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ABSTRACT

Experiments were carried out on the aspartate trans-carbamylase (ATCase) and the catalase of the thermophile Bacillus stearothermophilus. The ATCase of B. stearothermophilus was compared with that of the psychrophilic Vibrio psychroerythrus and with the published results of this enzyme from the mesophilic Saccharomyces cerevisiae. The ATCase activity of B. stearothermophilus is high in the early logarithmic phase of growth and falls rapidly to almost zero by the beginning of the stationary phase. The optimal temperature of 60°C for B. stearothermophilus ATCase is within its growth range. The psychrophilic ATCase activity increases in vitro above the temperature range for growth of the organism. The values of the energy of activation increase from the psychrophile through the mesophile to the thermophile. The enzyme of the thermophile loses only 50% of its activity during 24 hours storage at 60°C, in the absence of substrate but in its presence loses 90% of its activity in 20 minutes. The psychrophilic enzyme has 50% normal activity present after one hour at 37°C in presence of substrate, and its activity does not decline during 1 hour at 10°C in the presence of substrate. Cytidine triphosphate (CTP) may have a stimulatory effect upon the ATCase of B. stearothermophilus, at 50°C and 60°C. No conclusive evidence for feedback inhibition by CTP was found.

The catalase activity of a culture of B. stearothermophilus rises during the exponential and linear growth phases, to reach a maximum before the stationary phase when about half the total cell mass is produced. It then falls rapidly to zero before the stationary phase. Catalase activity is measurable in whole cells. Activity is increased after low speed centrifugation (500 g or 30,000 g) of broken cells. There is some loss of activity after high speed centrifugation (100,000 g for an hour), but no activity is measurable in the pellet.

The optimum temperatures for catalase activity in B. stearothermophilus and Escherichia coli are about the same (40°C). Similar optima were obtained in crude extracts and cell suspensions. The rate of increase in catalase activity, due to increase in temperature below the optimum, is greater in cell suspensions than in crude extracts of both species.

The energy of activation of the catalase of B. stearothermophilus is between 5,000 and 6,000 calories/mole in both crude extracts and cell suspensions. The energy of activation of heat inactivation is about 40,000 calories/mole in both crude extracts and cell suspensions. In contrast the energy of activation of E. coli catalase in crude extracts is less than half that of cell suspensions which is about 6,000 calories/mole. The energy of activation of heat inactivation of the catalase of E. coli in cell suspensions

is 4-5 times that in crude extracts, which is about 15,000 calories/mole. These findings are discussed in relationship to both the known differences in stability and composition of the cell membranes of the two organisms, and to their growth temperature ranges.

Evidence for the existence of two catalytic components in B. stearothermophilus is presented. One is even more heat labile than the catalase of E. coli and is possibly present in the external cell layers and the other is more heat stable than that of E. coli.

RESUME

L'aspartate transcarbamylase (ATCase) et la catalase d'une bactérie thermophile: Bacillus stearothermophilus, ont été étudiées. L'ATCase de Bacillus stearothermophilus a été comparée à celle d'une bactérie psychrophile: Vibrio psychroerythrus, ainsi qu'à celle d'une levure mésophile Saccharomyces cerevisiae. Chez Bacillus stearothermophilus, l'activité de l'ATCase est très importante au début de la phase logarithmique de la croissance, elle décroît rapidement et devient pratiquement nulle avec le début de la phase stationnaire. La température optimale pour l'activité de l'ATCase (60°C) est à l'intérieur des limites de croissance de B. stearothermophilus. Chez la bactérie psychrophile l'activité de l'ATCase in vitro, croît au delà de la limite de croissance de l'organisme. Les valeurs de l'énergie d'activation augmentent de la bactérie psychrophile à la mésophile, et de la mésophile à la thermophile. Les pertes d'activité chez la bactérie thermophile, sont de 50% après un passage de 24 heures à 60°C en absence de substrat, et de 90% après une période de 20 minutes à 60°C en présence de substrat. L'activité enzymatique de la bactérie psychrophile est de 50% après une période d'une heure à 37°C , elle n'est pas affectée par un passage d'une heure à 10°C en présence de substrat. Le triphosphate de cytidine (CTP) peut stimuler l'ATCase de B. stearothermophilus à 50°C et à 60°C . Il n'a pas été possible de montrer une rétro-inhibition par le CTP.

L'activité de la catalase d'une culture de Bacillus stearothermophilus croît au cours de la partie linéaire de la phase exponentielle de croissance, pour atteindre un maximum avant la phase stationnaire, lorsque la masse cellulaire produite est la moitié de la masse totale. Elle décroît rapidement et devient nulle juste avant la phase stationnaire. L'activité enzymatique est mesurée dans les cellules entières. L'activité des cellules cassées augmente après une centrifugation à faible vitesse (500 g ou 30,000 g). On observe une perte d'activité après une centrifugation à grande vitesse (100,000 g pendant une heure), mais aucune activité n'est mesurable dans le culot.

Les températures optimales d'activité de la catalase sont les mêmes (40°C) chez B. stearothermophilus et chez Escherichia coli. Des optima semblables sont obtenus avec l'extrait brut ou avec la suspension cellulaire. L'augmentation de l'activité de la catalase, dû à une augmentation de la température (en dessous de l'optimum) est plus élevée dans la suspension cellulaire que dans l'extrait brut, et ce, chez les deux espèces. L'énergie d'activation de la catalase de Bacillus stearothermophilus est de l'ordre de 5,000 à 6,000 calories/mole dans l'extrait brut et dans la suspension cellulaire. L'énergie d'activation de l'inactivation par la chaleur est de l'ordre de 40,000 calories/mole dans l'extrait brut et dans la suspension cellulaire. L'énergie d'activation de la catalase d'Escherichia coli est de 6,000 calories/mole dans la suspension cellulaire, alors que celle

de l'extrait brut est moitié moindre. L'énergie d'activation de l'inactivation par la chaleur de la catalase d'E. coli, est 4 à 5 fois plus élevée dans la suspension cellulaire que dans l'extrait brut, cette dernière est de l'ordre de 15,000 calories/mole. Ces données sont discutées en relation avec les différences connues dans la stabilité et la composition des membranes cellulaires, et avec les limites de la température de croissance des deux organismes.

Evidence qui favorise l'existence possible de deux catalases chez B. stearothermophilus est présentée. L'une des catalases est plus labile à la chaleur que celle de E. coli. Elle est probablement située dans les couches cellulaires externes. L'autre est plus stable que n'est la catalase d'E. coli.

INTRODUCTION

During the last two decades considerable interest has been shown in organisms that can grow in extreme environments. Attempts have been made to understand the means by which such organisms carry out metabolic processes in conditions which inhibit growth of the majority. Attention has centred on the sub-cellular and molecular levels of organisation to ascertain whether any differences between organisms growing in extreme environments and those growing in non-extreme situations might explain the success of the former group in the extreme conditions which are normal for them.

In the pages that follow the concept of an extreme environment is explained. There is a review of organisms growing in extremely hot places with special emphasis on bacteria. Those characteristics of thermophilic organisms which are held to be of selective advantage are discussed.

Definition of an extreme environment

In order to define extreme environments it is required to find a criterion or criteria applicable to all. Extreme environments have one or more factors outside the range in which most, but not all, living organisms grow and carry out life processes. Such factors are temperature, salinity, oxygen partial pressure or, more generally, oxidation-reduction potential (Eh), water activity ($.01 \times$ relative humidity), nutrient concentration, certain electromagnetic

radiations (light, U.V. infra-red), ionising radiations (cosmic rays, x-rays) and hydrostatic pressure. Now, if we examine environments having at least one environmental factor which is sub-optimal (or extreme) for most known living things, it becomes clear that only a few types of organisms live in them. Thus most organisms cannot exist above 60°C or below a pH of 3.0, nevertheless there are thermal waters above 60°C and acid situations of less than pH 3.0 in which a few species thrive (Brock, 1969). Life probably arose under the surface of water and was therefore protected from the U.V. radiation which could penetrate the Earth's atmosphere. Also it is thought that a layer of ozone was present near the surface of the Earth at this time, between 4×10^9 and 3×10^9 years ago (Bernal and Synge, 1972). Life possibly originated 3.6×10^9 years ago but the Earth is 4.6×10^9 years old (Miller and Orgel, 1974). However, some organisms are found growing on high mountains where more U.V. light and ionising radiations penetrate, than at low altitudes. Life arose on Earth in a reducing atmosphere (Bernal and Synge, 1972), consequently the first life was anaerobic. Later oxygen was formed by photosynthesis which appeared 3×10^9 years ago (Bernal and Synge, 1972), and possibly in other ways. Today some bacteria, including the Clostridia, of which there are about 100 species, are obligate anaerobes but in general life is aerobic today, and completely anaerobic situations support relatively few kinds of organisms. Few organisms can exist in saturated water moreover the water probably contained pyrimidines and purines which would absorb U.V. light.

salt solution, but the Dead Sea which is saturated with salts is in fact not dead in the sense that no life of any kind can live there. About eight species of bacteria and two species of blue-green algae are found there. There are no vertebrates nor vascular plants in any saline lake (Brock, 1969). Life is impossible without liquid water, yet a few species of animals and plants can exist in deserts by means of water storing adaptations and adaptations to prevent water loss. Most creatures would die if subjected to the hydrostatic pressure existing in the depths of the ocean, yet a few species of fish and some micro-organisms are found there (Brock, 1969). Environments low in a given nutrient; for example available nitrogen, can support only a few species capable of fixing atmospheric nitrogen. However this is a special case as other micro-organisms can feed off the nitrogen fixers or their nitrogenous products. In other words the nitrogen fixers alter the environment so that it no longer is so extreme. Bare rocks whose mineral content is unavailable to most organisms support only a few lichens (Stanier, et al., 1970, p. 747).

There are no vertebrates or vascular plants in thermal lakes and the upper temperature limit for other taxonomic groups is 40°C for eukaryotic algae (with one exception), 45°-51°C for animals, including protozoa, 56°-60°C for fungi and Cyanidium caldarum, a eukaryotic alga, 73°-75°C for blue-green algae and greater than 90°C for bacteria. Thus the more extreme the thermal environment the fewer the number of taxonomic groups which are represented (Brock, 1967a).

Brues in 1932 made a study of water beetles in N. American hot springs and found that the species diversity decreased as the temperature of the hot spring increased up to about 40°C. In 1936 Copeland found the same phenomenon amongst blue-green algae in Yellowstone Park hot springs. The temperature at which they were found ranged up to 75°C. He made similar findings for bacteria and fungi in high thermal environments and in very acid environments and for bacteria in environments rich in hydrogen sulphide (Brock, 1969).

Thus we can say that the factor common to extreme environments is the small number of species found; moreover, precise listing of organisms in any given extreme environment shows that there is a greatly reduced number of taxonomic groups present. Indeed almost pure culture of micro-organisms are found in some extreme environments (Brock, 1969).

The species living in extreme environments, having adapted to the conditions which exclude the majority of other organisms, may be found in large numbers (Brock, 1969), though this is not always so. Large numbers may be due to lack of competition for nutrients from other species (Brock, 1967b).

Environments having one limiting factor are rare (Brock, 1969). Combinations of limiting factors include high hydrostatic pressure, poor light and low nutrient supply in deep water; low temperature with bright light, a high level of near U.V. radiations and low pressure on high mountains, reducing conditions with high temperature and poor light in muddy waters in tropical countries. Experimentally it may

be possible to keep all factors constant and vary one. Hot springs are a natural extreme environment with a temperature gradient operating in otherwise near identical conditions of nutrient supply, light and oxygen concentration (Brock, 1967b).

Not only may more than one factor be limiting but the effect of a given factor may be indirect, through another factor. For example hydrostatic pressure and temperature both affect the solubility of gases, and therefore oxygen availability is affected. High temperature decreases the solubility of oxygen and the viscosity of water and increases its ionisation. High pressures inhibit thermal inactivation of some proteins (Brock, 1969).

Extreme factors in the environment can be excluded, detoxified, lived with by adaptation of molecular structure, and even depended upon (Brock, 1969).

Organisms found in extreme environments

General remarks. Brock (1969) referred to Vallentyne's observation that organisms which grow in extreme environments of all kinds have simple structures. In general prokaryotes are more abundant in extreme environments than are eukaryotes, though this is not true of extremely acid environments.

Non-photosynthetic prokaryotes can exist in extremely high thermal environments that are outside the range of photosynthetic prokaryotes. This has been observed in the same hot springs along the temperature gradient where other environmental factors, notably pH, light intensity and mineral content do not vary. Eukaryotes have nuclear and mitochondrial membranes and photosynthetic organisms have photosyn-

thetic membranes (in chloroplasts in eukaryotes). These membranes are thermolabile in all probability as nuclear membranes must be loosely enough constructed to allow the passage of RNA, and ribosomes and mitochondrial and chloroplast membranes must allow the passage of ATP. The hypothesis is that more rigid cell membrane construction is possible for prokaryotes as an adaptation to thermal environments and this would be selected for (Brock, 1969).

Definitions of the terms thermophile, mesophile and psychrophile

The term thermophile frequently applies to organisms growing above 45°-55°C, but it is more restricted when a given group is considered. Thus eukaryotic algae that can grow at 35°C are considered thermophilic, protozoa growing at 40°-45°C, blue-green algae growing at 50°C, fungi growing above 55°-60°C and bacteria growing above 55°C are all considered thermophilic. Bacteria have the greatest range. They are found growing at temperatures above 90°C but no species is known with an optimum temperature greater than 70°-75°C. Such bacteria can be classed as obligate, facultative or thermoduric. (thermotolerant). Obligate thermophiles cannot grow below 40°-42°C and have optimum temperatures of 65°-70°C. Facultative thermophiles can grow at room temperature and have maxima from 50°-65°C and thermoduric bacteria can grow at room temperature and have maxima at 45°-50°C (Farrell and Campbell, 1969). In earlier literature a thermophile was any microbe capable of growth at 55°C (Allen, 1953).

Mesophilic micro-organisms have optima of 25°-40°C, minima of 10°C and maxima of 45°C. Psychrophilic micro-organisms are capable of growth at 0°C but have optima up to 15°, 20° or 25°C (according to the authority). Organisms exist which die above 20°C.

These classes are somewhat arbitrary as growth conditions (nutrient supply, pH, Eh) may vary the temperature limits for a given organism (Farrell and Rose, 1967).

High temperature environments which support life.

It is interesting to reflect on the supposition that life first evolved at high temperatures on the cooling Earth. At that time high temperatures could not be regarded as extreme conditions except in a prophetic sense. This brings into perspective the attempt to define extreme environments. It is also interesting to bear in mind that adaptations to growth at high temperature may be either extremely ancient characteristics of life, retained, or new characteristics arising in mesophiles, from mutation and consequent natural selection of adaptations favourable to thermophily.

In addition to hot springs, geysers and fumaroles or steam vents, naturally occurring high temperatures occur in desert sands, sun-heated soils which may register 50°-70°C depending on the time of day and the darkness of the soil and piles of rotting humus (Allen, 1953). From, or from near, all these situations microorganisms have been isolated which are capable of growth at high temperatures.

Hot springs are of considerable interest for several reasons. Some are of great antiquity and the organisms in them are likely to have remained undisturbed by man for many centuries (Brock and Brock, 1966). Recent observations have shown that they do not vary in chemical, thermal and hydrological properties over many years (Brock, 1967a). Their effluent provides a temperature gradient as the waters gradually cool the farther from the spring they flow. Thus it is possible to determine the temperature maxima for different species in them and furthermore these organisms are growing in identical conditions of light, pH and nutrient supply. This means that the effects of temperature, the only variable, can be clearly gauged. However in different hot springs pH may vary from 1-9 or higher and the amount of nutrients, rare elements, hydrogen sulphide and radioactivity may vary. In some hot springs sulphur, or calcium carbonate or silica may be deposited. Evidence exists for hot spring activity in precambrian rocks, and it is possible that organisms living in them today may therefore show characteristics of primordial life better than other micro-organisms. Fossils of organisms similar to the Flexibacteriales living in hot springs today are known in Gunflint chert 2×10^9 years old, and these rocks were probably laid down in hot springs (Brock, 1967a).

Industrial situations causing high temperatures include piles of hay, manure piles containing plenty of straw, compost heaps, silage at 60° - 65° C (Allen, 1953), heaps of birds' nests, stored grain and sweating tobacco (Brock, 1969).

These would all be likely to contain a very high number of bacterial and fungal vegetative cells and spores, initially, but they have been shown to give rise to a selective growth of thermophilic organisms. Miede in 1907 first proved that the high temperature that could develop in haystacks was due to bacteria and fungi growing there, and not to purely chemical causes (Allen, 1953). Wallace and Sinha (1962) found species of Aspergillus, Penicillium and other fungi in stored grain: at first mesophiles were established and raised the temperature by their metabolic activity and then the thermophilic fungal species appeared. English, Bell and Berger (1967) found, in sweating tobacco, Bacillus subtilis, B. coagulans, B. megaterium, B. circulans, B. stearothermophilus and actinomycetes. All were thermophilic strains. Felsenstein et al. (1965) isolated from hay B. licheniformis which formed filamentous growth at 40°C, Streptococcus fradiae, Thermopolyspora polyspora which grew between 57° and 67°C, a thermophilic actinomycete and Moromonospora vulgaris which grew at 60°C. Thermophilic bacteria and yeasts and probably moulds are essential in the curing of cacao beans, when temperatures of 40°-50°C develop in the piles of pulp and beans scooped from the inside of the pods (Farrell and Rose, 1967).

Other industrially produced high temperature environments from which thermophilic organisms have been obtained include sugar solution from refineries (Allen, 1953), power plant effluents (Brock, 1969), and melted asphalt (Mateles et al., 1967).

They obtained a hydrocarbon utilizing thermophile from the latter source and also from steam condensate, waste refinery water, oil-water mixture, ditch water and the lawn of the Massachusetts Institute of Technology. Canned food, particularly when stored in the tropics, pasteurising milk and canned milk all support the growth of thermophilic organisms (Allen, 1953).

Other sources of thermophiles

Micro organisms found to be thermophilic when cultured in the laboratory have been obtained from many situations which are not hot. The first such organism was obtained by Miquel in 1879 from the Seine. He reported in 1888 that this organism could grow at 73°C and was incapable of growth at lower temperatures (Farrell and Rose, 1967). Since then thermophilic organisms have been obtained from freshly fallen snow, air, temperate as well as tropical soils, salt and fresh water both cold and thermal and raw milk (Gaughran, 1947). They have also been obtained from ocean bottom mud, mud under fresh water, sewage, feces of man and various animals including birds, frogs and fishes, rony bread, and soil deep in caves (Allen, 1953). This merely proves that the vegetative cells and spores of organisms capable of growth at high temperatures withstood the conditions existing in the source from which they were obtained. It is no proof that they were growing there. The distribution of such thermophilic organisms, however, is an enigma, as ocean bottom mud and soil deep in caves is far away from such likely sources as rotting vegetation, manure

and hot springs (Allen, 1953). This may simply be a measure of the thermophilically facultative possibilities of many bacteria, and the ubiquitous existence of spores.

Thermophilic bacteria

Bacteria provide examples of organisms growing at the highest temperatures known to allow life. Most are spore-forming bacilli but examples of non-sporulating bacilli are known. These include Lactobacillus thermophilus with an optimum of 50° - 62.8° C and a range of 30° - 65° C. Thermophilic sarcinae, staphylococci and streptococci (all Gram positive cocci) and spirochaetes have also been reported. Thermophilic actinomycetes from soils and compost heaps include species of Micromonospora and Thermoactinomyces. Thermophilic streptomycetes are also known and some isolates of Nocardia sebivorans and other pathogenic aerobic actinomycetes can withstand a temperature of 90° C for 10 minutes (Farrell and Campbell, 1969).

One thermophilic gram negative polar flagellated hydrogen oxidising organism is reported from petroliferous soil (McGee et al., 1967). The plates culturing it were placed in an atmosphere of 70% hydrogen, 20% oxygen and 10% carbon dioxide. The authors surmised that it could be a 'missing link' between anaerobic and aerobic life, dating from the time when radioactive decay liberated hydrogen into the earth's atmosphere, which was just becoming an oxidised one and therefore, for the first time, aerobic life was a possibility. Presumably oxidation of hydrogen with now available oxygen gives energy to fix carbon dioxide and produce carbohydrates and organic acids, themselves providing the substrates for oxidative phosphorylation.

