in the laboratory. Originally only four of her isolates grew at 30°C, and 13 at 35°C, but after four transfers on artificial medium, 8 strains grew at 30°C and all 21 strains grew at 37°C.

This confirms that the temperature limits on which bacteria have been classified are more apparent than real, and that unidentified substrates in the soil may make the maximum and minimum temperatures of a strain of bacteria under natural conditions different from those known in the laboratory.

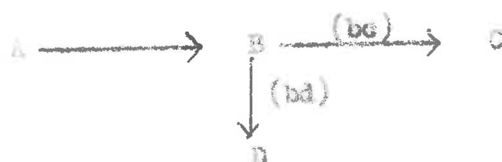
However, it is useful to have some sort of index to which to refer an organism, and Ingraham (1958) suggested that psychrophiles, mesophiles and thermophiles might be names used to classify organisms which differ in their heats of activation ($\Delta H^{\ddagger}$) or temperature co-efficients of growth. He showed that $\Delta H^{\ddagger}$ for a psychrophile was 9,020 Cals, and for a mesophile was 14,000 Cals. It will be interesting to find a figure for a thermophilic bacterium.

E. Behaviour of Thermophiles at "Normal" Temperatures.

If thermophiles are to be accepted as a normal part of the spectrum of life, we must explain why, if they can grow at high temperatures, they cannot grow at "normal" temperatures. Little

experimental work has been done on this, but several people have published speculations about it.

Gaughran (1947) suggested that at low temperatures growth ceased, due to a metabolic imbalance. This is borne out by the experimental results of Campbell & Williams (1953 [2]) who showed that this imbalance manifests itself by an increased requirement of essential metabolites:- e.g., in one strain of Bacillus stearothermophilis there is a shift in the minimum growth temperature from 46° to 56° on the addition of leucine and nicotinic acid to the medium. James (1953) said coupled reactions, the basis of cellular control, could be uncoupled by alteration of the equilibrum constants of the interdependent reactions with a change in temperature. Gaughran (1947) thought that the heats of reaction of some of the enzymes in thermophilic bacteria might be so high that they are unable to be activated at low temperatures. He proposed that in the event of two essential metabolites, C and D, being synthesized from a common precursor, as in the series



it could be visualized that the enzyme (bc) had a much higher activation energy than (bd). Then if at a minimum temperature, (bc) ceased to function, there would be an excess of metabolite D, and no essential C.

Johnson (1957) pointed out that at temperatures well below the optimum, the reaction rates of many enzymes no longer fit the Arrhenius

equation but seem to have higher activation energies than expected, e.g., March & Militzer (1956) showed that $\Delta H^{\ddagger}$ for inorganic pyrophosphatase from B. stearothermophilis was 21,000 between 45 and 70°C, and 34,000 below 45°C. Kavanan (1950) suggested that these changes in activation energy may be caused by low temperature reversible inactivation of the enzymes. Langridge (1963) pointed out that heats and entropies of activation were negative for cold inactivation and positive for heat inactivation, which is compatible with the concept of Hydrogen bond formation at low temperatures and Hydrogen bond breaking at high temperatures. Over bonding due to an intra-molecular hydrogen bonding at low temperatures could alter the enzyme structure so that active centres lose their specific configuration or are no longer exposed to the substrate, and there is, therefore, a loss of enzyme activity.

Gaughran (1947) also suggested that the permeability of all membranes may be impaired at low temperatures, thus causing metabolic imbalance due to the unavailability of substrates. Gaughran (1945) showed that the lipids of a stenothermophilic bacterium were largely saturated at all temperatures of growth and that they solidified at 37°C. He said that this would inhibit transport into the cell and was incompatible with metabolic activity in the cell at low temperatures. Dyer (1953) and Long & Williams (1960) have both investigated fats in B. stearothermophilis, but have arrived at conflicting conclusions, neither attempting to support Gaughran (1945).

Imsenecki & Solnzeva (1945) suggested that thermophilic bacteria carried a bacteriophage which became more lysogenic as the minimum

temperature was approached until there was no apparent growth. But there has been no phage isolated to date.

F. Behaviour of Thermophiles at High Temperatures.

Although more work has been done on the behaviour of thermophiles at high temperatures than at low temperatures, a satisfactory explanation of thermophilic growth has not been found yet. In view of the present concepts of protein chemistry, the problem of how they can grow at high temperatures is bewildering.

The two main theories which are being experimentally investigated are:-

(1). that proteins in thermophilic bacteria are more stable to heat than are proteins of mesophilic bacteria. Or that proteins in thermophilic organisms are stabilised by some protective substance.

(2) that in the event of an essential enzyme being heat labile the cells have the capacity to replace this enzyme as rapidly as it is broken down. Thus the theory is that the cells live in a dynamic equilibrium (Allen 1950).

These two theories will be discussed in turn.

G. Stabilised Protein Theory.

In order to investigate this theory, several proteins have been isolated and purified.

Koffler (1957) purified flagellin from the flagella of a mesophilic bacillus and a thermophilic bacillus. Flagella are organized aggregates of flagellin, which is a fibrous protein of the keratin,

myosin type. On heating, and under other extreme conditions, they disaggregate and lose their structure. This disaggregation can be detected by measuring the change in viscosity which it causes. Koffler found that the thermophilic and mesophilic flagellins disaggregate at 70°C and 50°C respectively. By mixing the two solutions of flagellin and measuring the temperatures of disaggregation he showed that there was probably no protective substance stabilizing the thermophilic flagella. The viscosity curve of the mixture changed at two points, at 50° corresponding to the mesophilic protein and 70°C corresponding to thermophilic protein. The thermophilic flagellin was much more stable than the mesophilic flagellin under other conditions which normally break hydrogen bonds, e.g., it was stable to 6 M urea and 10 M acetamide. There were fewer titratable groups in the thermophilic flagellin than in the mesophilic flagellin but potentiometric titration curves indicated that there was an excess of free carboxyl groups in the protein. The thermophilic flagellin was more stable to sodium dodecyl sulphate, which breaks salt linkages, than was the mesophilic flagellin. Manning & Campbell (1961) purified α-amylase from B.stearothermophilis. The enzyme is relatively heat stable from any source, but the thermophilic enzyme was stable, after it had been crystallised, for 1 hour at 90°. It had an optimum temperature for activity between 50° and 70°C. Like thermophilic flagellin, this enzyme was very stable to acetamide and urea. It differed from other microbial α-amylases, and from beef heart α-amylase in that it contained 2 - 2.5 times as many carboxyl groups, was very poor in basic amino

acids, and had 4.5 times as much proline (Campbell & Manning 1961). Investigation of the physical properties of the molecule showed that the enzyme existed in an extremely high degree of hydration which may, according to Manning, Campbell & Foster (1961), account for its resistance to heat and other agents which break hydrogen bonds. A solution of α-amylase has a large negative optical rotation, a property characteristic of unfolded proteins. It contains 4 di-sulphide bonds. Considering the large negative value for the optical rotation, the behaviour in the presence of reagents which break hydrogen bonds, the stability to heat, and the high proline content, Campbell et al have suggested that α-amylase has a non-helical structure, and is a random or semi-random coil, with its secondary structure due to s - s linkages between the two peptides which are necessary for its activity.

B. M. Davis (as long ago as 1897) suggested that thermophiles survived at high temperatures due to low grade protoplasmic organization.

Although there are few data, it is interesting to note:-

(1) that thermophilic flagellin (Koffler 1957) and thermophilic α-amylase (Manning & Campbell 1961) both contain an unusually large number of free carboxyl groups. Helinski & Yanofski (1962) and Henning & Yanofski (1962), working with the A protein of tryptophan synthetase in <u>Escherichia coli</u>, found two mutants with heat stable A proteins. Investigation

of the amino acid content of these proteins showed that in both cases a glycine, present in the wild type A protein, had been replaced by a glutamic acid. When the glycine was replaced by arginine the enzyme was heat labile.

(2) that Ties & Gross (1960) showed that collagens from warm water fish had a much higher imino acid content (as does thermophilic α-amylase) than collagen from cold water fish. They showed that the former collagen had a higher critical temperature for hydrogen bond rupture than the latter. They stated that this was support for the hypothesis that the molecular structure of collagens is maintained in large part by restrictions on changes in the secondary structure of the polypeptide chain imposed by the pyrrolidine rings of proline and hydroxyproline. It will be interesting to see if any other thermostable enzymes are rich in proline and resistant to agents which break hydrogen bonds.

Marsh & Militzer (1956) purified inorganic pyrophosphatase (a heat stable enzyme) from B.stearothermophilis and from yeast. They compared the kinetics of heat inactivation in the two enzymes and found that the entropy change during the inactivation of thermostable inorganic pyrophosphatase was much less than for the yeast enzyme (13 cal/mol/degree for the former and 71 cal/mol/degree for the latter). This suggests that the native thermostable enzyme, like α-amylase of Campbell et al (1961), may have been a less organised molecule than the

yeast inorganic pyrophosphatase. In addition, they showed from the heats of inactivation, by applying the equation of Stearn (1949), that only half as many hydrogen bonds were broken in the heat stable enzyme as were broken in the heat labile enzyme. This may be interpreted to mean that, like α-amylase and flagellin, inorganic pyrophosphatase depends on factors other than hydrogen bonding for the secondary structure of the protein, or that (Marsh and Militzer, 1956) the hydrogen bonding in the thermophilic protein is more effective.

Brown et al (1957) continued the work on inorganic pyrophosphatase and showed that although the optimal temperature for the activity of the enzyme from B.stearothermophilis was always 60°C, the stability of the enzyme varied with the temperature of growth: the enzyme became less stable as the temperature for growth of the organism was lowered. Other enzymes which show variation in heat stability with the temperature of growth are α-amylase (Campbell & Cleveland 1961) and Malic dehydrogenase (Nobell & Thompson 1957). A possible explanation of this phenomenon was put forward by Langridge (1963) who suggested that different iso-enzymes may have different degrees of heat stability, and that different iso-enzymes predominate depending on the temperature of growth. Aldolase has been purified from B.stearothermophilis by Thompson et al (1958), and Thompson and Thompson (1962). This is a thermostable enzyme, and its stability does not vary with the temperature of growth of the organism from which it has been isolated. Akaji and Campbell (1962) have isolated and purified ATP sulphurylase from Clostridium nigrificans and found that it was stable for 2 hours

at 65°. But no further findings have been published yet.

Although several proteins from thermophilic micro-organisms have been isolated and found to be heat stable in the purified state (at least two of these are known to be extra cellular), there is evidence that the thermophilic cell may contain many inherently heat labile proteins as well, e.g. pyruvic oxidase was stable in the whole cells of B.stearothermophilis, but labile when the cells were lysed with lysozyme (Militzer et al 1954). Stewart (1953) showed that α-alanine racemase was stable at 80° in whole spores of an aerobic bacillus, or in extracts of the spores, but completely lost its thermo-stability when it was partially purified. It seems that these enzymes are being protected by association with particulate structures in the cell, or with their neighbouring proteins. In support of stabilization of enzymes by their close association with other proteins is the work of Morita & Haight (1962). They showed that in whole cells, malic dehydrogenase was unstable at 78°C at atmospheric pressure, but had maximum activity at 101°C at 1,300 atmospheres. Early workers suggested that the enzymes of thermophilic cells are stabilised with a factor which is attached to the enzyme. However, to date there is no positive evidence to support this hypothesis. There appeared to ~~be no factor which was responsible for the heat stability of α-amylase,~~ aldolase, pyrophosphatase or flagellin.

Nakamura (1960) studied catalase from B.stearothermophilis. This enzyme was bound to a suppressor factor which had to be removed

by heating the extract to 65°, or by shaking it with charcoal before the enzyme became active. However, in the absence of the suppressor factor, the catalase was active at 0°C and had an optimal temperature at 60°C. In the presence of the suppressor, the optimal activity of the enzyme was reached at 65°C. The enzyme was more stable at 70° in the presence of the suppressor than in its absence, so Nakamura suggested that the suppressor might incidentally be acting as a stabiliser for the enzyme. But other enzymes are known which have to be heat activated, e.g. catalase from silk worm and tumour tissue (Nakamura 1961) and DPN-ases from many mesophilic organisms (Swatz et al 1956). It seems more likely that these suppressors are cellular control mechanisms rather than stabilising mechanisms.

Oates et al (1961) claimed that there was a factor present in the medium in which a thermophile had been grown which would allow the growth of a mesophilic sporing bacillus at 65°C. It is known that there is a very high degree of autolysis during the growth of a thermophilic organism, so the medium after growth must contain a high concentration of proteins and nucleic acids. So it is conceivable that transformation, or transduction (if Imsenecki's (1945) hypothesis that phage is present in thermophilic bacteria be true) may have occurred. But since the mesophile took several weeks to grow, it seems equally likely that the medium which had been sterilised by Seitz filtration, became contaminated by a thermophile.

H. Dynamic Equilibrium Theory.

Allen's "dynamic" theory (1950) was an attempt to explain how the thermobiotic cells survived in the event of some enzymes being unstable at the temperature of growth. In support of her theory Allen (1950) showed that both thermophilic and mesophilic cells died at the same rate (i.e. could not reproduce) if they were incubated at 55° in buffer, or in buffer + glucose. But the thermophiles were able to reproduce if incubated in buffer + glucose + a rich nitrogen source, while mesophilic cells still died.

Further evidence in support of her theory was produced by Hancock (1957). Working with an unidentified thermophilic bacillus, he observed the effect on several enzymes of incubating the whole cells at different temperatures. He showed that at 55°, the activities of catalase and β-galactosidase were lost when the cells were incubated in buffer, or buffer and glucose, but preserved if incubated in buffer, glucose, complete amino acid mixture, and a purine - pyrimidine mixture. He also showed that these conditions were optimal for the synthesis of the adaptive enzyme maltozymase, which he followed by the increase in enzyme activity with time, and by the uptake of C(14)-glycerol by the cell. It is interesting to note the difference between the activation energies for enzyme synthesis and enzyme activity. Hancock (1957) found that enzyme synthesis did not proceed below 30° and increased sharply above 37°, whereas the activation energy of inorganic pyrophosphatase (see page 6) was markedly reduced for temperatures above 45°.

Baker et al (1956) studying the minimal medium for growth of 150 strains of B.stearothermophilis were surprised by the large number of growth factors which were necessary, and the very large molecules which were apparently transported whole into the cells, e.g. biotin, thiamine, folic acid, nicotinic acid and either methionine or cyanocobalamin. Furthermore, the cells could use nutrients in very high initial concentrations, pointing to very high osmotic tolerance. They thought that all these facts were in direct support of the "dynamic theory" because they enabled the cell to absorb "building blocks" whole, thereby increasing the rate at which the cell could replace its enzymes. Gale & Koffler (1957) point out that "Death may be due to only one essential enzyme being heat labile, but the cell must have shop, tools and blue prints for its enzyme to be repaired and kept in usable condition". They suggest that thermophiles differ from mesophiles in that these components (structural, neuleic acid, enzymic etc.) are more stable in thermophiles, for if the ability of thermophiles to survive is merely due to rapid synthesis, why can the mesophiles not survive, too? The Q_{10} ratio applies to them, too, so at 70° their metabolism should be 81 times as active as at 30°.

Gaughran (1945) showed that in thermophiles fatty acids were mainly saturated, and therefore very stable. Dyer (1953) showed that the phospho-lipids of B.stearothermophilis were mainly sphingomyelins which are very stable at elevated temperatures, but this was not supported by Long and Williams (1960) who found that the phospho lipids consisted mainly of lecithins.

Wellerson and Tetrault (1955) investigated RNA in bacterial cells during growth and showed in a temperature study that the RNA concentration per cell reached a maximum at the optimal temperature for growth of mesophiles, but remained constant in thermophilic cells from 45° to the optimal temperature, after which the concentration fell sharply. They said nothing of the stability of RNA in thermophilic cells.

I. Significance of Oxygen in Growth of Thermophiles.

There is one simple difference between the two theories outlined above which is amenable to experimental investigation. The dynamic equilibrium theory involves larger amounts of energy to replace the continually disappearing enzymes. It is therefore surprising that little interest has been shown in the sources of energy, and the effects of oxygen in the growth of thermophilic bacilli, although almost every author who works with thermophiles mentions at some time that oxygen in large amounts is necessary for a good yield of thermophilic sporing bacilli. Imsenecki and Selnzeva (1945) reported that observations on thermophilic bacteria made during many years showed that aerobic thermophiles reproduce only in thin layers of liquid medium. Allen (1953) stressed the increased demand for oxygen by organisms growing at elevated temperatures. This demand, she said, was due to decreased solubility of the gas at elevated temperature. Baker et al (1955) showed that the solubility of oxygen in liquid medium was only one half as great at 60°C as at 30°C. Long and Williams investigated the effect of aeration on the production of vegetative cells and spores

of *B.stearothermophilis* at 37°C and 55°C. They found that at 55°C aeration with 300 ml air/min/litre medium yielded good growth of the organism, but sparging with nitrogen allowed no growth.

Miltimer et al (1954) showed that the enzyme pyruvic oxidase was very heat labile in the non-particulate state, but could be stabilised by the presence of pyruvate and oxygen.

These results confirm that oxygen is very important in the growth of thermophiles.

This thesis is concerned primarily with the importance of aeration in the growth of *B.stearothermophilis*, a stearothermophilic sporing rod, and in the use of glucose and amino acids as energy sources by the organism. It also includes investigations of the effect of temperature upon the growth of *B.stearothermophilis*, glycolysis, and some of the enzymes concerned with glucose degradation. The possibility that glutamic dehydrogenase and lactic dehydrogenase, but not alanine dehydrogenase, may be heat stable because they are protected by a stabilising substance is discussed.

CHAPTER II: METHODS

I. Propagating Bacillus Stearothermophilis

The organisms were maintained on ten ml agar slope cultures. The medium used was liquid medium C (Chapter III), solidified with 2% agar. The organisms were transferred once a month.

II. Growing Bacillus Stearothermophilis

The organism was innoculated into 2 x 10 ml glucose-free broth cultures (medium C) from the stock slope culture. The broth cultures were incubated over night at 63.5°, or at the desired temperature. They were poured into 5 litres of the medium to be used, which had been brought to the temperature of growth. During growth, the pH was controlled by continuous titration with 2N KOH controlled by a Radiometer Type TTT1 Titrator. Air or gas mixtures were blown into the medium through a sparger, and a condenser was fitted to the outlet to prevent excessive evaporation at high temperatures. At completion of growth the cells were harvested by centrifuging for 20 min. at 10,000 g in an M.S.E. Sp.624 centrifuge. The cells were routinely washed three times in distilled water.

III. Plating Bacillus Stearothermophilis

Special precautions had to be observed to prevent plates from drying out when incubated at 63°C.

A thick layer of agar (glucose-free growth medium solidified with 2% agar) was poured into each plate, set at 4°, and dried at 37°. After stroking the culture on to the agar, the plate was tied into

a plastic bag which contained a beaker of water and a little solid $NaHCO_3$. The whole was immersed in a water bath at 63.5° for 24 hours.

IV. Growth Curves

20 ml of a 16 hr. glucose-free culture were poured into 5 litres of medium at 63.5°, or at the desired temperature. Five ml aliquots were withdrawn immediately after innoculation, at half hour intervals during the lag phase, and every ten minutes during the logarithmic phase of growth. The turbidity of these samples was recorded as their optical density at 700 mμ. When appropriate, a glucose estimation, a pH reading, and a total count of cells was done on each sample. At the end of the logarithmic phase the cells were harvested, washed and freeze dried.

V. Glucose Estimations

Proteins were precipitated from the medium with 5% $ZnSO_4.6H_2O$ and 0.3N Ba $(OH)_2$ as described by Nelson (1944). Glucose in the filtrate was estimated using the copper reagent of Somogyi (1952) and the ammonium molybdate-arsenate reagent of Nelson (1944). Optical densities were read at 530 mμ. Standard glucose solutions were included in every estimation, within the range 0 to 100 γ glucose/2 ml.

VI. Total Count of Cells

This was done by an adaption of the method of Jones & Mollison (1948). Immediately after a sample had been drawn from the culture it was diluted by a series of ten-fold dilutions in distilled water to an estimated number of 200 cells/ml. The last four of these

dilutions were mixed with an equal volume of 6% molten agar-agar at 55°. One drop of the culture in 3% agar was dropped on to the stage of a haemocytometer and a cover slip pressed over the drop, forming a layer 0.1 m.m. thick between the stage of the haemocytometer and the cover slip. When set, the film of agar was removed from the stage under water and placed on a microscope slide. The thick radial edges of the agar were removed and the thin film allowed to dry on to the slide for 5 hrs. at room temperature before staining. The stain used was a variation of Manival's containing Patent Blue instead of Aniline Blue. The agar film was soaked in this stain for one hour at room temperature, then washed rapidly in water, absolute alcohol and mounted in Xam.

The number of cells in 50 fields on each slide was counted. A count near 50 cells/field was considered the most accurate, and the dilution yielding this count was used to calculate the number of cells/ml. of culture. One field covered a volume of 1.1×10^{-6} cc.

VII. <u>To Find the Mean Generation Time for B.stearothermophilis</u>

The mean generation time was calculated from the equation

$$M.G.T. = \frac{(ta - tb) \log 2}{\log a - \log b} \quad \text{(Wilson \& Miles 1955)}$$

where a = total no. cells at time ta

and b = total no. cells at time tb,

both times being during the logarithmic phase of growth.

VIII. Growth Rate

The growth rate was expressed by the constant k which is defined by the equation $\frac{dA}{dt} = kA$ where A is the absorbancy of the culture, and t is the time expressed in hours. The values for k were obtained from the linear portion of the semi-logarithmic plots of growth (Walker & Campbell 1963).

IX. Preparation of Cell-Free Extracts

Four methods were used -

1. Cells were broken on a Nossal Shaker: Two gram wet weight of cells was suspended in 10 ml. 0.01 M. K^+ phosphate buffer pH 7.4. Ten ml. of the suspension was mixed with 7 gm ballotini glass beads and was shaken for 3 x 20 sec. bursts, allowing 5 min. cooling time in ice between each burst. This method was 70 % efficient. The glass beads were separated from cell material by low speed centrifuging.

2. Cells were broken by sonic waves: One gram wet weight of cells was suspended in 5 ml. K^+ phosphate pH 6.2 0.01M, and was subjected to sonic disintegration for 10 min. This method gave 80% breakage.

3. Cells were allowed to autolyse: Cells were suspended in a concentration of one gram wet weight in 5 ml. K^+ phosphate buffer pH 5.2. They were incubated for 1 hour at 37°. The pH was then adjusted to 7.0 with 0.1M Na OH and allowed to stand for a further 10 min. The efficiency of this method was 40%.

4. With Lysozyme as Bubela (1964): 10 gm. wet weight of cells was resuspended to 42 ml. with 0.13 M tris HCl pH 7.5, and made 0.8% with respect to Na Cl, and 0.19 x 10^{-2} M with respect to Mg Cl_2. The mixture was incubated for 5 min. at 37°, and 50 mg. lysozyme (Sigma product 3 times crystallized from egg-white) was added. After incubating for a further 15 min. at 37° the suspension was cooled in ice for 5 min., and the protoplasts so formed were disrupted by one of two methods:-

a. The suspension was diluted to 200 ml. with ice cold distilled water. The protoplasts were broken within 30 mins. at 0°.

b. 50 mg digitonin (British Drug House Laboratory Reagent) was suspended in 2 ml. distilled water and added to the protoplasts. 90% of the protoplasts were broken in 10 mins.

The per cent breakage of the cells was found by estimating the protein concentration in the cell suspension before breakage, and in the supernatant after cells had been removed by centrifuging at 18,000 g. for 10 min. after breakage.

I. Protein Estimations

Throughout this study, protein estimations were done by the Biuret method with bovine serum albumin as standard (Gornall, Bardawill & David, 1949).

XI. Paper Chromatography

All paper chromatography was done by descending chromatography. In every case the glass tanks were equilibrated by lining three sides of the tank with Whatman 3mm paper and pouring 500 ml. solvent over the paper lining and into the bottom of the tank. After the sample had been applied to the chromatography paper it was hung in the tanks for 5-6 hours to equilibrate before the solvent was added to the trough. The length of the paper was 17" and if the solvent was to be allowed to run off the end, the lower edge of the paper was serrated with pinking shears to facilitate drop formation.

1. To separate Fatty Acids: The solvents used were -

   a. Secondary Butanol : pyridine : acetic acid : water (90:4:1:15 by vol.)

   b. Secondary Butanol : 1.8N ammonia (3:1 by vol.)

   c. Ethanol : water : 7.5N ammonia (8:1:1 by vol.)

   d. Formic acid : water : pentanol (10:40:50 by vol.)

To stain for Fatty Acids:

The method of Paskova & Monk (1960) was used -

Reagent I 0.075 brom cresol green
0.025 brom phenol blue
dissolved in absolute ethanol.

Reagent II 0.5 $KMnO_4$
$Na_2CO_3 \cdot 10H_2O$
in distilled water.

The reagents were mixed in the ratio 1:1 immediately before use. The acids showed as coloured spots on a blue background when the reagent was sprayed on to the paper.

2. To separate Sodium Salts of Fatty Acids: The following solvents were used -

a. N. propanol : conc. ammonia (S.G. 0.880) (70:30 by vol.) (Barreto & Barreto 1961).

The chromatograph was run for 17 hrs.

b. Ethanol : water : 7.5N ammonia (8:1:1 by vol.)

Sodium salts of fatty acids were stained by the method of Barreto & Barreto (1961). The chromatogram was dried thoroughly at 120° for 15 min. and then dipped into 0.1% chloranilic acid in ethyl ether. The excess stain was removed by washing the paper in ether. The sodium salts stained yellow and were clearly defined under U.V. light. The separation of the fatty acids and sodium salts of fatty acids is shown in Table I. Usually, the fatty acids were run as their sodium salts to overcome losses of the volatile short chain acids.

3. Solvents used to separate Amino Acids:

a. Phenol saturated with water

b. n-butanol : acetic acid : water (4:1:5 by vol.)

c. Secondary butanol : 1.8N ammonia (3:1 by vol.)

Good separation of amino acids was obtained by two way chromatography. Solvent c was developed twice in the one direction, drying the paper between each development and then solvent b was used at right angles

Legend for Table I.

Chromatography of Fatty Acids

The relative rates of movement of fatty acids in solvents described in the text (Method XI 1 and 2) are shown as the R succinate values.

TABLE I.

SOLVENT	XI 1.a.	XI 1.b.	XI 1.c. XI 2.b.	XI 1.d.	XI 2.a.
Lactate	0.80	4.0	3.08		
β-Hydroxybutyrate	0.80		3.07		
Acetate	0.77	2.8	3.04		
Glycollic	0.49			.60	
Butyric		2.4			
Cis-Aconitic	0.40				
Malic	0.28		0.82	.49	
Fumaric		2.2		.13	
Pyruvic	0.16				
Malonic	0.41				
Citric			0.17		
Na - Lactate			2.5		5.1
Na -β-hydroxybutyrate			2.7		5.3
Na - Acetate			2.6		5.5
Na - Butyrate			3.7		7.2
Na - Propionate			3.2		6.3
Na - Fumarate			1.1		1.3
Na - Malate			.35		0.7
Na - Glycollate			2.04		3.8
Na - Formate					4.4
Na - Pyruvate					5.3

to the first direction. Figs. 12 and 13 show separation of amino acids in this way. The amino acids were stained by dipping the paper into a solution of 0.1% Ninhydrin

1.0% Acetic Acid

0.1% Cd acetate

dissolved in acetone

Aromatic amino acids stained purple, imino acids stained orange, and other amino acids stained pink.

4. Separation of Polysaccharides (Black et al 1958)

N-propanol :ethyl acetate : water (7:1:2 by vol.)

5. Separation of Monosaccharides

Ethyl acetate : acetic acid : water (3:1:3 by vol.)

When development was complete, papers were dried at room temperature in an air draught in a fume cupboard. Glucose and other reducing sugars were sprayed with aniline - oxalate spray (Black et al 1958). 0.9 ml. aniline in 100 ml. 0.1 N oxalic acid was sprayed on to the paper, which was then heated for 10-20 min. at 100-105°. Reducing sugars showed as brown spots.

Radio-active spots were detected by radio autography, and by scanning strips of the chromatograph in the gas flow strip counter. (Method XIII 3.)

XII. Column Chromatography

1. Separation of fatty acids on silicic acid (an adaption of the method of Kinnary et al, 1955).

The silicic acid (Mallinckrodt 100 x mesh obtained from Mallinckrodt Chemical Works, New York) was washed free from small particles by repeatedly shaking in distilled water and pouring off the particles which did not immediately settle. The gel was dried at 100° for 48 hr.

4.92 ml. 0.5N H_2SO_4 was added dropwise, with constant mixing, to 8 gm. dried silicic acid. At the end of the addition, the particles were still well separated. This was made into a slurry by mixing with 45 ml. of chloroform (which had been equilibrated with 0.05N H_2SO_4), poured into a 2 cm column and allowed to settle. The column was washed with benzene (pre-equilibrated with 0.05N sulphuric acid).