Brock (1969) cultured a gram negative filamentous non-sporulating, yellow pigmented organism from alkaline hot springs at Yellowstone Park and Bloomington, Indiana. Its optimum temperature was 70°C and its range 40°-79°C, and it had a generation time of 50 minutes. It could use acetate as a sole carbon and energy source and ammonium as sole nitrogen source. The organism at first referred to as "YT1" was named Thermus aquaticus (Brock and Freeze, 1969). Its filaments enabled single-celled blue-green algae to grow in these waters, because they entangled them. These filamentous bacteria grew at 73°-75°C but in the laboratory when incubated at 70°C in enriched media they produced non-motile rods. At higher temperatures they were filamentous, as in nature. A similar organism has been isolated from hot tap water. If the same inocula were incubated at 55°C spore-forming bacilli developed, which suggested that the others are more extreme thermophiles. One strain was examined as to its sensitivities to anti-microbial agents and was found to share these with other gram negative bacilli (except that its sensitivity to penicillin was higher). From this it was concluded that there was no evidence for peculiar properties in cell envelope-membrane, ribosomes or respiratory mechanisms. Ramalay and Hixson (1970) described a non-pigmented organism in other respects resembling T. aquaticus.

Filamentous bacteria at Yellowstone also include some pink and white ones growing up to 88°C and forming mats visible to the naked eye (Brock, 1967b). At temperatures of over 90°C microscopic examination showed filamentous and rod shaped organisms. Brock considers these to be Flexi-bacteriales. Early reports were confused because fluorescent

microscopy was not available to distinguish the presence of chlorophyll in filaments less than 1 micron in diameter. Consequently Copeland may have described such organisms as algae (Brock, 1967b). Bauman and Simmonds (1969) found the polar lipid and fatty acid content of extremely thermophilic filamentous bacterial masses from two Yellowstone hot springs to resemble that of bacteria not blue-green algae and the carotene content was identical with that of bacteria. The iso-fatty acids had maximum chain lengths of 19 carbons, however, whereas those for bacteria are 15. This may be an adaptation to thermophily confirming greater strength with flexibility to the membranes. Brock (1967b) has also demonstrated by autoradiography that the filaments do not fix carbon dioxide. He proved the bacteria were actually growing in temperatures up to 95.5°C by immersing slides and pieces of string in the waters and removing them days later covered with growth, visible to the naked eye. He demonstrated, by filtering the water, that it contained no free-living bacteria; thus the bacteria had not become passively attached to the slides, but had grown on them. Bott and Brock (1969), irradiated, with U.V., such slides, at intervals, and always found significantly fewer bacteria on them than on the controls.

At the altitude of Yellowstone Park water boils at 92°C so some of these organisms are growing in superheated water. Whatever the difference between superheated water at 95°C and water at this temperature at sea level may be, Brock's (1967b) conclusion that there is no upper temperature limit for life, so long as there is liquid water available, seems

valid. As he pointed out, the highest temperature known to support life may be limiting in respect of pH, salinity, organic or other nutrients, and if this were not so life might be present even above this temperature.

Rod shaped and filamentous Sulphur bacteria grow at 90°-93°C in the Boulder Spring at Yellowstone Park at a pH of 8.9. The water contains 3 µg/ml of sulphide and these organisms have been shown to incorporate organic compounds only in the presence of sulphide. They are not living in optimum conditions as maximum uptake of organic compounds occurred at 80-90°C. They are likened to Beggiatoa (Brock et al., 1971).

A thermophilic photosynthetic purple sulphur bacterium exists at 60°C in Hunter Hot Springs, Oregon, and one also occurs in one spring in Yellowstone Park at 60°C (Brock, 1969).

Thermophilic spore-forming anaerobes include Desulfotomaculum (Clostridium) nigrificans, which is sulphate reducing (Campbell, 1965), C. thermosaccharolyticum, which can reduce nitrites but not nitrates (Ljungdahl, 1965) and C. thermoaceticum, which produces acetate from carbon dioxide, and C. thermocellum (Li, 1966). All have temperature optima of 55°C or higher. C. tartarivorum is an example of a facultative thermophilic spore former (Farrell and Campbell, 1969).

Loginova et al. (1966) described anaerobic cellulose decomposers obtained from soil in a pit in Yangantan. The temperature of the gas at the mouth of the pit was 69°-71°C and the organisms were cultured at 60°-65°C. They were anaerobic

but had aerobic symbionts and grew better when these were present. The symbionts were B. thermolactis, C. thermo-butyricum; B. stearothermophilus (thermophiles) and a mesophile B. cereus var. mycoides. B. lentus, another thermophilic symbiont was halotolerant although its mesophilic analogue was not. Longinova et al. obtained other species from the silt of Tashkent hot springs.

Spore-forming aerobes

In 1953 Allen published a review of aerobic spore-forming thermophiles which included an account of their isolation from soil, sub-aquatic mud (fresh and salt) and dried plant materials. She did not take samples from food of any kind because, she argued, food is in itself an enrichment medium and therefore, yields a limited range of organisms. She used enrichment media of great variety, but which fell into two groups, namely complex media of either yeast autolysate; (some with the addition of glucose or urea) or peptone, and defined mineral media containing one carbon source. The carbon sources were sugars, alcohols, amines, amino acids, carboxylic acids, aromatic compounds, heterocyclic compounds, paraffin, cellulose, agar, starch, inulin, pectin and chitin. All were incubated aerobically at 55-65°C. She obtained thermophiles in all cases except where the carbon source was formate, amines, tertiary butanol, ethylene glycol, phenol, indole, thioglycolate, barbiturate and paraffin. Growth was light if the carbon source was pectin and chitin and in pectin there

was little evidence of decomposition of the polymer. Only spore-formers grew, irrespective of whether the inoculum was pasteurised, and the type of inoculum did not influence the results. She found a progressively weaker growth on a mineral source medium with one carbon source, but could restore vigour with yeast autolysate which supplied growth factors. She described the organisms as 'protean' i.e. their characteristics depended on culture conditions and tended to change, but become more constant with continuous laboratory culture.

In 1948 Gordon and Smith had obtained cultures of all previously investigated presumed thermophilic spore-formers and some isolates from food. They cultured the organisms, noted their characteristics and were able to divide them into two main groups: - Bacillus coagulans, Hammer, which can grow in acidic media and lacks proteinases and nitrate reductase, and B. stearothermophilus (differing from B. circulans only in growing at 65°C) having proteinases and nitrate reductase.

As a result of her investigations Allen divided these organisms into four main groups, the two outlined above, and B. subtilis and B. sphaericus. Three other species described by earlier workers were not in the four groups, namely Denitrobacterium thermophilum Ambroc, B. thermocellulolyticus, Coolhaas, and Nitrosobacillus thermophilus, Campbell (Tables 1 and 2).

The B. stearothermophilus group was the most abundant in soil samples. Gordon and Smith had obtained it from soil, silage, grass cuttings, milk etc. and canned food. They had

obtained B. coagulans from all these (save grass cuttings) and B. sphaericus only from tinned food.

The characteristics of the other three species recognised by Allen as not falling into her four groups are set out in Table 11. Although cellulolytic thermophiles are likely to be obligate anaerobes, associated with aerobes and facultative anaerobes which play a secondary role, Allen noted that Coolhaas had grown thermophilic bacteria on cellulose agar aerobically, and had noticed a clear zone around the colonies. She also reported that a humidity of 98-100% was needed for the growth of cellulolytic bacteria aerobically.

Physiological and molecular basis of thermophily

Three explanations, not necessarily exclusive, have been put forward to explain the ability of thermophiles to live at high temperatures. It is suggested that thermophilic organisms have a high rate of metabolism which rapidly synthesises and replaces enzymes and other proteins and nucleic acids denatured by heat. In addition, at least some of their proteins, including enzymes, and those of ribosomes, are thermostable, and their cell membranes are more stable at high temperatures than those of mesophiles, in part as a result of high saturation of the fatty acid component of phospholipids. Evidence for these hypotheses follow.

Evidence for a high metabolic rate

One piece of evidence for a high metabolic rate in ther-

TABLE 2

Characteristics of other thermophilic spore formers (Allen)

	Nitrate Reduction to N ₂	Hydrolysis		NH ₄ ⁻ oxidation	Temperature	
		Gelatin	Starch		Cellulose	Min.
Denitrobacterium Thermophilum Ambroz	+	0	0	0	37°C	65-70°C
B. thermo- cellulolyticus Coolhaas	0	0	0	+	35°C	65°C
Nitrosobacillus thermophilus Campbell	0			NH ₃ ⁺ nitrate pH 9.5 opt.		

mophiles is the release into the growth medium during the logarithmic phase of growth of amino acids and substances yielding them upon degradation, including enzymes (Brock, 1969). Mesophiles release such substances only after logarithmic growth. The amino acids and proteins which are lost must be replaced at a higher rate to ensure logarithmic growth.

Other evidence adduced in support of a high metabolic rate is the "high need" for oxygen of some thermophiles (Brock, 1967a). The generation time of thermophilic spore-formers was found to be 24 hours at 55°C in broth without aeration, whereas when air was passed over cultures of a thermophilic organism, probably B. coagulans, at 55°C a generation time of 16 minutes was observed. For thermally facultative organisms the viable cell count and the total cell count were affected by available oxygen. The growth rate of B. circulans (B. stearothermophilus) was increased by aeration even of a 20 ml culture in a litre flask (Allen, 1953).

Farrell and Campbell (1969) remarked that maximum growth of B. stearothermophilus (measured by cell mass) was obtained when oxygen concentration reached that found in the mesophilic range of temperatures (143-200 micromolar). Maximum soluble oxygen concentration at 60°C is usually 140 micro molar. Above 140 micro molar oxygen growth is retarded. This observation demonstrates that one effect of a high temperature environment upon living organisms is mediated through the decreased solubility of oxygen and that the "high need" for oxygen is explicable by this. There would seem to be no reason to presume that it is evidence for a higher metabolic rate.

A high metabolic rate is suggested by the fact that death and destruction dominate the growth pattern at higher temperatures. Gaughran (1947) observed that many thermophiles have a high death rate at their optimum temperature. He compared total and viable cell counts and found also that at the end of the logarithmic phase total population curves showed a negative slope and a greater and greater divergence between total and viable cell counts. In other words the duration of time during which the cells can maintain the high replacement rate seems limited. He pointed out that autolysis is rapid, and as 'ghosts' are not counted in the total count, this reduces the apparent death rate.

Allen (1953) used the calculated temperature coefficients of life (growth) and death obtained by herself and others to make a comparison between E. coli, Lactobacillus delbrueckii and B. circulans, i.e. B. stearothermophilus. These organisms have increasing maximum temperatures of growth. The rate constants for growth and death were plotted on a logarithmic scale against the reciprocal of temperatures.

The Arrhenius equation $\frac{dk}{dT} = e^{-E/RT}$

R = gas constant
k = velocity constant
E = energy of activation
of molecules
T = temperature on absolute
scale

relating the rate of a thermochemical reaction to absolute temperature and including the energy of activation of the molecules (which increases with temperature) applies also to

reaction rates in living organisms. It can be applied to the logarithmic phase of growth of microbial cultures. Allén assumed that beyond the logarithmic phase the rate of the synthetic processes continued to be exponential relative to temperature, but the destructive processes played an increasing role. She estimated the extent of the destructive process at each temperature from the difference between the observed growth rate and the projection of the linear portion of the Arrhenius plot to that temperature. She found the death rates so computed were exponential and tentatively suggested that L. delbrueckii could grow at higher temperatures than E. coli because it had a higher temperature coefficient of growth, but that B. circulans could grow at a higher temperature still because it had a lower temperature coefficient of death.

Allen also found that the exponential death curves applied from the start in the growth of microbial cultures (the number of cells dying per unit time is proportional to the number remaining or death is a logarithmic function of time). She suggested that this could be explained by the heat inactivation of one 'critical catalyst' in the cell and not by the inactivation of components present in the cell in large amounts, such as respiratory enzymes. This "first order kinetics of death" lead Brock (1967) to conclude that because these thermal death curves can be interpreted as a result of a mono-molecular reaction, death by heat is therefore due to an effect on the cell membrane resulting in, initially, one rupture, rather

than an effect on ribosomes, enzymes or any other molecule or organelle present in large numbers. He considered that the heat stability of s-RNA and DNA would not be a problem, as those of mesophiles are stable given the ionic environment of the cell. He further pointed out that most organisms produce proteins, including enzymes, which are stable at their growth temperature (see next section) and stated that the ribosomes and the synthesizing machinery of thermophiles (as measured *in vitro* with artificial messengers) are known to be more heat stable than those of mesophiles. However, Farrell and Rose (1967) assert that one cannot assume that a monomolecular reaction is the cause of killing, on the basis of exponential survival curves.

Brock (1967b) made some interesting deductions from published values of growth rates of various organisms at their optimum temperatures under optimal growth conditions. He plotted, for an array of psychrophilic, mesophilic and thermophilic organisms, the logarithm of generations per hour against $\frac{1}{T}$. He found that the slope of the curve was much less than that found for E. coli at various temperatures by Ingraham, or indeed less than that for the organisms Allen had instanced. What this means is that thermophiles in fact do not grow as fast as one would predict from the Arrhenius equation, or, in other words, they are unable to take full advantage of the temperature at which they grow. Their metabolic rate is higher than that of mesophiles but

not as much as it theoretically could be. They have discarded growth efficiency in order to survive at all. Alternately, during evolution from primordial thermophiles, mesophiles have acquired greater possibilities, particularly in the structure of enzyme molecules and concomitant efficiency. Growth rate, temperature optima and thermal response are genetically fixed. Mutations altering these (by altering enzymes structure) would be selected in the right environment. It is probable that many mutations would be required to make big changes (e.g. a 40°C change in optimal growth temperature) as temperature sensitive mutants differ in temperature responses by only a few degrees.

Evidence for a higher resynthesis rate in thermophiles is provided by Bubela and Holdsworth who found that the turnover of labile RNA in B. stearothermophilus was 1 minute at both 40°C and 63°C. This compared with 5-6 minutes for E. coli at 40°C (Farrell and Campbell, 1969). The high rate of RNA turnover might be correlated with a high re-synthesis rate for proteins denatured by heat. The actual protein turnover rate was found to be 10 minutes at 0°C and 1-2 minutes at 63°C. Friedman (1968) suggested that B. stearothermophilus contains a ribonuclease with a low heat of activation and a protease with a high heat of activation.

Amelunxen and [redacted] (1968) found the thermostability of pyruvate kinase and glutamic oxaloacetic transaminase of B. stearothermophilus little more than that of B. cereus. Therefore the supply of these enzymes must depend upon continued

synthesis. The limited growth of B. stearothermophilus at 70-75°C might be attributable to such enzymes. Above a critical temperature the rate of inactivation would exceed the synthesis rate. At 80°C the organism will not grow although many of its enzymes show activity after 10 minutes heat treatment at this temperature.

Indirect evidence for a higher metabolic rate which makes a high re-synthesis rate possible is the fact that thermophiles are usually small, or at least slender. That is if they are longer than mesophiles they are also thinner. This gives a high surface/volume ratio which would facilitate exchange of materials with surroundings and support a higher re-synthesis rate. Allen (1953) noted that the size of individuals of the genus Bacillus decreases as the temperature range for growth increases, and that the thermophilic variants of B. cereus and B. megaterium have slender cells compared to those of the mesophilic forms from which they are derived. She also noted that the blue-green algae in waters at 70°C or above, in Yellowstone Park, are small-celled, compared to those in waters at or below 40°C. The sulphur bacteria in Boulder Spring had diameters of 0.15-0.2 µm (Brock et al., 1971). Only fungi were found able to grow at high temperatures and produce cells 10 microns or more in diameter (Allen, 1953).

Evidence for the thermostability of macromolecules

Proteins

There is no reason why a protein cannot be stable at

high temperatures provided the bonds which maintain the tertiary structure of its molecule remain intact. Koffler (1957) demonstrated that the proteins of B. stearothermophilus and three other thermophilic bacilli are more stable to heat coagulation than are those of the mesophiles Proteus vulgaris, E. coli, B. megaterium and B. subtilis. After 8 minutes heating at 60°C, at pH 7, well over half the mesophilic proteins coagulated and 0-4% of the thermophilic ones. Initial protein concentration and pH affected results but not the large differences between mesophilic and thermophilic proteins. Since these experiments could not entirely rule out the possibility that the proteins were stabilised by other factors present, rather than being intrinsically heat stable, Koffler decided to use highly purified extracts of flagella to test for intrinsic thermostability. He used the viscosity measurement of flagellar suspensions at different temperatures to show that flagella from E. coli are disintegrated into flagellin molecules at 50°C, but those of a thermophilic Bacillus are not disintegrated below 75°C. No stabilising or labilising substances could be demonstrated. The effect of urea and acetamide, which are known to break hydrogen bonds, lead Koffler to conclude that thermophilic flagellin contained more numerous or stronger hydrogen bonds than that of mesophiles. Titration curves for flagellin from mesophiles and thermophiles showed a striking difference between the total number of hydrogen ions bound at pH 2.5, and at pH 10.5, between the two groups. The fla-

gellin molecules of the thermophiles contained only about half as many ionisable groups as mesophilic flagellins. Koffler suggested that in thermophiles there is therefore less repulsion between similarly charged groups on the flagellin molecules than in mesophiles, and this would tend to prevent the disintegration of flagellae. All these experiments demonstrated the possibilities of molecular composition and structure as a basis for thermostability of macromolecules of thermophiles. They did not of course exclude the likelihood that in the living cell proteins in general are stabilised by metallic ions, other proteins, enzyme substrates and co-factors, or structural features of membranes. However with the possible exceptions of metal ions, these stabilising factors cannot operate on flagellins.

In 1973 Koffler reported that the polymer of flagellin from thermophiles begins to disintegrate at a temperature at which the alpha helical region of flagellin unfolds. He therefore concluded that it is this intramolecular nature of flagellin which is at least in part responsible for the thermostability of the polymer.

The flagella, apart from their basal body, consist of a spiral tubular filament of pure polymerized flagellin. Flagellin molecules from thermophiles spontaneously reassemble to form filaments over a wider range of temperature and pH than does flagellin from mesophiles. The polymers have twice the helical content of the monomers. However Koffler (1973) found that at 55°C or higher, flagellin from B. stea-
rothermophilus 2184 reassembled to form polymers with normal helical morphology, but below 50°C the polymers formed were shorter and straight.

Flagellin is a unique protein because it contains a few or no residues of cysteine, tryptophan, proline, histidine and tyrosine. Koffler found six tyrosine residues, one external, three well buried in the molecule of flagellin, and he presented evidence that the other two are concerned with self assembly and inter molecular thermostability, such that the polymer is more thermostable than the monomer. If he nitrated the external tyrosine residue of flagellin with nitromethane he found no change in its ability to polymerize nor in the thermostability of the resulting polymer. If the other two external tyrosine residues of flagellin were nitrated, the stability of the alpha helix remained unchanged but self assembled polymers had characteristics of mesophilic polymers. Thus they would form at pH 6 but not at pH 9 at 25°C, and the modified flagellin would not polymerize at 55°C and pH 6. The nitration of three tyrosine residues in flagellin produced both nitrated monomers, and dimers consisting of covalently linked nitrated monomers. Both the monomers and these dimers were capable of polymerization as above.

Koffler's work thus indicates that the protein of flagella which can have no stabilising factors other than, possibly, metal ions must owe its thermostability in thermophiles to intra and inter-molecular structure.

Enzymic proteins, as distinct from structural proteins, depend upon the presence, in the correct spatial configuration, of certain amino-acids for their catalytic activity. This active site of an enzyme may include a metal ion located in a non-protein prosthetic group; but in any event

thermostability is likely to be possible only if heat does not alter the active site conformationally by changing the shape of the rest of the molecule. Calcium and other divalent metal ions are known to stabilise enzymes to heat (Pfueller and Elliott, 1969), possibly because the ionic bonds they can form with ionised groups on the enzyme molecule are strong enough to resist conformational changes. It is known that thermophilic Fungi can grow at higher temperatures in the presence of calcium ions than in their absence (Brock, 1969). A too rigid enzyme molecular structure might however limit the accomodational possibilities of the enzyme vis-à-vis its substrate ("induced fit") and reduce its efficiency. Other enzyme attributes depending on its three-dimensional structure and its spatial adaptability (allostery, including feedback inhibition, and stimulation) are likely to be affected by adaptations necessary to thermophily.

The thermostable enzymes so far investigated fall into three groups (Farrell and Campbell, 1969). 1) Those which are stable at the temperature at which they are produced but not at slightly higher temperatures, 2) those which are stable at the temperature of production only if they are substrate-protected, 3) the highly heat resistant enzymes.

In group 1, Farrell and Campbell included the malate dehydrogenase of B. stearothermophilus 2184 which is produced at 65°C, slowly inactivated over 90 minutes at 75°C and rapidly in-

activated at 80°C, and the ATPase produced by the same organism. Its inorganic pyrophosphatase showed a lag period, but inactivation commences at 65°C. Some factor seemed to stabilise the enzyme for a time. The alpha amylase of thermophilically facultative strains of B. stearothermophilus and B. coagulans, the aldolase of B. stearothermophilus grown at 65°C and some peptidases were also included in group one. The heat stability of alpha amylase of B. stearothermophilus and aldolases are further discussed below.

In the second group of thermostable enzymes Farrell and Campbell included asparaginases from B. stearothermophilus and B. coagulans, both of which were less inactivated at lower temperatures than they were at the temperature of production in the absence of substrate. The pyruvate oxidase of B. stearothermophilus needed, as well as pyruvate, magnesium ions and oxygen for stability at the growing temperature. A membrane bound cytochrome-containing fraction from a thermophile was found to lose 50% of its activity after 90 minutes at 65°C. The biosynthetic L-threonine deaminase of B. stearothermophilus is partially stabilised at 70°C by threonine and pyridoxal phosphate (Thomas and Kuramitsu, 1971). A catalase from an unnamed thermophilic bacterium was inactivated at temperatures above 60°C unless an unknown protective factor was present (Nakamura, 1960). The crude extract of the enzyme had a temperature optimum of 65°C, but when treated with charcoal the resulting extract had a catalase temperature optimum of 60°C. Moreover below 55°C

the charcoal purified extract had greater activity than the crude extract. Nakamura explained this by a heat dissociable stabilising factor which was removed by the charcoal and which suppressed enzyme activity.

In the third group of thermostable enzymes Farrell and Campbell included a protease from B. thermoproteolyticus Rokko, α -amylases and glyceraldehyde-3-phosphate dehydrogenase from B. stearothermophilus. The protease and α -amylase being extra-cellular enzymes and therefore not protected from heat inactivation by intracellular factors, might be expected to show adaptations to thermophily in molecular composition. The protease, which had 50% activity after heating at 80°C for an hour and lost almost no activity at 50° and 65°C was stabilised by calcium. However an abundance of hydrophobic amino acids, including a high tyrosine content, within the globular molecule was thought to play a role in its thermostability. 8M urea had no effect on the stability of the enzyme at room temperature but accelerated its denaturation by heat, suggesting more or stronger hydrogen bonds might also be involved in the thermostability of the enzyme.

A somewhat detailed review on investigations of α -amylases, glyceraldehyde-3-phosphate-dehydrogenase and aldolases follows. It is included to indicate the type of investigations which have been made using purified enzymes, to attempt to elucidate the nature of heat stability at the molecular level. The overall conclusion seems to be that whilst stronger hydrogen bonds, -S-S- bonds, -SH groups and a high

content of hydrophobic amino acid residues may play a part, nothing is unequivocally established as conferring heat stability.

The α -amylases of B. stearothermophilus strain 1503-4 have been extensively investigated, but the existence of a markedly heat-stable one is not proven (Campbell and Cleveland, 1961; Campbell and Manning, 1961; Manning et al., 1961; Pfueller and Elliott, 1969 and Welker and Campbell, 1963 a-d). Pfueller and Elliott found this enzyme could hydrolyse starch at 70°C and above, provided calcium ions (or other metal ions) or bovine serum albumen were present. These were necessary at temperatures of 55°C upwards for protection from thermal denaturation. They found the enzyme to be cold labile (50% inactivated in 24 hours at 6°C) and completely stable at 25°C. They did not attempt to explain the cold lability in molecular terms, but considered it perhaps the most unusual property of the enzyme.

Cold lability and thermostability might be explicable if the enzyme molecule contains a high number of hydrophobic residues. Hydrophobic bonds are the only weak bonds involved in protein molecular structure that become weaker at lower temperatures. Hydrogen bonds, Van de Waal's forces (between uncharged groups) and ionic bonds (between charged groups) are strengthened at low temperatures (Hochachka and Somero, 1973).

Okasahara et al. (1970) found the alpha amylase of B. stearothermophilus Donk strain BS-1 to differ from that of B. subtilis in conformation, as evidenced by circular dichroism

measurements, in having a slightly higher content of hydrophobic amino acid residues, and in possessing one, not zero, cysteine residues in the enzyme molecule. Thermostability they found was dependent on the presence of calcium ions, and heat denaturation was retarded by bovine serum albumen. Endo studied a different strain of B. stearothermophilus and found an alpha amylase which retained 50% of its activity after heating for one hour at 90°C.

Amelunxen (1966) found the glyceraldehyde-3-phosphate dehydrogenase of B. stearothermophilus lost only 5.4 per cent activity after 10 minutes at 90°C. It even had some activity after 10 minutes at 100°C, if cysteine was present (Amelunxen and Lins, 1968). 8M urea only slightly lowered its activity at temperatures up to 50°C. At 55°C and 60°C a partially reversible rapid inactivation occurred. However rabbit muscle enzyme showed a large irreversible loss of activity at 30°C, and below, by treatment with 8M urea, which caused a large change in optical rotation (Amelunxen et al., 1970). This was taken as evidence that the thermophilic enzyme possibly had stronger hydrogen bonds (Amelunxen, 1967). In later papers (Singleton et al., 1969, Amelunxen, 1970) it was stressed that known physicochemical similarities between the thermophilic and mesophilic enzymes give few clues as to the mechanism of thermostability.

Singleton et al. (1969) compared the thermophilic enzyme to that of rabbit, pig and lobster muscle. They found no

striking amino acid differences, and therefore the content of hydrophobic side chains, which is greater than hydrophilic ones in these enzymes, does not seem to be correlated with thermophily. There are 4 SH groups in the thermophilic enzyme, all necessary for enzyme activity, but in the muscle enzyme there are 13 SH groups, 4 of which are necessary for enzyme activity. Therefore the suggestion was made that the active site is the same in both enzymes (Singleton et al., 1969). Substrate induced inactivation, that is inactivation by NADH in both the muscle and the thermophilic enzyme, was adduced in support of this, because inactivation was thought to take place at the active site (Amelunxen, 1966).

The inhibition of p-hydroxymercuribenzoate was found to differ between the thermophilic and muscle enzymes, in that it took place at higher temperatures in the thermophilic enzyme and was very marked but completely reversed by cysteine. There was total inhibition only, partially reversed by cysteine in the muscle enzyme. It was suggested that the 9-SH groups in the muscle enzyme not necessary to enzyme activity may be near the active site and attacked by PCMB. This was thought to reduce enzyme activity in some way (Amelunxen, 1967).

The thermophilic enzyme was thought to have a firmly bound carbohydrate in its molecule but this has been identified as the ribose in the cofactor nicotinamide adenine dinucleotide (Singleton et al., 1969). This cofactor is bound to the enzyme in the proportion of 4 moles of NAD to

one of enzyme. This is also true of the rabbit muscle enzyme. However removal of the NAD by charcoal had no effect on the thermostability of the thermophilic enzyme (Singleton et al., 1969). Thus the thermophilic enzyme would seem to possess thermostability by virtue of stronger hydrogen bonds and the loss of sulfhydryl groups.

Aldolases have been extensively investigated from the point of view of taxonomy and evolution. There are two main classes differing considerably in requirements, molecular weight and chemistry of the reaction. One, type 1, is found in eukaryotes except fungi; type 2 is found in prokaryotes and fungi. Type 1 is not inhibited by chelating agents and has a molecular weight of about 140,000, whereas type 2 is inhibited by chelating agents and has a molecular weight of only about 70,000. It is stimulated by exogenous metals. The suggestion is made that each type arose independently, but once evolved retained its properties throughout the phyla containing it (Freeze and Brock, 1970). The aldolases of B. stearothermophilus and Thermus aquaticus have been studied with a view to explaining, in molecular terms, their heat stability. Thompson et al. (1958) found that the thermostability of the B. stearothermophilus enzyme was only slowly decreased by cysteine. After 2 hours in the presence of cysteine 12% activity remained compared with over 99% activity remaining in the untreated enzyme after 1 hour at 65°C. Yeast or muscle aldolases were completely inactivated after 5 minutes at 65°C in the presence of cysteine. The cys-

teine instantaneously increased the activity of the B. stearothermophilus enzyme. Since cysteine prevents formation of -S-S- bonds, it seems possible that the thermostability of this enzyme is due to such bonds which prevent denaturation of the enzyme, but at the same time lower its activity. Kinetic studies by these workers showed that the cysteine treatment lowered the activation energy, E, the free energy of activation, ΔG , and the heat change, ΔH , and raised the entropy of activation, ΔS . They postulated that a mild denaturation could account for the effects of cysteine, which also decreases the K_m tenfold at 30°C, less at higher temperatures. Presumably a mild denaturation giving a looser structure makes binding of enzyme and substrate easier. Thompson (1962) later found when studying a purified preparation of the enzyme that it was no longer activated by cysteine. Also it was substrate specific for fructose 1,6 diphosphate in contrast to yeast and muscle aldolases which can also use fructose 1 phosphate. He further found that hydrazine inhibits the thermophilic aldolase and not the yeast or muscle aldolase and explained this and the difference in substrate specificity by assuming different sites on the enzyme molecules of the two aldolases, that on the thermophilic enzyme being blocked by the hydrazine bonded cleavage products, unlike that on the yeast and muscle enzyme. Once again there is indication that thermostability is attained at a price, in this case loss of substrate versatility. Thompson (1962) found that the thermophilic aldolase was

stable on treatment with carboxypeptidase, whereas muscle aldolase lost 90% activity. As Richards and Rutter (1961) had found that yeast aldolase was similar to B. stearothermophilus in this respect this suggests either a difference in terminal amino acid, or a different spatial arrangement of the molecule.

Freeze and Brock (1970) have recently investigated the aldolase of Thermus aquaticus, which has optimal activity at 95°C. It is substrate specific for fructose 1,6 diphosphate, and is activated by cysteine, even when purified. Cysteine also labilizes the enzyme to heat, but at an even higher temperature than that of B. stearothermophilus. The enzyme is substrate protected in the presence of cysteine, but in its absence substrate labilises the enzyme a little to heat. Presumably the partial denaturation postulated as the effect of cysteine, which makes the enzyme more heat labile is prevented by a conformational change resulting from binding of the substrate. The K_m of the enzyme is reduced by cysteine but only threefold.

Freeze and Brock (1970) found the T. aquaticus aldolase to be metal ion dependent, which is characteristic of type 2 aldolase. They thought that this metal ion dependency derives from a metal at the active site, very lightly bound and removable by EDTA, but not other chelating agents and that metal ion activation derives from a loosely bound enzyme metal ion complex, possibly at a site other than the active one. They found that the purified aldolase of B. stearothermophilus is not activated by exogenous metals, but is metal ion dependent.

The molecular weight of the T. aquaticus aldolase was found to be 140,000, about twice that of other type 2 aldolases, and the same as that of rabbit muscle aldolase, a type 1 enzyme (Freeze and Brock, 1970). However if treated with cysteine (6×10^{-3} M) the enzyme preparation gave two elution peaks from gel filtration with Sephadex G-200, one of 140,000 molecular weight and one of 70,000. Both fractions responded identically to EDTA and cysteine (inhibition and stimulation of activity, respectively). It was concluded that the aldolase was heterogeneous. Two other class 11 aldolases are known with molecular weight of 140,000, those of Anacystes nidulans and Saprospira thermalis; this indicates that the larger molecular weight is not a characteristic of heat stable enzymes. Both the molecular species of T. aquaticus were assayed at 80°C.

Freeze and Brock conclude that the active site of the thermostable enzyme has been unchanged by evolution as it resembles other type 11 aldolases in response to inhibitors and activators and catalytic activity, but the rest of the molecule has been altered in the process of acquiring heat stability.

Thermostability of cell membranes of thermophilic organisms

Many enzymes are membrane-bound and are reduced in activity by sonic treatment. Friedman (1968) refers to the heat labilising effect of sonication upon a membrane bound amino-acid activating enzyme prepared from protoplasts of B. stearothermophilus. Protoplasts stable to osmotic shock and heat and cold are formed by B. stearothermophilus sponta-

neously in culture and T. aquaticus protoplasts, produced by lysozyme, are stable in distilled water, and boiling water for one hour (Brock, 1969). In the hot springs at Yellowstone Park spheroplasts were presumed to have been formed spontaneously by the pink filamentous bacteria growing there at near boiling point (Brock, 1969). Such thermostable cell membranes may contribute to the thermostability of membrane bound enzymes in these organisms. There is some evidence that the thermostability of some membranes is correlated with the presence of longer branched and straight chain fatty acids than are found in mesophilic organisms (Friedman, 1968; Bauman and Simmonds, 1969).

There is also evidence that the lipids of thermophilic microorganisms remain constant in saturation at different growth temperatures whereas those of mesophilic microorganisms increase in saturation as the temperature of growth rises (Gaughrin, 1947). The phospholipids of B. stearothermophilus are almost entirely spingomyelins which have a higher melting point than most phospholipids (Koffler, 1957). Almost all lipids of bacilli and other gram positive organisms occur in the cell membrane.

Consideration of the thermostability of membranes, possibly conferred by the branched nature or saturation of their lipids, leads one to a consideration of the activity of membrane bound enzymes, particularly permeases. Uptake of many metabolites depends upon permeases, and it may be that the ability to grow at high temperatures, in part due to the type of lipid produced, carries with it the restriction of growth to those temperatures at which the lipid molecules are able to permit conformational changes in the permeases. Farrell and Rose

Cell Walls

There is little information available on the differences in the composition of cell walls of mesophiles and thermophiles. Farrell and Campbell (1969) reported the glycopeptide content higher and the teichoic acid content lower in cell walls of B. stearotherophilus and B. coagulans grown at 55° than at 37°C and an unusual polymer of a teichoic acid-like compound in the cell walls of these organisms when grown at 55°C. Their cell walls had a higher lipid content than those of most mesophiles.

The cell wall synthesising particulate enzymes of B. stearotherophilus incorporate radioactive material into glycopeptides at 37°C, 55°C and 65°C. The system in B. megaterium for synthesising glycopeptides was maximally active at 22°C and was gradually inactivated at 30°C and 37°C (Farrell and Campbell, 1969).

The extreme thermophile YT-1 (T. aquaticus) investigated by Brock (1969) was presumed to have a peptidoglycan wall and, although a gram-negative organism, showed high sensitivity to penicillin. In 1970, Brock and Edwards reported periplasmic pouches running transversely around the cell of T. aquaticus, formed by regular connections between the peptidoglycan layer and outer cell envelope. He considered it possible that, by making retention of periplasmic constituents more secure, they help growth at high temperatures. The extremely thermophilic sulphur bacteria in Boulder Spring (Brock et al., 1971) also have periplasmic pouches between the very distinct plasma membrane and the cell wall.

This, however, lacks a morphologically distinct peptidoglycan layer and he emphasised that the cell envelope of these sulphur bacteria is quite different from any other mesophilic or thermophilic bacteria.

Growth requirements of thermophilic aerobic spore-forming bacilli in culture

Reference has already been made to the enriched media used by Allen (1953) to isolate the thermophilic spore-formers. She also states that growth declines on mineral media with one carbon source, but improves on addition of yeast autolysate. She maintains that thermophilic bacteria have 'extensive synthetic powers' but that at high temperatures accumulation of acid, or low utilization of a carbon source can stop growth. Hence thermophiles require addition of growth factors (e.g. vitamins, amino-acids) for continued vegetative growth. Thus Allen found one strain of B. sphaericus and two of B. coagulans would grow on a rich mineral medium and several carbon sources. The addition of biotin and riboflavin was necessary to support the growth of "large numbers of thermophiles of all types". However the addition of large amounts of calcium (12 mgms%) was as efficacious as the biotin and riboflavin, in many cases. Cations are concerned in stabilising enzymes to heat, particularly calcium, which would explain why extra calcium could substitute for biotin and riboflavin, the cell could utilise stabilised enzymes to make its own vitamins.

Campbell and Williams (1953) distinguished three groups of temperature effects on growth requirements of microorganisms. In one group the temperature had no effect, in a second group more growth factors were needed at higher temperatures and in a third group more growth factors were needed at lower temperatures. The presence of a thermolabile enzyme on the biosynthetic pathway of a vitamin or other growth factors could explain the need for more growth factors at higher temperatures. The presence of an enzyme with a high heat of activation might explain the requirement for more growth factors at lower temperatures. Temperature lability of apo-repressors or altered affinity between inducers and apo-repressors at high or low temperatures may also limit the growth range.

Genetics of thermophily and psychrophily

McDonald and Matney (1963) had evidence that the ability to grow at 55°C can be transferred from strain 164 of B. subtilis to strain 164S^r. The frequency of transformation was 10⁻⁴. The recipient strain was streptomycin resistant, but only 10-20% of the transformants (55⁺) were still streptomycin resistant. This indicated a linkage between the streptomycin sensitive (S^s) locus and the 55⁺ locus in the donor strain. The authors stated that facultative thermophiles are mostly killed by sudden transfer from 48°C to 55°C. Obligate thermophiles on the other hand are equally viable at 44°C or after a shift from 44°C (near minimum) to 68°C.

The B. subtilis strains transformed in the above study were facultative thermophiles which were, before transformation, sensitive to a sudden change of temperature to 55°C. The ability to be an obligate thermophile, i.e. to withstand a sudden temperature change was thus the characteristic transferred. One can speculate that a mutation or mutations resulted in a structurally more rigid but less heat sensitive enzyme or enzymes occurring when the 55°C characteristic was acquired in the donor strain, during its evolution.

McDonald and Matney regarded the boundary between mesophilism and thermophilism to be between 44°C and 52°C. Facultative thermophiles can have both types of metabolism between 44°C and 52°C. Campbell (unpublished work) obtained a heat labile enzyme (alpha amylase) in a facultative thermophile grown below 46°C and a heat stable enzyme in cells grown above 50°C.

Zuber (1973) pointed out that there are two possible ways in which a facultative thermophile can adapt to heat changes. It can use different biochemical pathways dependent upon the quantities and activities of enzymes at different temperatures. Or it can produce thermostable and thermolabile varieties of enzymes at high and low temperatures respectively. He examined various enzymes produced by facultative strains of B. stearothermophilus, which could grow at 37°C and at 55°C. He obtained evidence of thermostable and thermolabile variants of enzymes produced at the higher and lower temperature. The enzymes included an amino-peptidase,

a protease, some enzymes of the glycolytic pathway and of the citric acid cycle. He found in Bacillus caldattenax a long lag phase when cells were transferred from 37°-70°C during which thermolabile enzymes disappeared and thermostable enzymes were formed. The reverse process was also demonstrated.

Zuber, having demonstrated the existence of variants of enzymes in thermo-facultative organisms speculated that there might be two genes, and two regulators. Presumably the activator for the thermostable enzyme would be inactive below a certain temperature and that for the thermolabile enzyme would be thermolabile itself. Repressors would present the reverse situation. Zuber also suggested that only one gene might be present but that temperature affected transcription or translation such that a different enzyme variety was produced. Thirdly temperature, he argued, could affect directly the enzyme molecule once produced. Thus heat could alter the phenotypic expression of the genetic code.

Referring to the experiments of McDonald and Matney it is therefore possible that the transformation involved the transference of gene(s) coding for one or several key thermostable enzymes. Other possibilities are the transference of the gene coding for a thermostable RNA synthetase, thus affecting transcription, or that for ^athermostable ribosomal enzyme, thus affecting translation. These mutations would, however, not confer thermophily unless all other necessary enzymes and indeed structural proteins were thermostable at

the temperature of production. Another theory as to the possible means by which growth temperature characteristics are altered by mutation is referred to below, but it concerns a change to psychrophily.

Olsen and Metcalfe (1968), using ultra violet irradiation, obtained mutants of Pseudomonas aeruginosa whose growth range was shifted from 11°-44°C to 0°-32°C. The mutation rate was 1 per 10⁸. They surmised that a limited number of genetic loci were involved in the mutation and proceeded to test this by transduction with pseudomonas bacteriophage Px4. They used a tryptophan-requiring auxotrophe as the recipient. They found a frequency of transduction for psychrophily, or for tryptophan independence of 2.5 x 10⁻⁷ to 6 x 10⁻⁸ per viable phage. About 50% of the psychrophily were cotransduced for tryptophan independence, and 50% of the tryptophan independent isolates were psychrophiles. The fact that tryptophan independence and psychrophily were cotransduced in 50% of the mutated P. aeruginosa was taken as supporting evidence of their theory that a limited number of genetic loci controlled psychrophily, and suggested that those loci regulating cell division were involved.

Repression and Induction at different temperatures

Some interesting observations on repression and induction of enzymes in E. coli at different temperatures have been reported (Farrell and Rose, 1967). Although many of these concern low temperatures they help to an understanding of the molecular basis of temperature effects in general.

β . galactosidase was synthesised at the same rate between 20° and 43°C in two strains of E. coli ML30, one constitutive and one fully induced by isopropylthio- β -galactoside. Below 20°C the rates of enzyme synthesis decreased. Glucose repression of β . galactosidase in this strain occurred at low temperatures at which growth was detrimentally affected. A mutant E. coli was constitutive for β . galactosidase at 43°C but needed an inducer at 14°C. Possibly changing affinity of the inducer for the repressor protein at different temperatures explains the results. A temperature sensitive mutant of E. coli required histidine at low temperatures: furthermore it was proved that it produced a phospho-ribose ATP pyrophosphatase 1000 times more sensitive to feedback inhibition by histidine at 37°C than that of the parent. As the temperature fell below this the inhibition increased in the mutant to the extent that exogenous histidine had to be supplied. This demonstrates that the affinity of small molecules for allosteric proteins is affected by temperature.