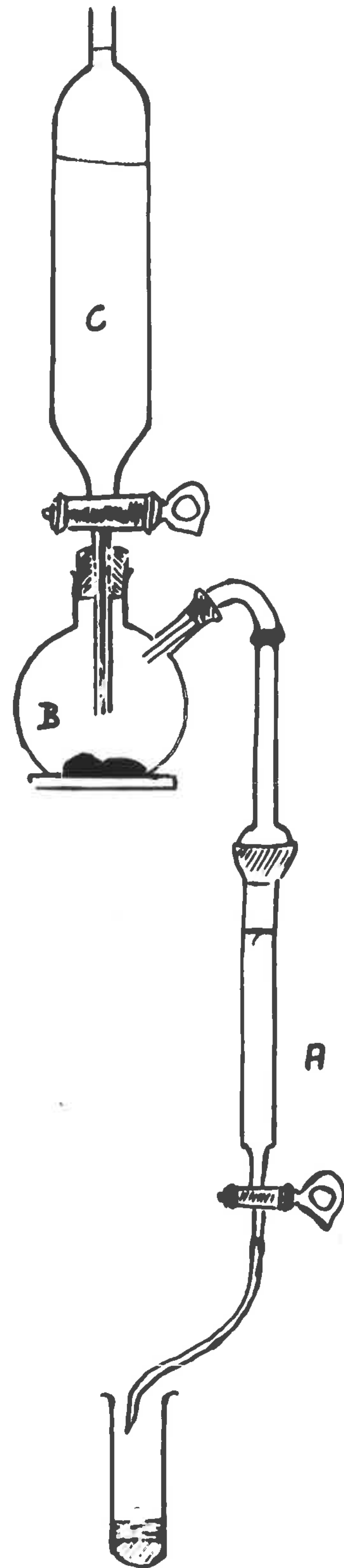
The carboxylic acids to be separated were pipetted on to an ashless filter paper pad which was dropped on to the top of the washed silicic acid column. The acids were separated and eluted from the column by a gradient of benzene to ether (both solvents being previously equilibrated against 0.05N H_2SO_4).

Fig. I shows the apparatus used.

Two ml. fractions were collected from the column into 2 ml. distilled water for the first 600 ml., and then 5 ml. fractions were collected for a further 500 ml. Each fraction was titrated with 0.003N Na OH (CO_2 free) using an automatic titrator (Radiometre type TTTI). The fraction was mixed with a stream of CO_2-free N_2 during the titration to facilitate partition of the organic acids from the organic solvents into the aqueous layer. The column was standardised by running known amounts of known acids on the column, singly, and in

Legend for Fig. 1.

Apparatus used for gradient elution of Carboxylic acids from a silica gel column. The carboxylic acids, on a disc of ashless filter paper, were placed on top of the gel in column A. Column A, flask B (capacity 250 ml.) and the tube joining the two were filled with benzene. Funnel C was filled with ether which was allowed to run slowly into flask B during development of the column. The contents of flask B were mixed continuously with a magnetic stirrer. The flow rate of ether into B determined the rate of flow from column A. Two ml. fractions were collected at the rate of 1 ml in 2 min. for the first 600 mls, and then 5 ml fractions were collected.


C
B
A

groups, in varying concentrations.

Fig. 2 shows the pattern of elution of the following acids: butyric, propionic, acetic, fumaric, pyruvic, succinic, lactic and β-hydroxy butyric, glycollic and malic. After elution and titration, the sodium salts of the acids were evaporated to a small volume, and their identity confirmed by paper chromatography.

2. Separation of Lactate from Glucose on a Dowex-1-Formate 200 - 400 mesh ion exchange column. (Dow Chemical Co., Midland, Michigan).

Preparation of Dowex-1-formate. Dowex-1 was stirred once with 2N Na OH for 30 min., then washed with distilled water until the pH of the water did not change when mixed with the resin. The Dowex was then stirred with 8N formic acid for 30 min. Excess formic acid was removed by washing with water. The resin was dried at 100° and stored in air tight bottles.

$C_{(14)}$-u-glucose + lactate, and glucose + $C_{(14)}$-2-lactate were placed in two columns 1 cm x 6 cm, washed well with 0.01M glucose, followed by 50 ml. water. The lactate was eluted from the column with 10 ml. 8N formic acid, or with 12 ml. 0.2M ammonium formate pH 4.0. Table 2 shows that the glucose and lactate were separated adequately, and that the 0.2M ammonium formate pH 4.0 gave a more quantitative return of lactate than did 8N formic acid.

3. Removal of Amino Acids from Neutral Solutions:

Neutral solutions were passed through Amberlite CG 120 100 - 200 mesh in the H^+ form. (Rohm & Hass, Philadelphia). Amino acids were

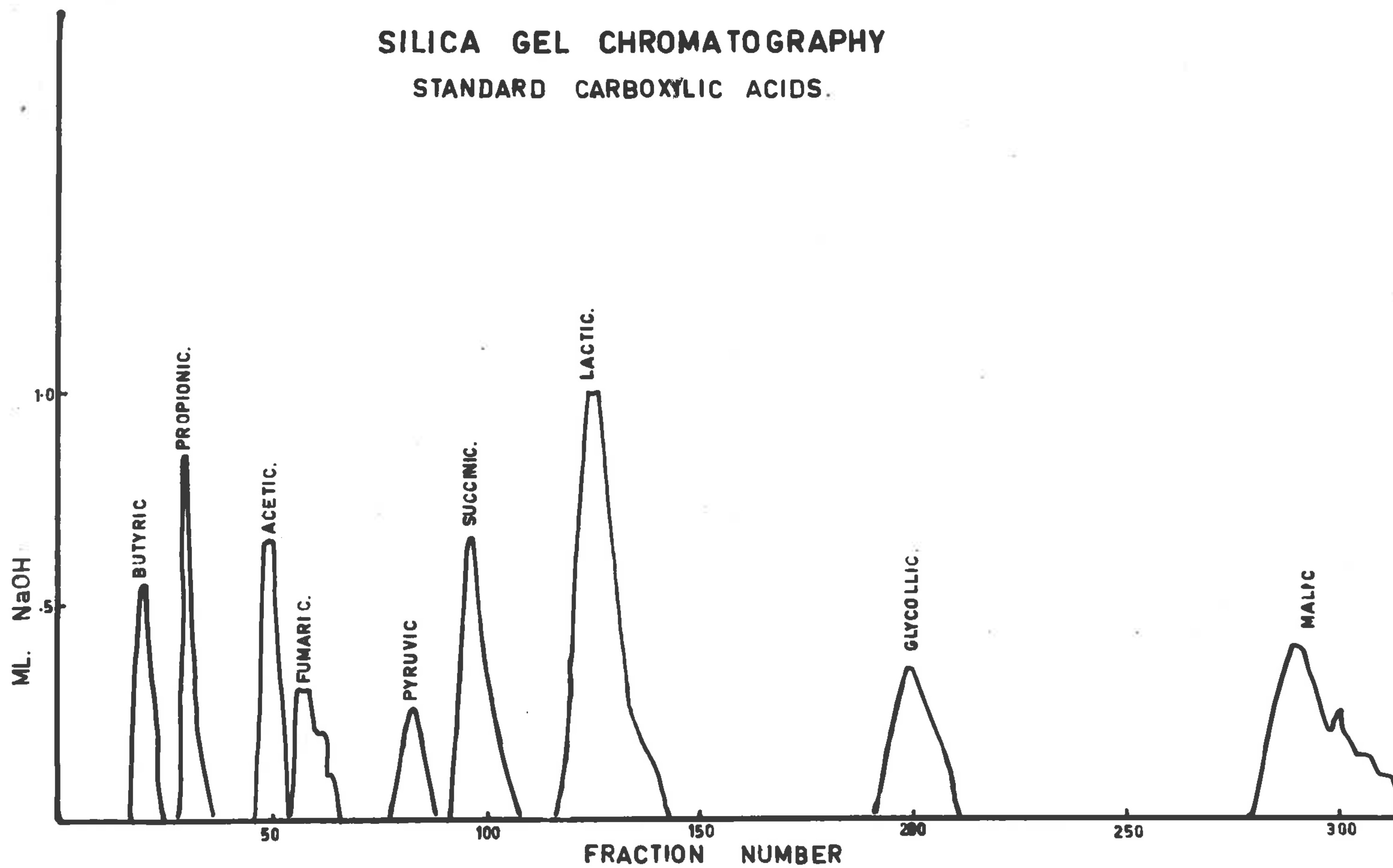
Legend for Fig. 2.

Chromatography of Carboxylic Acids

Known amounts of standard acids were separated on a silica gel column, with a benzene-ether gradient elution as described in Method XII 1.

5 µ	equivalents of		Butyric acid.
10 µ	equivalents of		propionic acid
12.5 µ	"	"	acetic acid
5 µ	"	"	fumaric acid
10 µ	"	"	pyruvic acid
20 µ	"	"	succinic acid
50 µ	"	"	lactic acid
12.5 µ	"	"	glycollic acid
10 µ	"	"	malic acid

were placed on the column. The 2 ml fractions collected were titrated against 0.003 N NaOH, and the amount of NaOH used was plotted against the fraction number.



SILICA GEL CHROMATOGRAPHY
STANDARD CARBOXYLIC ACIDS.
ML. NaOH
1·0
·5
BUTYRIC
PROPIONIC.
ACETIC.
FUMARI C.
PYRUVIC
SUCCINIC.
LACTIC.
GLYCOLLIC
MALIC
50
100
150
200
250
300
FRACTION NUMBER

TABLE 2.

Elution of ^{14}C - Lactate from a Dowex-1-Formate Column

	Eluted with 8 N formic		Eluted with 0.2M formate pH 4.	
	glucose	lactate	glucose	lactate
Glucose + $C_{(14)}$-2-Lactate (0.1 µc.)	0.004 µc	0.087 µc	0.001 µc	0.1 µc
$C_{(14)}$-u-Glucose (0.1 µc + lactate	0.104 µc	0.002 µc	0.101 µc	-

$C_{(14)}$-u-glucose + lactate, and glucose + $C_{(14)}$-2-lactate were placed on Dowex-1-formate as described in the text. The lactate was eluted from the column with 10 ml. 8 N formic acid or with 12 ml. 0.2M ammonium formate pH 4.0. The separation and recovery of glucose and lactate are shown.

eluted from the 6 cm. x 1 cm. columns of resin with 20 ml. 2N ammonium hydroxide.

XIII. Methods for Counting $C_{(14)}$ Compounds

All $C_{(14)}$ compounds were obtained from the Radio-Chemical Centre, Amersham, England.

Where possible, radio-activity was measured by liquid scintillation techniques.

1. Scintillation Counting:

The apparatus used was Ekco type N664 A Scintillation Detector (Ekco Electronics Co.) : Ekco type N530 Automatic Scaler Pulse Analyser Type N102 (Dynatron Radio Ltd., Maidenhead, Berks).

The photo multiplier was operated at 1100 volts, and the pulse analyser was set at a threshhold of 10 volts with a channel width of 30 volts. The scintillation detector was kept at 2°to reduce electronic "noise".

(1) The following liquid, an adaption of that of Bray (1960) was used except when counting glass fibre paper discs:

Naphthalene (British Drug House, A.R. Grade) 60 g.

2.5 di-phenyl-oxazole (P.O.P.) (Packard Inst. Co., Scintillation Grade) 4 g.

1,4 bis 2 - (Methyl 5 di-phenyl-oxazole) benzene (Packard Inst. Co., Scintillation grade) 0.2 g.

Methanol absolute (May & Baker, Laboratory Reagent) 100 ml.

Ethylene Glycol (British Drug House, A.R. Grade) 20 ml.
Dioxane (British Drug House, A.R. Grade) - up to 1 litre

For every sample, 5 ml. scintillation liquid was placed in a scintillation pot, and a background count was done for 300 sec.

An appropriate amount of $C_{(14)}$ labelled sample was added to the pot and counted for a further 300 sec. Then 50 µl Internal Standard ($C_{(14)}$-4-labelled Cholesterol in toluene = 0.01 µc/ml or 0.1 µc/ml) was added to the pot and counted for a further 300 sec. to detect quenching.

Results were expressed as µ curies based on the standard counts.

(2) To count $C_{(14)}O_2$ collected in 5N NaOH, a small volume was pipetted on to a disc of glass fibre paper 1" in diameter. The disc was dried under an infra red lamp for only the length of time necessary to ensure that the paper was quite dry. The dry disc was placed in the following scintillation liquid -

2:5 diphenyloxazole	3 g.
Toluene	1 litre.

5 ml. of liquid was used in a pot, the narrow neck of which had been removed to facilitate entry of the fibre disc.

2. End-window Counting of Powdered $C_{(14)}$ Samples:

To assay powdered samples labelled with $C_{(14)}$, e.g. $BaC_{(14)}O_3$, or

acetone dried $C_{(14)}$ labelled cells, the material was compressed into a 1 cm. planchet to form an infinitely thick layer, i.e. in excess of 30 mg. of material. The radio activity on the planchet was determined using a type N 620 shielded end window Geiger Müller tube (Ekco Electronics Ltd.). The count obtained for each sample was corrected for detector resolving time, and then for background radiation. The corrected counts were converted to absolute radio activities by using, for $BaC_{(14)}O_3$, a factor determined experimentally with $BaC_{(14)}O_3$ of known specific activity (Chick 1963) and for cell material by using a Standard Planchet containing $C_{(14)}$ perspex.

3. <u>Detection of $C_{(14)}$ Compounds after Paper Chromatography:</u>

a. Radio-active substances were placed on Whatman No. 1 paper in 1" bands, 2" apart. At completion of development the paper was cut into 1½" strips down the line of development. The radioactive areas were detected on these strips by passing them through an Actigraph 2 radioactive paper-strip scanner mounted with a type C14 windowless gas flow detector (Nuclear Chicago Corporation). A reference mark was made on the paper with a drop of a solution of $C_{(14)}$-4-Cholesterol.

b. On two dimensional chromatograms, and when large amounts of $C_{(14)}$-lactate or $C_{(14)}$-glucose were being purified by paper chromatography, the radioactive areas were detected by radio-autography. Reference marks were made in three corners of the chromatogram with radioactive ink. Kodirex No-Screen

X-ray film (Kodak (Australasia) Pty. Ltd., Melbourne) was exposed to the chromatogram in lead lined exposure holders. Exposed films were developed with Kodak D19 developer. The developed film was placed over the chromatogram so that the reference marks matched, and the radioactive areas were outlined on the chromatograms.

XIV. Preparation of $C_{(14)}$-3, 4-Glucose.

The method was adapted from the method of Bernstein et al (1955). A chicken was starved for two days, given a little feed at the end of the second day, then fasted till morning. The chicken was then given 250 mg. glucose by stomach tube. 30 min. later, another 250 mg. glucose was given and 70 µc $NaHC_{(14)}O_3$ in 2 ml. 0.1M Na HCO_3 was injected intro peritoneally. A further 250 mg. glucose and 70 µc $NaHC_{(14)}O_3$ were given every 30 min. for $3\frac{1}{2}$ hr., i.e. a total of 500 µc $NaHC_{(14)}O_3$ and 2 gm. glucose. The chicken was killed. The liver was removed into cold 60% perchloric acid, and homogenised in a Lourdes Homogeniser. After removing the protein precipitate by centrifuging, the glycogen was precipitated by pouring the supernatant into three volumes of cold ethanol. The glycogen precipitated from the ethanol while standing at 4°C for 4 hrs. The glycogen was collected by centrifuging, was washed twice with ethanol, twice with di-ethyl ether, and dried in vacuo. There was 0.6669 g. glycogen.

Radioactivity of the glycogen was found by counting at infinite thickness in an end-window counter. It was calculated that -

$$0.9 \times 10^{-3}\ \mu c/mg\ C_{(14)}\ \text{compound}$$

$$= 660\ \text{counts/min. at infinite thickness}$$

$$\text{Glycogen activity} = \frac{13,050}{660} \times 0.9 \times 10^{-3}\ \mu c/mg$$

$$= 1.97 \times 10^{-2}\ \mu c/mg$$

but there was 0.6669 g. glycogen

∴ there was a total of 13.2 μc glycogen

The glycogen was hydrolysed by dissolving it in 2N H_2SO_4 and heating in a boiling water bath for 1 hr. After cooling, the sulphate was removed with Ba CO_3. The precipitated Ba SO_4 was washed twice with water, and the washingswere added to the hydrolysed glycogen (supernatant).

The glucose was purified by paper chromatography, first in n-propanol: ethyl acetate: water (7:1:2 by vol.) and on a second chromatograph in ethyl acetate: acetic acid: water (3:1:3 by vol.). The glucose was located by radio-autography, and eluted from the paper with 50% ethanol in water. It was evaporated to dryness in a rotary evaporator at 30°.

To prove that the glucose was labelled in 3 and 4 positions, it was degraded to lactic acid by Lactobacillus casei as described by Wood et al (1945).

The Lactobacillus casei was grown in the following medium:

Glucose	1%
Yeast Extract	0.5%
Tryptone	0.5%
Sodium acetate	0.6%

50 ml. of a 24 hr. old innoculum were used in 1.5 litres of medium. The culture was grown for 24 hours at 38°C. The cells were harvested, washed three times in isotonic saline, and suspended in four times their weight of water.

The reaction mixture for degrading the glucose was

2 wet weight of <u>Lactobacillus casei</u>

0.06 m. Na H CO_3

0.5 g. Ca CO_3

purified glucose (4 μc and 200 mg.)

The final volume was 30 ml.

Fermentation was judged complete when there was no further evolution of gas from $NaHCO_3$ as lactic acid was formed. The cells were removed from the medium by centrifuging and the lactate was separated by ion exchange chromatography with Dowex-1-formate (Method XII 2). The lactate was purified by paper chromatography and degraded, as in Method XVII.

V. <u>The Analysis of the End Product of Glucose Metabolism after Growth.</u>

After completion of growth the cells were separated from the medium by centrifugation. The medium was made pH 8.0 with Na OH, and concentrated 50 times in a rotary evaporator. Five ml. of the concentrated medium was made 2.7 N with respect to H_2SO_4, keeping it cool in an ice bath. The acidified extract of the medium was intimately mixed with 8 g. celite (which had been previously washed with ether and dried at 100°C overnight) by grinding them together in a mortar. The mixture was transferred to a 38 m.m. soxhlet thimble and extracted with di-ethyl

ether for 24 hr. The ether was removed from the extracted material by evaporating under a stream of nitrogen, and the fatty acids were separated on a silicic acid column as Method XII 1.

XVI. Analysis of the End Products of Glucose Metabolism in Resting Cell Suspensions.

The apparatus used is illustrated in Fig. 3.

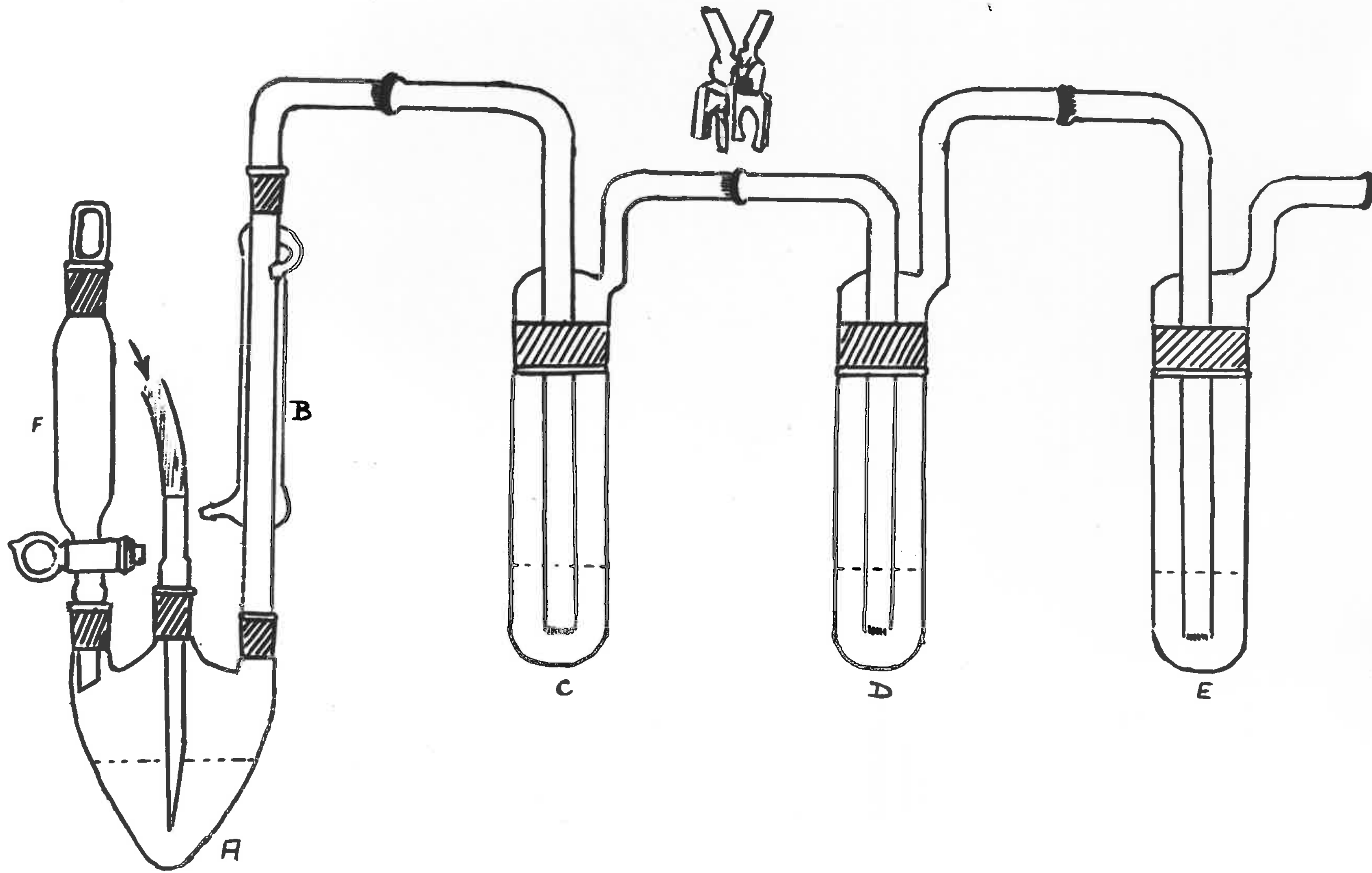
Into vessel A were placed 20 mg. $C_{(14)}$-u-glucose, 6 μc., 20 mgm. Ca CO_3 as buffer (Phosphate buffer interfered with chromatography later), 1 g. wet weight of cells and water to 30 mls. The mixture was incubated at 60°C, anaerobically, with a stream of oxygen-free N_2: CO_2(95:5) blowing through the solution. A condenser B was placed in the exit neck of the flask to prevent evaporation of water and volatile end products. The gas was bubbled through a sparger into cold ethanol in tube C to collect any volatile products which did escape through the condenser. The gas was then passed through two spargers in vessels D and E, both of which contained ethanolamine in methyl cellosolve (1:2 by vol.) to absorb CO_2.

After 20 min. vessel A was immersed in ice, and 3 ml. 5N perchloric acid was added from funnel F to stop the reaction. The system was sparged with N_2 + CO_2 mixture for a further 10 min. The methyl cellosolve and ethanolamine mixture was counted by scintillation methods (Method XIII 1 (1)). The ethanol in vessel C was added to vessel A and the whole was centrifuged to remove the cells and precipitated protein from the supernatant.

Legend for Fig. 3

Apparatus used for the analysis of the end products of glucose metabolism in resting cell suspensions.

The use of the apparatus is described in Method XVI.


F
B
A
C
D
E

The procedure carried through at the end of the reaction is illustrated in Fig. 4.

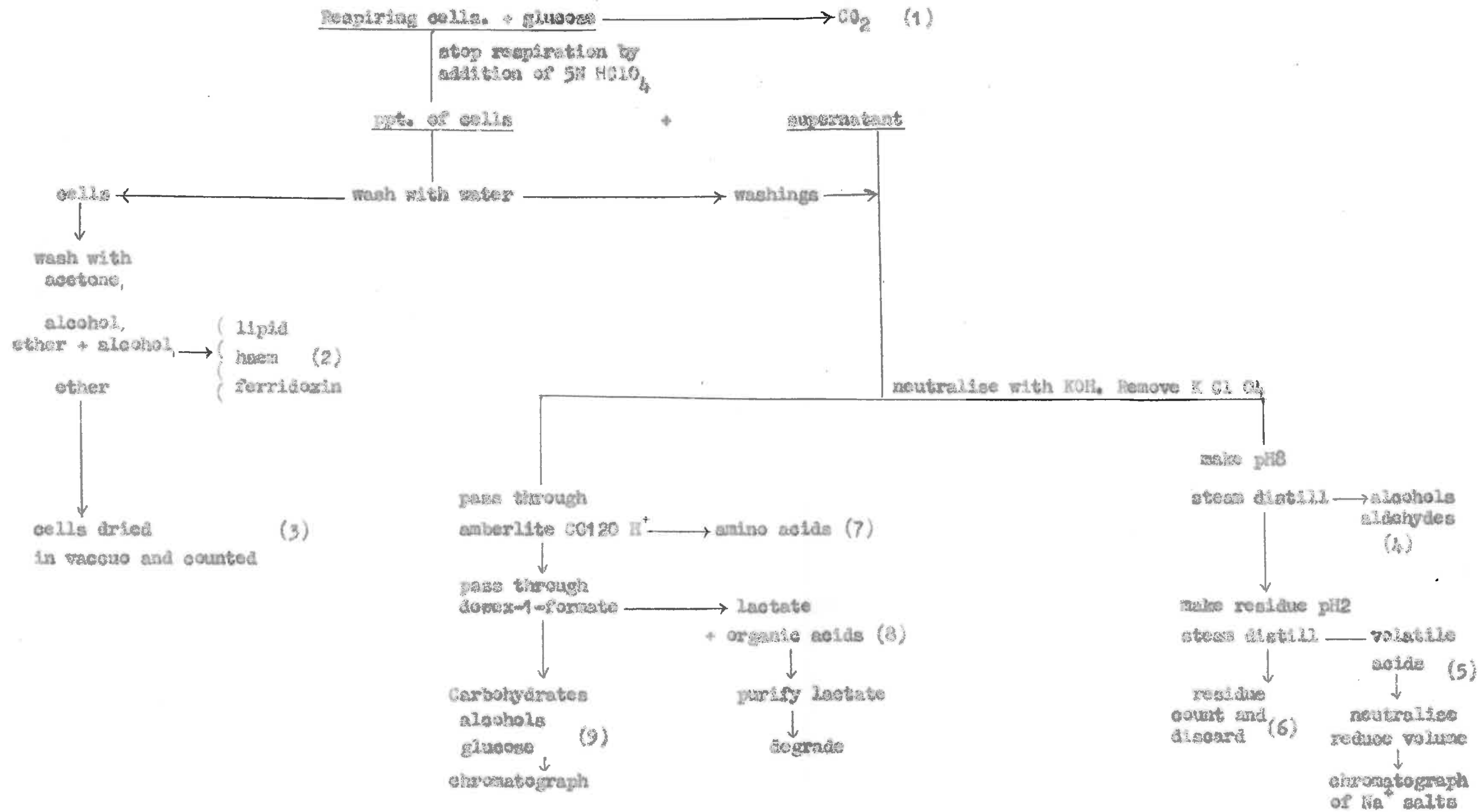
The cells were washed with two small volumes of water, which were added to the supernatant. The cells were then washed with acetone, ethanol, ether and ethanol, and ether, and dried in vacuo over caustic soda. The cells were analysed for radioactivity by end-window counting as in Method XIII 2. The acetone and ethanol washings were evaporated to a small volume under a stream of nitrogen, assayed for radioactivity by scintillation methods (Method XIII 1(1)), and investigated for lipid.

The supernatant was neutrallized with 5N KOH and left at $4^{o}C$ overnight to precipitate the potassium perchlorate. Half the supernatant was passed through Amberlite CG 120-H^{+} ion exchange resin to remove amino acids (Method XII 3), and then through a Dowex-1-formate column to remove lactic acid and other organic acids (Method XII 1). The sugars and alcohols which passed freely through both columns were chromatographed in n propanol: ethyl acetate: water (7:1:2 by vol.), and were investigated in the Actigraph 2 radioactive paper-strip scanner (Method XIII 3 a). Glucose was run as a marker and was stained with aniline-oxalate spray (Method XI 5).

The amino acids eluted from the Amberlite CG 120 with 2N NH_4OH, and the organic acids eluted from the Dowex-1-formate (Method XII 2) were assayed for radioactivity by scintillation methods (Method XIII 1a). The organic acids were then evaporated to dryness

FIG. IV.

Scheme for analysing the end products of glucose metabolism in a resting cell suspension of *B. stearothermophilis*.



on a rotary evaporator at 40°C. which removed the volatile organic acids, and the lactic acid which remained was purified by paper chromatography (Method XI 2b), and degraded to its constituent carbon atoms (Method XVII).