Other examples of temperature effects on enzyme synthesis cited by Farrell and Rose are: -

glutamate decarboxylase in E. coli WT. Inducible at 37°C
partly constitutive at 30°C

tryptophanase in E. coli WT. inducible at 30°C
, not inducible below 15°C

Alkaline phosphatase in E. coli B mutant - repression by inorganic phosphate decreases between 20° and 40°C

Ornithine carbomoyl transferase and argino-succinase in
E. coli mutant - repression by arginine decreases between
20° and 42°C

Explanations for these repression phenomena are either a
thermolabile apo-repressor or decreased affinity between
apo- and co-repressor at higher temperatures.

It is possible that the induction and repression of
the enzymes of thermophilic organisms are affected in part
by temperature effects on inducers and repressors. Clearly
a thermophile must be able to regulate enzymes synthesis
and possibly thermostable apo-repressors are produced,
making cell regulation possible at the temperature of growth.

Summary of the introduction

Thermophilic organisms have been isolated from numerous
locations and have been found growing in natural and man-made
not situations. Their abundance in some hot places suggests
that they are remarkably well adapted for life at high temp-
eratures. This adaption is largely determined by their proteins,
though the intra-molecular characteristics related to their
heat stability are not unequivocally determined, and vary
between one protein and another. Extra-molecular factors
are required in some cases for heat stability. The membranes
of thermophiles are more heat stable than those of mesophiles
possibly due to lipid composition.

Enzymes and the concept of the Energy of Activation

Most chemical reactions taking place in living cells are catalysed by enzymes. The enormous importance of enzymes is that they reduce the energy of activation of a chemical reaction thereby making possible at temperatures and pH values conducive to life, reactions which would not otherwise take place, at measurable velocities.

Arrhenius (Giese, Cell Physiology, 1968) introduced the concept of energy of activation to explain thermal quotients of two for a rise in temperature of 10°C. According to his calculations an increase in velocity of a reaction of 100% could not be attributed to increase in kinetic energy of molecules. The velocity depended, he argued, on the number of molecules of reactant attaining a high enough energy level to change chemically. He found experimentally that a graph of the natural logarithm of the velocity of a chemical reaction against the reciprocal of the absolute temperature was a straight line. The slope of this line (a), which measures the change in velocity with temperature, he related on theoretical considerations to E (the energy of activation expressed in calories per mole) by the equation

$$a = \frac{E}{R}$$

R = gas constant

Because the graph is a straight line and has negative slope

$$\ln K = b - \frac{a}{T} \quad \text{and} \quad \frac{d \ln K}{dT} = -\frac{a}{T^2}$$

K = velocity

b = intercept on x axis

$$\text{Therefore} \quad \frac{d \ln K}{dT} = -\frac{E}{RT^2}$$

T = absolute temperature

$$\text{Integrated } \frac{K_2}{K_1} = e^{-\frac{E}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)}$$

K_1 = velocity at T_1

K_2 = velocity at T_2

T_1 = lower temperature

T_2 = higher temperature

$$\text{or } \ln \frac{K_2}{K_1} = \frac{E}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)$$

$$E = R \times \frac{\ln K_2 - \ln K_1}{\frac{1}{T_1} - \frac{1}{T_2}} \quad \text{calories/mole}$$

$$\text{and } E = 2.303 \times R \frac{\log_{10} K_2 - \log_{10} K_1}{\frac{1}{T_1} - \frac{1}{T_2}} \quad \text{calories/mole}$$

Arrhenius then calculated the proportion of molecules having the energy of activation before and after a 10°C rise in temperature, using the Maxwell-Boltzmann distribution law.

$$\frac{N_1}{N_0} = e^{-(E/RT)}$$

N_1 = number molecules having energy equal to or greater than the energy of activation of the reaction

N_0 = total number of molecules

He found that there was a disproportionate increase in this number.

The energy of activation is related to the enthalpy change, ΔH , by the formula $\Delta H - RT = E$. ΔH is very large compared to RT , ($R = 1.987$ calories/mole/degree and $T = 273^\circ - 343^\circ$ in our experiments. Therefore $RT \approx 600$ cal./mole at these temperatures). Therefore determination of ΔH is an approximate measure of E . Comparison between ΔH of the same reaction under different conditions can therefore be informative.

The mechanism of enzyme catalysis is presumed to involve

the formation of an intermediate complex of the enzyme molecule and the reactant(s). Both are altered in special configuration, bonds are broken and other bonds are made by movement of electrons, and the product(s) are formed. The steady state or absolute reaction rate theory of Eyring (Giese, 1968, p. 248) supposes the formation of an activated complex of the reactants. It can be applied to the theory of the intermediate enzyme substrate complex. When representing the thermodynamic quantities relating to the formation of the enzyme substrate complex, it is customary to write the thermodynamic equation as $\Delta G^* = \Delta H^* - T\Delta S^*$ where ΔH^* = enthalpy of heat content, G^* = free energy and S^* = entropy of the activated intermediate complex of enzyme and substrate. According to the absolute reaction rate theory

$$K = \frac{k k' T}{h} e^{-\frac{\Delta G^*}{RT}}$$

K = rate constant for formation of active complex
 k = transition coefficient (probability factor concerned with ES → E+S or P)
 k' = Boltzman constant $\left(\frac{R}{N}\right)$

h = Plank's constant $q = hv = \frac{hc}{\lambda}$ and $h = \frac{q}{\nu} = \frac{q\lambda}{c}$
 q = quantum energy (ergs)
 ν = frequency c = velocity of light
 λ = wave length

and thus the rate varies inversely and exponentially with ΔG^* . Catalytic efficiency is therefore increased if ΔG^* is reduced. It is apparent that E is a valid index of the comparative ability of enzymes from different organisms to lower the energy barrier, ΔG^* , to a given reaction only if the entropy is the same.

According to the collision theory:

$$K = PZe^{-E/RT} \quad (\text{J.G. Morris, 1968, p. 249})$$

where P is a constant, the probability of colliding molecules having both the correct orientation towards each other and the necessary energy (E) to react, and Z is the collision frequency in unit volume, depending on mass and radii of the reactants' molecules, temperature and concentration. As $\Delta G = \Delta H - T\Delta S$ and $\Delta H \approx E$ the above equation can be written

$$K = PZe^{-(\Delta G + T\Delta S)/RT}$$

and K varies inversely and exponentially with ΔG . However (p. 248) Morris states that the collision theory is applicable only to reactions between gases.

INTRODUCTION TO THE EXPERIMENTAL INVESTIGATION

In order to shed light upon adaptation to thermophily I investigated the temperature effects on the activity and stability of two enzymes of B. stearothermophilus. I chose the anabolic enzyme aspartate transcarbamylase (ATCase) because it is on the pathway of pyrimidine synthesis, is therefore essential to growth and cell division, and so must function adequately at the optimum temperature of growth of this organism. As Farrell and Campbell (1969) reported that some enzymes of thermophilic organisms are thermostable at the temperature of production only if substrate protected it was also of interest to ascertain whether ATCase was stabilized or labilized to heat by its substrate. Amelunxen and Lins (1968) reported little difference in thermostability of glutamic oxaloacetic transaminase in B. stearothermophilus and B. cereus (a mesophile). A transaminase might be involved in the buildup of proteins, or in other words is anabolic. Therefore there is no a priori reason for ATCase to be thermostable.

The other enzyme studied, catalase, is present in all aerobic organisms except the Lactobacillaceae. It is a catabolic enzyme, regarded as protective as it dissociates toxic hydrogen peroxide into water and oxygen (Stanier et al., 1970, p. 663). Hydrogen peroxide is a byproduct of oxidase activity in cells. Presuming the protective function of this enzyme to be a necessary concomitant for growth of B. stearothermophilus

it was likely to be informative to study its thermostability and activity at different temperatures. Amelunxen and Lins (1968) had found the pyruvate kinase of B. stearothermophilus and B. cereus to differ little in thermostability, indicating that one cannot assume all catabolic enzymes of thermophiles are thermostable.

Catalase is rapidly inactivated by its substrate (Maehly and Chance, 1954) but its stability in absence of substrate can be investigated. This was of especial interest in whole cells, which have catalatic activity. One can presume that the effects of temperature in vivo are similar to those on a suspension of cells in vitro.

Brock (1969) composed an Arrhenius plot of growth rates of different organisms at their optimum temperatures. The slope of this graph is a measure of the energy of activation of growth of microorganisms in general. This slope is lower than that of the mesophile E. coli growing at different temperatures. As growth is a function of the activity of many enzymes I determined the energy of activation of the ATCase of B. stearothermophilus and compared it with that of Vibrio psychroerythrus and the published results of that of Saccharomyces cerevisiae.

Friedman (1968) suggested that in B. stearothermophilus a protease with a high energy of activation, in conjunction with a ribonuclease having a low energy of activation, might explain the equally rapid turnover of labile RNA at 40°C and 63°C although the turnover of protein was much higher at 63°C than at 0°C. These enzymes are catabolic ones. I have

compared the energy of activation of the catalase of B. stearo-thermophilus and of E. coli in both crude extracts and cell suspensions, and also the energy of activation of heat in-activation, to find out whether there are differences which might relate to the differences in optimum growth temperatures.

Further information on the enzyme studied

ATCase couples carbamyl phosphate to aspartic acid to form carbamyl aspartic acid (CAA), and is the first enzyme specific to the pyrimidine synthesis pathway. CAA is converted by a series of reactions into uridine diphosphate (UDP) and uridine triphosphate (UTP) and cytidine triphosphate (CTP) can be formed from the latter. These nucleotide phosphates act as feedback inhibitors. The enzyme has been isolated and purified from a number of organisms, and usually contains allosteric subunits in addition to the catalytic subunit.

O'Donovan and Neuhard (1970) state that CTP inhibits ATCase from Escherichia coli, Salmonella typhi-murium, Pseudomonas aeruginosa and P. fluorescens, Enterobacter (Aerobacter) aerogenes, Serratia marcescens, Citrobacter freundii, Rhodopseudomonas spheroides, Halobacterium cutirubrum. They also state that UTP inhibits ATCase from P. aeruginosa, P. fluorescens, Neurospora crassa and Saccharomyces cerevisiae, and also state that no feedback inhibition of ATCase has been found in Bacillus subtilis and Streptococcus faecalis. The

ATCase of Vibrio

costicolus does not exhibit feedback inhibition (Kushner, private communication).

Catalase, a heme protein enzyme, is present in all living organisms possessing a cytochrome system (Deisseroth and Dounce, 1970). Its presence in all organisms except a few bacteria is taken as evidence that it is evolutionarily ancient (Nicholls and Schonbaum, 1963). As there is evidence that the young Earth had an anaerobic reducing atmosphere and that life evolved under these conditions (Bernal and Synge, 1972) it cannot be among the enzymes that were first evolved. It is not present in obligate anaerobes e.g. the Clostridia or the Chlorobacteriaceae, which may be descended from the earliest, aerobic, forms of life, non-photosynthetic and photosynthetic respectively. >

Catalase has been widely regarded as having a protective role against toxic hydrogen peroxide which is produced by aerobic dehydrogenations e.g. by flavoprotein oxidases (Thomas et al., 1973, p. 136). However these authors state that systems producing hydrogen peroxide in aerobes are not now thought to be numerous, and surmise that the situation might have been different in their ancestors, in which case catalase activity in present day organisms is to be regarded as vestigial. Against this approach is the fact that almost the only aerobic organisms lacking it, the microaerophilic Lactobacillaceae, possess flavoprotein peroxidases which destroy hydrogen peroxide by using it to oxidise organic substrates (Stanier et al., 1970, p. 664). The term peroxidase usually applies to a heme

protein enzyme, but unlike catalase peroxidases cannot catalyse the destruction of hydrogen peroxide 'catalytically' using a second molecule as a hydrogen donor and liberating oxygen. They use an organic hydrogen donor, which thus becomes oxidised (George, 1952). Moreover, catalase can act 'peroxidatically' too especially when substrate concentration is low (less than 10^{-4} molar), and there is evidence that this is its major mode of catalysis in Micrococcus lysodeikticus (Chance, 1952).

In vitro catalase can function in the absence of hydrogen peroxide. The substrates are hydroperoxides, e.g. methyl or ethyl hydroperoxides. The affinity of catalase for these alkyl peroxides is much lower than for hydrogen peroxide (Thomas et al., 1973).

In rat liver and kidney catalase is found in peroxisomes together with oxygen-linked dehydrogenases which yield hydrogen peroxide (uric acid oxidase, D-amino acid oxidase and L- α -hydroxy acid oxidase). Present also are flavin oxidases yielding hydrogen peroxide (Thomas et al., 1973). Frederick and Newcomb (1969) state that in animal peroxisomes the peroxidatic activity of catalase is more important, but in plant peroxisomes it is the catalytic reaction which is more important. In some plants hydrogen peroxide is released by the oxidation of glycollate, $\text{CH}_2\text{OH-COO}^-$, a product of photosynthesis, excreted by the chloroplasts. The peroxisomes are frequently located close to the chloroplasts. The other product of glycollate oxidation, glyoxylate, may be reduced to glycine which

in turn is oxidised. This is thought to account for the increased uptake of oxygen in the light, termed photo-respiration. As glyoxylate and hydrogen peroxide can react non enzymatically to produce formic acid, carbon dioxide and water, it is thought that catalase plays a role in photo-respiration by immediately removing the hydrogen peroxide and preventing this non-enzymatic reaction (Zelitch, 1972).

Nichols and Schonbaum (1963) state that the highest concentration of catalase in plants is found in the chloroplasts, though Frederick and Newcomb (1969) found no evidence that catalase is located mainly in the chloroplasts of tobacco leaves. It is possible that catalase plays a role in the production of oxygen in photosynthesis because the photolysis of water yields free OH radicals which might give rise to hydrogen peroxide, which, in turn, would be broken down by catalase. However Nichols and Schonbaum, having regard to the nature of inhibitors of the oxygen-evolving step in photosynthesis, do not believe that catalase can be involved. Thomas et al., (1969) report that there is some evidence that a flavoprotein enzyme is involved (this could produce hydrogen peroxide to be broken down by catalase), but that very little is known about the enzymes associated with oxygen evolution in photosynthesis. Cheniae (1970) does not mention catalase in a paper entitled 'Evolution of oxygen in photosynthesis'.

One reason why the protective role of catalase has been questioned is the occurrence of people who lack catalase, or

have an altered catalase of very small activity (Takahara, 1952). These people have not been shown to have any metabolic defect, though they are very susceptible to dental infections and tissue necrosis in the jaw, nasal cavity, sinuses, tonsils and pharynx. This is thought to be caused indirectly by streptococci and pneumococci which lack catalase and produce hydrogen peroxide in sufficient quantities to oxidise haemoglobin to met-haemoglobin. Presumably their flavoprotein peroxidase activity is not high enough to destroy enough of the hydrogen peroxide they produce.*

Catalase is regarded as having a protective role in the prevention of oxidation of haemoglobin to methaemoglobin, but Keilin and Hartree (1945), found catalase did not protect haemoglobin from oxidation by slowly generated peroxide. They also found that the possession of catalase by bacteria did not prevent them being killed by the glucose oxidase of Penicillium notatum. Clayton (1960a), found that catalase is not constitutive in Rhodospseudomonas, though inducible by oxygen. However, Rhodospseudomonas is a facultative aerobe, so that the non-constitutive character could be used as an argument that catalase does have a protective function and is induced when required. It is known to be induced by hydrogen peroxide, and there is evidence that when induced by air (oxygen) induction is through the intermediary hydrogen peroxide (Clayton, 1960b).

* However it is possible that the catalase activity of tissues other than blood (kidney, spleen, liver) is not as greatly reduced in acatalasemic people as is that of blood. (Takahara 1971).

MATERIALS AND METHODS

Description of strains and culture methods

Bacillus stearothermophilus

National Research Council strain B 1027 and 9001. Both are from the American Type Culture Collection strain 12016. Although ATCC 12016 is listed as "Bacillus sp. obligate thermophile" the N.R.C. received it as B. stearothermophilus.

Strain N.R.C. B 1027 was supplied as frozen cells (by A. Matheson) which had been grown at 65°C from a 3% inoculum in copiously aerated batch cultures of 100 litres of Trypticase Yeast Salts (TYS) Medium (Pace and Campbell, 1967). They had been harvested in early logarithmic phase, after 3-3½ hours growth. The absorbance at 650 nm was 1.3 in a 10 mm cuvette in a Coleman Junior Spectrophotometer. This strain was used for investigations concerning the effect of temperature on ATCase activity.

Strain N.R.C. 9001, was used for all catalase investigations and the ATCase activity at different times in the growth cycle. Stock cultures were grown on TYS agar slants at 55°C, stored at 5°C and sub-cultured once a fortnight. Lyophilised cells were used to start an inoculum on some occasions. An inoculum was grown up in TYS in a 25 ml conical flask in a shaking water bath at 60°C. Cells were obtained by adding a 1-10% inoculum to 1-4½ litres of TYS plus 1.5% glucose and incubating in a water bath at 60°C. Aeration with moistened

air at 60°C was maintained by means of a sparger, and a stirred was positioned over the sparger to aid turbulence. The growth chamber was a large conical flask. It was also possible to use a large beaker with baffles. The top was covered with foil through which the stirrer and sparger passed through an opening protected with cotton wool. Contamination was not a problem given the large inoculum and high growth temperature. This method gave better aeration. Cells were harvested after 3-5 hours in late linear phase and washed twice with 0.13 M phosphate buffer pH 6.8.*

Escherichia coli. ATCC 11303 (S.E. Luria, strain B).

Stock cultures on nutrient agar slants were grown overnight at 37°C, stored at 5°C and sub-cultured once a month. 20 ml of nutrient broth inoculated from a stock slant were incubated overnight at 37°C on a shaker. This inoculum was added to 1 litre of nutrient broth, incubated on a reciprocating shaker at 37°C, harvested in mid-logarithmic phase and washed twice with phosphate buffer.

Vibrio psychroerythrus N.R.C. 1004. A psychrophilic marine bacterium isolated from flounder eggs, in Norway. A polar flagellated gram negative rod classified and named by D'Aoust and Kushner (1972). Stock cultures on slants of Psychrophile Salts Medium (PSM, D'Aoust and Kushner, 1971) were grown at 10°C for 3 days, stored at 5°C and sub-cultured once a month. 25 ml of PSM inoculated from a stock slant were incubated at 10°C on a rotary shaker until the medium was red (2-3 days).

500 ml of PSM plus the above 5% inoculum were incubated on a * A growth curve for B. stearothermophilus, N.R.C. 1001, is shown in fig. 23, p. 103.

rotary shaker at 10°C for 3 days. Cells were washed twice in buffer containing salts as in the medium.*

Preparation of crude extracts for ATCase activity measurements

Vibrio psychroerythrus. Cells were centrifuged and washed twice in growth medium without tryptone. They were then taken up in 0.01 Molar Tris buffer, pH 7.4, to which had been added sodium chloride and magnesium chloride in the same concentrations as in the growth medium. The presence of the salts prevented spontaneous lysis. The suspension was then lysed by sonication of 30 seconds at power level 1 on the Branson Sonifier. After centrifuging at 12,000 g for 15 minutes the supernatant was used for enzyme assay.

Bacillus stearothermophilus. Frozen washed cells supplied by Dr. A. Matheson were taken up in 0.01 Molar Tris buffer, pH 7.4, and lysed by incubation at 37°C for one hour with lysozyme, in the ratio 1 : 10,000 enzyme to wet cells, by weight. After centrifuging at 27,000 g for 30 minutes the supernatant was used for enzyme assay.

Escherichia coli. Cells were centrifuged and washed twice in 0.01 M tris buffer, pH 7.4. To obtain the crude extract the cells were taken up in Tris buffer again and sonicated for three 20-30 second intervals, with cooling in ice between, at power level 8 in the Bransen Sonifier. After centrifuging at 12,000 g for 15 mins. the supernatant was used for enzyme assay.

* A growth curve of V. psychroerythrus is shown in fig. 22,

Preparation of cell suspensions and crude extracts for catalase activity measurements.

Bacillus stearothermophilus and Escherichia coli.

Cells harvested in late logarithmic phase of growth were centrifuged and washed twice in phosphate buffer. If possible enzyme assay was carried out the same or the following day, in which case they were stored at 5°C in the refrigerator.

Activity was not reduced by such storage, though it was by prolonged storage at 5°C. Activity was measurable after freezing, though it was found in one case to be reduced by 25% after 6 days and in another not to be affected by this length of time at -20°C. Pure catalase is stated by the handbook "Worthington Enzymes", published by the Worthington Biochemical Corporation, to be unstable, if frozen, but stable for 6-12 months at 5°C.

Cell suspensions were made by suspending the washed cells in approximately 1-10 times their weight of phosphate buffer 0.013 molar pH 6.8. Crude extracts were prepared by breaking the cells in such cell suspensions in the Braun homogeniser. Ballotini beads of 0.1 mm diameter were used and four bursts of 30 seconds at the higher speed were found to break almost all cells. The extract was centrifuged at 20,000 g for 20 minutes and the supernatant was used for assays. Assays were carried out immediately as very dilute solutions of catalase are unstable (Herbert, 1955) and the amount of catalytic activity in our preparations was sometimes low.

Heat treatment for catalase stability investigations

The enzyme preparation was added to preheated buffer in a constant temperature bath, shaken for one minute (or the time of heat treatment if less) and left for the required time. It was then cooled rapidly to 0°C.

Butanol and Toluene treatment

Cell suspensions were washed with 10% v/v butanol or toluene by shaking for 30 minutes on a reciprocating shaker. Afterwards, the washed cells were either centrifuged at 30,000 g for 20 minutes and taken up in fresh buffer or used without centrifuging for activity determinations.

Method of assay of ATCase activity

The product of the enzyme action, carbamyl aspartic acid (CAA), was estimated colorimetrically, using a Beckman DB spectrophotometer, after the method of Gerhard and Pardee (1969). This was based on the estimation of carbamylamino acids by Koritz and Cohen (1954) and Fearon (1939).

Results were calculated as specific activity or micro-moles of CAA/mg protein/minute.

A standard curve was prepared for every assay (Fig. 1).

The reaction mixture consisted of 0.1 ml of crude extract and 0.4 ml of substrate. The enzyme reaction was stopped by addition of the colour reagent.

<u>Substrate</u>	Carbamyl phosphate	10^{-2}	Molar
	Aspartic acid	3×10^{-2}	Molar
	Tris base buffer	5×10^{-2}	Molar

Adjusted to pH 7.4 (after Kaplan et al., 1967).

The higher concentrations of aspartic acid follows from the fact that the K_m for this substrate is higher than for carbamyl phosphate (Shepherdson and Pardee, 1960).

A blank containing the assay mixture only was set up for every time interval and every temperature. After addition of the colour reagent, 0.1 ml of enzyme is added. There is a non-enzymic reaction between the two substrates; in addition hydrolysis of carbamyl phosphate, yields substances containing the ureide grouping, which also develop colour (Weitzman and Wilson, 1966 and Shindler, private communication).

Checks on the method

Two concentrations of enzyme in crude extracts of E. coli and B. stearothermophilus were assayed for ATCase activity. In both cases the slope of the time course was linearly related to enzyme concentration, showing that enzyme activity was being measured (Figs. 2a and 2b).

Assays using the B. stearothermophilus enzyme showed a falling away from linearity after 3 minutes incubation of the assay mixture.

Assays using boiled crude extract had no activity.

FIGURE 1

Standard curve for carbamyl aspartic acid. Absorbance at 560 nm after addition of the colour reagent.

Stock 10^{-3} Molar carbamyl aspartic acid was diluted to give concentrations from $0.1-0.5 \times 10^{-3}$ M. These were incubated to develop colour in the same way as the assay mixtures after the colour reagent was added.

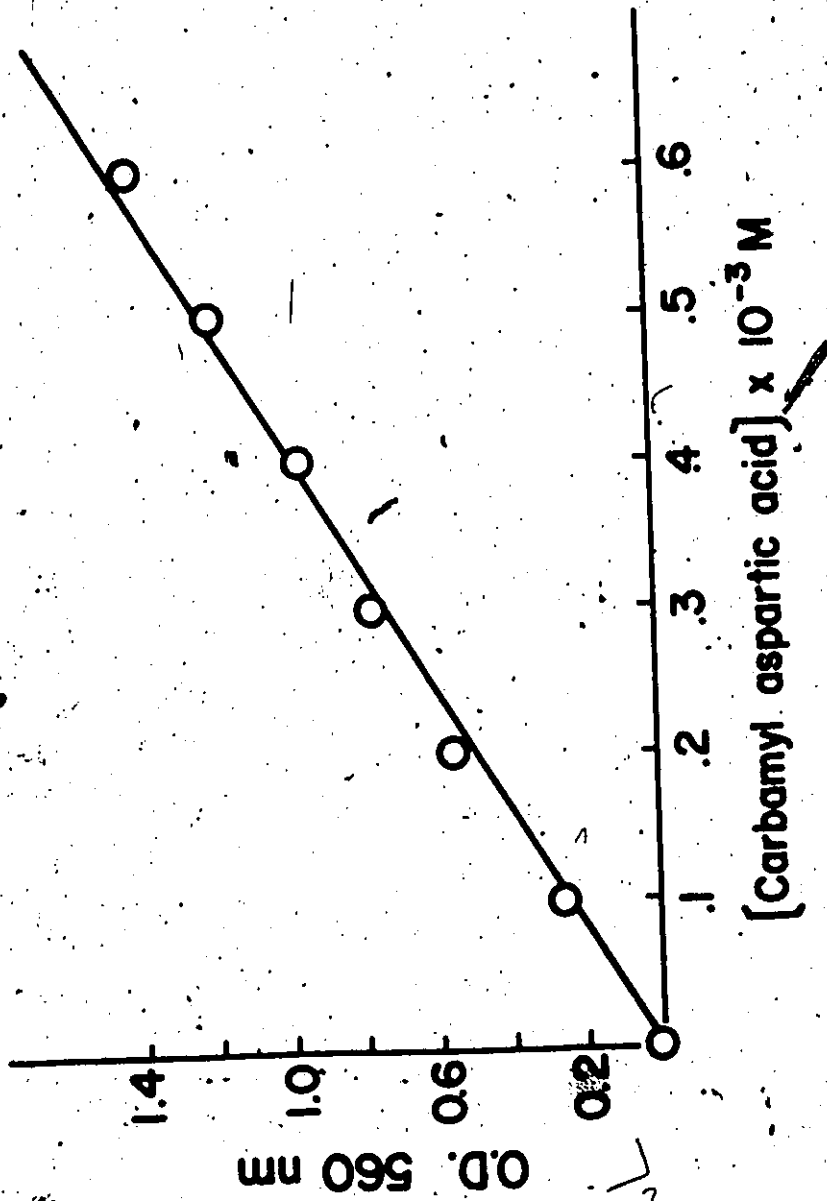


FIGURE 2a

ATCase activity of a crude extract of B. stearothermo-
philus.

Time course with two strengths of enzyme.

Concentration of CAA proportional to absorbance at
560 nm.

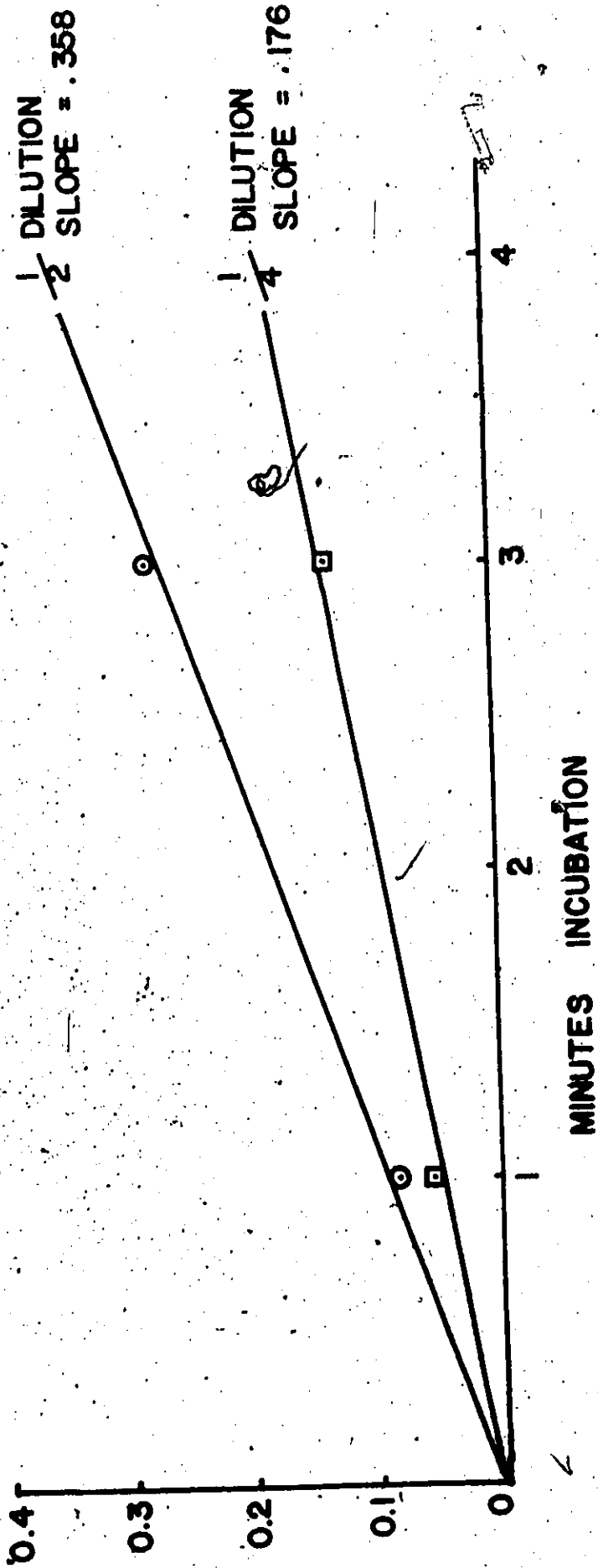


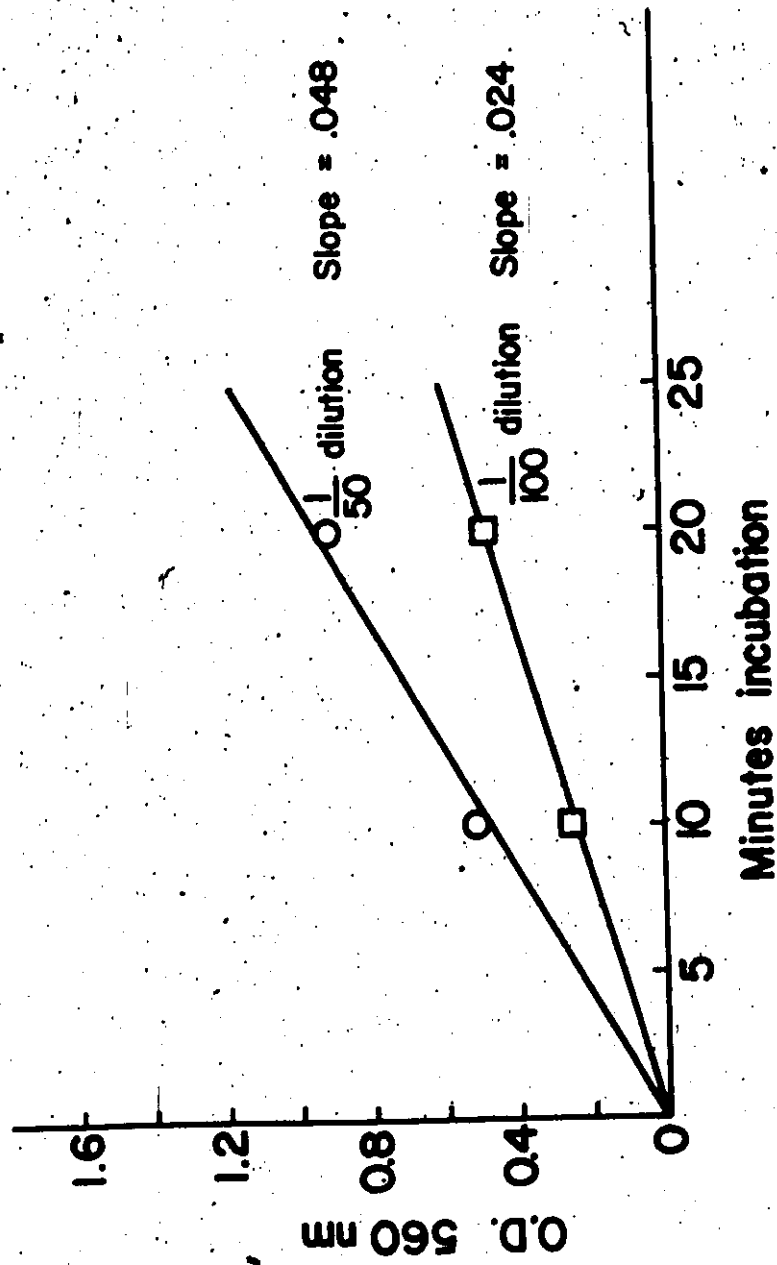
FIGURE 2b

ATCase activity of a crude extract of E. coli.

Time course with two strengths of enzyme.

Concentration of CAA proportional to absorbance at

560 nm.



Methods of assay of catalase activity

Two colorimetric methods were employed and a Beckman DB spectrophotometer used to determine absorbance.

Method 1, after Beers and Sizer, 1952.

The decrease in substrate concentration was measured by the decrease in absorbance at 240 nm (Fig. 3). The assay mixture contained 0.05-1.0 ml of enzyme preparation in a total of 10 ml of hydrogen peroxide in phosphate buffer pH 6.5-7. An aliquot was placed in the cuvette of the spectrophotometer. Alternatively aliquots were removed at intervals into sodium azide solution of such strength that the final concentration was 10^{-3} molar. This stopped the reaction.

Fig. 4 shows a time course of the activity of the enzyme. It is evident that the rate of reaction decreases progressively after 2 minutes.

Substrate. Stock 30% hydrogen peroxide, stored in a dark bottle in the refrigerator, was diluted to 3% strength with phosphate buffer and desired dilutions made with buffer, and kept on ice.

Fig. 5 shows activity of the enzyme v. substrate concentration. Activity ceases to bear a linear relationship to substrate concentration above 0.02 M hydrogen peroxide. Above 0.05 M catalase activity declines. This is attributable to the formation of inactive complexes between the enzyme and hydrogen peroxide which is increasingly favoured by higher concentrations of substrate (Maehly and Chance,

1954). Therefore, it is best to use a concentration of substrate lower than that giving maximum velocity. At such a concentration, where substrate concentration and velocity of enzyme reaction are linearly related, it is presumed that the formation of inactive complexes does not appreciably affect the kinetics of enzyme reaction.

It was decided that the best concentration of substrate was 0.02 M. A substrate concentration of 0.01 M was used for some experiments in which the assays were conducted over short periods of time and therefore substrate was not limiting.

Enzyme concentration

Fig. 4 shows that the slope of the line (velocity) for 0.1 ml crude extract is double that for 0.05 ml crude extract. Therefore, as rate of decrease in absorption at 240 nm bears a linear relationship to enzyme concentration, it can be presumed to be a measure of its activity (Fig. 6).

It is not possible to produce a true enzyme concentration v. activity curve. This is because such a curve can be obtained only by using excess substrate. It is deemed advisable to use high concentrations of enzyme, as in dilute solutions the enzyme is less stable (Maehly and Chance, 1954).

Assays at high temperature

Although no fall in optical density at 240 nm was observable in a hydrogen peroxide solution maintained at 60°C

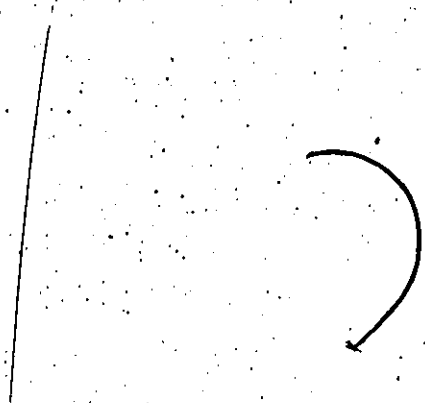


FIGURE 3

Standard curve for hydrogen peroxide.

Absorbance at 240 nm.

Temperature = 0°C.

Concentration of hydrogen peroxide is linearly related
to absorbance at 240 nm.

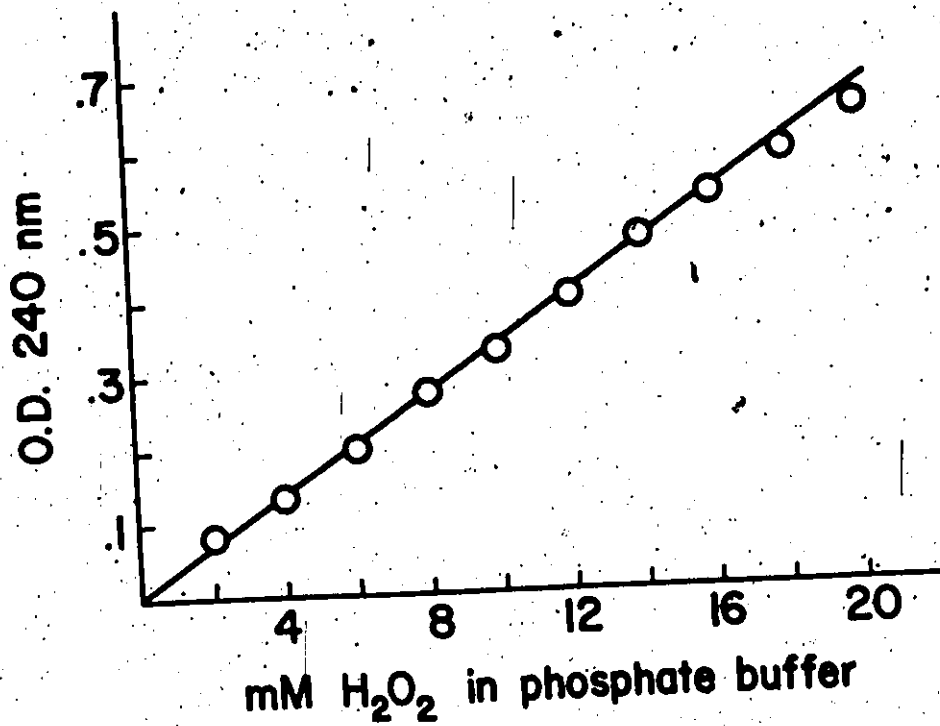


FIGURE 4

Time course of catalase activity of a crude extract of B. stearothermophilus. Decrease in absorbance at 240 nm
Temperature = 0°C.

Substrate 0.01 M H₂O₂ in phosphate buffer pH 7.

Slope is calculated from the straight part of the line.

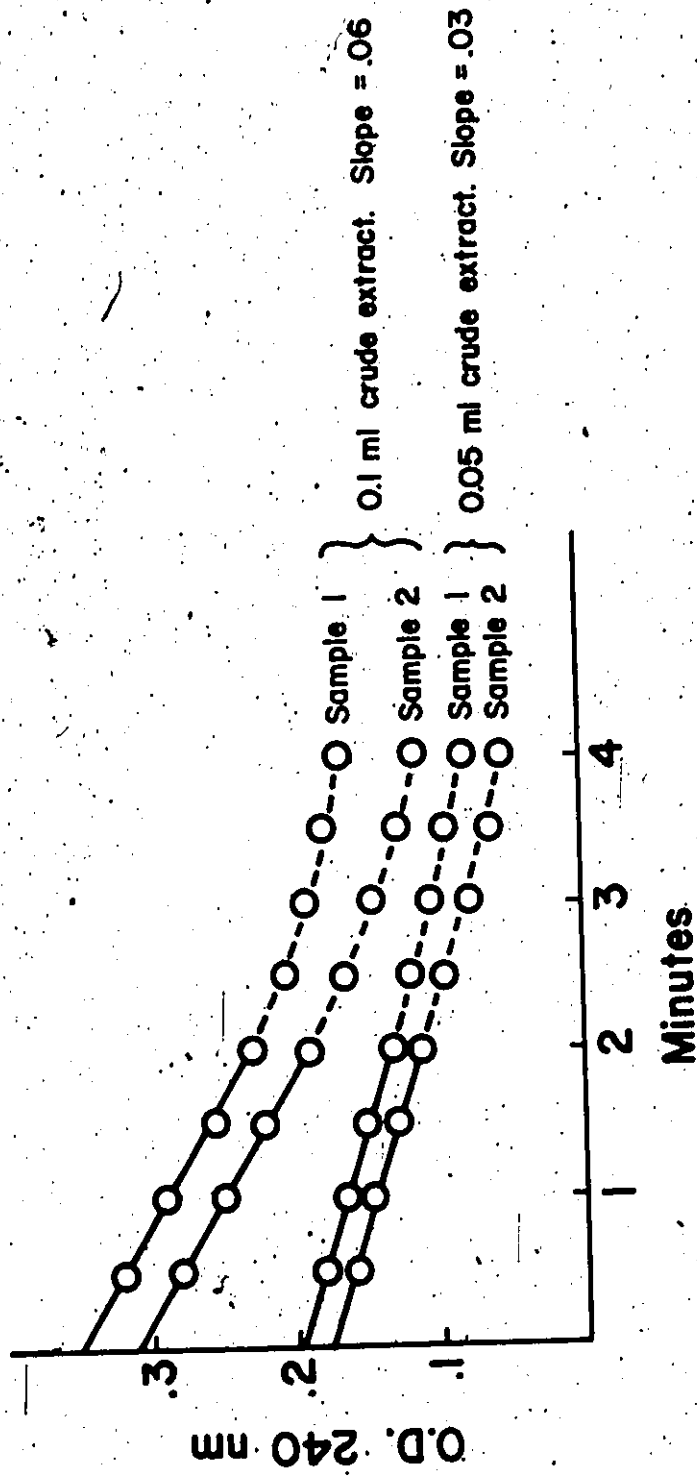


FIGURE 5

Catalase activity of B. stearothermophilus at different substrate concentrations.