The second half of the supernatant was made pH 8 and steam distilled in a Markham still. This removed alcohols and other non-acid volatile substances. The distillate was examined for radioactivity, for $C_{(14)}$-ethanol, and for acetoin, 2, 3-butylene glycol and diacetyl (Methods XVIII and XIX).

The residue after distillation was made pH2 with H_2SO_4, and steam distilled again. The volatile organic acids were collected in 70 ml. distillate, which was adjusted to pH8 and reduced to a small volume in the rotary evaporator. The sodium salts of the volatile acids were chromatographed (Method XI 2b). The residue was assayed for radioactivity and discarded.

XVII. Degradation of Lactic Acid.

Lactic acid was degraded to acetic acid and CO_2 by the method of Katz et al (1955).

2 ml. 5% $KMnO_4$ in 2N H_2SO_4 was added to 1 ml. lactic acid containing 100 m Mole carrier lactic acid. The reaction was carried out in a clinbritic pot with a centre well cemented in place with Araldite plastic cement and the pot was closed with a skirted rubber cap. A slight vacuum was drawn, and the mixture was heated at 80° for 30 min. After cooling, NaOH or ethanolamine in methyl cellosolve was added to the centre well of the clinbritic pot to absorb $C_{(14)}O_2$.

The acetate was purified by steam distillation at pH 3. The distillate was neutralised and evaporated to dryness in a rotary evaporator at 30°C. The $C_{(14)}$-acetate was acidified with a little unlabelled acetic acid, dissolved in a small volume of ethanol, transferred to flask A of Fig. 3, readjusted to pH7 and dried. The acetate was degraded by the method of Phares (1951). 50 mg. Na Azide was added to the flask and 0.5 ml. 100% sulphuric acid (A.R. Sulphuric: fuming sulphuric 3:1 by vol.) was run in through the funnel F (see Fig. 3). The mixture was heated to 70°C in a water bath for 1 hr. The flask was cooled in ice and the CO_2 was swept into 5N NaOH or ethanolamine in methyl cellosolve (1:2 by vol.) in flask B and C with a stream of nitrogen.

5 ml. 10N KOH was added to flask A and the methylamine was distilled into 3 ml. of 0.4 $N.H_2SO_4$. The methylamine was degraded to CO_2 with chromic acid, and the CO_2 was collected either in 5N NaOH or in ethanolamine in methyl cellosolve.

$C_{(14)}O_2$ collected in ethanolamine was counted by scintillation methods (Method XIII 1a). $C_{(14)}O_2$ collected in 2N NaOH was made to a known volume and a portion was pipetted on to glass fibre paper and counted by liquid scintillation, as described in Method XIII 1b.

XVIII. Detection of Radioactive Ethanol in Aqueous Solution

Carrier ethanol (0.01 g. moles) was added to solutions suspected of containing $C_{(14)}$-ethanol. Iodoform was formed from ethanol according to the following equation:-

$$CH_3CH_2OH + 3I_2 + 4Na\ OH \longrightarrow CHI_3 + H.COONa + 3NaI + 3H_2O$$

For the reaction to go to completion two-fold excess iodine and six-fold excess NaOH was needed (Vögel 1959). Iodine solution in potassium iodide, and 10 N sodium hydroxide were added dropwise and alternately to the aqueous ethanol until there was no further precipitate of iodoform. The iodoform was removed from the solution by centrifuging, was washed once with a little cold distilled water, and dried in a dessicator over caustic soda. It was dissolved in a little chloroform and assayed for radioactivity by scintillation counting (Method XIII 1a).

XIX. Colour Test for Acetoin, 2:3 Butylene Glycol and Diacetyl.

To 5 ml. aqueous solution to be examined, 1 ml. of 0.5% aqueous creatine and 1 ml. of 5% α-naphthol were added in that order. (The α-naphthol was redistilled under nitrogen and dissolved in 2.5N NaOH immediately before use). The colour was allowed to develop for 10 min. for diacetyl, or for 1 hr. for acetoin and 2.3 butylene glycol (Westerfield 1945).

XX. Detection of Poly-β-hydroxy-Butyrate.

B.stearothermophilis cells were grown in an adaption of the medium of MacRae & Wilkinson (1958), i.e.

polypeptone	0.5%
yeast extract	0.3%
$K_2H PO_4$	0.3%
KH_2PO_4	0.1%
Na Cl	0.3%
Na_2SO_4	0.1%

$MgCl_2\ 6H_2O$ 0.01%

$MnCl_2\ 4H_2O$ 0.001%

acetic acid (glacial) 15 ml.

40% Na OH to neutralise

2% glucose sterilized separately.

Poly-β-hydroxy butyrate was extracted by the method of Law & Slepecky (1961). Cells were harvested after 7 hours' growth, washed once, then 14 g. wet weight was suspended in 1 litre sodium hypochlorite solution (prepared by the method of Williamson & Wilkinson, 1958). After 1 hr. at 37°C, lipid granules were centrifuged, washed in water, acetone and alcohol. Poly-β-hydroxy butyrate was dissolved by extraction with three small aliquots of boiling chloroform. The hot chloroform solution was filtered, and the filtrate used for assay. The chloroform was removed at 30°C under a stream of nitrogen, and 10 ml. conc. H_2SO_4 was added to the residue. This was heated for 10 min. at 100° in a boiling water bath, cooled, and after thorough mixing, a spectrum was run between 220 mμ and 280 mμ using H_2SO_4 as reference. β-hydroxy butyrate was converted to crotonic acid which absorbs at 235 mμ.

XXI. Thunberg Technique.

The method used was that described by Umbreit et al (1949).

In the main tube 0.2 mole Na^+ K^+ phosphate buffer pH 7.4, 0.1 m mole substrate and 2×10^{-6} mole redox dye (Methylene blue, 2, 6-dichlorophenolindiphenol, or Benzyl viologen) were placed.

One ml. cell suspension (1 gm wet weight in 8 ml. water) was placed in the side arm. The tube was evacuated for 10 min. on a water pump to remove all the oxygen. When the evacuated tubes were incubated at 60° the reaction mixture in the tube "boiled over" into the side arm. This was overcome by filling the tube with nitrogen : carbon dioxide mixture (95:5). The tubes were incubated with occasional shaking until the dye was 90% reduced; or for 2 hrs.

XXII. Assay Methods for Enzymes Investigated

1. Hexokinase (Goldman & Blumenthal, 1964)

This assay couples hexokinase with glucose-6-phosphate dehydrogenase.

Reaction mixture contained -

Tris - HCl pH 7:5	320 µ mole
$MgCl_2$	8.0 µ mole
NADP	1.0 µ mole
Glucose	1.0 µ mole
ATP	1.0 µ mole

Glucose-6-phosphate dehydrogenase

Enzyme, and water to 3 ml.

The reaction was followed by measuring the increase in optical density at 340 mµ as the NADP was reduced.

2. Glucose-6-Phosphate Dehydrogenase (Goldman & Blumenthal, 1964)

The reaction mixture contained -

Glucose-6-phosphate	1.0 μ mole
Tris - HCl pH 7.5	320 μ mole
NADP	1.0 μ mole

Enzyme, and water to 3 ml.

The reaction was followed by measuring the increase in optical density at 340 mμ as the NADP was reduced.

3. Phospho Hexose Isomerase

a. To measure the rate of the enzyme action when catalysing fructose-6-phosphate ⟶ glucose-6-phosphate the system was linked with glucose-6-phosphate dehydrogenase and the rate of reduction of NADP followed at 340 mμ.

The reaction mixture contained -

Tris HCl pH 7.5	320 μ mole
NADP	1.0 μ mole
Fructose-6-Phosphate	8.0 μ mole

Crude Enzyme and water to 3 ml.

It was found that B. stearothermophilis contained a very active glucose-6-phosphate dehydrogenase so crystalline glucose-6-phosphate dehydrogenase was not added.

b. To measure the rate of enzyme action when catalysing the reaction glucose-6-phosphate ⟶ fructose-6-phosphate the following method was used (Stanbury 1961)

Glucose-6-Phosphate	2 μ mole
Tris H Cl. pH 7.4	20 μ mole

Crude enzyme and water to 1.5 ml.

The reaction was stopped by adding 3.5 ml. 8.3N HCl followed by 1 ml. alcoholic resorcinol (Roe (1934)). The mixture was heated to 80°C for 10 min., cooled and the optical density read at 540 mμ. Phospho hexose isomerase was measured by the rate of formation of fructose-6-phosphate from glucose-6-phosphate.

4. Glucose dehydrogenase

The reaction mixture contained -

Tris HCl pH 7.5	320 μ mole
Glucose	1.0 μ mole
NADP or NAD	1.0 μ mole
Enzyme, and water to 3 ml.	

The reaction was followed by measuring the rate of reduction of NADP or NAD at 340 mμ.

5. Aldolase (Goldman & Blumenthal 1964)

This assay is based on the conversion of dihydroxyacetone phosphate to glycerophosphate with an accompanying oxidation of $NADH_2$. The assay mixture contained -

Tris HCl pH 7.5	320 μ mole
Fructose 1-6 di Phosphate	1.0 μ mole
Enzyme, and water to 3 ml.	

After incubating for 10 min. at the required temperature, the reaction was stopped by lowering the pH to 4 with HCl, and boiling for 10 min. This was done to free the mixture of a very active $NADH_2$ oxidase. Precipitated protein was removed by

centrifugation. The pH was raised to 7.5 with NaOH. 3.0 µ mole $NADH_2$, commercial crystalline $\mathcal{L}$-glycerophosphate dehydrogenase and triose phosphate isomerase were added. The amount of $NADH_2$ oxidised was a measure of the activity of aldolase. A change in optical density of 0.207 at 340 mµ is equivalent to 0.1 µ mole $NADH_2$ oxidised.

6. Phospho-Fructo-Kinase.

This was linked with the aldolase assay above. Added crystalline aldolase ensured the breakdown of fructose 1,6-di-phosphate to triose phosphate as it was formed.

The reaction mixture contained -

Tris HCl	pH 7.5	320 µ mole
$Mg\ Cl_2$		8.0 µ mole
ATP		1.0 µ mole
Fructose-6-phosphate		8.0 µ mole

Crystalline rabbit muscle aldolase.

As for the aldolase assay the reaction was stopped after 10 min. by lowering the pH to 4, boiling for 10 min. and centrifuging to remove precipitated protein. The amount of dihydroxyacetone phosphate formed was determined by measuring the oxidation of $NAD\ H_2$ at 340 mµ in the presence of added triose phosphate isomerase and glycerophosphate dehydrogenase.

7. Pyruvic Kinase.

The activity was measured by the method of (Bücker &

Pfleiderer 1955). The pyruvate formed by the action of the enzyme was reduced to lactate in the presence of an added excess of crystalline lactic dehydrogenase and $NADH_2$. The rate of oxidation of $NADH_2$ was measured by recording the decrease in optical activity at 340 mμ.

The reaction mixture contained -

$NADH_2$	0.45 μ mole
Phospho enol pyruvate	2.34 μ mole
ADP	0.69 μ mole
$Mg\ SO_4$	24 μ mole
K Cl	220 μ mole
Tris HCl. pH7.6	150 μ mole

Crystalline lactic dehydrogenase 18,000 units/mg.

This enzyme was not always added as the organism contained a very active lactic dehydrogenase.

8. Lactic Dehydrogenase.

(1) When measuring the activity of the enzyme catalysing the reduction of pyruvate the reaction mixture contained -

Na pyruvate	2.34 μ mole
Tris HCl pH 7.6	150 μ mole

Enzyme, and water to 3 ml.

The oxidation of $NADH_2$ was measured spectrophotometrically at 340 mμ.

(2) A second assay method was used to follow the oxidation of lactate and the accompanying reduction of NAD at 340 mμ. The reaction mixture contained -

Na lactate 50 μ mole

NAD 2 μ mole

and glycine + Na OH buffer pH 9.6 180 μ mole.

To draw the reaction to completion for the estimation of lactate the glycine buffer was replaced by the following:-

3.8 g. glycine, 5 ml. hydrazine hydrate, 2 ml. 0.1M EDTA and 8.0 ml. N.Na OH was made to 100 ml., 1.5 ml. of this mixture was used per cuvette instead of the glycine buffer used above (Holdsworth 1964).

9. Alcohol Dehydrogenase - was measured by the method of Caiger et al (1962).

The reaction mixture consisted of 4.7 g. glycine, 2.5g. NaOH and 7.3 ml. of twice distilled ethanol, made to 150 mls. with water.

1.8 ml. of this was used per cuvette

2 μ mole NAD was added

and crude enzyme and water made the volume to 3 mls.

The activity of the enzyme was followed spectrophotometrically by recording the reduction of NAD at 340 mμ.

10. 6-Phosphogluconic Dehydrogenase

The reaction mixture contained -

6-Phosphogluconic acid		1.0 μ mole
Tris HCl	pH 7.5	320 μ mole
NADP		1.0 μ mole

Cell-free extract and water to 3 mls.

The activity of the enzyme was followed spectrophotometrically at 340 mμ as the NADP was reduced to $NADPH_2$.

11. <u>Glutamic Dehydrogenase</u> (Holdsworth 1964)

The activity of this enzyme was measured by the reduction of 4,5 di methyl thiazoloyl tetrazolium bromide (MTT) in the presence of glutamic acid and NAD.

The reaction mixture consisted of

Na^+ K^+ $PO_4^{\equiv}$ buffer pH 8.0	0.025 m mole
NAD	3 μ mole
Na glutamate	0.2 m mole
MTT	0.4 mgm

Crude extract of <u>B. stearothermophilis</u>, and water to 2 ml.

After incubating the mixture for 3 min. the reaction was stopped by the addition of 0.2 ml. T.C.A. (40% W/V) and 5 ml. 5% pyridine in dioxan (v/v). After removing the precipitate by centrifuging the colour was read at 580 mμ. The activity of the enzyme was proportional to the optical density.

Other amino acid dehydrogenases were assayed by replacing the glutamate by the same concentration of the appropriate amino acid.

CHAPTER III: SELECTION OF GROWTH MEDIUM

When looking for a suitable medium in which to grow B. stearothermophilis for our purposes, the following points were considered.

a. The medium should be complete in order that the cells would have their full number of constitutive enzymes.

b. It should support rapid growth of cells in a good yield.

c. The pH should remain constant throughout growth. It was soon found that if the pH were allowed to drop to pH5.5 the cells rapidly lysed on exhaustion of the carbon source.

d. The medium should be simple to prepare, and inexpensive.

Medium A.

The medium in which the organism was originally being kept in our department was Medium A. It had the following composition -

Sodium citrate	3%
KH_2PO_4	0.75%
$MgSO_4\ 7H_2O$	1%
$CaCl_2$	0.375%
Na molybdate	0.125%
m-inositol	0.025%
Choline-Chloride	0.005%
Thiamine - HCl.	0.005%
p-amino Benzoicacid	0.0025%
Tween 80 (10%)	25 ml.%
Metal solution	25 ml.%
Sucrose	3%

Difco casamino acids	2%
dl-Tryptophan	0.05%

<u>Metal Solution</u>

EDTA	5 g.
$Mn\ SO_4\ H_2O$	6.15 g.
$Zn\ SO_4\ 7H_2O$	11 g.
$Fe\ SO_4\ 7H_2O$	1 g.
$Co\ SO_4\ 7H_2O$	0.3 g.
$CuSO_4\ \ 5H_2O$	0.04 g.
H_2BO_3	0.06 g.
KI	0.001 g.
$(NH_4)_6\ Mo_4O_{24}$	0.06 g.

made to 2 litres.

In medium A, growth was very sparse after 24 hours at 55°. The medium was not simple to prepare.

<u>Medium B1.</u>

Allan (1954) stressed that growth at elevated temperatures needed a good supply of oxygen, and a high concentration of salts, especially divalent ions, and in particular Ca^{++}.

Medium B1 was made to meet these requirements: it had the following composition.

casein hydrolysate	0.5%
peptone	1.5%
yeast extract	0.5
KH_2PO_4	0.1%

$K_2H\ PO_4$	0.1%
NaCl	0.2%
$MgSO_4$	0.1%
$MnCl_2$	0.0005%
$CaCl_2$	0.1%
Metals Solution as for medium A	
Mg acetate	0.1%
Glucose	1%

To prevent oxidation of glucose, and precipitation of Ca^{++} salts, these solutions were autoclaved separately, and added to the bulk of the medium immediately before it was cultured.

Growth in medium B1 yielded a much more dense suspension of cells after 24 hr. at 55° than medium A had done. Using medium B1, experiments were done to find the optimal temperature for growth, i.e. the greatest yield of cells in the shortest time.

For this purpose 1 litre medium was innoculated with 10 ml. culture grown overnight at the temperature to be investigated. The culture was aerated with air, and samples were removed hourly to measure the optical density. The organism was grown at 55°, 60°, 62°, 63°, 64°, 65°, 68°. It was decided that the organism should be grown at 63.5°, and an estimate of the mean generation time (M.G.T.) of the organism at this temperature, and in this medium was found (Methods VI and VII).

The M.G.T. was 22.5 min. in this medium at 63.5°C. The pH fell rapidly during growth in this medium, and the cells lysed when pH5.2 was reached.

Medium B2.

In order to prevent the pH from falling, medium B2 was buffered with 10% solid $CaCO_3$ and the 0.1% $CaCl_2$ used in medium B1 was omitted. In medium B2 the M.G.T. was 14.4 min, and the pH did not fall until late in the logarithmic phase of growth. Subsequent work suggested that the more rapid growth in the presence of Ca CO_3 may have been due to the ample supply of CO_2 in medium B2. This subject is discussed in Chapter IV D 2.

Adding solid $CaCO_3$ to the medium was not a satisfactory way to control the pH, however, as it could not be separated easily from the cells when they were harvested, and much of the phosphate in the medium probably precipitated as calcium salts.

Medium B3.

This variation used only 0.05% $CaCl_2$, and 2% Na β-glycerophosphate as buffer. With medium B3 it was found that the M.G.T. was 8.4 min, and the pH dropped slowly during growth. However the Ca^{++} salts still precipitated out on incubation at 63.5°.

Medium B4.

In this medium, citrate was added as a chelating agent to try to keep the Ca^{++} salts from precipitating. The medium had the following composition.

polypeptone	2%
yeast extract	80 ml/l.
Na lactate	0.5%
glucose	0.5%

KH_2PO_4	0.1%
$K_2H\ PO_4$	0.1%
$MgSO_4$	0.1%
Mg acetate	0.1%
Na citrate	0.1%
$Ca\ Cl_2$	0.005%
Na β-glycero phosphate	2%
Metals (as medium A)	10 mls/l.

The Ca^{++} salts did not precipitate out appreciably, and the pH dropped at the end of the logarithmic phase of growth. The M.G.T. was 12 min., and 3 g. wet weight of cells were obtained per litre of medium.

Medium C

At this stage, it was decided that automatic control of pH would enable a simpler medium to be used. A Radiometer type TTTI titrator was obtained and medium C, an adaption of the medium of Long & Williams (1959) was prepared as follows -

Polypeptone	0.5%
Basamin Busch	0.3%
$K_2H\ PO_4$	0.3%
$KH_2\ PO_4$	0.1%
glucose	0.2%

The pH was kept at 6.8 by titrating the medium with 2N KOH during growth.

Growth in this simply prepared medium was rapid, and the yield could be increased by adding more glucose at intervals during growth.

Basamin-Busch, a yeast autolysate, has a high concentration of inorganic salts. It was therefore unnecessary to supplement the medium further.

In medium C the M.G.T. was 4.5 min, and there was a yield of 14 g. wet weight of cells. When the glucose was exhausted there was rapid lysis of the cells, but this could be prevented by adding more glucose while harvesting. However this was not practicable in most of this work as we were concerned with glucose metabolism and wanted to know the exact amount of glucose present in our cells during the experiments.

Therefore, medium C was chosen for the work reported in this thesis, either with or without the glucose, designated respectively medium C and glucose-free medium C.

CHAPTER IV: GROWTH AND ENERGY METABOLISM

A. Growth in the presence of Glucose

1. The Effect of Oxygen on Growth of B. stearothermophilis

It was mentioned previously that almost every author who has published work with B. stearothermophilis has stressed that oxygen in very large amounts is necessary for good growth of the organism. Allen (1953) mentioned that thermophiles can grow in the absence of oxygen providing that glucose is present. We found that our strain of B. stearothermophilis grew in a still deep culture in the presence of glucose at 60°. We therefore asked, "How important is oxygen for the growth of B. stearothermophilis at 63.5°?"

The questions we wanted to answer were

(1) would the cells grow in the absence of oxygen, or with very little oxygen and

(2) if so, whether the metabolism of the cells differed markedly under aerobic and anaerobic conditions.

The first question could be answered simply by doing a series of growth curves in different O_2 tensions, noting the rate of increase in cell population.

The second question could be partially answered by correlating growth rate with glucose utilisation, and by examining the end products of glucose metabolism to see if they differed in the presence and absence of oxygen.

A series of growth curves was done, all in the same medium (Medium C, Chapter III) but aerating with different concentrations

of oxygen. Five litres of medium were used for each growth curve, and each was sparged with a known mixture of nitrogen, carbon dioxide, and oxygen.

Oxygen-free nitrogen + carbon dioxide (95:5) was supplied (with a laboratory analysis report) by the Commonwealth Industrial Gases Ltd., Bourke Road, Alexandria, Sydney, Australia. Carbogen (oxygen: carbon dioxide 95:5) was obtained from Commonwealth Industrial Gases Ltd., Jervois Street, Torrensville, S. Australia.

The culture was sparged with 51. gas/min. and the proportion of gases used was measured in two flow meters. The proportions were:-

1. 100% nitrogen + carbon dioxide mixture
2. 80% nitrogen + carbon dioxide, 20% carbogen
3. 60% nitrogen + carbon dioxide, 40% carbogen
4. 0% nitrogen + carbon dioxide, 100% carbogen
5. air
6. 100% nitrogen (free of oxygen and of carbon dioxide)

When growing in an atmosphere of 100% carbogen, the culture was incubated in a flask, the bottom of which consisted wholly of scintered glass. The gas was forced through the scintered glass, and left no doubt that the medium was thoroughly oxygenated.

Samples taken at intervals during growth were assayed for remaining glucose (Method V), and the absorption at 700 mμ was measured (i.e. a measure of the number of cells /ml. of medium). After harvesting, the cells were freeze dried, stored over phosphorus pentoxide for 16 hours, and weighed. The amount of 2N KOH necessary

to keep the pH constant during growth was noted, and the medium was examined for end products of glucose metabolism as described in Method XV. Fig. 5 shows the graphs of growth (% absorption of the medium at 700 mμ plotted v. time) and glucose utilisation (expressed in mg./100 ml medium v. time), when cultures were sparged with gases containing different amounts of oxygen. The growth rate "k", as defined in Method VIII, was obtained from the slope of the growth curve. It can be seen that neither "k", nor the rate of utilization of glucose changed by increasing the oxygen from 0 to 40%. However, in 100% carbogen "k" was less although the total yield of cells was greater. There was no growth at all if the culture was sparged with CO_2 free Nitrogen.

Table 3 summarises the data obtained from these growth curves. It shows that the length of the lag phase and of the logarithmic phase do not vary very much with changes in oxygen concentration. The percentage glucose converted to acid was calculated from the buffering capacity of the medium and the amount of KOH necessary to keep the pH constant during growth, assuming that 2 mole mono-carboxylic acid were formed from 1 mole glucose. The amount of glucose converted to acid, according to this calculation, did not vary significantly up to 40% oxygen, but was slightly less in 100% oxygen. This latter finding might be due (partly) to chemical oxidation of glucose. However, twice the dry weight of cell material was produced aerobically with carbogen as was produced anaerobically.

Legend for Fig. 5.

The growth and utilisation of glucose in varying concentrations of oxygen.

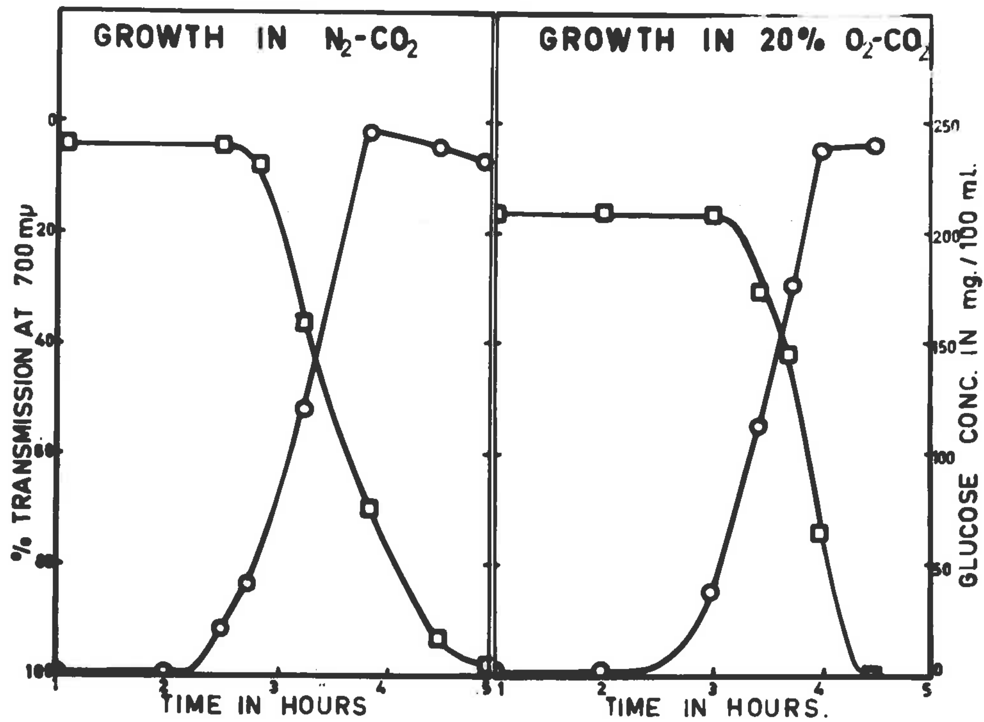
B. stearothermophilis was grown in Medium C Chapter III. Cultures were gassed at the rate of 5 l./min/5 l. medium.

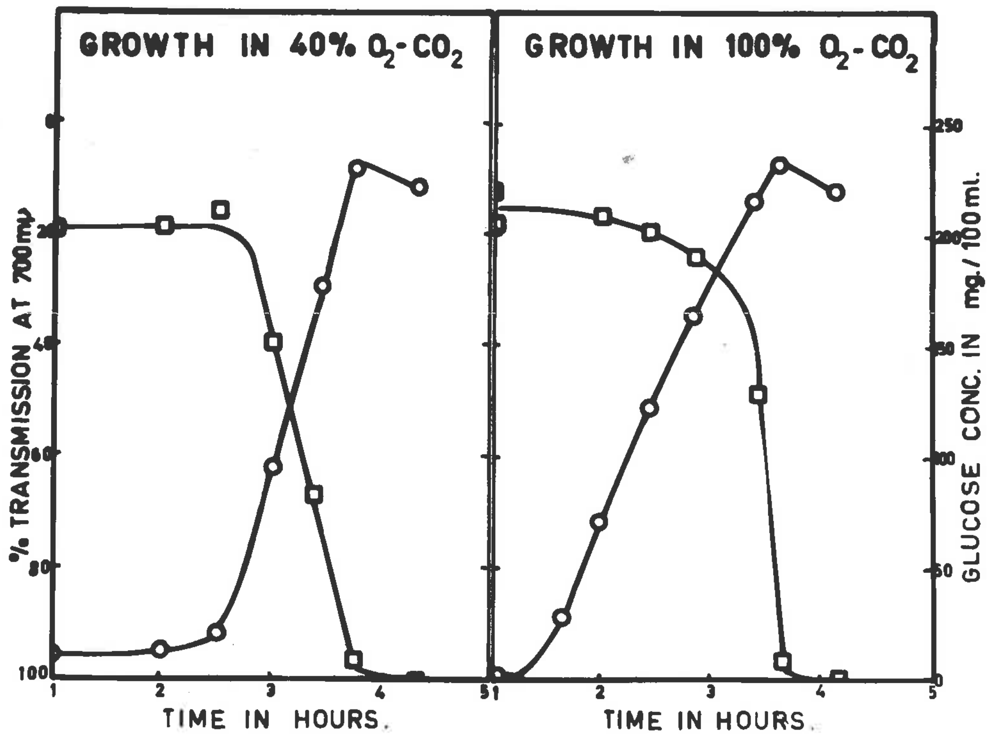
Samples were taken at intervals, and the % transmission measured at 700 mμ. The glucose concentration was estimated on each sample by Method V.

The % transmission at 700 mμ (an estimate of the cell density) and the glucose concentration in mgm/100 ml medium were plotted against time of growth in hr.

O—O—O = % transmission at 700 μm

□—□—□ = glucose concentration in mgm/100 ml medium.


GROWTH IN N2-CO2
GROWTH IN 20% O2-CO2
% TRANSMISSION AT 700 mμ
GLUCOSE CONC. IN mg./100 ml.
TIME IN HOURS
TIME IN HOURS.