UV absorption method

H_2O_2 in phosphate buffer pH 7

Temperature = 0°C

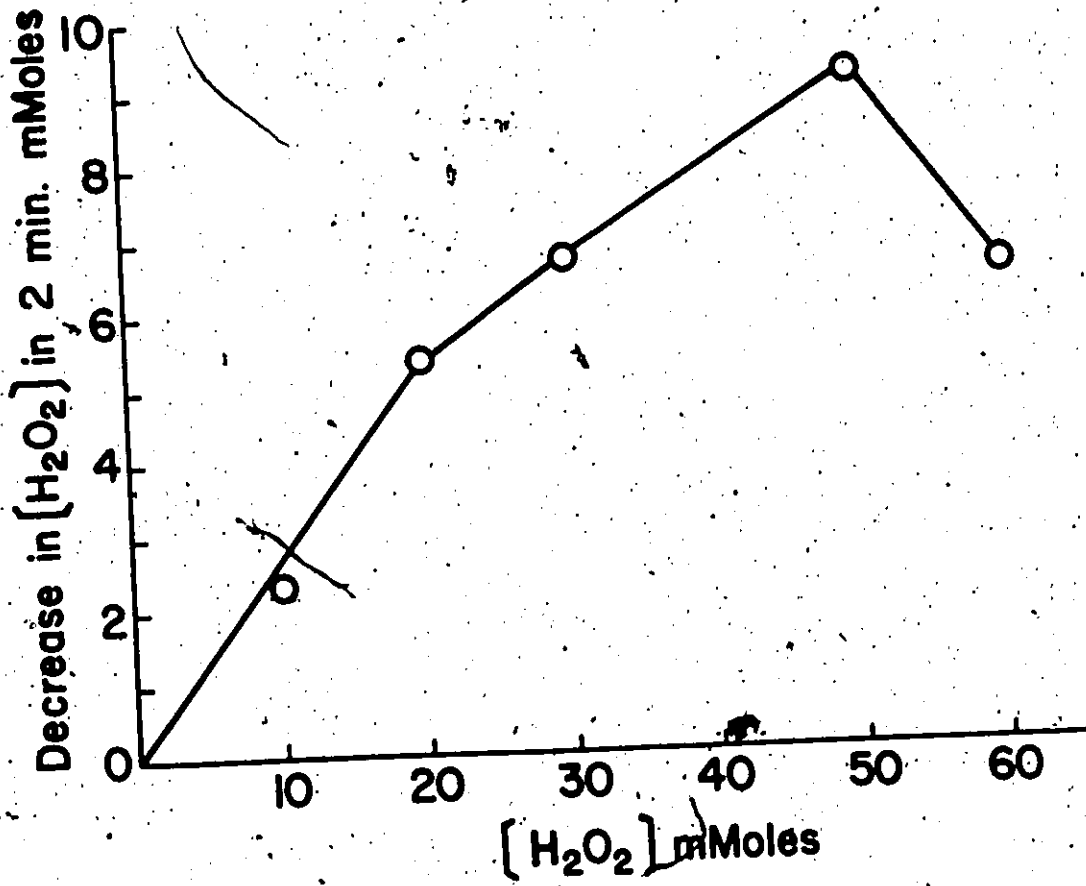


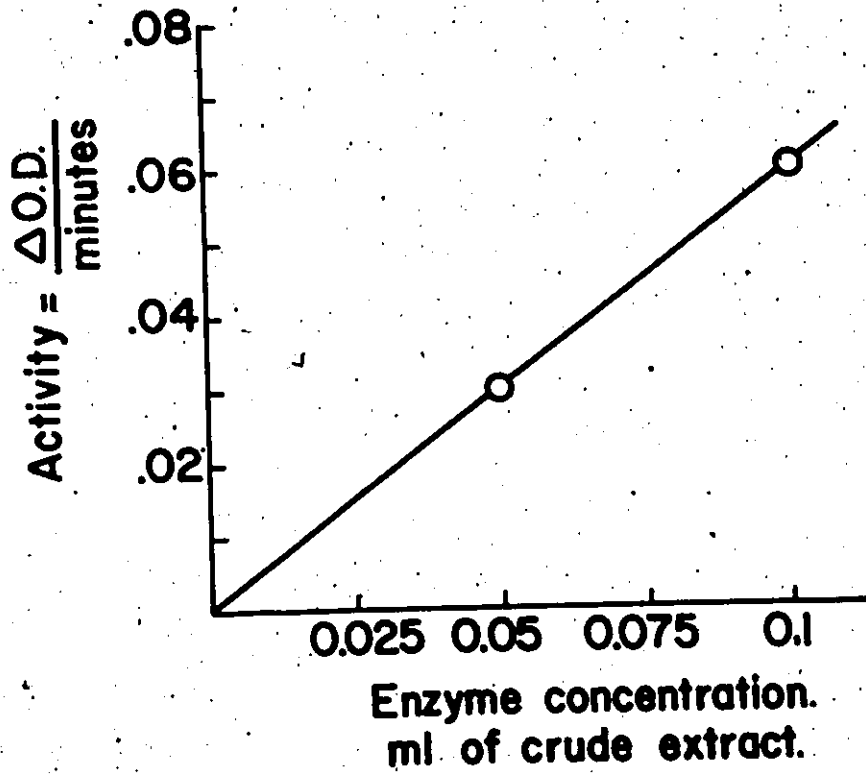
FIGURE 6

Catalase activity of B. stearothermophilus at different enzyme concentrations.

UV absorption method

Substrate = 0.01 M H_2O_2 in phosphate buffer pH 7

Temperature = 0°C



for 4 minutes, it was not possible to measure enzyme activity at high temperatures. Assays at temperatures above 40°C showed inaccuracies due, it was presumed, to hyperchromic shift caused by heat denaturing of the nucleic acids present. At 40°C quadruplicate samples gave variable readings and above this temperature, although activity was present (evinced by bubbling), no change in optical density (or a positive change) occurred.*

Method 2, after Chantrenne, 1955.

Absorbance at 410 nm of a saturated solution of titanium sulphate in 2N sulphuric acid containing hydrogen peroxide is linearly related to hydrogen peroxide concentration (Fig. 7). It is sometimes necessary to make dilutions of the coloured solution to give optical density readings in the required range (Fig. 8).

It was ascertained that sufficient titanium sulphate was present to develop full colour with all concentrations of hydrogen peroxide used. Cloudiness which developed in the titanium sulphate solution after addition of phosphate buffer was removed by standing or centrifugation.

Assays using crude extract or cell suspension which had been boiled for 10 minutes never showed catalatic activity up to a temperature of 80°C.

Fig. 9 shows a time course of the activity of catalase in a cell suspension of B. stearothermophilus, and of the activity of Worthington catalase from beef liver. This latter parallels that of the cell suspension, confirming *As this work involves enzyme assays at high temperatures, this method, although simple, is often impracticable.


FIGURE 7

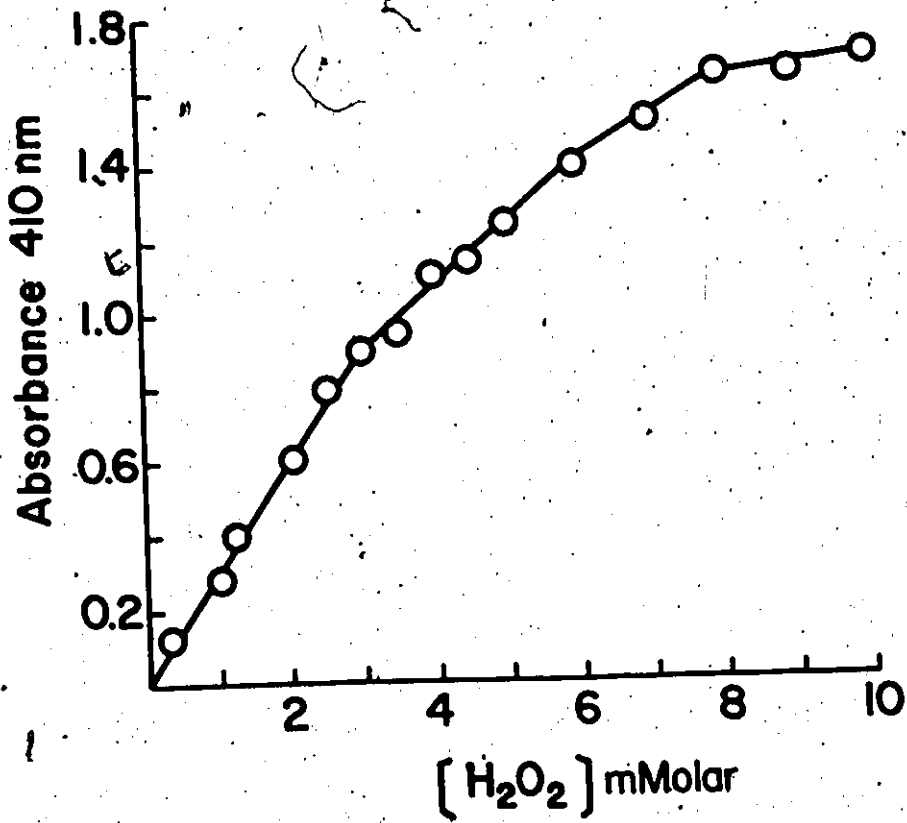
Standard curve for hydrogen peroxide

Absorbance at 410 nm in saturated titanium sulphate.

1 ml of 1-10mMolar hydrogen peroxide in phosphate buffer pH 7 added to 2 ml of saturated titanium sulphate in 2N. sulphuric acid.

Final concentration of hydrogen peroxide is 1/3 of that shown.





~ FIGURE 8

Standard curve for hydrogen peroxide using diluted colour reagent.

After addition of 1 ml of 0-20 mMolar hydrogen peroxide in phosphate buffer pH 7 to 2 ml of saturated titanium sulphate, the mixture is further diluted with 9 ml of saturated titanium sulphate : phosphate buffer, 2:1 to give final concentration shown in first line of figures. Original concentration before addition to titanium sulphate is shown in the second line of figures.

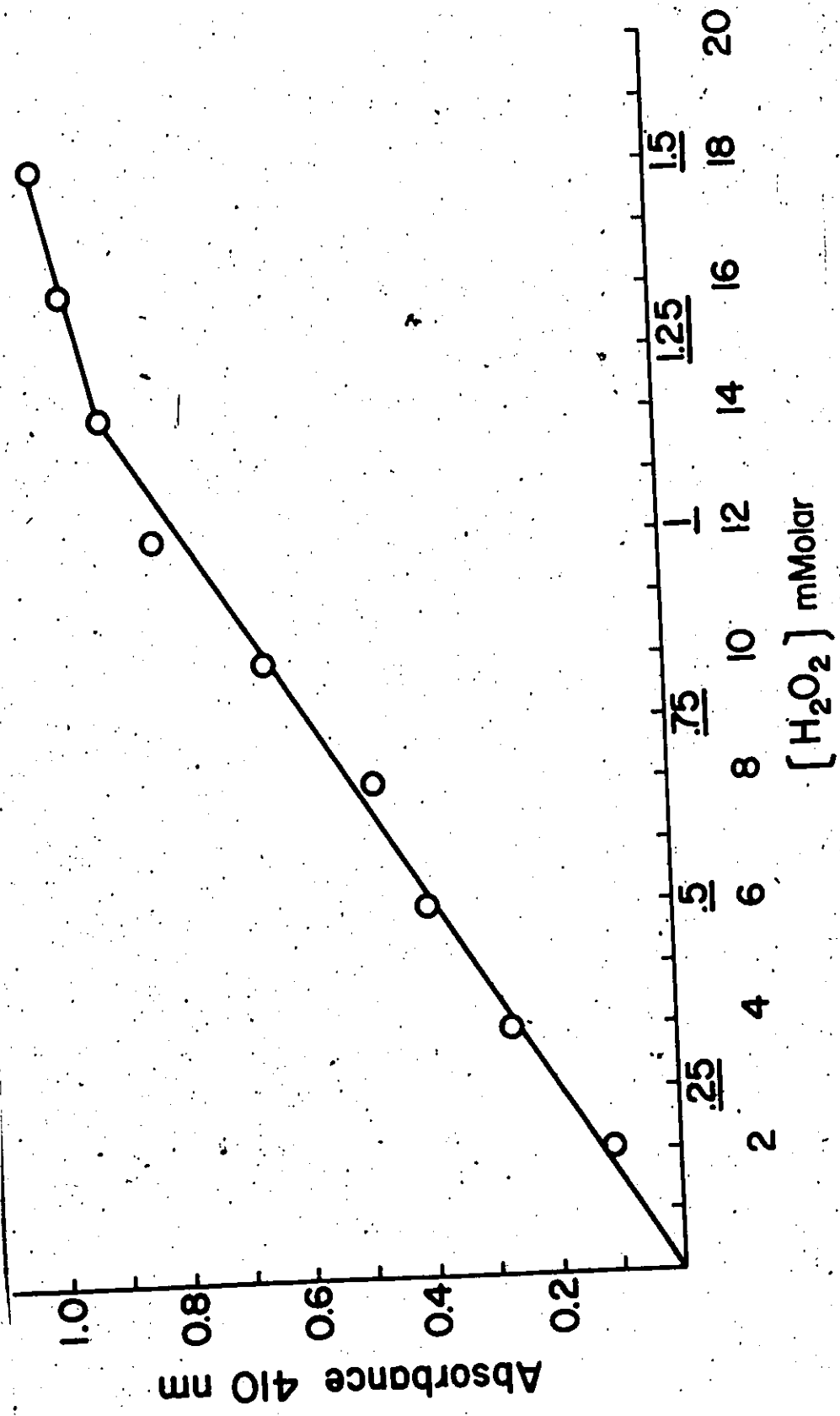
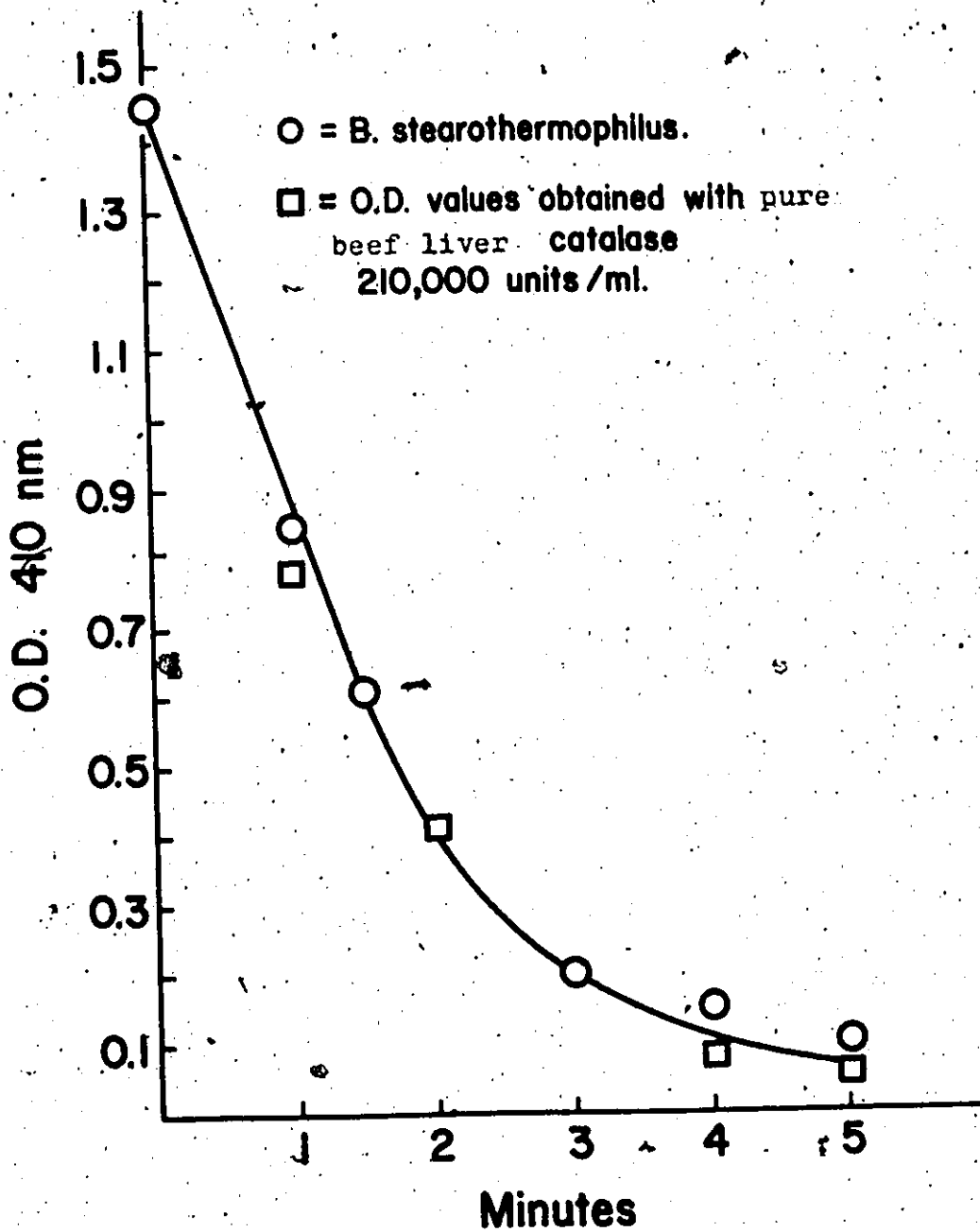


FIGURE 9

Time course of catalase activity of a cell suspension of B. stearothermophilus. Decrease in absorbance at 410 nm of a mixture of saturated TiSO_4 : aliquot of assay mixture, 2:1.

Substrate = .01 M H_2O_2 in phosphate buffer pH 7
Temperature = 25°C



that enzyme activity is being measured. In fact because of the concentration of catalase arbitrarily chosen in this experiment, the curves were identical.

Fig. 10 shows catalase activity of a cell suspension of B. stearothermophilus at different substrate concentrations. The inactivation of the enzyme by the hydrogen peroxide is not demonstrable below 0.02 M hydrogen peroxide (cf. ultraviolet absorption method). In practice assays using 0.02 M hydrogen peroxide gave optical density readings in the required range, if the colour reagent was diluted, after addition of the aliquot of assay mixture, as explained above. Seah and Kaplan (1973) subsequently demonstrated a broad optimum at 0.01 M hydrogen peroxide using purified catalase from baker's yeast.

Fig. 11 shows that the slope of the graph of activity v. time for 0.1 ml cell suspension is twice that for 0.05 ml cell suspension. Fig. 12 shows that zero time velocity calculated by extrapolation is linearly related to enzyme concentration. Fig. 13 shows enzyme concentration v. activity curves. From these results, it is seen that under certain experimental conditions of substrate concentration and length of assay (up to 0.02 M hydrogen peroxide and up to 5 minutes duration) it is safe to assume that activity is proportional to enzyme concentration.

Duration of assay

Fig. 9 demonstrates the rapid reduction in enzyme activity with time, by the change in slope of the graph. Measurable)

FIGURE 10

Catalase activity of a cell suspension of B. stearothermo-
philus at different substrate concentrations.

Titanium sulphate method.

H₂O₂ in veronal acetate buffer pH 6.5

Temperature = 25°C

Velocity units = Δ H₂O₂ nMole/litre/minute

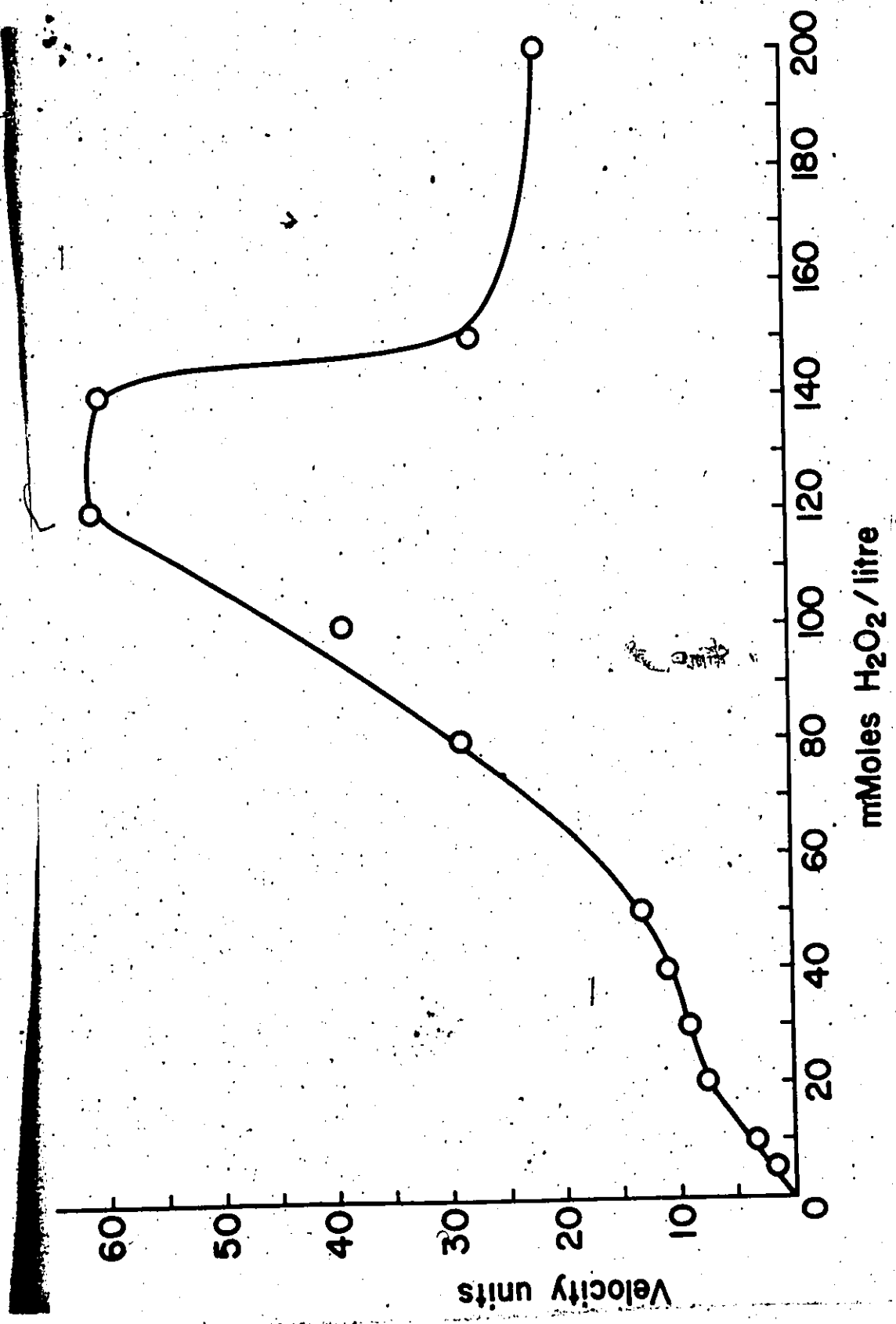


FIGURE 11.

Time course of the catalase activity of a cell suspension
of B. stearothermophilus.

Titanium sulphate method

Substrate 0.01 M hydrogen peroxide

Temperature = 0°C

Decrease in log O.D.

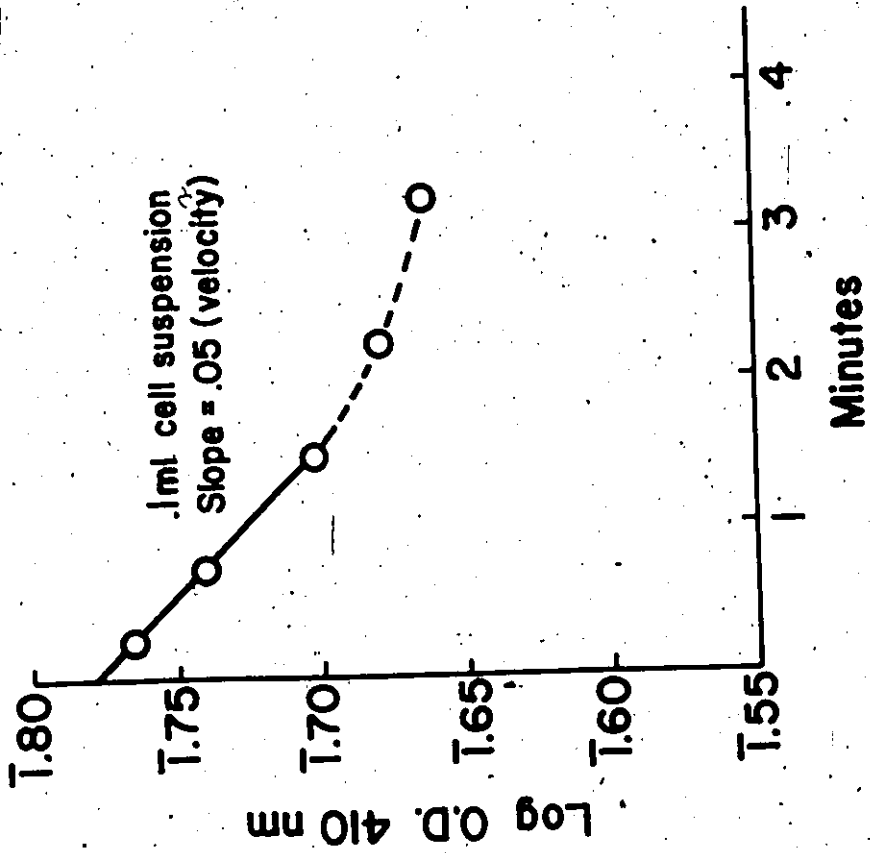
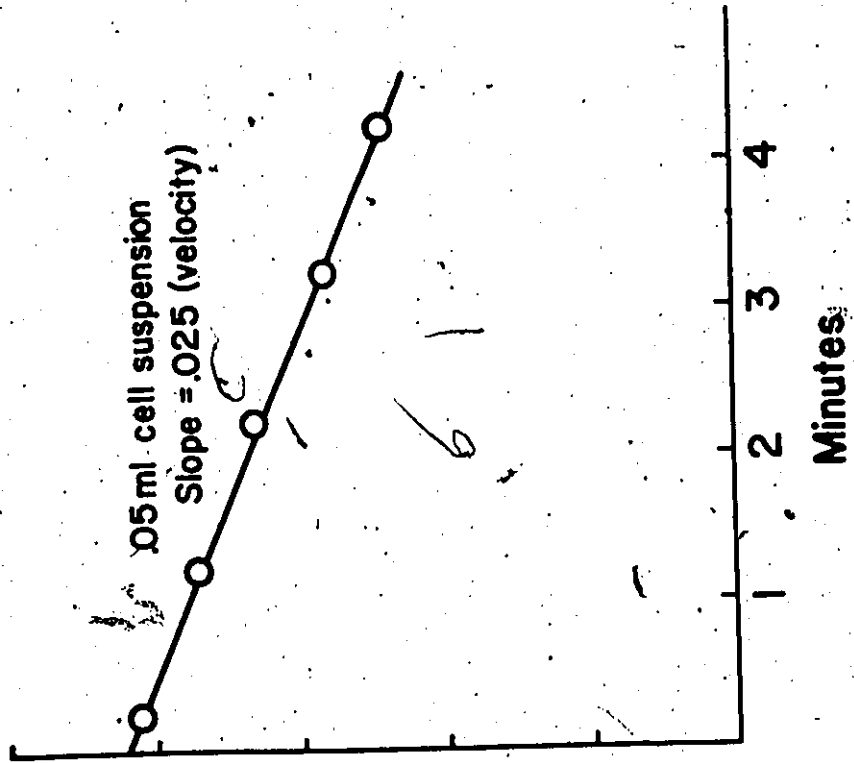


FIGURE 12

Catalase activity of a cell suspension of B. stearothermo-
philus. Extrapolation of log velocity at successive times
to log velocity at zero time.

Velocity = $\Delta \log \text{OD} / \text{minute}$. Titanium sulphate method.

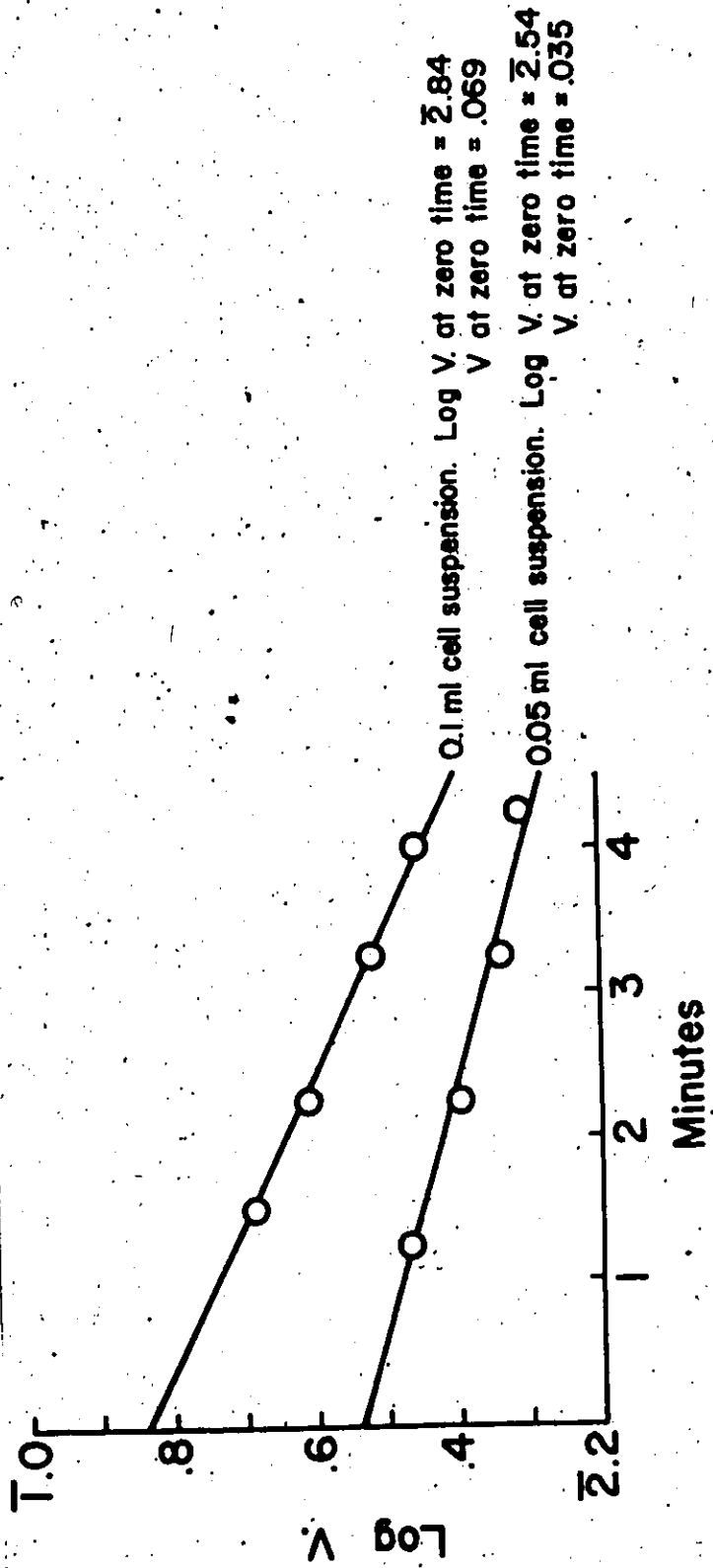


FIGURE 13

Catalase activity of cell suspensions of B. stearothermo-
philus at different enzyme concentrations.

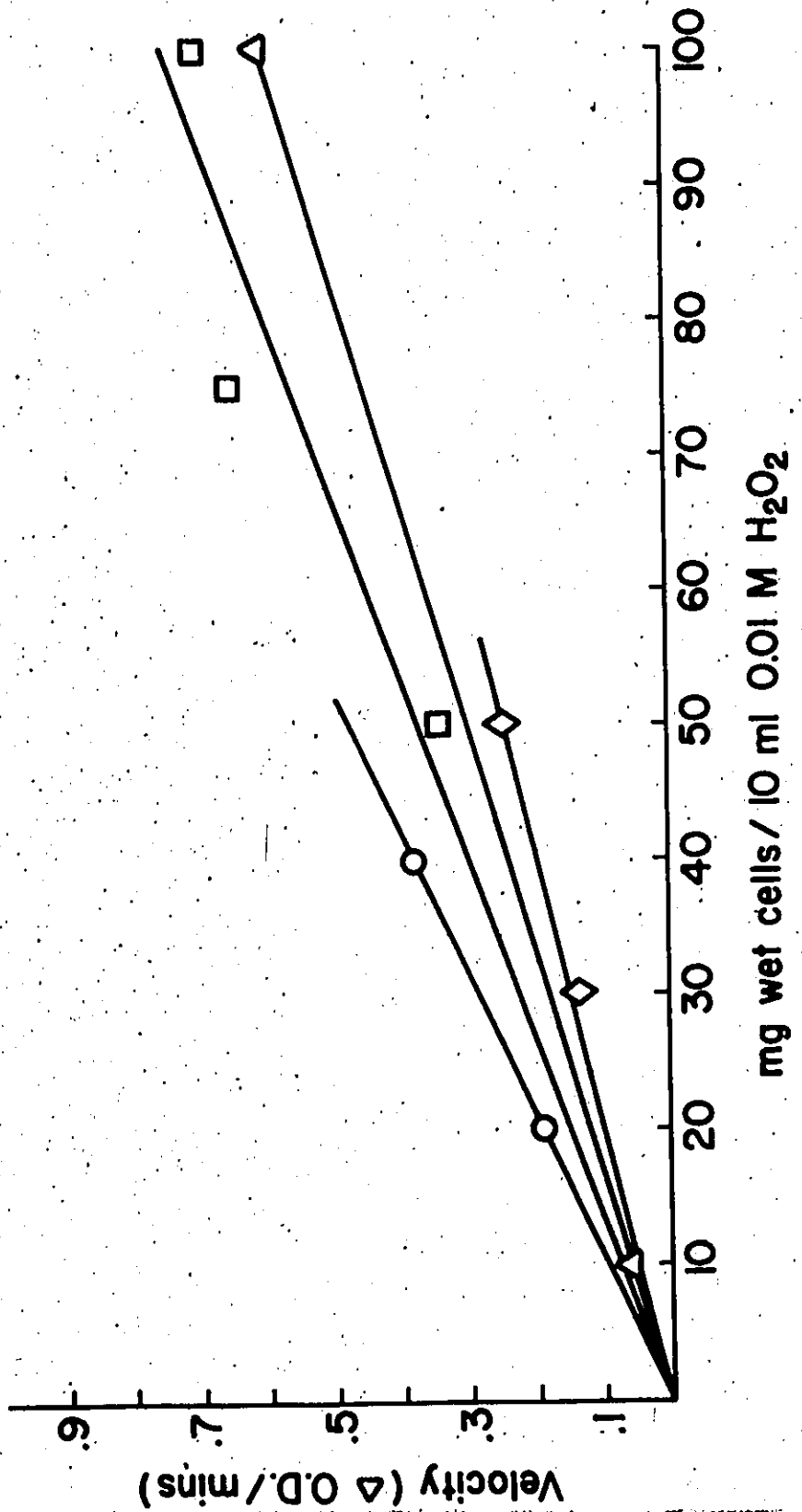
Titanium sulphate method

Substrate 0.01 M H_2O_2

- ◇ unbuffered H_2O_2 . $0^\circ C$
- unbuffered H_2O_2 . $25^\circ C$
- H_2O_2 in phosphate buffer pH 7. $0^\circ C$
- △ H_2O_2 in phosphate buffer pH 7. $25^\circ C$

i.e.

- △ washed cells stored overnight in refrigerator
- same cells stored a further 24 hrs in refrigerator
fresh cell suspension.
- ◇ same suspension as △ stored 3 days in refrigerator
- same cells as others stored 9 days at $-20^\circ C$
new suspension



changes with assays of duration of 30 seconds are possible with extracts of high catalase activity. It was found more practicable to measure the optical density at zero time and at one minute intervals up to 5 minutes. Velocities calculated (see below) were extrapolated back to zero time. This makes comparison of enzyme activity for varying parameters more valid.

Velocity units

1. Rate of destruction of hydrogen peroxide (use standard hydrogen peroxide concentration v. optical density curve).
2. After Sumner and Somers, 1947. Change in the logarithm of the optical density. (\log_{10} optical density at zero time, $-\log_{10}$ optical density at time x) per minute. This figure is proportional to the log of the reciprocal of the fraction of hydrogen peroxide remaining, (because optical density up to 1.0 is proportional to hydrogen peroxide concentration), and is therefore a measure of rate of activity when divided by number of minutes.

Calculation of zero time velocities

If velocities at successive times are plotted against time a rectangular hyperbola results, but if the log of velocities is plotted against time a straight line is obtained, and log zero time velocity found by extrapolation (Fig. 12). The actual velocity is therefore the anti log of this figure. By these means inaccuracies due to the rapid inactivation of catalase with time, are minimised.

Effect of heat on hydrogen peroxide

Substrate blanks in three minute assays at temperatures up to 80°C showed no change in hydrogen peroxide concentration. It was therefore presumed that destruction of hydrogen peroxide measured at all temperatures used was due to enzyme activity, and that substrate blanks were not necessary.

pH optimum for catalase

Buffer strength

Phosphate buffer was made by adjusting 0.0125 M K_2HPO_4 to desired pH by the addition of solid KH_2PO_4 . This was used for most of the experimental work. It was found that as low as 0.0004 M buffer maintained the pH of an assay mixture for 5 minutes (assays were never longer) and that 0.0125 M buffer maintained it for 30 minutes. Phosphate buffers of higher strength develop cloudiness when added to the saturated titanium sulphate, but this was removed on centrifuging. Veronal acetate buffer was used for some experiments to determine optimum pH. This was made by mixing equal quantities of $\frac{M}{7}$ sodium diethyl barbiturate (veronal or barbitol) and $\frac{M}{7}$ sodium acetate (kept as stock), diluted $\frac{1}{10}$ and pH adjusted by addition of 0.1 M hydrochloric acid if necessary (i.e. for pH values below 9.6).

Fig. 14a shows the catalase activity of a cell suspension of B. stearothermophilus at different pH values maintained by phosphate and citrate buffers. Fig. 14b is the same as Fig. 14a but using veronal acetate buffer. Fig. 15 shows the catalase activity of a crude extract of B. stearo-

FIGURE 14a

Catalase activity of a cell suspension of B. stearothermo-
philus at different pH values.

Titanium sulphate method

Substrate 0.01 M H_2O_2

Temperature = 25°C

Velocity units = Δ mMoles H_2O_2 /litre/minute

Citrate and phosphate buffers used.

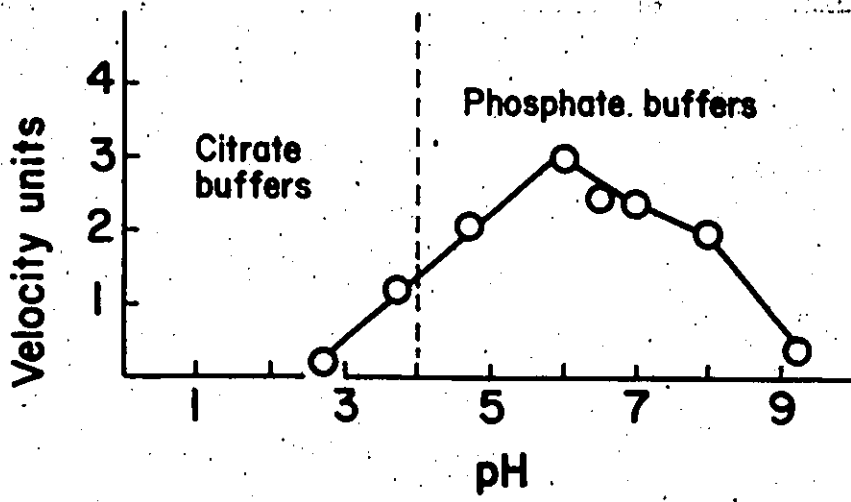


FIGURE 14b

Catalase activity of a cell suspension of B. stearothermo-
philus at different pH values.

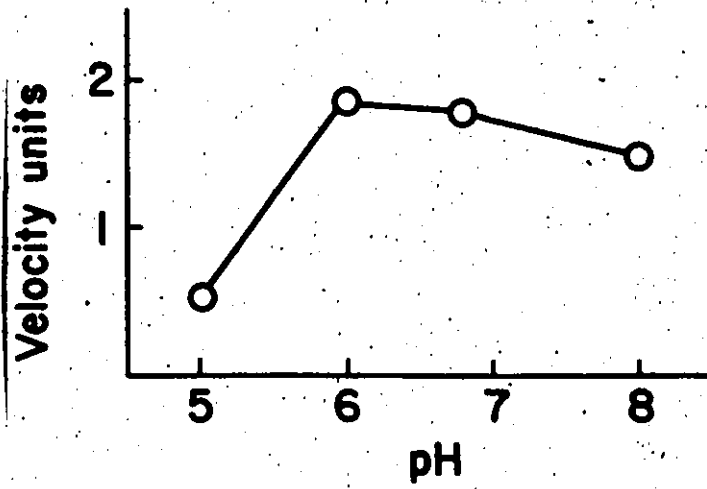
Titanium sulphate method

Substrate 0.01 M H_2O_2

Temperature = 25°C

Velocity units = Δ mMoles H_2O_2 /litre/minute

Veronal acetate buffers used.