GROWTH IN 40% O2-CO2
GROWTH IN 100% O2-CO2
% TRANSMISSION AT 700mμ
GLUCOSE CONC. IN mg./100ml.
TIME IN HOURS.
TIME IN HOURS.

Legend for TABLE 3.

The length of the lag phase, and the logarithmic phase were calculated from Fig. 5, the graphs of growth rates, and glucose utilisation plotted against time.

The amount of 2N KOH necessary to keep the pH constant throughout growth was measured, and the percentage glucose converted to acid was calculated from the amount of KOH used, assuming that 2 mole mono-carboxylic acid is produced from 1 mole glucose.

The dry weight of cells was obtained by freeze drying the cells after harvesting and washing, drying over phosphorous pentoxide for 16 hr., and weighing. The weight of dried cell material per 1 gm mole glucose was calculated from the results obtained.

The growth rate "k" is defined in Method VIII.

TABLE 3.

The Effect of Oxygen on the Growth of *B. stearothermophilis*

	100% N_2-CO_2	80% N_2-CO_2 20% Carbogen	60% N_2-CO_2 40% Carbogen	100% Carbogen
Length of lag phase	1.75 hr	2.5 hr.	2.25 hr.	2.5 hr
Length of log phase	1.5 hr	1.5 hr.	1.5 hr.	2.25 hr.
Amount Glucose used	9.5 gm.	11 gm.	10 gm.	10 gm.
Amount KOH used	4.0 gm.	4.3 gm.	4.0 gm.	-
∴ Amount Glucose converted to Acid	86%	81%	82%	-
Dry weight cell material produced	1.1 gm.	1.4 gm.	1.8 gm.	2.3 gm.
Weight cell material per 1 gm mole glucose (Y glucose)	21.1 gm.	23 gm.	32.4 gm.	41.5 gm.
Growth Rate, "k"	0.83	0.81	0.90	0.92

After the cells had been harvested, the end products of glucose metabolism were isolated from the media in which they had been grown. The carboxylic acids produced during growth were isolated (Method XV) and separated on silica gel columns (Method XII 1).

Figs. 6, 7, 8, illustrate the separation of the organic acids produced under the different conditions. Table 4 shows the relative amount of each acid formed at each concentration of oxygen. An extraction of the medium before growth showed no appreciable amount of any organic acid present. The acids were identified by comparing the pattern of elution from the columns with the pattern obtained with a set of standard acids (Fig. 2). The principal acids which were eluted were lactic and acetic. The identity of these acids was confirmed by paper chromatography (Method XI. 2); Lactate was also confirmed enzymatically by showing that it was a substrate for commercial crystalline lactic dehydrogenase (Method XXII. 8. 2). A third small peak, present in extracts of all the media after growth may have been succinic acid. Fig. 8 shows that when succinic acid was added to the column as well as the unknown acids, the third peak did not separate from the succinic acid, which probably indicates that the third peak was identical with succinic acid. A fourth peak, representing an acid which was only present when the organism was grown in 100% carbogen, was eluted from the column at the same place as pyruvic acid. There was no confirmation of this identity.

The results of the analysis of the acidic end products of metabolism support the evidence obtained from the growth curves, viz.

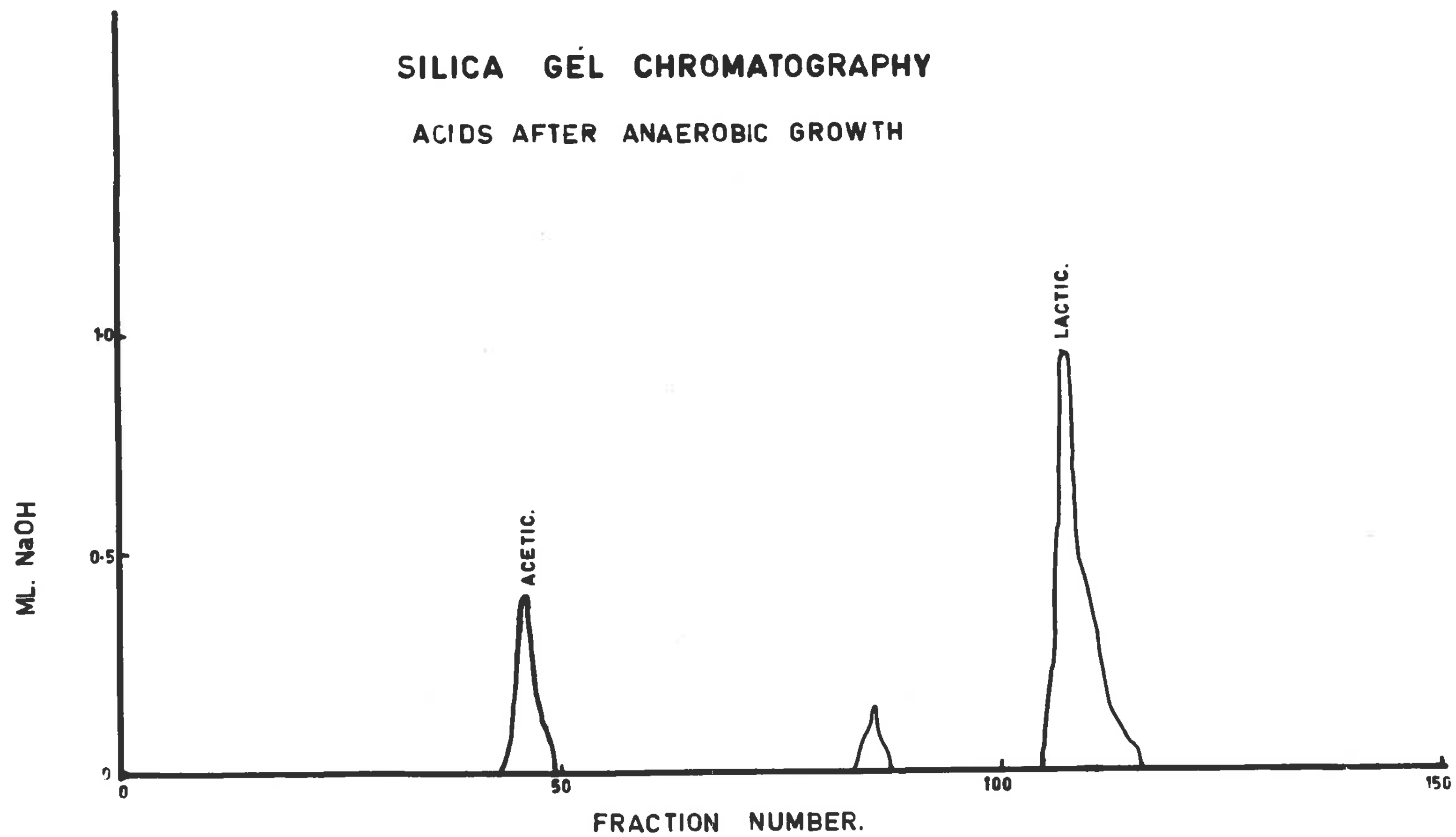
Legend for Fig. 6.

Silica gel chromatography of the organic acids produced during growth in 95% nitrogen + 5% carbon-di-oxide.

The acids were extracted from the growth medium by evaporating it to a small volume at pH 8.5, then, after making it 2.7 N with respect to sulphuric acid, mixing it with celite and extracting with ether for 24 hrs.

The acids were separated on a silica gel column by gradient elution with benzene-ether as described in Method XII 1.

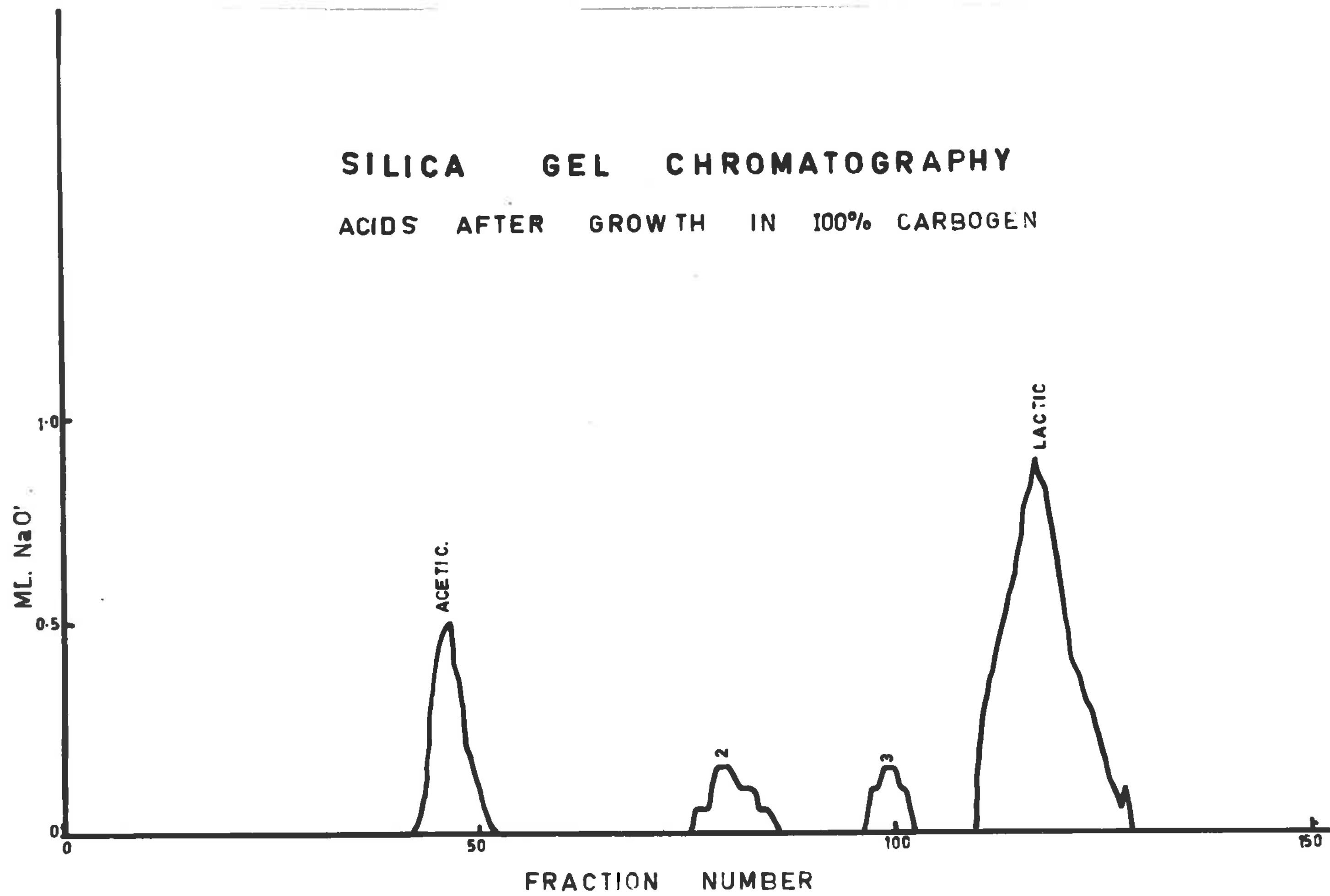
Acids were identified by comparison with fig. 2, by paper chromatography (Method XI 1, 2) and by enzyme assay.


SILICA GÉL CHROMATOGRAPHY
ACIDS AFTER ANAEROBIC GROWTH
LACTIC.
ACETIC.
ML. NaOH
1·0
0·5
0
0
50
100
150
FRACTION NUMBER.

Legend for Fig. 7.

Silica gel chromatography of the organic acids produced during growth in carbogen.

The method for extracting the acids from the growth medium, for separating, and identifying them is described on Legend for Fig. 6.



SILICA GEL CHROMATOGRAPHY
ACIDS AFTER GROWTH IN 100% CARBOGEN
ML. NaO'
1·0
0·5
0
ACETIC.
2
3
LACTIC
0
50
100
150
FRACTION NUMBER

Legend for Fig. 8.

Silica gel chromatography of the organic acids formed during growth in air.

The method for extracting the acids from the growth medium, for separating and identifying them is described in the Legend for fig. 6.

10 μ equivalents of succinic acid was placed on the column with the acids extracted from the medium, in an attempt to identify the small peak removed from the column at fraction 95.

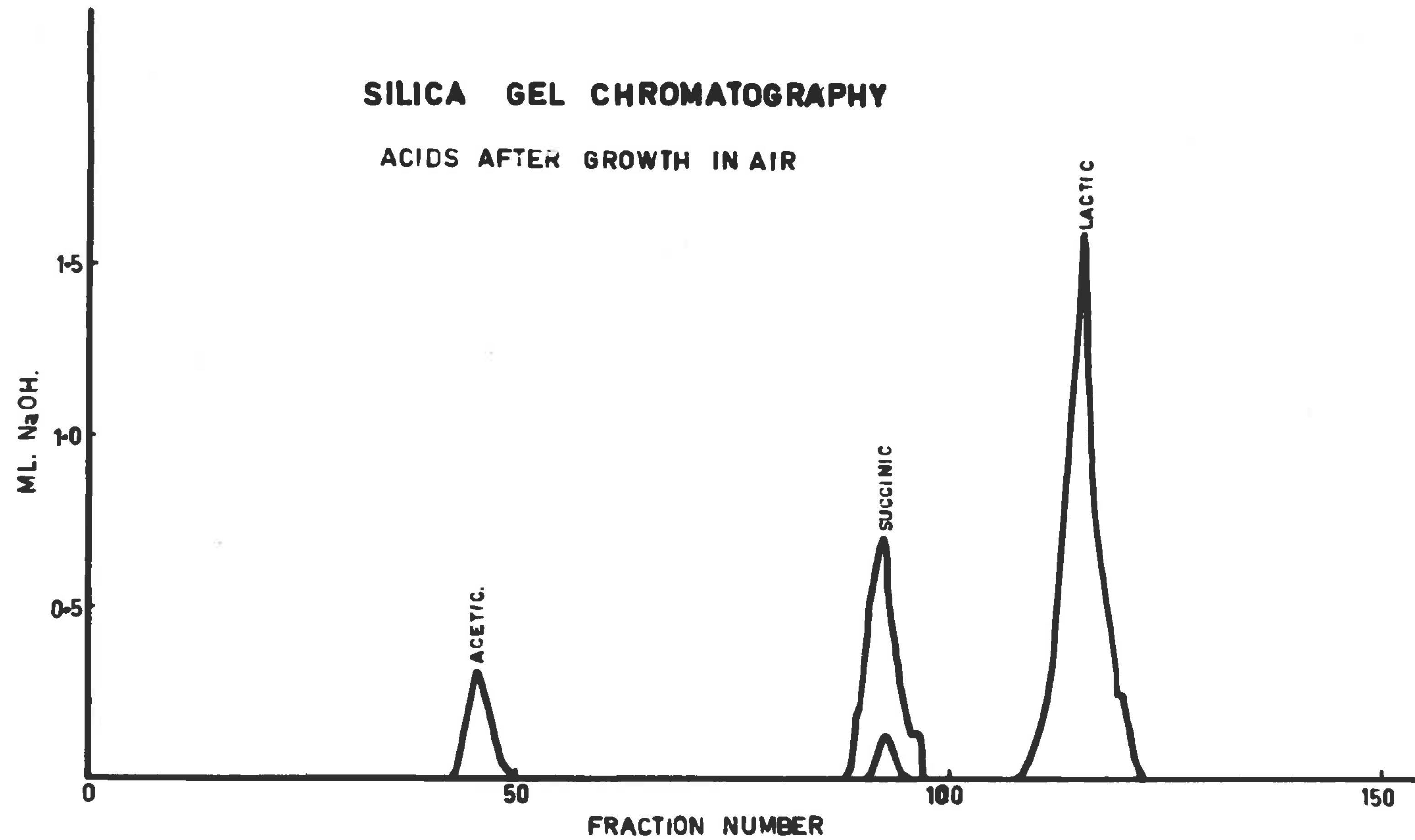

SILICA GEL CHROMATOGRAPHY
ACIDS AFTER GROWTH IN AIR
ML. NaOH.
1·5
1·0
0·5
ACETIC.
SUCCINIC
LACTIC
0
50
100
150
FRACTION NUMBER

TABLE 4.

Carboxylic Acids Produced from Glucose During Growth

	100% N_2-CO_2	80% N_2-CO_2 20% Carbogen	60% N_2-CO_2 40% Carbogen	100% Carbogen
% Glucose converted to acid (From Table 3)	86%	81%	82%	-
% Lactic Acid	84%	85%	83%	64%
% Acetic Acid	14.5%	12%	14%	22%
Acid unaccounted for	1.5%	3%	3%	14%

Table 4 is a summary of the data illustrated in Figs. 6, 7 and 8. Carboxylic acids were extracted from the medium with ether after growth in N_2-CO_2, 20% carbogen, 40% carbogen and 100% carbogen, and separated on a silica gel column as described in Method XII 1.

The % glucose converted to acid was obtained from Table 3. Lactic and acetic acids are expressed as a percentage of the total organic acids produced from glucose.

glucose utilization was little different in the presence or absence of oxygen. The acid identified (but not confirmed) as succinic acid was the only member of the di- or tri- carboxylic acids present, suggesting that oxygen probably did not stimulate Kreb's cycle in the presence of glucose.

In an attempt to account for the glucose not converted to carboxylic acid during growth (less than 10) the medium was made alkaline with Na OH and distilled. The distillate was examined for ethanol, and for di-acetyl or acetoin (Methods XVIII and XIX). Ethanol could not be detected by distillation or by the formation of iodoform. But the test for di-acetyl was positive. There was no attempt to assay di-acetyl quantitatively because some of this very volatile substance would doubtless be lost during growth and rapid aeration at 63.5°.

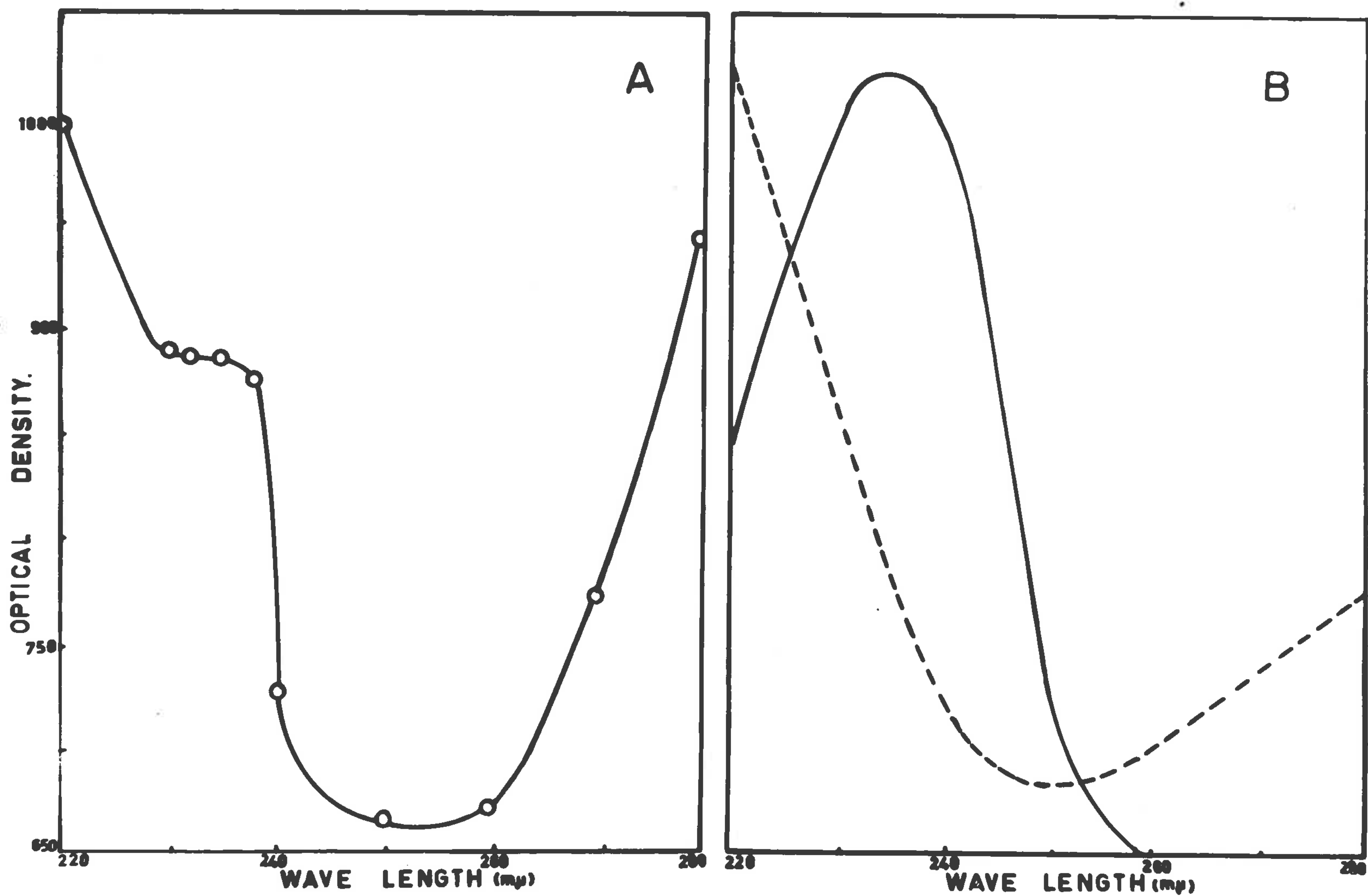
Since poly-β-hydroxy butyrate is a common storage product in the Bacillus species, B. stearothermophilis cells were examined for it by Method XX. MacRae & Wilkinson (1961) published the spectrum of a sulphuric acid hydrolysate of poly-β-hydroxy butyrate, and of a contaminant which interferes with the assay. These spectra are shown in Fig. 9. On the same figure is the spectrum 220-280 mμ obtained when the hypochlorite insoluble, hot chloroform soluble fraction of B. stearothermophilis was hydrolysed in concentrated sulphuric acid. It can be seen that there is a peak at 235 mμ, and the spectrum suggests that both poly-β-hydroxy butyrate and the interfering substance were present. However, when grown in our

Legend for Fig. 9.

Evidence for the presence of poly-β-hydroxy butyrate in *B. stearothermophilis.*

poly-β hydroxy-butyrate was extracted from the cells, and measured as described in Method XX.

Fig. 9A is the spectrum of the substance extracted from *B. stearothermophilis.* Fig. 9B is the spectrum given by pure poly-β-hydroxy butyrate (continuous line), and the spectrum of an interfering substance which is obtained from *B. subtilis* by the same extraction procedure. Fig. 9B are spectra published by MacRae & Wilkinson (1958).


A
B
OPTICAL DENSITY.
750
650
220
240
WAVE LENGTH (mμ)
220
240
WAVE LENGTH (mμ)

normal medium (medium C) no suggestion of poly-β-hydroxy butyrate was found.

Proof that no products of glucose (e.g. poly-β-hydroxy butyrate, fats, teichoic acids, polysacharides) were incorporated into the cells during growth was obtained by growing the cells anaerobically with 50 μc. $C_{(14)}$-U-glucose/2 l. medium. After the cells had been harvested, washed and dried, they were dissolved in formamide and counted by liquid scintillation techniques (Method XIII, 1a). The cells were found to contain only 1.2 μc $C_{(14)}$ compounds, while 48.6 μc $C_{(14)}$ remained in the medium. 4% by weight of the cell was fat (extracted from the dried cells by continuous extraction with ethanol for 24 hours, then di-ethyl ether), and the fat accounted for 0.0018 μc $C_{(14)}$.

2. Nitrate as a Terminal Electron Acceptor

Many strict or facultative anaerobic bacteria can use nitrate as a source of oxygen. One of the criticisms of the conclusions drawn from our work on growth curves done in different concentrations of oxygen is that oxygen may become so insoluble at 63.5° that it is not in fact available to the cells, even in the presence of 100% carbogen. If this were so, then the amount of growth would be limited by the amount of energy which could be produced by the anaerobic metabolism of glucose, and the lactate would be produced almost quantitatively from pyruvate in order to regenerate the reduced pyridine nucleotides. But *B. stearothermophilis* can use nitrate as

an electron acceptor (Wilkes 1963), and nitrate becomes more soluble with an increase in temperature. Therefore, in the presence of nitrate it is theoretically possible for glucose to be completely oxidised through the tri-carboxylic acid cycle since the pyridine nucleotides could be regenerated through an electron transport chain instead of during the reduction of pyruvate to lactate. If this were to happen then there should be a greater amount of energy produced from glucose, reflected in a greater cell yield, and a smaller yield of lactate, when the organism was grown in a nitrate medium than when grown anaerobically.

A growth curve was done using medium C with the addition of 0.1% sodium nitrate. The cultures were sparged with 95% nitrogen, 5% carbon dioxide. In addition to measuring the % transmission at 700 mμ, and glucose concentration of the samples collected during growth, a nitrite estimation was done on the samples by the method of Rider & Mellon (1949) (Method XXXIII). It can be seen from Fig. 10 (bottom right) that the shape of the growth curve resembled that of anaerobic growth rather than that in 100% carbogen. In fact, "k" = 22.5 which is greater than "k" for aerobic growth. 8.8 g. of glucose was used and 1.2 g. dry weight of cells per 1 g. mole glucose were formed. This is comparable with the weight produced during growth in the absence of oxygen, and in air (Table 3), but less than the weight of cell material produced during growth with 40% and 100% carbogen. 95% of the glucose was converted to acid,

Legend for Fig. 10.

Growth of *B. stearothermophilis*

Graph at top of fig. is a plot of the increase in cell density with time when *B. stearothermophilis* is grown in the absence of glucose, with air.

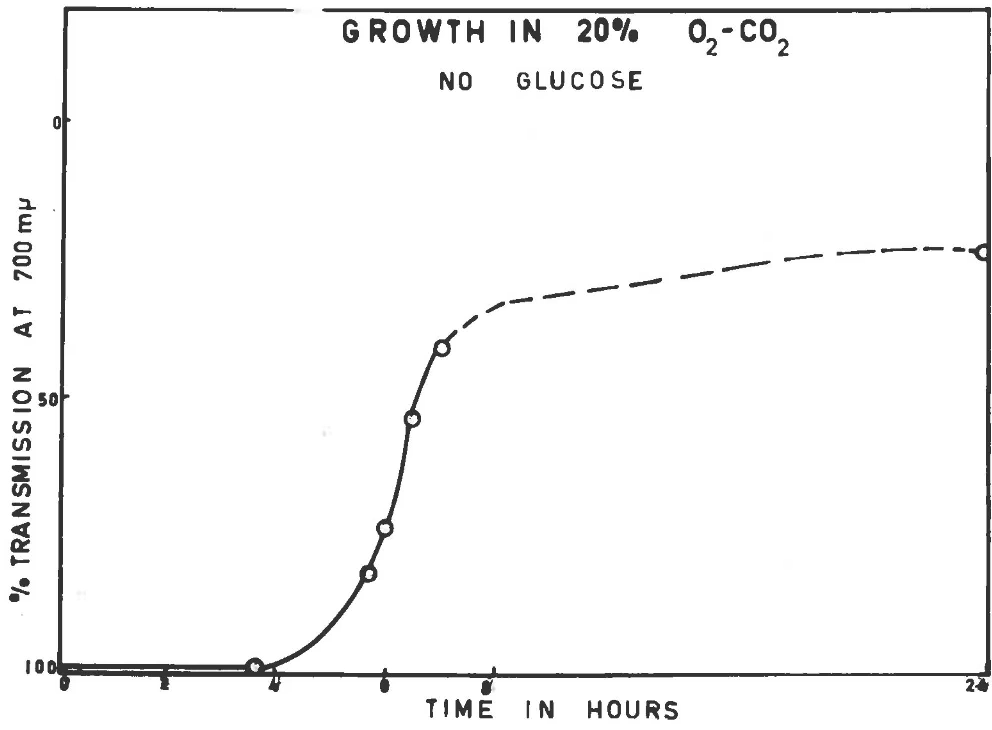
Bottom left is a graph of anaerobic growth in the absence of glucose, but with 0.1% KNO_3.

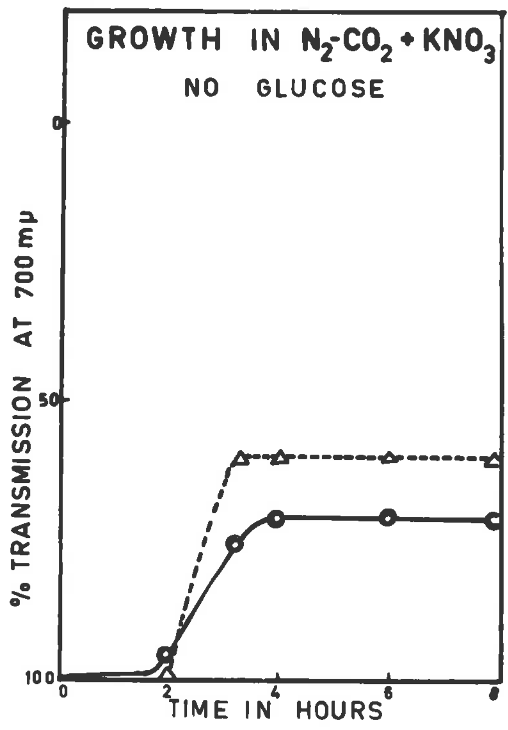
Bottom right is anaerobic growth in the presence of both glucose and nitrate.

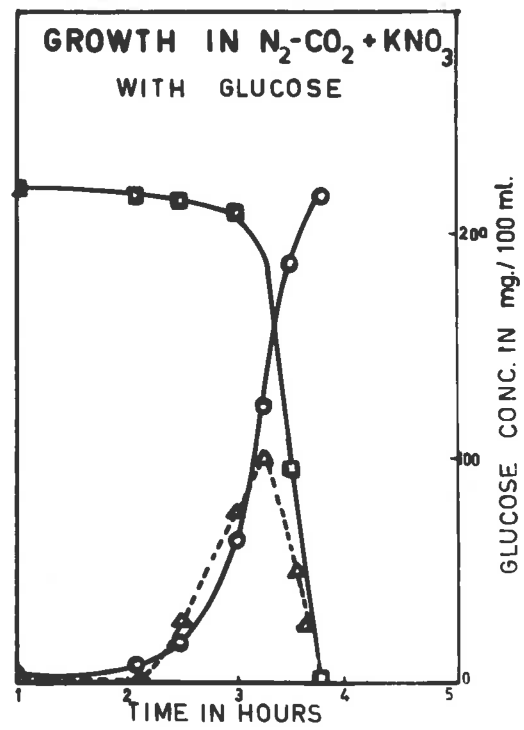
○—○—○ = % transmission at 700 mμ of samples extracted from the medium during growth.

□—□—□ = glucose concentration in mg/100 ml of samples extracted during growth.

△----△--△ = concentration of nitrite in the medium as estimated by the method of Rider & Mellon (1949).


GROWTH IN 20% O_2-CO_2
NO GLUCOSE
% TRANSMISSION AT 700 mμ
0
50
100
0
2
4
6
8
24
TIME IN HOURS


GROWTH IN N_2-CO_2 + KNO_3
NO GLUCOSE
% TRANSMISSION AT 700 mμ
0
50
100
0
2
4
6
8
TIME IN HOURS


GROWTH IN N_2-CO_2 + KNO_3
WITH GLUCOSE
GLUCOSE CONC. IN mg./ 100 ml.
200
100
0
1
2
3
4
5
TIME IN HOURS

and of this, 90% was lactic acid. Nitrite accumulated in the growth medium during the lag phase, but disappeared during the logarithmic phase of growth.

It seems from these data that B. stearothermophilis is not able to use nitrate as an electron acceptor for the complete oxidation of glucose under the conditions used for the experiment.

3. The Catabolism of Glucose by Resting Cell Suspensions

It has been shown that lactate was the principal end product of the metabolism of glucose during the growth of B. stearothermophilis, no matter how much oxygen or nitrate was present. A series of experiments was done with resting cell suspensions to find if lactate were still the major end product, to find the pathway by which the glucose was being used, and whether this pathway supplied enough energy for growth. The contribution of various pathways of glucose metabolism was estimated by following the fate of each of the carbon atoms of the glucose molecule (Dawes & Holmes (1958)). Variously labelled glucose solutions were used, viz. $C_{(14)}$-1-glucose, $C_{(14)}$-2-glucose, $C_{(14)}$-3,4-glucose, $C_{(14)}$-6-glucose and $C_{(14)}$-u-glucose, and in each instance the percentage contribution of the $C_{(14)}$ was traced in each of the three carbon atoms of lactate, the major end product.

A resting cell suspension was made by harvesting the cells during the logarithmic phase of growth, washing them three times and resuspending in distilled water. The cells were incubated with each of the labelled glucose solutions, and the end products were collected

as described in Method XVI and in Fig. 4. Each of the differently labelled glucose solutions was carried through the extraction procedure in the absence of cells to find how much glucose was degraded chemically under the rigorous conditions of the experiment. Cells with $C_{(14)}$-U-glucose were incubated aerobically in a stream of carbogen, as well as anaerobically. All the other incubations were done anaerobically.

Table 5 shows the results of the analysis of the end products after incubation. As found during growth experiments, non-volatile acid was the main end product. This was identified as lactic acid by paper chromatography (Method XI 2), the radio-active spots being detected by analysis on the gas flow strip counter (Method XIII 3a) and identified by comparison with standards run on the same paper (Fig. 11). A very small second peak produced on aerobic incubation corresponded with succinic acid on the accompanying chromatogram.

The volatile organic acid fraction was neutralised with NaOH, evaporated to a small volume and chromatographed as the sodium salts (Method XI 2). Passage through the gas flow strip counter showed that the only detectable spot was one corresponding with sodium acetate.

The non-volatile, non-acidic fraction obtained after passage through Dowex-1-formate (Method XII 2) was reduced in volume by rotary evaporation, and chromatographed by Method XI 4, thus separating the saccharides. The radioactive spots were detected

Legend for TABLE 5.

The catabolism of glucose in resting cell suspensions was investigated, using $C_{(14)}$-1-glucose, $C_{(14)}$-2-glucose, $C_{(14)}$-3, 4-glucose, $C_{(14)}$-6-glucose and $C_{(14)}$-u-glucose. The reaction mixture contained 60 mg $C_{(14)}$-glucose, 6 μc., 60 mg. Ca CO_3 as buffer, 3 gm wet weight of cells made to 30 ml. with water. The flasks were gassed with N_2 + CO_2 during the 20 minute incubation, after which the reaction was stopped by cooling to 0° and adding 15 m mole perchloric acid, and the flasks were gassed for a further 10 minutes. $C_{(14)}$-u-glucose was also incubated as above in an atmosphere of carbogen. CO_2 was collected in ethanolamine, and volatile substances in cold ethanol. The cells and reaction mixture were analysed after incubation, as described in Fig. 4 and Methods XVI.

TABLE 5.

End Products of Glucose Catabolism

Product	$C_{(14)}$-u-Glucose (anaerobe)	$C_{(14)}$-1-Glucose	$C_{(14)}$-2-Glucose	$C_{(14)}$-3,4-Glucose	$C_{(14)}$-6-Glucose	$C_{(14)}$-u-Glucose (aerobe)
CO_2	1.1%	0.9%	0.04%	2.3%	0.7%	1.0%
Alkaline steam volatiles	1.1%	0.1%	0.21%	Nil	.24%	1.4%
Volatile Organic Acids	2.1%	0.1%	0.21%	Nil	.14%	1.4%
Non-volatile acids	60%	50%	70%	50%	70%	50%
Cells	1.8%	1.5%	-	5.1%	0.6%	1.1%
Acetone Solubles from the Cells	1.9%	-	5.0%	5.1%	12%	1.2%
Amino Acids	2.5%					2.5%
Non-volatile, non-acid remainder	30%	40%	20%	30%	20%	30%

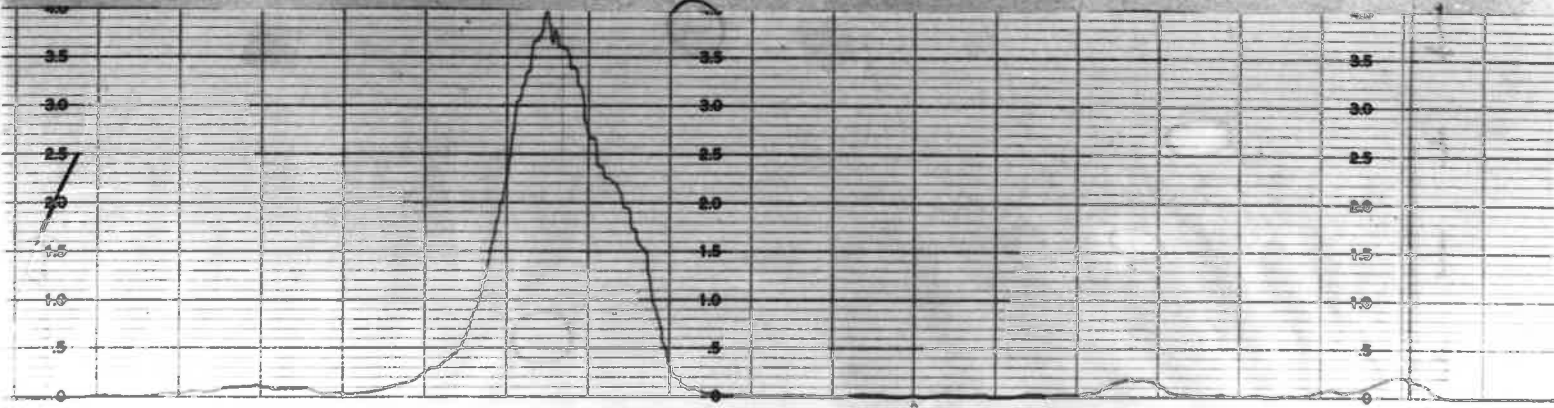
Legend for Fig. 11

The Identification of non volatile organic acids formed by B. stearothermophilis during the catabolism of $C_{(14)}$-u-glucose.

Organic acids which had been purified by elution from Dowex-1-formate with 8 N formic acid were separated by paper chromatography (Method XI 1).

Marker spots run on the chromatogram were stained, the method of Paskova & Monk (1960) (Method XI 1).

The strip on which the test solution had been run was cut longitudinally and run through the gas flow strip counter (Method XIII 3). The trace of radioactivity on the strip, recorded automatically, is shown.



by the gas flow strip counter and identified by comparing them with a range of saccharides run on the same chromatogram. Most of $C_{(14)}$ remaining was glucose. The second radioactive spot did not stain with analine-oxalate spray, and was probably a degradation product of glucose, which is not very stable at 60°. If it were a sugar, it was probably a mono-saccharide with a smaller R_f value than glucose, or a di-saccharide with a greater R_f value than cellobiose, e.g. a di-glucose linked 1:1.

Examination (by Method XVIII) of the substances produced during the incubation, which were steam volatile from an alkaline solution, showed that there was no ethanol present.

The lactate formed was purified by elution from Dowex-1-formate with 8 N formic acid (Method XII 2 2), then after evaporating the acid to dryness to remove the volatile organic acids, it was dissolved in a small volume, and purified further by paper chromatography (Method XI 1C). The lactate was detected on the paper by radio-autography (Method XIII 3 6) and eluted with 100 ml water.

Degradation of the lactate (Method XVII) showed that C_3 and C_4 of glucose became the carboxyl group of lactate, C_2 became C_2 of lactate and C_1 and C_6 became C_3 of lactate (Table 6).

TABLE 6.

Degradation of $C_{(14)}$-Lactic Acid

<table>
<tr><th>Lactate</th><th>$C_{(14)}$-1-glucose</th><th>$C_{(14)}$-2-glucose</th><th>$C_{(14)}$-3,4,glucose</th><th>$C_{(14)}$-6-glucose</th></tr>
<tr><td>CH_3</td><td>99%</td><td>2%</td><td rowspan="2">7.4%</td><td rowspan="2">97%</td></tr>
<tr><td>$CHOH$</td><td>2%</td><td>90%</td></tr>
<tr><td>$COOH$</td><td>0.02%</td><td>5.0%</td><td>92%</td><td>4.3%</td></tr>
</table>

Lactate was isolated after incubation of $C_{(14)}$-glucose with washed cell suspensions by Method XII, 1. Lactate was degraded by the method of Katz et al (1955) (Method XVII).

. Growth in the absence of Glucose

After it had been established that *B. stearothermophilis* would grow both anaerobically and aerobically in the presence of glucose, experiments were done to find if glucose were the only source of energy being used under these conditions. The growth medium was very rich in nitrogenous substances such as amino acids, purines and pyrimidines, which are known to be sources of energy for some bacteria. It is known that many of the amino acids can be fermented under anaerobic conditions (Barker 1961) as well as being oxidised aerobically, so *B. stearothermophilis* was examined for its capacity to metabolise these substances.

a. Growth curves were done in medium C (Chapter III) as before, but without the addition of glucose. The culture was sparged with 95% nitrogen + 5% carbon dioxide, with 19% oxygen, 76% nitrogen + 5% carbon dioxide, or with 95% nitrogen + 5% carbon dioxide in the presence of 1% KNO_3 in the medium. Fig. 10 shows that *B. stearothermophilis* grew slowly but to a fairly high cell density (% transmission = 30) in the absence of glucose if oxygen was present. Nitrate supported growth in the absence of both oxygen and glucose only to 70% transmission at 700 mμ. It was shown that nitrate accumulated in the medium and did not disappear as it had done when the organism was grown in the presence of nitrate and glucose (Fig. 10, bottom right). *B. stearothermophilis* did not grow at all in the absence of both glucose and an oxygen source.

It was shown that the single addition of the amino-acids, glutamic, proline, histidine or arginine to the glucose-free medium during growth in oxygen, all gave an increased yield of cells.

b. Two-way electrophoresis and chromatography (Method XI 3) were used to find which amino acids were utilised during growth. B. stearothermophilis was grown aerobically in a medium containing 0.4% KH_2 phosphate buffer pH 6.8, 0.3% basamin and 1% casamino acids. The medium was chromatographed before and after growth, placing 10 µl medium on the Whatman paper. Electrophoresis in pyridine : acetic acid:water in the ratios 30:100:4870 by vol. separated the basic, acidic and neutral amino acids. Chromatography at right-angles to the direction of electrophoresis gave separation of the neutral amino acid.

After growth, proline and arginine could not be detected, but all the other amino acids seemed uniformly decreased, as might be expected since they were being used for synthetic purposes as well as for energy.

c. The evolution of CO_2 from amino acids was investigated manometrically.

A resting cell suspension was prepared from a culture grown in glucose-free medium.

Warburg cups with two side arms were used. Tris-HCl pH 7.8, 320 µ mole, and amino acids (1 µc and 10 µg) were placed in the main compartment. A complete mixture of $C_{(14)}$ labelled amino-acids

(Chorella hydrolysate) was used. Cells, 0.2g wet weight, were placed in one side arm, and 0.25 ml. 5N perchloric acid in the other. The total volume in the cups was 2.5 ml. The cups were incubated at 44°. (At higher temperatures there was some difficulty in using manometry because liquid distilled over from the heated cups into the manometers). The rate of endogenous respiration was determined, and then the organisms were tipped into the substrate. After an appropriate time the reaction was stopped by adding perchloric acid to the incubation mixture, and the CO_2 output was measured. Oxygen consumption was measured in cups containing NaOH to absorb CO_2 in the centre well.

The results showed that there was a gas exchange. 8.06 µl CO_2 was evolved and 7.16 µl O_2 taken up in 30 min. at 44°. In this time, 4.01 of the amino acid $C_{(14)}$ was given off as $C_{(14)}O_2$. It was shown by two-way chromatography (Method XI 3) followed by radio-autography (Method XIII 3) of the amino acids before and after incubation that proline and arginine were completely used up, and the other amino acids were partially used, except aspartic acid which appeared to accumulate (see Figs. 12 and 13).

Further evidence that B. stearothermophilis was able to use amino acids as a hydrogen source was gained by using the Thunberg technique (Woods 1936). Table 7 shows that all the amino acids tested could be oxidised with a concomitant reduction of one of the

Legend for Fig. 12.

Chromatography of $C_{(14)}$ labelled amino acids before incubation with washed cell suspension of B. stearothermophilis.

Two way chromatography was done by Method XI 3.

The spots were elucidated by radio autography. (Method XIII 3)

AUTORADIOGRAPH OF AMINO ACIDS.

BEFORE INCUBATION

ALA

LEUC

ISO LEUC

VAL

TYR

PRO

ALA

THREO

SER

GLYC

ARG

GLU

LYS

ASP

ALKALINE SOLVENT

ACID SOLVENT

Legend for Fig. 13.

Chromatography of $C_{(14)}$ labelled amino acids after incubation with a resting cell suspension of B. stearothermophilis.

The incubation mixture contained $C_{(14)}$-u-amino acids 10 mg and 1 μc.; tris-HCl, pH 7.6, 320 μ mole; .2 gm wet weight of cells. Protein was precipitated, after completion of the reaction, with 0.5 m perchloric acid, and removed by centrifuging.

The supernatant was chromatographed by Method XI 3, and the spots were elucidated by radio autography (Method XIII 3).

AUTORADIOGRAPH OF AMINO ACIDS

AFTER INCUBATION

LEUC
βALA
ISO LEUC
VAL
TYR
ALA
TREO
GLYC SER
LYS
ASP
GLUT
ACID SOLVENT
ALKALINE SOLVENT

Legend for TABLE 7.

The Thunberg Technique for Investigation of Amino Acid Dehydrogenases. Woods (1936)

Tubes contained 0.2 m mole Na^+K^+ phosphate buffer, pH 7.4, 0.1 m mole amino acid, 0.5 u mole redox dye (Benzyl viologen, Methylene blue, or 2, 6 dichloro-phenol-indophenol). In the side arm was 1 ml suspension of cells (2 gm wet weight per 10 ml buffer). After evacuating and replacing the vaccuum with nitrogen the suspension was equilibrated at 63.5° for 5 min, then tipped and the whole incubated until 90% decolourisation. A control for endogenous substrate was run in the absence of any added amino acid.

TABLE 7.

Substrate	Medium in which cells grown	Benzyl Viologen	Me. Blue	Indophenol
Glycine	Medium C, Chapter III	>90 min.	>90 min.	30 min.
Alanine	"	75 min.	15 min.	20 min.
Valine	"	-	-	60 min.
Leucine	"	60 min.	60 min.	20 min.
Serine	"	-	10 min.	-
Glutamic acid	"	-	3 min.	>120 min.
Lysine	"	-	>3 hr.	10 min.
Arginine	"	-	>3 hr.	20 min.
Histidine	"	-	>3 hr.	15 min.
Ornithine	"	-	>3 hr.	3 hr.
Phenyl-alanine	"	-	>3 hr.	30 min.
Tryptophane	"	-	>2 hr.	30 min.
Proline	"	1.5 min.	<1 min.	2 min.
Glucose	"	-	4 min.	-
Blank	"	No decol.	No decol.	No decol.
Aspartic acid	Glucose free Medium C	-	4 min	-
Glutamic acid	"	-	120 min.	>120 min.
Glutamine	" (lysed cells)	-	4 min.	-

redox dyes tested. Generally 2, 6-dichlorophenolindophenol was reduced most quickly. Methylene blue could be reduced in the presence of alanine, leucine, serine and aspartic acid, and if the cells were lysed, with glutamine. Glutamic acid was only oxidised by cells which had been grown in the absence of glucose. Later, however, it was shown by enzyme assay (Method XXII 11) that glutamic dehydrogenase was acting in cells grown in the presence of glucose, but there was apparently no glutamic acid permease present (Chapter V G1). The metabolism of proline was very interesting. Benzyl viologen, methylene blue and 2,6-dichlorophenolindophenol were reduced very rapidly in its presence. The reaction mixture was chromatographed after proline had been incubated with whole cells of B. stearothermophilis (Method XI, 3, b.). There were three radioactive substances present. One corresponded with proline on the chromatogram, one with glutamic acid, and there was one unidentified substance which ran between glutamic acid and proline, perhaps glutamic semi-aldehyde. The proline solution did not reduce the dyes in the absence of the cells. The problem has not been pursued further.

The evidence points to the fact that B. stearothermophilis can oxidise amino acids, and can use them as an energy source for growth if oxygen, or some other electron acceptor, is present. But the organism cannot use amino acids as a source of energy for growth in the absence of oxygen.

C. The tricarboxylic acid cycle in *B. stearothermophilis*

It was found that acetate, when added to glucose-free medium, increased the cell yield of *B. stearothermophilis*. If 10 g. sodium acetate were added to 5 l. medium, the cell yield was 14 g. wet weight of cells. 6 g. wet weight of cells per 5 litres were produced when the organism was grown with no other energy source than the amino acids in the medium. The organism could only use acetate if grown at pH 5.8 or below. Acetate would probably be partly ionised, and more easily absorbed at this pH. During growth there was a rise in pH as the acetate was used, leaving Na^+ in the medium. The pH was kept at 5.8 by titrating with 2N HCl.

When lactate was added to the glucose-free medium used above, it did not increase the cell yield.

The fact that acetate was being used for energy, suggested that under some circumstances the tricarboxylic acid cycle might function in *B. stearothermophilis*.

The hypothesis that acetate could be oxidised in the tricarboxylic acid cycle, but that the cycle was not used in the presence of glucose, was tested manometrically, using resting cell suspensions of the organism. The evolution of CO_2 from acetate, accompanied by an oxygen uptake, was interpreted as indicating that the tricarboxylic acid cycle was functioning. Manometer cups with two side arms were used. The cups contained 320 μ mole Tris-HCl, pH 7.5, and 20 μ mole substrate. 0.4 g. wet weight of cells which had been grown either in

the presence of glucose or with acetate were placed in one side arm and 0.25 ml of 5 N perchloric acid in the other side arm. 0.2 ml. of 2N NaOH was placed in the centre well of one set of cups to absorb CO_2, and the total volume was made to 2.5 mls. with water. The substrates used were $C_{(14)}$-1-acetate, $C_{(14)}$-2-lactate, $C_{(14)}$-u-glucose, $C_{(14)}$-1-acetate + lactate, acetate + $C_{(14)}$-2-lactate, and $C_{(14)}$-1-acetate + glucose.

Table 8 shows that when $C_{(14)}$-1-acetate was incubated with acetate grown cells there was a large gas exchange and 74.6% of the label was evolved as $C_{(14)}O_2$. But when $C_{(14)}$-1-acetate was incubated with glucose grown cells there was little gas exchange and only 1.58% of the label was evolved as $C_{(14)}O_2$. However, when $C_{(14)}$-1-acetate + glucose were incubated with acetate grown cells, there was a large gas exchange but little $C_{(14)}O_2$, suggesting that glucose was preferentially oxidised. There was no CO_2 evolved from glucose if glucose grown cells were used. There was a greater gas exchange with $C_{(14)}$-2-lactate as substrate with acetate grown cells than with glucose grown cells. But lactate appeared to interfere with the oxidation of acetate by acetate grown cells.

It seems that the tricarboxylic acid cycle functioned in cells which had been grown with acetate as the energy source, even when glucose was the substrate. But cells grown with glucose as energy source were not able to oxidise acetate.

Legend for TABLE 8.

Manometric Investigation of the Kreb's Cycle

Cells were grown with acetate or glucose as energy source, in Medium C, Chapter I. They were incubated in manometric cups with two side arms. The cups contained 320 µ mole Tris-HCl ph 7.5, 20 µ mole substrate, 0.2 ml of 2N NaOH in the centre well, 0.4 gm wet weight of cells in one side arm and 0.25 ml 5 N. perchloric acid in the other side arm. Water brought the total volume to 2.5 mls. After equilibration, the rate of endogenous respiration was measured, then the cells were tipped into the main compartment. At the end of the incubation time the acid was tipped into the reaction mixture to precipitate cells and to evolve any trapped CO_2.

TABLE 8

Manometric Investigation of Kreb's Cycle

Substrate	Energy source during growth in Medium C.	O_2 Uptake	CO_2 Evolution	$C_{(14)}O_2$ as % of original $C_{(14)}$
$C_{(14)}$-1-acetate	Acetate Glucose	24 µl. 1.5 µl.	23.3 µl. 1.6 µl.	74.6% 1.58%
$C_{(14)}$-2-lactate	Acetate Glucose	8.4 µl. 1.5 µl.	8.3 µl. 1.6 µl.	0.51% 0.02%
$C_{(14)}$-u-glucose	Glucose	-	-	0.03%
$C_{(14)}$-1-acetate + lactate	Acetate	5.4 µl.	3.9 µl.	2.32%
Acetate + $C_{(14)}$-2-lactate	Acetate	5.8 µl.	4.1 µl.	0.01%
$C_{(14)}$-1-acetate + glucose	Acetate	39.9	37.5	0.02%

D. Discussion of Chapter IV.

The results show quite conclusively that B. stearothermophilis will grow in the absence of oxygen if glucose is present. This conclusion is at variance with almost every other author. Most authors have based their results on growth curves drawn by plotting viable counts of the cultures over the period of growth, e.g., Gaughran (1947) showed that the viable vegetative cell count decreased with a decrease in the surface to volume ratio of the culture. Baker et al (1955) showed that oxygen was only half as soluble at 60° as at 30°, and Neilson et al (1959) stated that "it is now known that many early workers under-estimated the importance of adequate aeration of cultures". Long & Williams (1960) stress that there is an increased oxygen demand due to decreased solubility of the gas at elevated temperatures, and showed in proof of their statement that a culture of B. stearothermophilis grew well if sparged with air at the rate of 500 ml/min/litre of medium, but grew not at all if sparged with nitrogen alone.

There are several reasons why our results might differ from those mentioned above:

1. It was said that oxygen was necessary for growth because cells taken from anaerobic culture showed a lower percentage viability than did cells taken from an aerobic culture. Neilson et al (1957) showed that viable counts on thermophilic cultures are not very satisfactory by the ordinary Miles & Misra method, and unless conditions are controlled very strictly, are not reproducible.

In this thesis it has been shown that the mean generation times (calculated by the method of Ingraham (1958)) are the same, regardless of whether the culture is sparged with nitrogen + CO_2 or with air. If two cultures have the same increase in total counts over the same period of time, and the mean generation times of the two cultures are the same, then each culture must contain the same percentage of viable organisms at any given time. Therefore, despite the fact that fewer cells from an anaerobic culture form colonies on a plate than do cells from an aerobic culture, it seems that the cultures have an equal number of cells that can reproduce in the environment of the growth medium, whether oxygen is present or not.

A cell taken from an anaerobic culture and placed on an aerobic plate may have difficulty in coping with the change in environment. Hancock (1957) suggested that in the thermophile with which he worked catalase was an adaptive enzyme. I have found that cells of B. stearothermophilis could produce O_2 from H_2O_2 if they had been grown aerobically, but not if they had been grown anaerobically. Nakamura (1960) showed that catalase in B. stearothermophilis needed to be activated before use. Therefore, it is possible that some anaerobically grown cells cannot reproduce on an aerobic plate because they are unable to produce catalase quickly enough, or because they lack some other factor which might protect them from the effects of oxygen in their new environment.

2. As discussed earlier, Long & Williams tested B. stearothermophilis for anaerobic growth by sparging the culture with nitrogen. They had no carbon dioxide in the medium. Work reported in this thesis showed there was no growth when the culture in glucose medium was sparged with CO_2-free nitrogen (Chapter IV A) but growth in $N_2 + CO_2$ (95:5 by vol) was comparable with growth in air. It seems possible that CO_2 might be the limiting factor rather than oxygen.

Borek & Waelsch (1951) showed that if they raised the growth temperature of Lactobacillus arabanosus from 26° to 37°, phenyl alanine and tyrosine had to be added to the medium. By demonstrating that these amino acids were not necessary if they increased the CO_2 tension at 37°, and that they were necessary if they decreased the CO_2 tension at 26°, they proved that the effect was probably due to the lack of CO_2 which is essential for synthesis of phenyl alanine.

CO_2 is 72% as soluble in water at 60° as at 35° (Langridge 1963). However, although CO_2 is necessary for growth of B.stearothermophilis there was only a very small amount of CO_2 absorbed by the cells when incubated with $NaHC_{(14)}O_3$ (see Table 3).

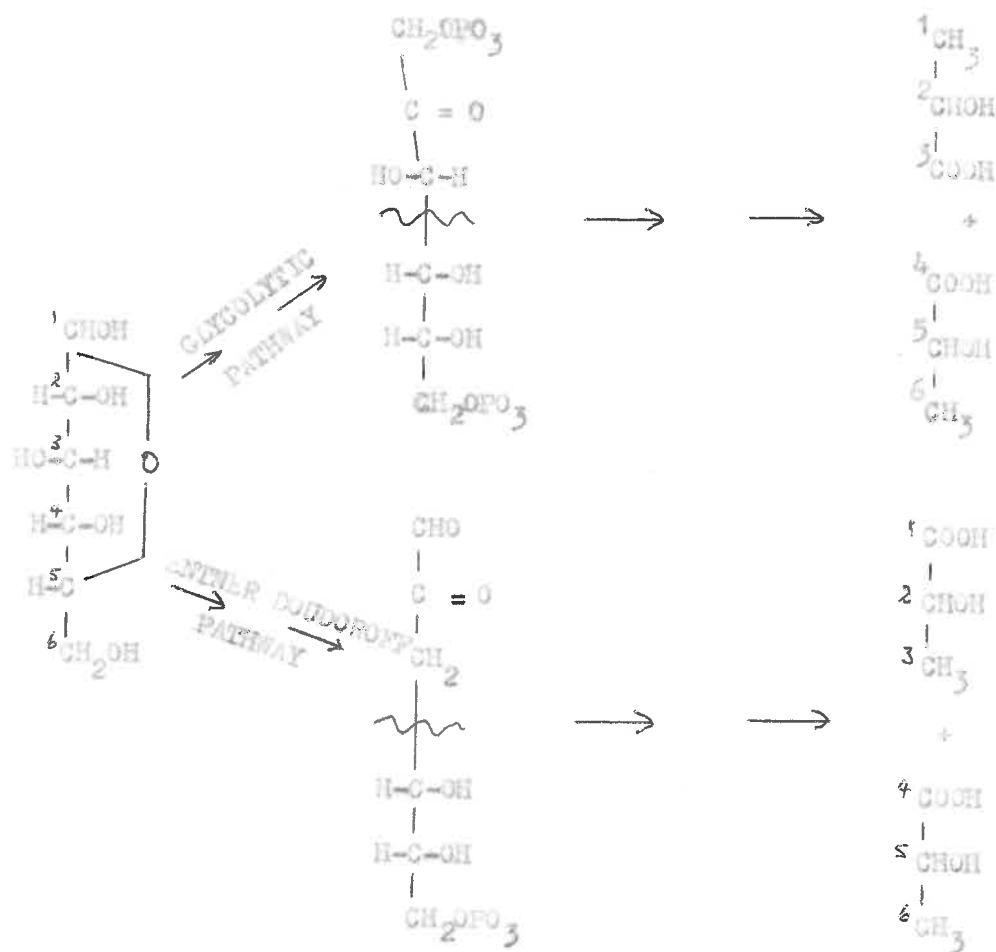
3. Earlier workers did not control the pH of their cultures during growth. The work reported in this thesis was done at controlled pH and may account for the different results obtained. The pH drops very rapidly to 5 during growth. This accelerates cell lysis and, in addition, would make CO_2 even less soluble than

at 6.8, the pH at start of growth.

The results of experiments in which resting cell suspensions of B. stearothermophilis were incubated with $C_{(14)}$ labelled glucose suggest that the organism derives energy from glucose via the glycolytic pathway. Evidence in favour of this hypothesis is:-

(a) There is a negligible amount of CO_2 produced from glucose during the incubation. Therefore the hexose-mono-phosphate shunt does not operate since the first steps in this pathway involve the evolution of CO_2 from C_1 of glucose. So glucose must have been degraded by the glycolytic pathway, the Entner Doudoroff pathway, or by some pathway not yet discovered.

(b) There is an almost quantitative conversion of glucose to lactate. The labelling pattern of the lactate shows that C_1 and C_6 of glucose become the C_3 of lactate, and $C_{3,4}$ of glucose becomes the carboxyl group of lactate. This is congruous with the labelling pattern of lactate when formed via the glycolytic pathway. In the Entner Doudoroff pathway C_1 and C_4 of glucose become the carboxyl groups of lactate, and C_3 and C_6 the C_3 of lactate, i.e.



The small quantities of volatile acids and alcohols isolated from the reaction mixture after incubation of the organism with $C_{(14)}$ glucose may not be significant, because the quantities of these substances varied with small changes in pH during steam distillation. However, the labelling of these 2-carbon compounds is consistent with their formation from pyruvate preformed via the glycolytic pathway from glucose (Table 5), e.g. there were no labelled volatile substances formed from $C_{(14)}$-3, 4-Glucose.

To prove that B. stearothermophilis could use the glycolytic pathway, several key enzymes were assayed (Method XXII). It was shown that hexokinase, phospho-hexose isomerase, phospho fructo kinase, aldolase, pyruvic kinase, and lactic dehydrogenase were all present and active in cell free extracts of the organism (see Table 9). It was interesting to find that hexokinase had a much higher specific activity in cells harvested in the logarithmic phase of growth than those harvested in the stationary phase.

Also present in the extracts were glucose-6-phosphate dehydrogenase, and 6-phospho gluconic dehydrogenase. These enzymes were, in fact, more active in cell free extracts, than was phospho hexose isomerase, the enzyme in the glycolytic pathway which competes with them for the substrate, glucose-6-phosphate. It is interesting to speculate about the conditions under which they would be used and why there was no CO_2 evolved from glucose under the conditions I used (i.e. 6-phospho-gluconic dehydrogenase was not active). Glucose-6-phosphate dehydrogenase and 6-phospho gluconic dehydrogenase were both NADP specific. Was it because NADP H_2 could not be reoxidised that these enzymes could not be used? Was growth in the presence of glucose so rapid that even when 20% oxygen was being supplied the culture was virtually anaerobic?

Evidence showed that despite reports that some of the enzymes of the Kreb 's cycle were present in B. stearothermophilis cells (Militzer et al 1949) (e.g. Malic dehydrogenase and enzymes that could oxidise citrate, α-keto glutarate and succinate) the organism did not appear

Legend for TABLE 9.

The Activities of Some Enzymes concerned with the Degradation of Glucose

Cell free extracts of *B. stearothermophilis* were prepared by mechanical disintegration as described in Method IX. 1.

Enzymes were assayed according to Methods XXII, and these activities were calculated for activities at 34°C.

Activity is expressed as µ mole substrate converted in 5 min/mg. protein.

TABLE 9.

The Activities of Some Enzymes concerned with Degradation of Glucose

Enzyme	Activity μ mole substrate converted in 5 min/mg protein at 34°C
Glucose dehydrogenase	Nil
Hexo-kinase	
(in logarithmic phase)	0.108
(in stationary phase)	0.064
Phospohexose isomerase	0.058
Aldolase	0.105
Phospho-fructo kinase	0.106
Pyruvic Kinase	0.12
Lactic dehydrogenase	40.0
Alcohol dehydrogenase	0.83
Glucose-6-Phosphate dehydrogenase	0.16
6-Phospho-gluconic dehydrogenase	0.34

to be able to use Kreb 's cycle during growth with glucose. Was this, too, a result of an inadequate oxygen supply? The experimental evidence suggests that it is not the only factor since cells grown in the presence of glucose could not produce CO_2 from acetate, yet cells grown in the presence of acetate could. This is not due to an inhibitory effect of glucose or glucose metabolites on the enzymes of Kreb 's cycle for the cells could produce CO_2 from glucose if they had been grown in the presence of acetate. This suggests either that glucose inhibits the synthesis of one or more of the enzymes of Kreb 's cycle or that the electron transport system does not form during growth with glucose as the energy source. This latter suggestion could also explain why the hexose mono-phosphate shunt did not operate. Packed cells of B. stearothermophilis are more highly coloured when grown in the absence of glucose than in its presence. But Militzer et al (1950) claim that cytochromes b, c and a were present in their strain of the organism, and they had used glucose for energy source during growth of the cells.

The Krebb's cycle is probably also involved in the production of energy from amino acids. Many of them (including proline, arginine and histidine) which increased the growth yield of B. stearothermophilis, (Chapter IV B.a) are oxidised through glutamic to α-keto glutarate. There was an exchange of O_2 and CO_2 when cells grown in the absence of glucose were incubated with an amino acid mixture.

It may be significant that growth in the presence of glucose is at least 5 times more rapid than with any other energy source tried. Oxygen, which is very insoluble at elevated temperatures, could be rapidly used, keeping the medium almost anaerobic. Then, if there is a defective cytochrome system, it is more likely to be the result of an oxygen lack caused by rapid growth with glucose, than a direct inhibition by glucose or its metabolites.

CHAPTER V: THE INFLUENCE OF CHANGE IN TEMPERATURE ON B. STEAROTHERMOPHILIS

Chapter IV showed that the use of glucose by B. stearothermophilis was in no way extraordinary. In fact, it appeared to use glucose by the glycolytic pathway. It is interesting to speculate about whether a thermophile derives all its energy from conventional mechanisms, or whether it may be able to use the heat in its environment to supplement the usual energy sources. After all, some bacteria have adapted themselves so that they can use light energy.

Many authors have related the yield of cellular material directly with the energy which the organism was able to derive from its energy source, e.g. Bauchop & Elsden (1960) showed that both Streptococcus faecalis and Saccharomyces cerevisae are able to produce 20 g. dry weight of cells when using 1 g. mol glucose during growth in a complete medium. Streptococcus faecalis produced 10 g. dry weight of cells when 1 g. mol arginine (the only energy source available) was catabolised during growth. In fact 10 gm. dry weight of cell material appeared to be formed for every gm-mole ATP which could be gained from the substrate under conditions where the substrate was only being used as an energy source. Other examples are described by Senez (1962).

Table 3 shows that B. stearothermophilis produced 21 g. dry weight of cells for 1 gm-mole glucose used during anaerobic growth or in the presence of 20% oxygen, 80% $N_2 + CO_2$ at 63.5°C. If B. stearothermophilis were able to derive some energy from the heat of its environment the cell yield per 1 gm-mole glucose should be

greater at elevated temperatures than at lower temperatures. Therefore, a series of growth curves was done over the range of temperatures in which _B. stearothermophilis_ would grow in one medium, viz. 37° to 75°.

A. The Influence of Temperature on Growth of _B. stearothermophilis_

These growth curves were done in glucose medium (Chapter III, C) as described in Method II, and gased with $N_2 + CO_2$(95:5 by vol) at the rate of 5l/min. They were grown anaerobically so that any change in growth yield could not be influenced by an increased solubility of oxygen at lower temperatures.

The inoculum for each growth curve was incubated in glucose-free medium at 60° and 20 ml. inoculum was added to 5 l. medium at the temperature required. At lower temperatures it was found that the lag period was very long, e.g. at 45° the lag period was 11 hrs. The lag could be reduced without altering the rate of growth in the logarithmic phase by adding a much larger inoculum, or an inoculum which was itself in the logarthmic phase of growth.

At each temperature, the rate of growth, the rate of glucose utilisation, and the yield of cells were measured as described in Methods IV and V. The pH was always kept constant at pH 6.8 during growth.

Table 10 shows that at temperatures between 45° and 63° the dry weight of cell material / 1 gm-mole glucose was unchanged. But

Legend for TABLE 10.

Growth of *B. stearothermophilis* at Temperatures from 37° to 75°.

The rate of growth is the slope of the curve measured during the logarithmic phase of growth when the optical density at 700 mμ was plotted against time in hr.

Y glucose is the dry weight of cell material produced per 1 gm Mole glucose.

TABLE 10.

Growth of *B. stearothermophilis* at Temperatures from 37° to 75°.

Temp. °C.		$\log_{10}$ Growth Rate	Y Glucose
37	0.003215	approx. 0.005	<4
47	0.003125	0.88	21
50	0.003096	1.3010	23
53	0.003067	1.38	21
55	0.003049	1.64	20
57	0.003030	1.74	19
59	0.003012	1.83	21
62	0.00299	1.95	22
63	0.002976	1.987	20
63.5	0.002960	1.93	21
68	0.002933	1.79	20
70	0.002915	1.58	8
75	0.002874	0.063	<4

at temperatures above 63° and at 37°, the cell yield per 1 gm-mole glucose was less than that between 45° and 63°.

At 37° eight days passed before growth was detectable by a slight increase in optical density. It was confirmed microscopically that this turbidity was due to bacteria. These Gram-positive rods were able to grow rapidly when transferred to medium at 63.5°, so it was concluded that they were B. stearothermophilis and not a contaminant. Two g. glucose were used in eight days, but the cell yield was less than 0.1 g. wet weight.

The rate of growth was expressed as the slope of the line drawn when O.D. at 700 mμ was plotted against time during growth. The slope was measured near the end of the logarithmic phase of growth, i.e. half way along the plot of the logarithmic phase.

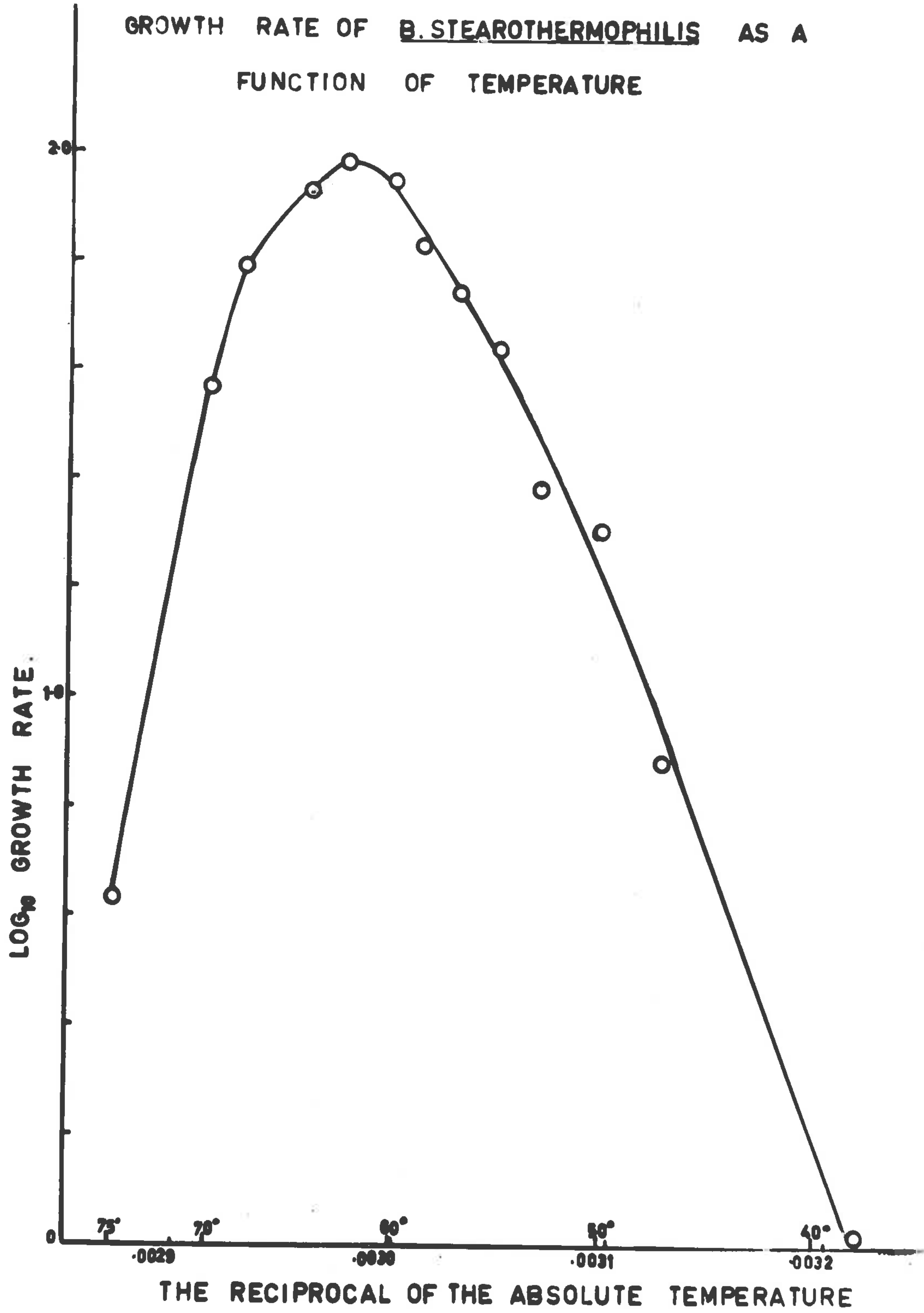
As mentioned in Section D, Chapter I, Ingraham (1958) has proposed that organisms might be classified into psychrophiles, mesophiles and thermophiles, depending on their heats of activation ($\Delta H^{\ddagger}$), or temperature co-efficients of growth. He showed that $\Delta H^{\ddagger}$ for a psychrophile was 9,020 cals and for a mesophile was 14,000 cals. To find a figure for B. stearothermophilis the $\log_{10}$ (growth rate) was plotted against the reciprocal of the absolute temperature (see Fig. 14). From this graph the $\Delta H^{\ddagger}$ for growth was determined using the Arrhenius equation, viz.

Legend for Fig. 14.

Growth Rate of *B. stearothermophilis* as a Function of Temperature.

Growth rate was calculated from the slope of the curve during the logarithmic phase of growth at each temperature, when the optical density at 700 mμ of samples taken during growth was plotted against time.

Data is shown in Table 10.



GROWTH RATE OF B. STEAROTHERMOPHILIS AS A FUNCTION OF TEMPERATURE
2·0
1·0
0
LOG10 GROWTH RATE
75°
70°
60°
50°
40°
·0029
·0030
·0031
·0032
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$$\text{Slope of curve} = \frac{\Delta H^{\ddagger}}{2.303\ R}$$

where R is the gas constant. The slope of Fig. 14 was measured on the linear part of the curve between 50° and 57° as

$$\frac{1.825 - 1.325}{0.0001} = \frac{0.5}{0.0001}$$

Then from the above equation

$$\Delta H^{\ddagger} = \frac{0.5}{0.0001} \times 2.303 \times R$$

$$= \underline{23,000\ \text{cal.}}$$

The heat of inactivation of growth (ΔH) can also be found from Fig. 14.

ΔH = ((-Slope of the curve after the optimal temperature of growth has been exceeded) x 2.303 R) + $\Delta H^{\ddagger}$ = 187,000 cal.

However, there are not enough points on this part of the curve to make this an accurate figure.

B. The Effect of Change in Temperature on Glucose Degradation during Growth.

The temperature coefficient for the degradation of glucose during growth was determined by a similar method to that used for the coefficient of growth. Glucose concentration in the medium was estimated in each sample taken during growth at each temperature. These concentrations were plotted against time (as in Fig. 5). The rate of utilisation of glucose was expressed as the slope of the curve during the logarithmic phase of growth, and the $\log_{10}$ of these rates were plotted against the reciprocals of the absolute temperatures. The curve resembled that for growth (Fig. 14) except that the optimal temperature for the rate of breakdown of glucose was higher than that for growth, viz. 68°.

The energy of activation for glucose degradation was calculated from the slope of this curve, using the Arrhenius equation as before. $\Delta H^{\dagger}$ for glucose degradation during growth = 24,000 cal.

It is still a mystery why growth of B. stearothermophilis is so slow at 37°, and too slow to detect at temperatures lower than 37°. One of the theories put forward to explain this is that the cells cannot use their energy source (in the case of glucose) at these temperatures, either because the enzyme systems are inactive, or because glucose cannot be transported across the cell membrane.

These two possibilities were tested.

C. The Influence of Temperature on the Use of Glucose by Washed Cell Suspensions of *B. stearothermophilis*

It was shown in Chapter IV that glucose is converted to lactate almost quantitatively by *B. stearothermophilis* under anaerobic conditions. In these experiments the amount of lactate formed in a given time was used to indicate the rate of degradation of glucose. Preliminary experiments showed that lactate formation from glucose was a linear function of time, and that after 10 min. the reaction had not reached completion at 30°, 40°, 50° or 60° under the conditions used. Therefore, the washed cell suspension (prepared as Method II) was incubated with glucose for 10 min. at each temperature, and the lactate isolated.

The reaction was carried out in a clinbritic flask fitted with a skirted rubber cap. One ml. of organisms which had been harvested in the logarithmic phase of growth (1 gm. wet weight of cells) was injected into the temperature equilibrated flask containing 1 μc 2 mg. $C_{(14)}$-u-glucose, and either 2 mg. Ca CO_3 as buffer and 1 ml. water, or 1 ml. medium C (i.e. phosphate buffer and complete amino acids). The mixture was agitated by blowing $N_2 + CO_2$ (95:5 by vol) through it. After 10 min. the reaction was stopped by plunging the flask into ice and adding 0.2 ml. 0.5 N perchloric acid. The precipitated protein was removed and the perchloric acid neutralised with 2N KOH. The potassium perchlorate was removed by centrifuging after standing at

4° overnight. The $C_{(14)}$-lactate was isolated from the supernatant on a Dowex-1-formate column as described in Method (XII 2), and counted by liquid scintillation (Method XIII 1 a).

Fig. 15 shows a plot of the $\log_{10}$ (total counts of $C_{(14)}$-lactate formed from $C_{(14)}$-U-glucose in 10 min) against the reciprocal of the absolute temperature at which the lactate was formed. It can be seen that both in the presence and in the absence of amino acids the rate of increase in utilisation of glucose with temperature below 25° was slight, whereas there was a marked increase in the rate as the temperature rose above 30°, shown by the increased slope of the curve.

The rate of lactate formation increased much more steeply in the presence of phosphate and amino acids, than when the incubation mixture was only buffered with $CaCO_3$. This was not due to growth in the presence of the amino acids, but to autolysis in their absence. In fact, at 50° the O.D. at 700 mμ dropped to half of the initial value in 10 minutes when washed, anaerobically grown cells were incubated with glucose in the absence of amino acids.

Does the change in rate of utilisation of glucose above 30° mean that if we were patient we would be able to detect growth at temperatures between 30° and 37°? Was the slow rate of lactate formation below 25° due to decreased rate of transport of glucose across the cell walls?

The first question was unanswered. The second was tackled in two ways, viz.

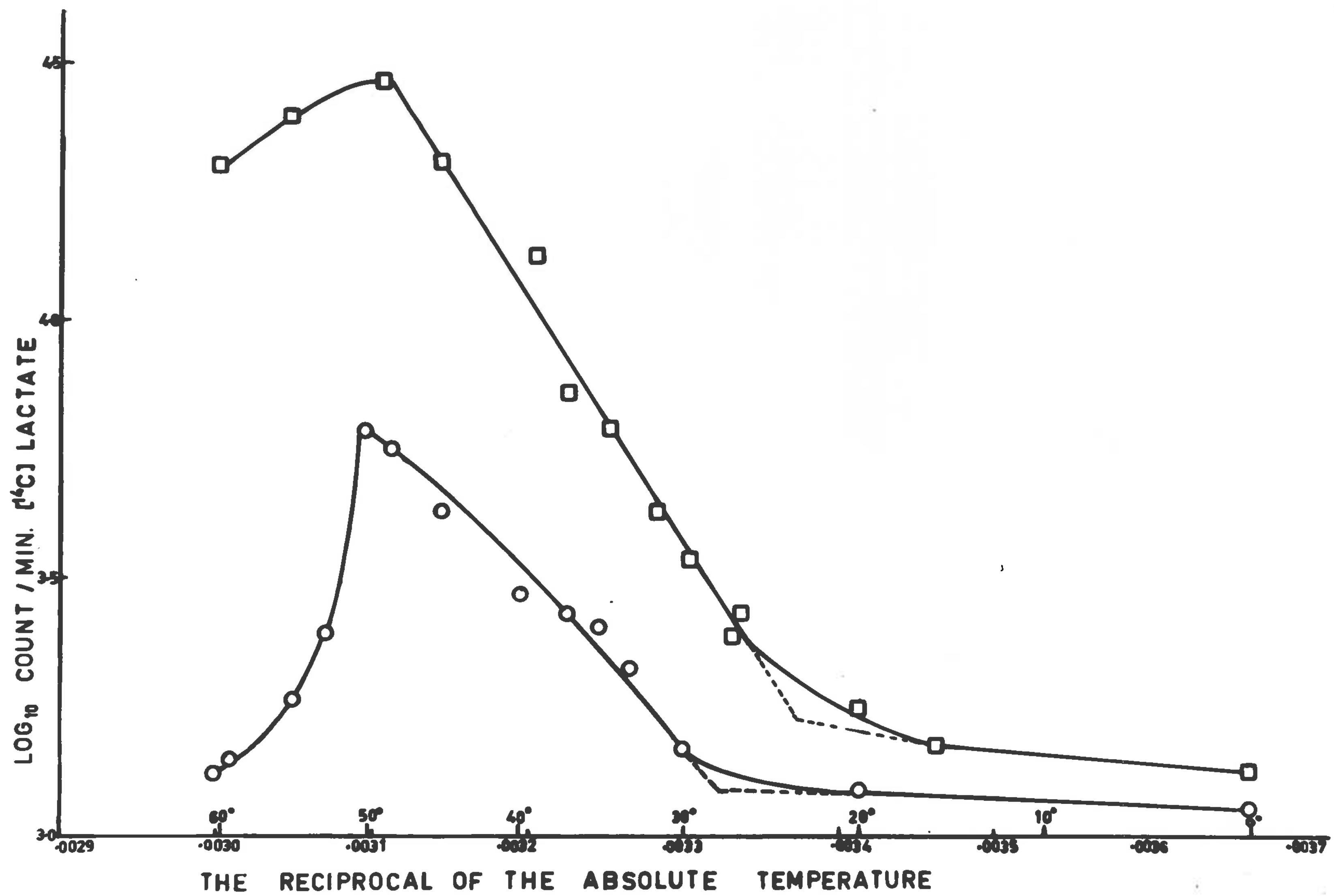
Legend for Fig. 15.

The rate of glycolysis as a function of temperature.

The rate of glycolysis at each temperature was estimated as the amount of lactate formed from glucose in 10 min.

O—O—O = rates obtained when the washed cell suspension of *B. stearothermophilis* was incubated with glucose, and $CaCO_3$ buffer.

□—□—□ = rates obtained when washed the cell suspension was incubated with glucose in the presence of Medium C, Chapter III.



LOG10 COUNT / MIN. [14C] LACTATE
4·5
4·0
3·5
3·0
60°
50°
40°
30°
20°
10°
0°
·0029
·0030
·0031
·0032
·0033
·0034
·0035
·0036
·0037
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(a) by looking directly at glucose transport into whole cells

(b) by looking at the effect of temperature on glucose utilisation in cell-free extracts of <u>B. stearothermophilis.</u>

D. Transport of Glucose into the Cells.

Unfortunately, we were able to gain no quantitative measure of glucose transport because we could not overcome the problem of autolysis mentioned above.

½ g. wet weight of cells were incubated with 0.5 mg. $C_{(14)}$-U-glucose, 2 µc in a total of 2 ml. K^+ phosphate buffer pH 6.6, 0.05 M. at the temperature required (0° - 50°). After 1 min. transport was stopped by adding 2 ml. ice cold uranyl acetate 2 x 10^{-3} M pH4. (Cirillo & Wilkins 1964) and the whole placed in an ice bath. The cells were separated from the incubation mixture by filtering through a cellulose acetate membrane. It was evident that there had been cell lysis because the medium became viscous, the filtrate frothed profusely, and it took 30 min. to filter the cells. This lysis was not caused by the uranyl acetate.

In an endeavour to overcome cell lysis, 1 gm. wet weight of cells were incubated as above, and separated from the residual glucose by centrifuging at 18,000 g. for 10 min. The cells were washed three times with ice cold 10^{-3} M. uranyl salt. Less than ½ gm. wet weight of cells remained after the third wash, although all the cells were packed hard after centrifuging at 18,000 g.

The cells were dried, dissolved in formamide and counted by scintillation techniques. $C_{(14)}$-lactate in the medium was isolated and counted because it was assumed that this lactate must have been

a product of degradation of some glucose that had been transported into the cell. The results showed that glucose was taken up at all temperatures, but no quantitative results could be obtained because we could not overcome the problem of cell lysis.

E. The Effect of Change in Temperature on Glucose Degradation by Cell-free Extracts of B. stearothermophilis.

These experiments were done in a similar way to the experiments with washed cell suspensions (Section C).

Cell-free extracts were prepared from cells harvested in the logarithmic phase of growth by disintegration on the Mossal Shaker (Method IX 1). Whole cells were removed from the extract by centrifuging at 18,000 g, and the extract was checked microscopically to see that no whole cells remained. 25 mgm protein (about 1 ml. extract) was incubated in each clinbritic flask with 1 mgm. $C_{(14)}$-u-glucose (1 uc). ATP (0.001 m Mole) ADP (0.01 m Mole), NAD (0.01 m Mole) and Mg Cl_2 (0.04 m Mole) were added to the incubation mixture. The buffer was 1 ml. glucose free medium C. After 10 min. incubation at the appropriate temperature the reaction was stopped as before.

The $C_{(14)}$-lactate was eluted from the Dowex-1-formate column with 0.2M ammonium formate pH 4.0.

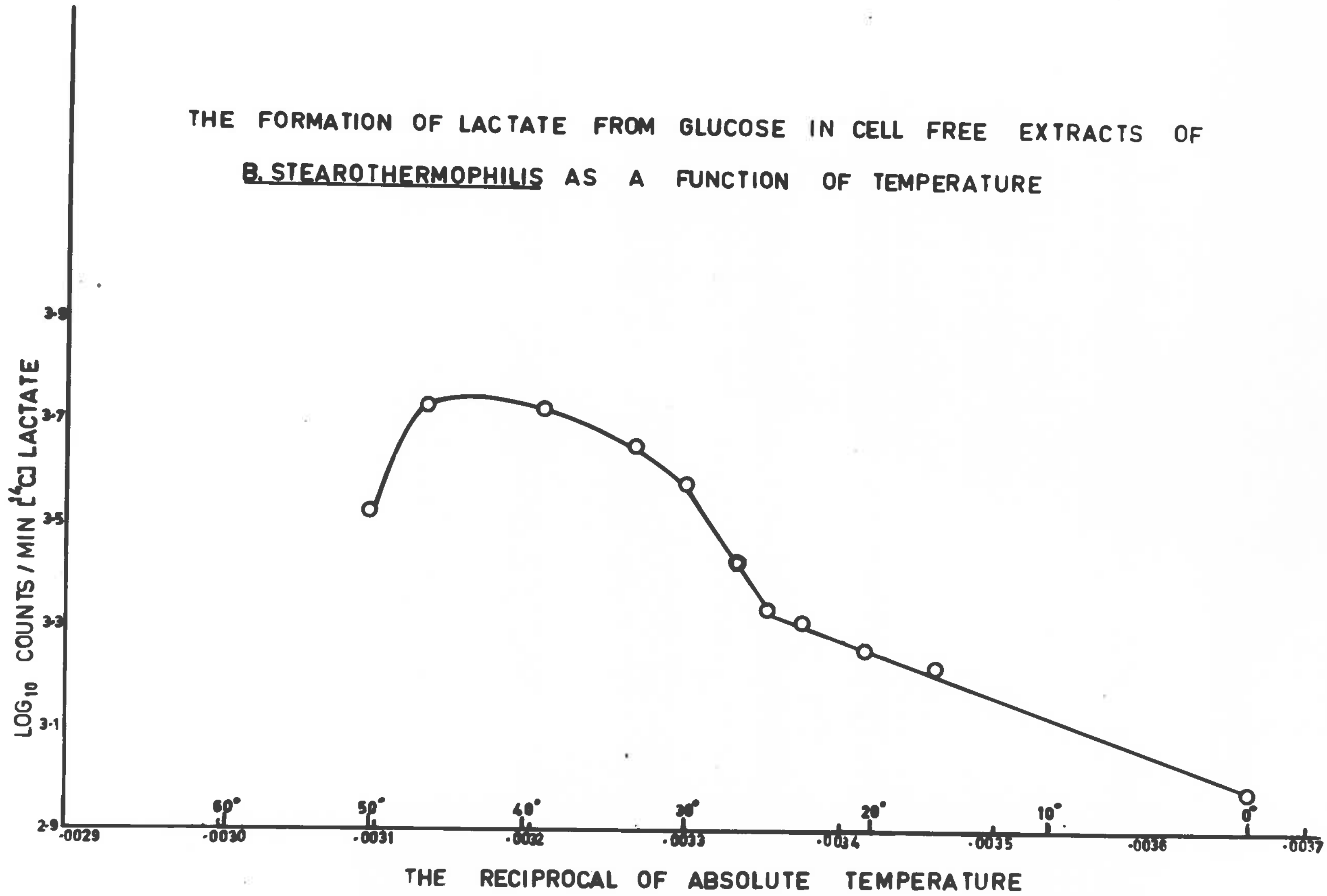
Fig. 16 shows a plot of $\log_{10}$ (lactate formed from glucose in 10 min.) against the reciprocal of the absolute temperature. This experiment was repeated four times, and each set of results showed the response to temperature increased between 26° and 28° in the same way that the response changed in whole cells. The slope decreased again above 35°. This is probably a function of the instability of one or all of the enzymes in cell-free extract involved in the

Legend for Fig. 16.

The Formation of Lactate from glucose in cell free extracts of *B. stearothermophilis* as a function of temperature.

At completion of reaction, at each temperature, the $C_{(14)}$ lactate was separated from the $C_{(14)}$-glucose by Method XII 2. Lactate was eluted from the Dowex-1-Formate column with 0.2 m ammonium formate, pH 4.0.

Lactate was counted by scintillation Method XIII, 1,b.


THE FORMATION OF LACTATE FROM GLUCOSE IN CELL FREE EXTRACTS OF
B. STEAROTHERMOPHILIS AS A FUNCTION OF TEMPERATURE
LOG10 COUNTS / MIN [14C] LACTATE
3·9
3·7
3·5
3·3
3·1
2·9
60°
50°
40°
30°
20°
10°
0°
·0029
·0030
·0031
·0032
·0033
·0034
·0035
·0036
·0037
THE RECIPROCAL OF ABSOLUTE TEMPERATURE

degradation of glucose to lactate. The slope of the curve was greater at temperatures below 25° than it was in whole cells (c.f. Fig. 15).

This change in response of glucose utilisation to temperature did not appear to be due, or only partly due, to a decreased permeability of the B. stearothermophilis cells to glucose at low temperatures.

Some of the enzymes concerned with glucose catabolism were examined to find if they showed a response to temperature similar to that of the complex process of degrading glucose to lactate.

F. The Influence of Temperature on Some Enzymes concerned with Glucose Degradation in *B. stearothermophilis*.

The data in Sections A to E show that

(1) growth ceased at temperatures below 37°C, and

(2) that there was a change in the rate of glycolysis with temperature reflected in a sudden change at 30°C in the slope of the curve when $\log_{10}$ (rate of glycolysis) was plotted against the reciprocal of the absolute temperature. This change in rate was demonstrated not only in whole cells of *B. stearothermophilis* but, to a lesser extent, in cell free extracts. It was concluded therefore that it was not wholly due to a change in the rate of transport of glucose into the cells.

Some enzymes from the glycolytic pathway were investigated to see if they showed the same change in rate of activity with temperature.

Unless otherwise stated crude extracts of *B. stearothermophilis* cells, which had been harvested in the logarithmic phase of growth, were prepared by mechanical disintegration (Method IX 1). The enzymes were assayed as described in Method XXII. The activities of Hexokinase, glucose-6-phosphate dehydrogenase, phospho-hexose isomerase, and lactic dehydrogenase were studied by measuring the change in optical density at 340 mμ when the enzyme was incubated with its substrate and either NAD, NADP, or $NADH_2$. A Shimadzu

recording spectrophotometer with constant temperature control was used. The other enzymes were incubated with their reaction mixtures in a constant temperature water bath, and the end products estimated after the reaction had been stopped.

In all the assays the substrate (at the desired temperature) was added to the temperature equilibrated incubation mixture at zero time to start the reaction. The enzyme was added to the blank and test cuvettes immediately before the substrate.

1. Hexokinase

The activity of hexokinase was measured by Method XXII 1 over a temperature range of 18° to 56°. The rate of change in optical density at 340 mμ at each temperature was calculated at zero time, at 3 min. and 7 min. The rate was calculated by measuring the slope of the tangent to the trace on the recording spectrophotometer chart at each of the times indicated, i.e. the number of enzyme units per mg. protein, where a unit is the amount of enzyme which will catalyse a change in optical density of 1 in one minute.

Fig. 17 shows the $\log_{10}$ (specific activity of hexokinase at zero, 3 min. and 7 min.) plotted against the reciprocal of the absolute temperature. It can be seen that the change in activity with temperature does not show the break demonstrated for glycolysis in Figs. 16 and 15.

$\Delta H^{\ddagger}$ for hexokinase at zero time was 8,800 cal. between 25° and 32°.

Legend for Fig. 17.

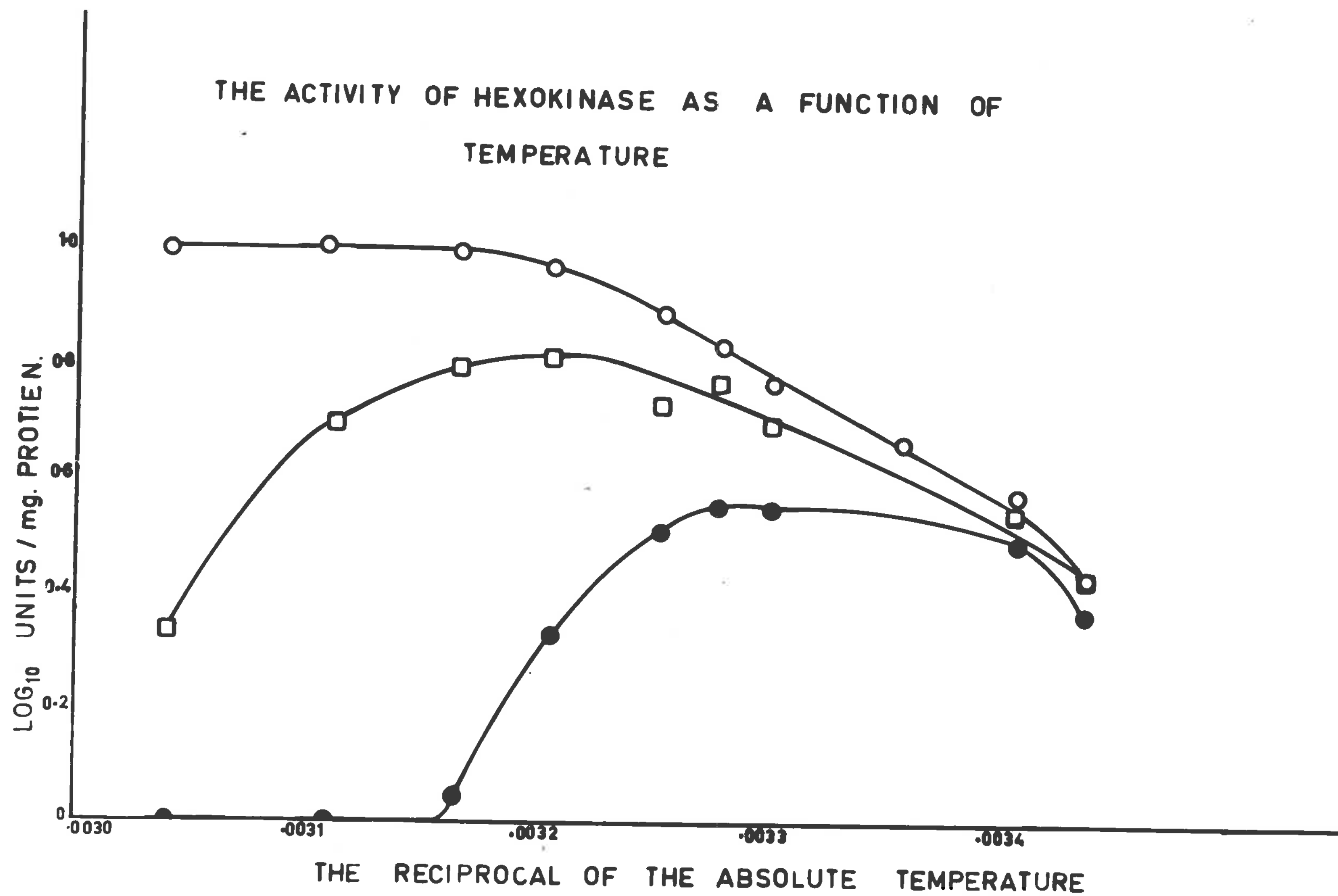
The Activity of Hexokinase as a function of Temperature.

Hexokinase was assayed at each temperature according to Method XXII, 1, and the activity calculated as described in Section F. 1, Chapter V.

○—○—○ = activity at zero time.

□—□—□ = activity after 3 min.

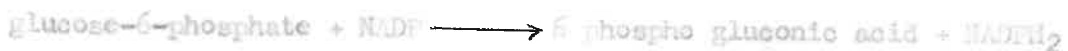
●—●—● = activity after 7 min.


THE ACTIVITY OF HEXOKINASE AS A FUNCTION OF TEMPERATURE
LOG10 UNITS / mg. PROTIEN.
1·0
0·8
0·6
0·4
0·2
0
·0030
·0031
·0032
·0033
·0034
THE RECIPROCAL OF THE ABSOLUTE TEMPERATURE

It is also apparent from fig. 17 that hexokinase is not a heat stable enzyme. At 43°C it had lost 18% of its activity in 3 min. and 96% of its activity after 7 min. This effect would be the same if the enzyme were heat stable but one or all the substrates for the reaction were depleted in 7 min. But it is probably a loss of enzyme activity due to heat inactivation since only one third of the NADP in the reaction mixture was reduced. Equi-molar ratios of glucose, NADP and ATP were added to the reaction mixture.

The reaction being observed was

glucose + ATP $\xrightarrow{Mg^{++}}$ glucose-6-phosphate



glucose-6-phosphate + NADP $\longrightarrow$ 6 phospho gluconic acid + $NADPH_2$

$\therefore$ the ratio of glucose:ATP:NADP = 1:1:1 and if only $\frac{1}{3}$ NADP was used then only $\frac{1}{3}$ glucose and ATP should have been used. However it is known that there is an active ATPase present in B. stearothermophilis (Marsh & Militzer (1956)).

There was no glucose dehydrogenase present when assayed by the method of Goldman & Blumenthal (1964). An excess of ATP in the reaction mixture inhibited the hexokinase. 5 µmole ATP caused complete inhibition, and this precluded the addition of excess ATP.

The assay of hexokinase depended on the simultaneous presence of glucose-6-phosphate dehydrogenase in the crude enzyme. Therefore this enzyme was investigated to find if the apparent instability of hexokinase was in fact an expression of the instability of glucose-6-phosphate dehydrogenase.

2. <u>Glucose-6-phosphate dehydrogenase.</u>

This enzyme was assayed by Method XXII 2. The activity was measured by recording the change in optical density at 340 mμ as NADP was reduced. The specific activity was calculated as described above for hexokinase (Chapter V.A.a). Fig. 18 is a plot of $\log_{10}$ (specific activity of glucose-6-phosphate dehydrogenase) against the reciprocal of absolute temperature.

It can be seen that this enzyme is far more heat stable than was hexokinase. In fact at 60° it had lost only 28% of its activity after 3 min. and 83% after 7 min. But it had lost all its activity after 3 min. at 65°.

The plot of $\log_{10}$ (specific activity of glucose-6-phosphate dehydrogenase) against the reciprocal of absolute temperature did not fall on a smooth curve as it did in the other systems shown. There appear to be at least three peaks of activity (although the line in Fig. 18 has been drawn as a smooth curve) at 36°, 47° and 62°. Starch gel electrophoresis of the crude extract of <u>B. stearothermophilis</u> showed that there were 6 bands with glucose-6-phosphate dehydrogenase activity. The enzyme was detected by flooding the starch gel with the following mixture

3 (4, 5 di methyl thiazolyl 1.2) 2, 5 di phenyl tetrazolium Bromide (Nutritional Biochemicals Corp. Cleveland, Ohio)	20 mg.
NAD	5 mg
Na Azide	50 mg

Legend for Fig. 18.

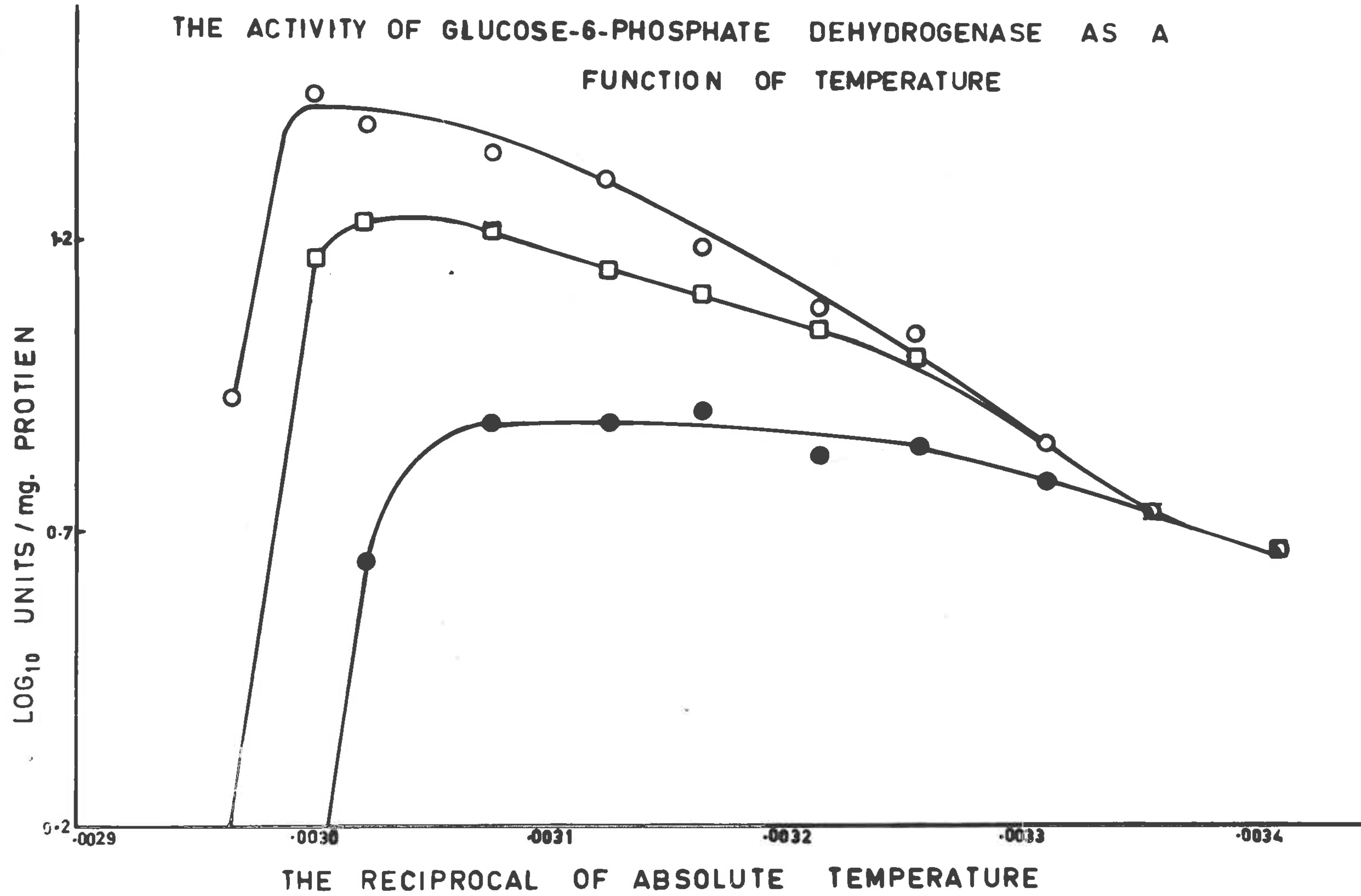
The activity of Glucose-6-phosphate dehydrogenase as a function of temperature.

Glucose-6-phosphate dehydrogenase was assayed at each temperature according to Method XXII, 2, and the activity was calculated as described for Hexokinase in Section F. 1, Chapter V.

○—○—○ = activity at zero time

□—□—□ = activity after 3 min.

●—●—● = activity after 7 min.


THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AS A FUNCTION OF TEMPERATURE
1·2
0·7
0·2
LOG10 UNITS / mg. PROTIEN
·0029
·0030
·0031
·0032
·0033
·0034
THE RECIPROCAL OF ABSOLUTE TEMPERATURE

Tris buffer pH 7.5, 0.1 M	8 ml
glucose-6-phosphate 1.0 M	5 ml.

This was warmed to 45°C, and mixed with an equal quantity of molten 2 agar-agar at 45°.

The starch gel on to which the above mixture had been poured was allowed to stand at room temperature until blue colour developed at the sites of enzyme activity.

No work was done to correlate the peaks of activity shown in the temperature curve (Fig. 18) with the bands separated by starch gel electrophoresis.

$\Delta H^{\ddagger}$ for this enzyme at zero time was 11,702 cal.

3. Phospho hexose isomerase

This enzyme was originally assayed by Method XXII 3a which measured the rate of formation of glucose-6-phosphate from fructose-6-phosphate. However the equilibrum of the reaction apparently favoured the formation of fructose-6-phosphate and fig. 19 shows that the activity of the enzyme increased only slightly with increase in temperature. This is the result one would expect with increase in temperature if the equation for the free energy of reaction is considered i.e. $\Delta G^{0} = -RT \ln K$. where K for this reaction

$$= \frac{\text{glucose-6-phosphate}}{\text{fructose-6-phosphate}}.$$

If the equilibrium of this reaction is <1, then ΔG^{0} becomes positive, and an increase in temperature T will decrease the rate of formation of glucose-6-phosphate from

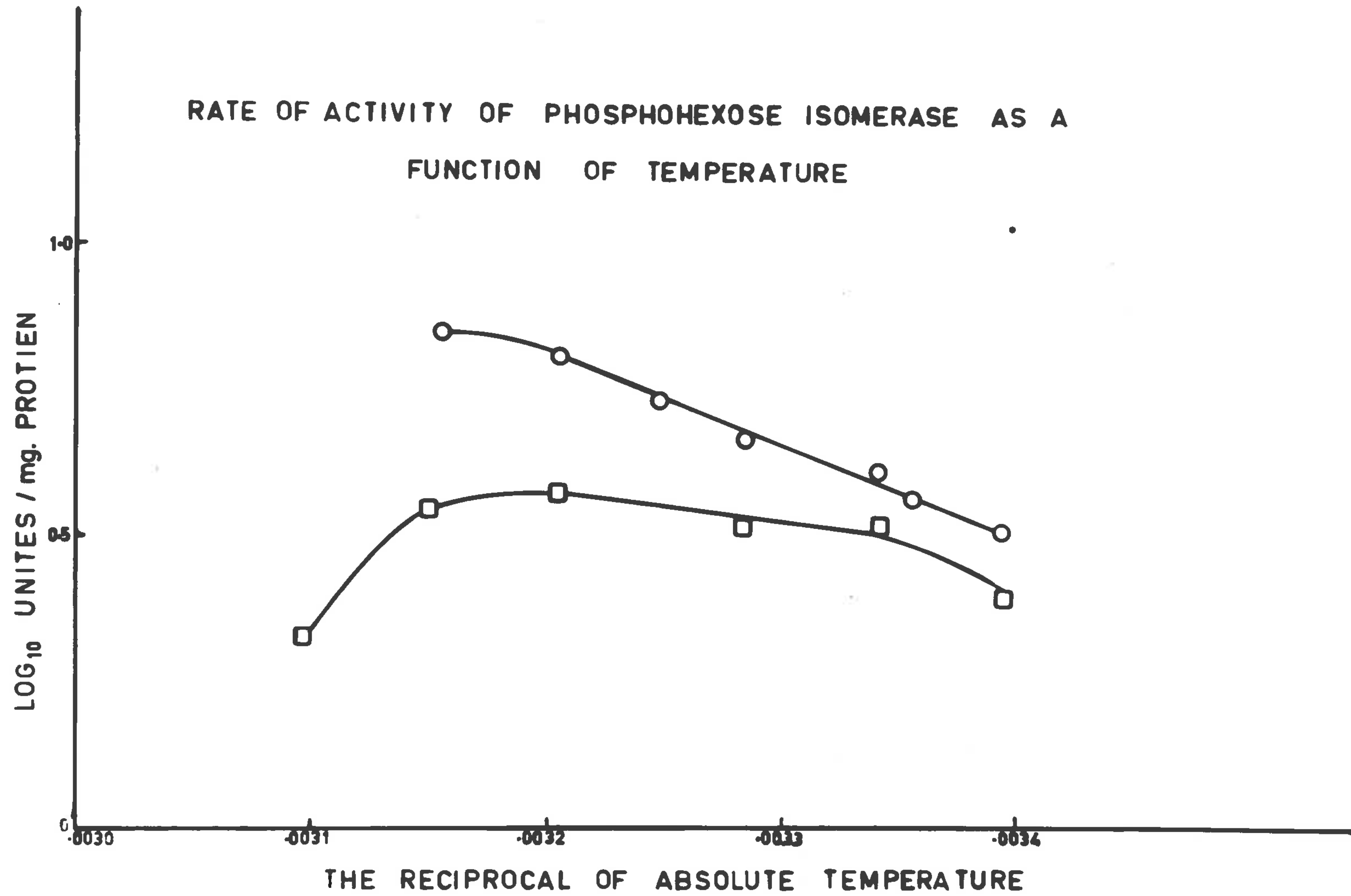
Legend for Fig. 19.

The activity of Phosphohexose isomerase as a function of temperature.

Phosphohexose isomerase was assayed at each temperature according to Method XXII, 3,a., and the activity was calculated as described for hexokinase in Section F. 1, Chapter V.

○—○—○ = activity at zero time

□—□—□ = activity of the enzyme after 3 min. at the temperature of the assay.



RATE OF ACTIVITY OF PHOSPHOHEXOSE ISOMERASE AS A FUNCTION OF TEMPERATURE
1·0
0·5
0
LOG10 UNITES / mg. PROTIEN
·0030
·0031
·0032
·0033
·0034
THE RECIPROCAL OF ABSOLUTE TEMPERATURE

fructose-6-phosphate, even if there is excess fructose-6-phosphate present.

When the enzyme was assayed by measuring the rate of formation of fructose-6-phosphate from glucose-6-phosphate by the method of Roe (1934) the activity of the enzyme rose steeply between 0 and 50°.

[illegible]. Phospho-Fructo kinase and Aldolase

These enzymes were assayed by Method XXII 6 and XXII 5 respectively. These assays differed from those for hexokinase, and glucose-6-phosphate dehydrogenase in that the enzyme activity could not be followed during the reaction, but could only be estimated from the amount of product which had been formed when the reacion was stopped; in these cases after 10 min. The temperatures used were 0, 18°, 22½°, [illegible]°, 30°, 34°, 39°, 45°, 50° and 56°. For both these enzymes a plot of the $\log_{10}$ (amount of end product formed in 10 min.) against the reciprocal of absolute temperature showed no break in the rate of change in activity between 0 - 40°C.

For phosphofructo kinase the maximum amount of end product was formed at 39° and none was formed at 56°. As for hexokinase this apparent instability of the enzyme may have been an artifact. If ATPase in the crude extract had been very active then ATP, which was needed in substrate amounts for the reaction, would have become limiting. Excess ATP inhibited the reaction.

Aldolase catalysed the formation of the maximum amount of triose phosphate in 10 min. from fructose-1, 6-di phosphate at 55° and none at 65°.

5. <u>Lactic Dehydrogenase</u>

This was assayed by Method XIII B(1). The change in optical density at 340 mμ as $NADH_2$ was oxidised by pyruvate was recorded by the Shimadzu recording spectrophotometer. The rate of the reaction was calculated as described for hexokinase (Chapter V [illegible]).

Lactic dehydrogenase was comparatively heat labile. At zero time its activity was 30% less at 47° than it had been at 45°. However it was interesting to note that at any temperature up to 50°, the activity at 7 min. was identical with that at zero time. This was unexpected since the heat stability of a protein is a function of time as well as temperature. However it can be explained by supposing that the enzyme was partly inactivated in the interval between the addition of enzyme to the cuvette, and the addition of the substrate (Na pyruvate), and that the substrate stabilised the enzyme. This supposition will be examined in the following section.

As with other enzymes investigated, the change in rate of the reaction with temperature did not show a break between 20-40° when $\log_{10}$ (activity of lactic dehydrogenase) was plotted against the reciprocal of absolute temperature.

. Experiments to Investigate the Heat Stability of some Enzymes in *B. stearothermophilis.*

Two methods were used to test the inactivation of enzymes to heat

(1) The enzyme was assayed at the temperature being investigated.

(2) The enzyme was incubated for a given time at the temperature to be investigated, and then assayed at 30°. This latter method is inferior since an enzyme might be reversibly inactivated at elevated temperatures, and regain its activity when returned to 30°.

1. Lactic Dehydrogenase

The activity of lactic dehydrogenase (measured by Method XIII [illegible]) varied significantly with the method by which the crude extract was prepared.

At 30° the activity of a fresh extract prepared by mechanical disintegration (Method IX 1) was 40 units/mg. But an extract prepared by lysis with lysozyme (Method IX 4 a) was only 2 units/mg. An extract when prepared by either method which had been stored overnight at -20° had lost all activity.

In addition the crude enzyme prepared by lysis with lysozyme was more heat labile than that prepared by mechanical shaking. The former was completely inactivated after it had been heated in phosphate buffer (0.01 M pH 7.4) for 5 min. at 50°. The crude extract prepared by mechanical disintegration had lost only 60 of its activity under identical conditions. The original protein concentrations were 8 mg/ml.

It could be argued that there was loss of activity in the extract due to proteolysis. To test this possibility, the protein concentration of the extract was increased by addition of 16 mg/ml of Bovine serum albumin (Sigma fraction V). This did not afford protection to the enzyme during the enzyme assay. In addition, it seems unlikely that proteolysis was occurring because lactic dehydrogenase activity was not lost if the extracts were kept at room temperature for 8 hours.

The crude extract prepared by mechanical disintegration was treated with the sodium salt of perfluoro-octanoic acid (1:1000 w/w perfluorooctanoic acid : organisms in the original suspension). The extract was centrifuged for 1 hr. at 100,000 g. and the supernatant was assayed by a method similar to that used for glutamic dehydrogenase (Method XXII 11). Glutamate was replaced by 0.1 M Na lactate and the buffer was adjusted to pH 8.0. The assay was done at 30°.

The enzyme treated in this way was active at 30°, but lost 90 of its activity when heated for 2 min. at 50°C. If Na lactate in in a concentration of 0.01M was present while the enzyme was being treated at 50°, only 40% activity was lost in 2 min. This supports the suggestion put forward in Section [illegible] of this Chapter that lactic dehydrogenase lost activity when placed in a buffered solution in a cuvette at 47°, but was stabilised by the addition of its substrate; in that case pyruvate.

The fact that the enzyme could be labilised by freezing and thawing, and by treatment with perfluoro octanoic acid suggests that after mechanical disintegration the lactic dehydrogenase was relatively stable because it was attached to a cell fragment.

2. Glutamic Dehydrogenase

The pattern for loss of activity with temperature shown with this enzyme was similar to that with lactic dehydrogenase, although glutamic dehydrogenase was always more stable than lactic dehydrogenase.

These cells were grown in Medium C (Chapter III) with 0.1 glutamate as the energy source instead of glucose, and sparged with air.

Whole cells, and cell-free extracts prepared by mechanical disintegration, were tested for 10 min. at 65° with no change in activity when assayed by method XXII 11.

If the extract was kept at -20° for 16 hr. and centrifuged for 1 hr. at 100,000 g after it thawed, the enzyme, which was in the supernatant, had lost some of its heat stability. In fact, after heating the undiluted extract for 1 min. at 65°, only 86% of its activity remained. The presence of glutamate (0.01 M final concentration) during the incubation protected the enzyme to some extent, e.g. 62% of the original activity remained after 5 min. at 65°. The presence of NAD in the incubation mixture instead of glutamate did not protect the enzyme significantly.

On the contrary, if the cell free extract was frozen rapidly in solid CO_2, and thawed soon afterwards by placing in a 50° water

bath, the 100,000 g supernatant was still stable for 5 min. at 65°. However when the extract, freshly prepared by mechanical disintegration, was treated with perfluoro octanoic acid as in Section F. 1 (Chapter V), 63% of its activity was lost after heating at 65° for 5 min.

Whole cells grown in glucose medium C (Chapter III) had no glutamic dehydrogenase activity. However when they were broken by mechanical disintegration, glutamic dehydrogenase activity was comparable with that from cells grown in glutamate. Evidently transport of glutamate into the cells of B. stearothermophilis is governed by an adaptive enzyme. This explains why whole cells would not reduce any redox dye in the presence of glutamate (see Chapter IV B).

When whole cells which had been grown in glucose were incubated at 65°, and then assayed at 55°, there was an increase in glutamic dehydrogenase with the time the cells were left at 65°. This was due to autolysis of the cells.

J. Alanine Dehydrogenase

This was assayed by Method XXII 11 replacing glutamate with alanine. Alanine dehydrogenase retained its activity in the crude extracts which had been either stored at -20° for 16 hrs, thawed and centrifuged at 100,000 g, or treated with perfluoro octanoic acid as in Section F 1 (Chapter V). It was stable for 15 min. at 65°.

4. Other Enzymes.

Other enzymes which were assayed at 30° after they had been incubated under various conditions were

a. Pyruvic Kinase. This was assayed by the method of Bücher & Pfleiderer (1955). After it had been heated for 10 min. at 60°, the activity was still the same as it had been before incubation. But it lost 50% of its activity on heating at 65° for 10 min.

b. $NADH_2$ dehydrogenase. This was assayed by measuring the rate of change of optical density at 340 mμ when 0.1 M $NADH_2$ was mixed with a crude extract of B. stearothermophilis. The activity of this enzyme was unaltered after it had been heated at 60° for 15 min. In fact some $NADH_2$ dehydrogenase activity remained after a crude extract had been heated at pH 4 in a boiling water bath for 5 min.

c. Alcohol Dehydrogenase. This was assayed by the method of Caiger 1962, (Method XII 9) and did not lose any of its activity on being heated at 60° for 10 min. But the activity fell rapidly when heated above 60°.

In addition the stability of enzymes investigated in Section E of this chapter were noted,

i.e. glucose-6-phosphate dehydrogenase reached a maximum activity at 60° at zero time, and lost only 28% of its activity after 3 min. at 60°.

Aldolase had a maximum temperature at 55°.

Hexokinase and Phospho fructo kinase were both relatively unstable to heat, the former losing 96% of its activity after 7 min. at 43°, and the latter having an optimal temperature for activity at 59°.

B. Discussion of Chapter V.

This study of the effect of temperature on B. stearothermophilis was originally done to test the unlikely possibility that the organism might be able to derive some energy from the heat of the environment. However the dry weight of cell material produced per 1 gm. mol glucose did not vary between 40° and 68°, so it was concluded that in the medium used, the energy for growth was derived only from glucose, and by conventional methods.

However, several interesting questions arose from these data, viz.

1. The question many people have asked: why does growth cease at low temperatures?

2. The calculation of $\Delta H^{\ddagger}$, the thermal increment for the systems studied, raises the question of the significance of $\Delta H^{\ddagger}$ in complex systems and in enzyme systems.

3. The variation in the heat stability of the systems studied was of interest.

1. Why does growth cease at low temperatures?

It took 8 days to demonstrate growth at 37°. Is this really the minimum temperature for growth? There was no growth at 35° in ten days, but may there not have been growth if it had been incubated for 50 days? It seems unlikely for several reasons.

It has been shown that amino acid incorporation, or enzyme synthesis ceases at low temperatures, e.g. Bubela (1964) showed

that amino acids were not incorporated into <u>B. stearothermophilis</u> below 37°. Halpern (1961) showed adaptive synthesis of glutamic acid decarboxylase in <u>Escherichia coli</u> ceased at 30° although active at 37°, and Hancock (1957) showed both incorporation of glycine into <u>B. stearothermophilis</u> and the synthesis of catalase and other adaptive enzymes ceased below 37°. But all these processes depend on energy, and here we have shown not only that formation of lactate from glucose is slow below 30°, but that glucose utilisation and growth are "uncoupled" at 37°. (Section A.1)

This "uncoupling" could have been caused by

a. glucose degradation by extra cellular enzymes,

b. the cell being unable to use the ATP formed from glucose inside the cell for energy for growth, or

c. ATP not being formed during the breakdown of glucose inside the cell.

In a and c the energy would be dissipated as heat. (If the theory that life at normal temperatures evolved from thermophilic life be true, then is this a forerunner of our temperature regulating mechanism?)

Unlike glycolysis when studied as a whole, single enzymes from the glycolytic pathway showed a linear response to temperature between 0 and 40°C when plotted as log (activity of the enzyme) against the reciprocal of absolute temperature. But no coupled reaction in the

sequence was examined. There are several reasons why a series of reactions, or a coupled reaction might cease to function at low temperatures.

a. As the temperature decreases the size of the intra molecular bonds would become less until it can be visualised that an active centre is either distorted, or has become too different in size from the substrate to be able to react with it. However Longridge (1963) says that this type of response is associated with a greater $\Delta H^{\ddagger}$ at low temperatures than at higher temperatures e.g. March & Militzer (1956) showed that pyrophosphatase has $\Delta H^{\ddagger}$ 34,000 cal. below 45°, and 21,000 cal. above 45°. The systems for growth and glycolysis (Sections A, B, C) and for amino acid incorporation (Bubela 1964) showed a marked decrease in $\Delta H^{\ddagger}$ below 30°.

b. If each enzyme in a chain of reactions has a different $\Delta H^{\ddagger}$ (or change in rate of reaction with temperature) then the ratios of their activities will be different at every temperature studied. Therefore it can be seen that only in a limited temperature range will the activities be such that the reaction will be balanced, and the sequence run smoothly.

c. The temperature of ionisation of the various buffering systems in a cell, and at the enzyme interfaces might be such that at low temperatures the enzymes no longer have a suitable pH for activity.

Unfortunately we were unable to differentiate between glucose transport and glycolysis. But evidence suggests that even if transport is involved in the cessation of growth at low temperatures it is not the only factor since glycolysis almost ceased in cell free extracts as well as in resting cell suspensions below 30° (Section E).

2. The second aspect of this work on temperature studies is a consideration of $\Delta H^{\ddagger}$, which is described by Johnson, Eyring & Polissar (1954) as the thermal increment, the heat of activation, or temperature characteristic for the enzyme action or system.

Work in this thesis suggests that in biological systems the figure for $\Delta H^{\ddagger}$ is only true for a reaction proceeding in whole and fully active cells, i.e. in this Chapter the figures for growth, and glucose utilisation during growth are the only valid ones. (Sections A 1 and B)

However in resting cell suspensions $\Delta H^{\ddagger}$ for glycolysis varied tremendously with the conditions in which the cells were incubated, viz. $\Delta H^{\ddagger}$ for glycolysis in cells incubated with

glucose and $CaCO_3$ buffer was	14,000 cal.
with amino acids and phosphate was	20,700 cals
and in cell free extracts was	12,000 cals.

The rate of glycolysis, over 10 min. interval (from which $\Delta H^{\ddagger}$ was estimated) was least where the enzymes in the pathway were likely to be destroyed most quickly. When enzymes were assayed in cell free

systems $\Delta H^{\dagger}$ became greater and greater the nearer the measurement was taken to zero time (see figs. 17 and 18). A true $\Delta H^{\dagger}$ could only be obtained if the amount of active enzyme were known at each reading. Obviously the stability of an enzyme to heat is a function of the time at which the enzyme is exposed to heat. Therefore, until the denaturation time is less than the time it takes for the enzyme to reach equilibration with its substrate, the rate of the reaction at the instant when the enzyme - substrate equilibrum is reached should be the true rate.

However in a whole dynamic cell (i.e. one living in an environment in which it can grow) one does not expect to get this denaturation, and $\Delta H^{\dagger}$ for growth, and glycolysis during growth (Sections A and B) are more likely to be characteristic for this organism.

Heats of inactivation seem to be more meaningful and the heats of inactivation for the enzymes measured were the same at each time investigated (e.g. see fig. 18), although the maximum temperature for activity of the enzymes varied with time.

Ingraham (1962) showed theoretically from the equation of Johnson & Lewin (1946)

viz. $$\text{Growth} = \frac{cTe^{-\frac{\Delta H^{\dagger}}{RT}}}{1 + e^{\Delta S/R}\, e^{-\Delta H/RT}}$$

where $\Delta H^{\dagger}$ = heat of activation

ΔH = heat of inactivation

ΔS = entropy of inactivation

R = gas constant

and c = a constant

(which fits a curve such as fig. 14)

that by changing $\Delta H^{\ddagger}$ from 15,000 to 120,000 they did not alter the maximum temperature for growth significantly (in fact from 40.89°C to 45.65°C).

However it seems that changes in heat of inactivation (in other words the stability of the system) would have a much more marked effect on the maximum temperature of reaction.

The following equations of Johnson, Eyring & Polissar (1954) were considered.

(1) $$K_{Tmax} = \frac{\Delta H^{\ddagger} + 2RTmax}{\Delta H + \Delta H^{\ddagger} - RTmax}$$

(2) $$\log_e K = -\frac{\Delta H^{\dagger}}{RT} + \frac{\Delta S}{R}$$

where $\Delta H^{\ddagger}$ = heat of activation

ΔH = heat of inactivation

ΔS = entropy of inactivation

Tmax = the maximum temperature, i.e. the temperature at the turning point of the curve of the log growth rate against the reciprocal of absolute temperature (as fig. 14) and K is the equilibrum constant for activation and inactivation of the system at that temperature.

Then if $\Delta H^{\dagger}$ = 15,000 cal and ΔS = 40 cal/deg/mol

when ΔH = 1,035,000 cal, Tmax = 37

ΔH = 463,000 cal, Tmax = 47

ΔH = 220,000 cal, Tmax = 57

ΔH = 124,000 cal, Tmax = 67

It seems therefore that ΔH has more influence on the maximum temperature than $\Delta H^{\ddagger}$. But these values are much higher than one would expect for an enzymically catalysed system.

If the above equations are considered with $\Delta H^{\ddagger} = 15{,}000$ cal and $\Delta H = 150{,}000$ cal,

Then when $\Delta S = 33$ $T_{max} = 40$

$\Delta S = 31.5$ $T_{max} = 50$

$\Delta S = 27.4$ $T_{max} = 80$

i.e. small changes in ΔS appear to cause large changes in the maximum temperature.

This supports the hypothesis of Davis (1897) that enzymes are more stable at high temperatures if they are not highly organised, and the experimental findings of Campbell et al (1961) that α-amylase is a random coil, and heat stable pyrophosphatase from B. stearothermophilis has a lower entropy of inactivation than the yeast pyrophosphatase (see Section F, Chapter I).

From the data available (fig. 14) and Section A, Chapter V, ΔS for growth of B. stearothermophilis is approximately 45.

3. However, lactic dehydrogenase and glutamic dehydrogenase, two of the three enzymes which I studied with respect to their heat stability (Section G, Chapter V) appeared to be stable because they were bound to a substance which was removed (or destroyed) by freezing overnight at -20°C, or by treatment with perfluoro octanoic acid. This suggests that they were stabilised by association with

a hydrophobic substance, perhaps a phospholipid fragment of the cell. Conversely alanine dehydrogenase was not affected by freezing and thawing, or by perfluoro octanoic acid. It would be interesting to examine these enzymes more fully, and to compare the figures for entropy of inactivation for the three enzymes. Perhaps lactic dehydrogenase and glutamic dehydrogenase are more highly organised molecules than the heat stable alanine dehydrogenase, and have to be held in their three dimensional configuration by association with a more stable moiety.

CHAPTER VI: DISCUSSION AND CONCLUSIONS

The work reported in this thesis deals with two main topics. Firstly, the metabolism of B. stearothermophilis concerned with the production of energy was investigated; and secondly, an attempt was made to add to the slowly accumulating knowledge about how B. stearothermophilis can thrive at 60°, but fails to grow below 37°.

Despite the fact that many papers have been written to the effect that B. stearothermophilis is a strict aerobe (Imsenecki & Solneneva (1945), Militzer et al (1949), Allen (1953), Baker et al (1955), Long & Williams (1958)), the results reported in Chapter IV of this thesis, on balance studies and labelling patterns of the end products of the metabolism of $C_{(14)}$ glucose, show conclusively that, even in the presence of oxygen, oxidative pathways for the break down of glucose were not used. The reason for the difference in our results from those of other authors is discussed in Section D, Chapter IV. Another difference in our results from those of other workers was the rate of growth, or Mean Generation Time found for B. stearothermophilis in our medium. Neilson (1959) said, "In spite of the generally accepted concept that thermophilic bacteria have rates of multiplication commensurate with the high temperatures at which they grow, data are not available to substantiate this suggestion". When B. stearothermophilis was grown in medium C Chapter III, stirred vigorously with air, total cell counts done on samples taken during incubation showed that the mean generation time

for the organism was 4.5 min. However, the mean generation time varied markedly with the composition of the media. Chapter III lists several media in which we grew B. stearothermophilis. Although each medium was at the same pH (initially), each was complete with respect to amino acids, and each had glucose as the energy source, the mean generation times varied from 20 min. to 4.5 min. This difference undoubtedly accounts for the many mean generation times reported in the literature e.g. Allen (1953) quoted a time greater than 16 min., Imsenecki & Solnzeva (1945) found the time to be 16 min., and Neilson (1959) reported a mean generation time of 12 min.

During growth of B. stearothermophilis, lactate was the major end product of glucose metabolism. Investigation of the labelling pattern of the lactate, the lack of CO_2 produced from glucose, and the enzyme present showed that glucose was probably metabolised via the glycolytic pathway. If this is true there would have been a net gain of 2 mols ATP per 1 mol glucose during growth.

In 1960 Bauchop & Elsden postulated that where a carbon source was used for energy alone, i.e. was not incorporated into the cell, there was a constant amount of cell material produced for each gm. mol ATP formed from the substrate. This constant has now been found for a number of micro organisms including Escherichia coli, Streptococcus faecalis, Aerobacter aerogenase, and a yeast Saccharomyces cereviseae. Bauchop and Elsden (1960) and Senez (1962) have shown that in different organisms using the same substrate, 10.5 gm ($\pm$2.2 gm)

dry weight of cell material was produced for every 1 gm mole ATP which the organism was able to gain from the substrate. They used the nomenclature

Y substrate = yield of cells per 1 gm mole of substrate

and Y ATP = yield of cells per 1 gm mole ATP.

Then in organisms using the glycolytic pathway (Strep. faecalis, Sacch. cereviseae) Y glucose = 21 gm. and Y ATP = 10.5 gm. Where heterolactate fermentation was carried out via the hexose monophosphate shunt, one mole ATP was produced per mole glucose and Y glucose = 8.6. For the one organism (Strep. faecalis) using different substrates they showed 2 mole ATP were produced from glucose, the Y glucose = 22 and therefore the Y ATP = 11; 1.67 mole ATP were produced from ribose, the Y ribose = 21, and therefore Y ATP = 12.6; and 1 mole ATP was produced from arginine, the Y arginine was 10 and therefore Y ATP = 10.

Our experimental data adds further confirmation to the growing evidence that Y ATP = 10.5 is a universal figure applying to micro-organisms growing in a complete medium and not incorporating their substrate into the cell material.

We showed that glucose was not being incorporated into the cell during anaerobic growth (Section A, Chapter IV), and that it was probably being catabolised via the glycolytic pathway. Table 3 shows that 21 gm dry weight of cell material was produced per 1 gm

mol glucose. If the glycolytic pathway was used, then the net gain of ATP from glucose was 2 mole. ∴ if Y glucose = 21 gm, Y ATP = 10.5 gm. This result was the same when the organism was grown anaerobically, in air, or in 20% carbogen.

However when using 40% carbogen, Y glucose = 30 gm, and in 100% carbogen Y glucose = 40 gm. This suggests that there were 3, and 4 moles ATP formed respectively, for every mole glucose used. (Section A1, Chapter IV). It was shown in Section B Chapter IV that B. stearothermophilis could utilise amino acids for energy when grown aerobically, and it is suggested that 1 mole ATP arose from a substrate other than glucose. When the organism was grown in 100% carbogen, 20% of the glucose was converted to acetate. Therefore the fourth mole ATP could have come from the reaction acetyl-PO_4 → acetate + ATP. An enzyme which would catalyse this reaction was not demonstrated.

The fact that only 4 mole ATP was formed per mole glucose used under aerobic conditions suggests that the tricarboxylic acid cycle was not being used, for if glucose were completely oxidised, the number of molecules of ATP produced would be nearer 24 than 4.

Militzer et al (1951 and 1952) have shown that some of the enzymes of the tricarboxylic acid cycle (e.g. aconitase, malic dehydrogenase, succinic dehydrogenase) are present in extracts from B. stearothermophilis, but no evidence was presented that they were part of the oxidative cycle. They may have been concerned with synthetic reactions. Experimental evidence that the tricarboxylic

acid cycle did not function in cells which had been grown in glucose was given in Section C Chapter IV. However, the cycle did appear to be active if the cells were grown slowly using acetate, or amino acids as an energy source. It was not clear whether the important factor allowing the production of an active oxidative cycle was the slow growth, or the absence of glucose. As glucose did not inhibit the tricarboxylic acid cycle in cells which had been grown in acetate, it was postulated that when glucose was present in the growth medium, the cell material was synthesised so rapidly that there was always an oxygen lack, which lead to suppression of the Kreb's cycle, and oxidation by other oxidative methods. Section C Chapter IV presents results showing that the tricarboxylic acid cycle was not active in resting cells after growth in glucose, but when present after growth in acetate, the cycle was not suppressed by glucose. It seems, therefore, that there was a defect in synthesis at the enzyme level during growth, rather than a direct suppression. Perhaps one of the essential enzymes for the tricarboxylic acid cycle or other oxidative pathway is an adaptive one, synthesised in response to a high oxygen tension, or a substrate formed by an oxidative reaction.

Holdsworth (1965) has shown that if both nitrate and oxygen are present during growth, less lactate is formed and there is a larger cell yield. This suggests that oxygen lack during growth is the important factor in causing a defect in the oxidative pathways.

In an extension of the work on energy production in *B. stearothermophilis*, the organism was grown at various temperatures from 37° to 72°, and the yield of cells per 1 gm mole glucose was calculated. The Y glucose was the same (21 gm) for all temperatures between 40° and 65° inclusive. Outside these limits glucose disappeared from the medium, but although there was some growth, the cell yield was very much less than 21 gm per gm mole glucose.

We interpreted the fact that Y glucose was the same from 40° to 65° to indicate that in this medium heat energy was not used by *B. stearothermophilis*. This was not an unexpected result since, although there is a large store of potential energy in a liquid culture at 63.5°, it would need an as yet undiscovered enzyme mechanism to concentrate and convert the energy into a "high energy" chemical bond which could be used in synthetic reactions.

It was interesting to find that Brown, Militzer & Georgi (1957) grew a strain of *B. stearothermophilis* at several different temperatures and found that

at 40° the yield was 20 gm. cells,

at 50° the yield was 55 gm. cells,

at 60° the yield was 61 gm. cells, and

at 70° the yield was 27 gm. of cells.

However, they did not state how closely they controlled their growth conditions, nor how much of their energy source was used at each temperature. If they incubated the culture for the same length of

time at each temperature, and then harvested the cells, the yield would be an expression of the rate of growth, and not yield in terms of the amount of substrate used.

By plotting the $\log_{10}$ (rate of growth), for a range of temperatures, against the reciprocal of the absolute temperature, the heat of activation of growth ($\Delta H^{\ddagger}$) was found (Section A Chapter V). Ingraham (1958) suggested that micro organisms might be classified into psychrophiles, mesophiles, or thermophiles depending on their heats of activation for growth. He showed that a psychrophile had $\Delta H^{\ddagger}$ = 9,020 cal. and a mesophile 14,000 cal. $\Delta H^{\ddagger}$ for B. stearothermophilis, a strict thermophile, was 23,000 cals. It does seem that these activation energies are distinct from one another. However, these three figures were found for organisms which have very different growth parameters, viz. a strict thermophile which only grows between 37° and 68°C, a mesophile which grows between 8° and 44°C, and a psychrophile which grows from 0° to 30°C. There are an infinite number of bacteria with an infinite number of growth parameters and it seems unlikely that they will fall into three clear cut groups. However there may be a characteristic $\Delta H^{\ddagger}$ for each single organism. It was shown by Hansen (1933) that in E. coli the growth rate changed markedly in different media, but the temperature increment always remained the same.

The high energy of activation for growth of B. stearothermophilis suggests that a certain minimum temperature is needed before the

reactions necessary are sufficiently rapid for growth to occur. This might explain the phenomenon that the organism will not grow at "normal" temperatures, i.e. below 37°.

Investigation of the energy of activation for some of the processes necessary for growth revealed that $\Delta H^{\ddagger}$ for protein synthesis in B. stearothermophilis is 23,000 cal. (Bubela 1964), and for glycolysis is 24,000 when measured during growth (Section B Chapter V). Both amino acid incorporation (Bubela 1964), and glycolysis almost cease at low temperatures (below 37° and 30° respectively). This is reflected in a change in the slope of the curve when $\log_{10}$ (rate of glycolysis, or amino acid incorporation) is plotted against the reciprocal of the absolute temperature.

Because the activation energies for these processes are high, it is feasible that growth does not occur at low temperatures because some of the vital processes can not be activated. However, against the theory that a minimum temperature is required for activation is the finding that when the $\log_{10}$ (rate of activity) of some of the enzymes of the glycolytic pathway are plotted against the reciprocal of absolute temperature, there is a linear response from 0°C (or room temperature) to the temperature of maximum activity. The enzymes investigated were not inhibited at temperatures below 30°. The only enzyme which has been reported to be inhibited at low temperatures is catalase (Nakamura 1960). The fact that complex

processes are inactive at low temperatures, but the constituent enzymes for these processes are not inhibited at low temperatures suggests that the balance of reactions which normally pertains in an active cell is disturbed. Reasons why a decrease in temperature might cause metabolic imbalance are discussed in Section H Chapter V.

Gaughran (1947) suggested that thermophilic bacteria could not grow at low temperatures because the essential metabolites could not be transferred across the cell membrane. Our experiments, designed to gain direct knowledge about the influence of temperature on glucose transport, failed because we could not control cell autolysis. (Section D Chapter V) However, a plot of $\log_{10}$ (rate of glycolysis in cell free extracts) against the reciprocal of the absolute temperature revealed that in the absence of a cell membrane, glycolysis was still inhibited below 27°C, although less so than in whole cells. Therefore lack of glycolysis in whole cells at low temperatures is not wholly due to lack of transport into the cell. If the supply of energy at low temperatures is inadequate, however, this will indirectly affect transport of nutrients into the cell, because active transport is an energy requiring process. Furthermore, transfer of substrates across the cell wall may not be the only transport problem at low temperatures. During synthesis of new cell material, energy and reducing power, as well as substrates, have to be transferred across intra cellular membranes, and carried to the appropriate parts of the cell. These processes may be disturbed at low

temperatures, too.

There is still no decisive reason why B. stearothermophilis can not grow rapidly below 37°. But since this organism is organised so that it can grow at 60°, it does not seem any more surprising that it does not grow below 37° than that E. coli will not grow below 8°.

The last aspect of thermophilic life to be considered was how B. stearothermophilis was able to grow well at high temperatures.

Davis (1897) suggested that thermophilic bacteria were able to survive because they had a low grade of protoplasmic organisation. Theoretical calculations concerning the influence of changes in the energy of activation, the energy of inactivation, and the entropy of the system showed that small changes in ΔS (entropy) caused large changes in the optimum temperature for growth, and the lower the figure for ΔS, the higher the optimal temperature (Section C Chapter V). This supports the theory of Davis, but it is dangerous to build theories on the results of kinetic calculations, when there is no practical evidence for the results.

At the enzymic level, Manning et al (1961) showed that α-amylase, an extra cellular heat stable enzyme, is probably a random coil (see Section G Chapter I), and March & Militzer (1956) showed that entropy changes during the inactivation of inorganic pyrophosphatase from B. stearothermophilis were smaller than entropy changes for the corresponding enzyme from yeast. There is evidence that some

of the enzymes in *B. stearothermophilis* are stable because the organism is organised in such a way that they are protected by association with cellular structures, e.g. Morita & Haight (1962) were able to protect Malic dehydrogenase in whole cells from destruction at 101°C by increasing the atmospheric pressure, and Nakamura (1960) showed that catalase was protected by a factor associated with the enzyme. Many of the enzymes examined in cell-free extracts have appeared at first sight to be heat stable, but have proved to be unstable on partial purification, e.g. Militzer et al (1951, 52) showed this was so with pyruvic oxidase. We showed (Sections G 1 and 2, and Section H, Chapter V) that glutamic dehydrogenase and Lactic dehydrogenase were probably being protected in crude extracts of *B. stearothermophilis* by association with a cell fragment, e.g. a phospho lipid fragment.

However, some intra cellular enzymes do appear to be inherently heat stable, e.g. inorganic pyrophosphatase (Marsh & Militzer 1956), aldolase and ATPase of Militser were purified many times and retained their heat stability. Alanine dehydrogenase did not lose its activity when treated in the same way in which Lactic dehydrogenase and glutamic dehydrogenase were treated to separate them from their hydrophobic stabilising factors.

Although many enzymes seem to be heat stable, or stable when in association with cell particles, if only one vital protein were heat labile, *B. stearothermophilis* would not be able to survive at 60°.

Allen (1953) formulated her "dynamic theory" to explain how the cell could live if one, or some, of the enzymes were heat labile. (Section F,(2) Chapter I).

Data presented in this work (Sections C & D Chapter V) confirm the finding of Allen that whole cells lose their activity with respect to glycolysis very rapidly when incubated in an inorganic buffer, but not so quickly if the buffer contains amino acids, and a yeast autolysate. Allen interpreted this to mean that the enzymes were being continually replaced when substrates were present from which to synthesise them. Bubela (1964) showed in support of the "Dynamic" theory that nucleic acid, and protein turn over in <u>B. stearothermophilis</u> were much more rapid than in a mesophile, <u>E. coli</u>, at 40°. The growth rates of the two organisms were comparable at this temperature.

There seems no single factor which enables <u>B. stearothermophilis</u> to live at elevated temperatures. Evidence is accumulating to show that some enzymes are stable to heat: some are stable because they are associated with a heat stable moiety, and it is possible that some are being continually resynthesised as they are destroyed by heat.

Conclusions

1. The strain of *B. stearothermophilis* which we investigated did not require oxygen for growth when glucose was present. Carbon di oxide rather than oxygen appeared to be a limiting factor when a culture was gassed with nitrogen.
   However when *B. stearothermophilis* was grown in the absence of glucose, oxygen was essential.
2. Glucose was catabolised by glycolysis, and lactate was the principal end product of metabolism.
   The tricarboxylic acid cycle was not functional in resting cell suspensions which had been grown with glucose as an energy source, but was active if the cells had been grown with amino acids or acetate as the energy source.
3. The minimum temperature for growth was 37°, growth was most rapid at 63°, and the maximum temperature for growth was 74°. Change in temperature between 40° and 68° did not influence the yield of cells per 1 gm Mole glucose, but growth and glycolysis were uncoupled at 37° and above 68°. The heat of activation for growth was 23,000 cal.
4. Plots of $\log_{10}$ (rates of reaction) against the reciprocal of absolute temperature, revealed that there was a sudden decrease in the slope of the curve for glycolysis at 30°, in whole cells and, to a lesser extent, in cell free extracts. This change in slope was not reflected when enzymes of the glycolytic pathway

were assayed over a range of temperatures, and plotted in the same way. Therefore glycolysis did not appear to cease at low temperatures because the enzymes were individually inactive.

5. At high temperatures glycolysis in resting cell suspensions was heat labile, unless a rich nitrogen source was supplied. In cell free extracts glycolysis, and some enzymes of the glycolytic pathway were heat labile, and were not protected by increasing the protein concentration during incubation. Others were heat stable in crude extracts. Glutamic dehydrogenase and lactic dehydrogenase appeared to be stable because they were being protected by a heat stable factor.

6. It was suggested that growth ceased at a minimum temperature because the balance of the rates of enzymes in a complex series of reactions was disturbed; and was able to proceed at 63° because some proteins were heat stable, but others which were heat labile, were being continually repaired. This latter conclusion supports the theory of Allen, (1950)

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