5

FIGURE 15

Catalase activity of a crude extract of B. stearothermo-
philus at different pH values.

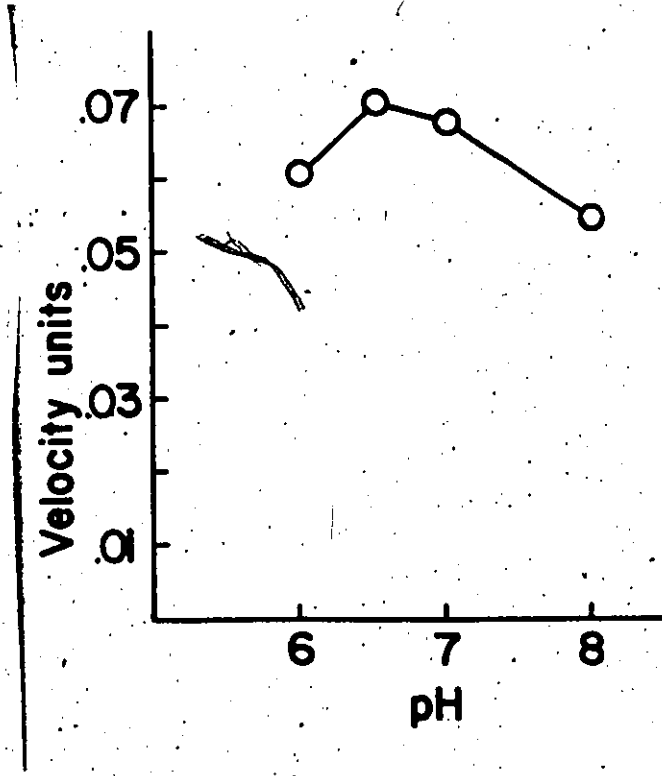
UV absorption method

Substrate = .01 M H_2O_2

Temperature = 0°C

Velocity units = Δ O.D. 240 nm/min

Phosphate buffers used



thermophilus at different pH values maintained by phosphate buffer.

In an unbuffered assay using whole cells the pH was 7.0 at the start and 3.4 at the finish (at 5 minutes). Clearly buffering is important for accurate measurements of activity, yet the optical density change over 2 minutes at 25°C varied little from that of a buffered control. Fig. 13 shows that at 0°C an unbuffered assay gave better linearity with enzyme concentration than at 25°C. Few et al. (1957) found that lysates of M. lysodeikdikus were far more sensitive to pH than whole cells and presumed this was due to the ability of cells to regulate their internal pH over a fairly wide range of external pH values. For this reason our assays of crude extracts were carried out only over the pH range 6-8. It is concluded that the catalase activity of B. stearothermophilus in whole cells or crude extracts can be satisfactorily assayed at pH 6.5-7.

RESULTS OF ATCase INVESTIGATIONS

ATCase activity during the growth cycle of *B. stearothermophilus*

ATCase activity is high in early logarithmic phase and falls throughout the logarithmic phase and linear phase of growth, becoming almost zero in the stationary phase (Fig. 16).

ATCase activity of crude extracts of *B. stearothermophilus* and *V. psychroerythrus* at different temperatures

Fig. 17a is a plot of the ATCase activity of crude extracts of *B. stearothermophilus* at temperatures from 37°C to 80°C. The maximum activity, at 60°C, was taken to be 100% although in one of several experiments the specific activity at 70°C was higher than at 60°C.

As the rate of activity of the thermophile enzyme started to decrease after 3 minutes incubation of the assay mixture, this time was chosen to determine its optimum temperature.

Fig. 17b shows the specific activity of the ATCase of *V. psychroerythrus* at temperatures up to 37°C. It is difficult to define the optimum temperature as it can be seen from Fig. 18 that the specific activity after 15 minutes incubation of the assay mixture is the same at 25°C as at 37°C. At this latter temperature the specific activity increases with time up to 37 minutes of incubation of the assay mixture and then falls. The specific activity of the enzyme at 25°C however falls slowly from 15 to 65 minutes incubation of the assay mixture, when it is approximately the same as that of the assay mixture which has been incubated at 37°C.

FIGURE 16

ATCase activity in crude extracts of *S. stearothermophilus* during the life of a culture.

Specific activity = $\mu\text{moles CAA/mg protein/minute}$.

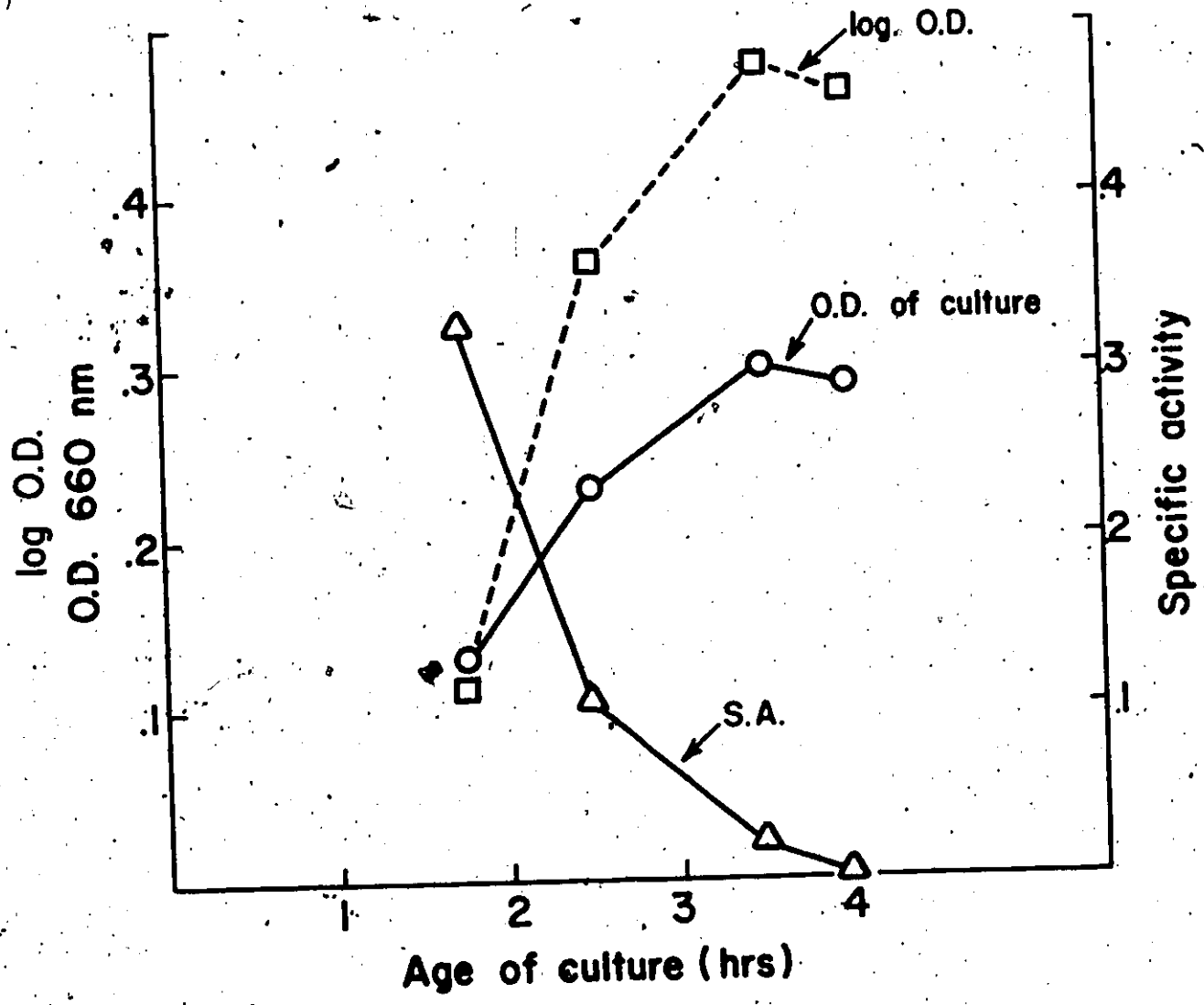


FIGURE 17a

ATCase activity in crude extracts of B. stearothermophilus at different temperatures.

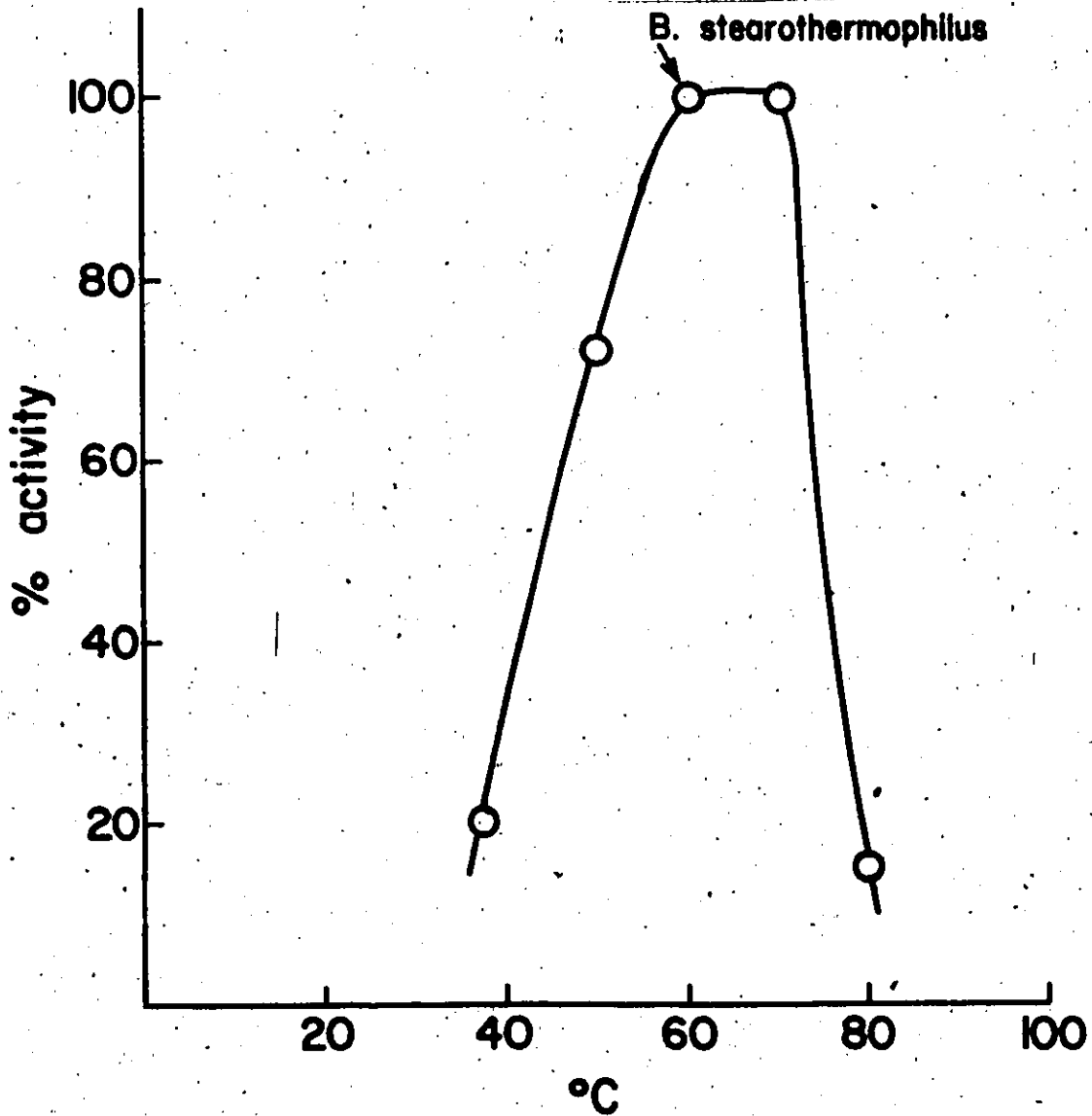


FIGURE 17b

ATCase activity in crude extracts of V. psychroerythrus at different temperatures.

Incubation time = 25 minutes and 35 minutes.

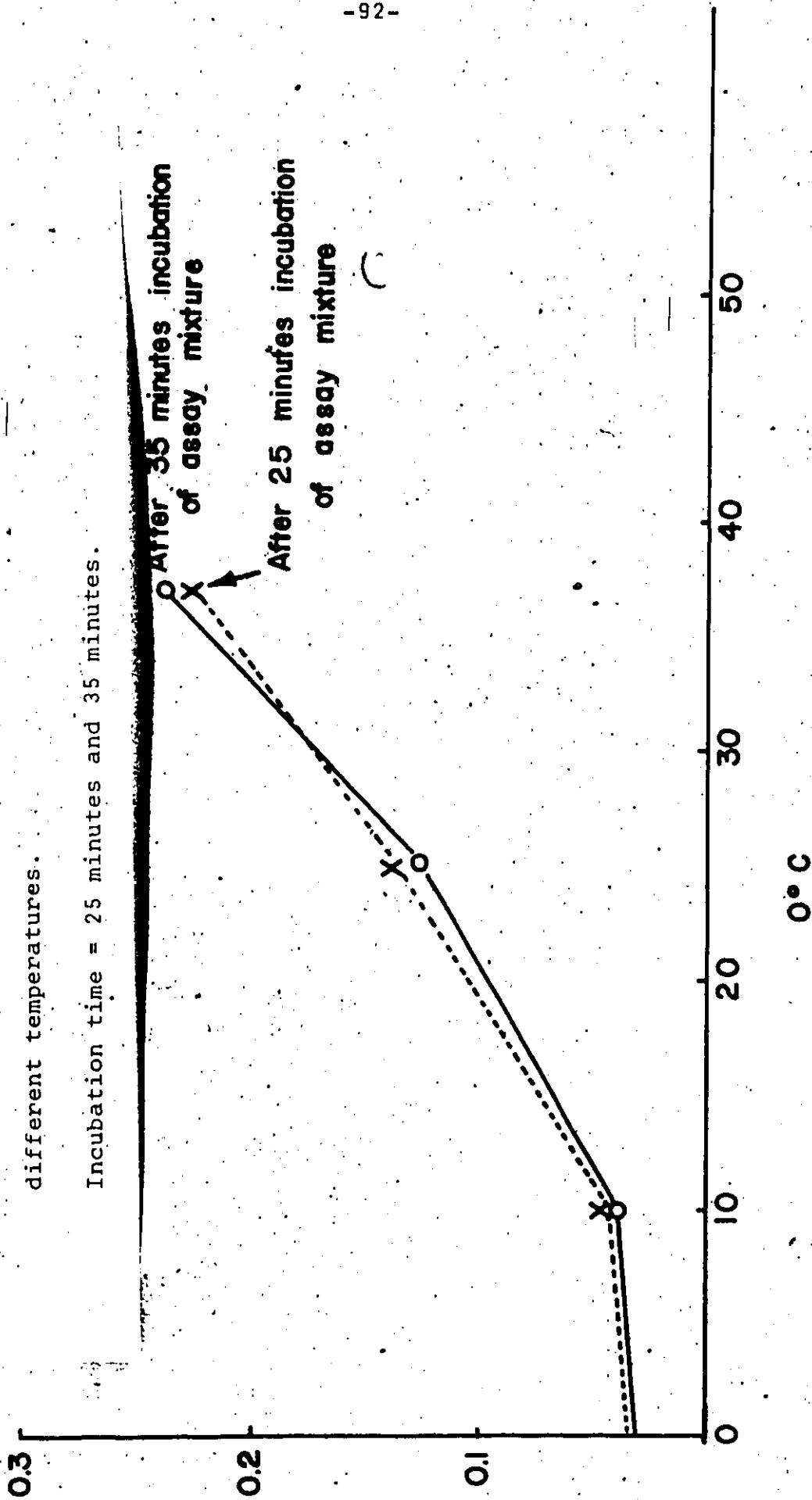
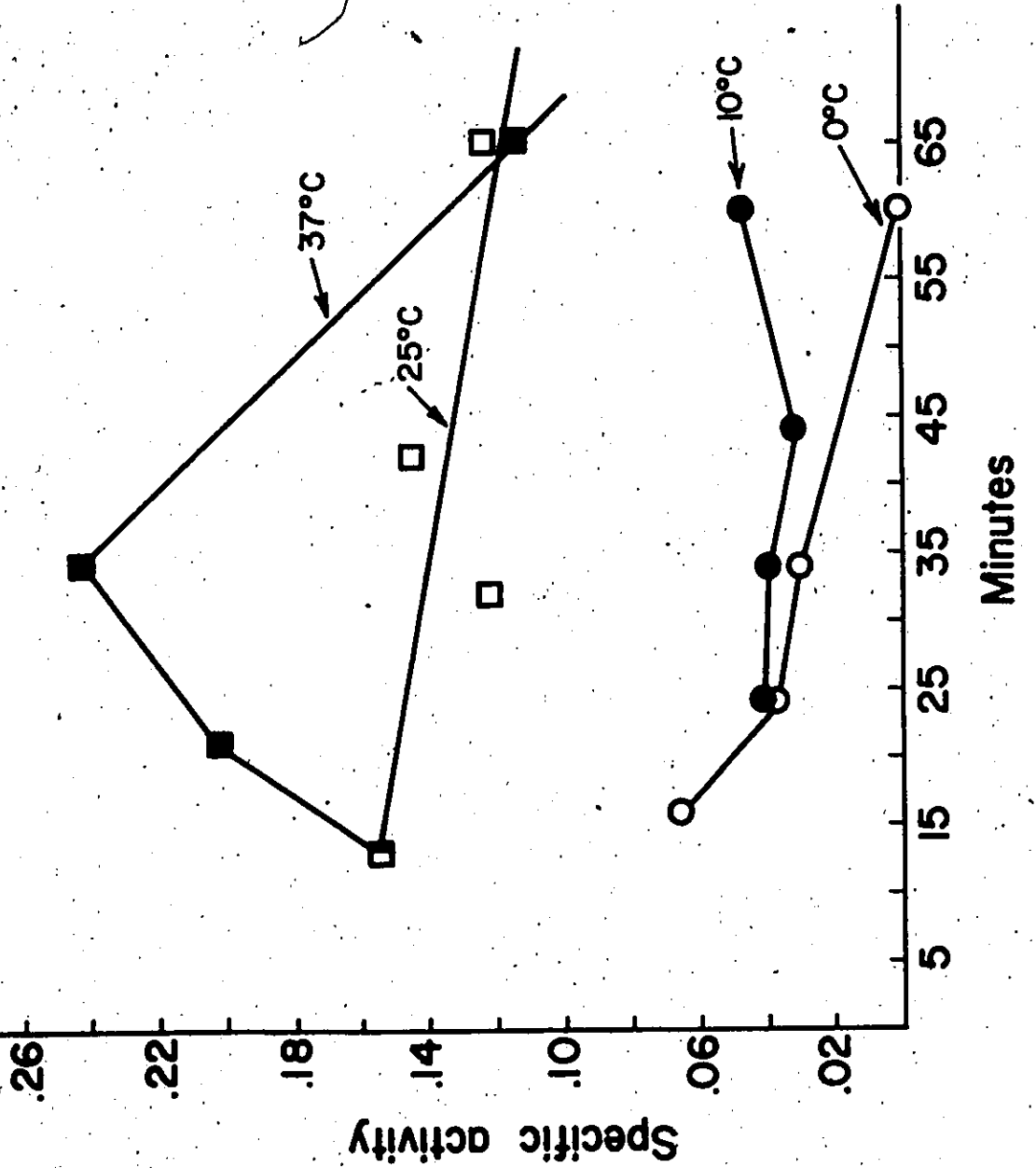


FIGURE 18

Variation of the specific activity of ATCase in a crude extract of Vibrio psychroerythrus with time of incubation of assay mixture, at different temperatures.



Arrhenius plots are shown in Fig. 19 from which energies of activation were calculated. As there are only three points on the plot for the B. stearothermophilus enzyme its value would seem to be $E = 13,900$ cal/mole (average of two values). That for the V. psychroerythrus ATCase is $E = 9,600$ cal/mole, and for S. cerevisiae is $E = 11,250$ cal/mole (Kaplan and Messmer, 1969).

FIGURE 19

Arrhenius plots of ATCase activity in crude extracts of B. stearothermophilus and V. psychroerythrus.

Energy of activation, E, calculated from the formula

$$E = 2.303 \times R \frac{\log k_2 - \log k_1}{\frac{1}{T_1} - \frac{1}{T_2}}$$

where R = gas constant

k_1 = specific activity at T_1

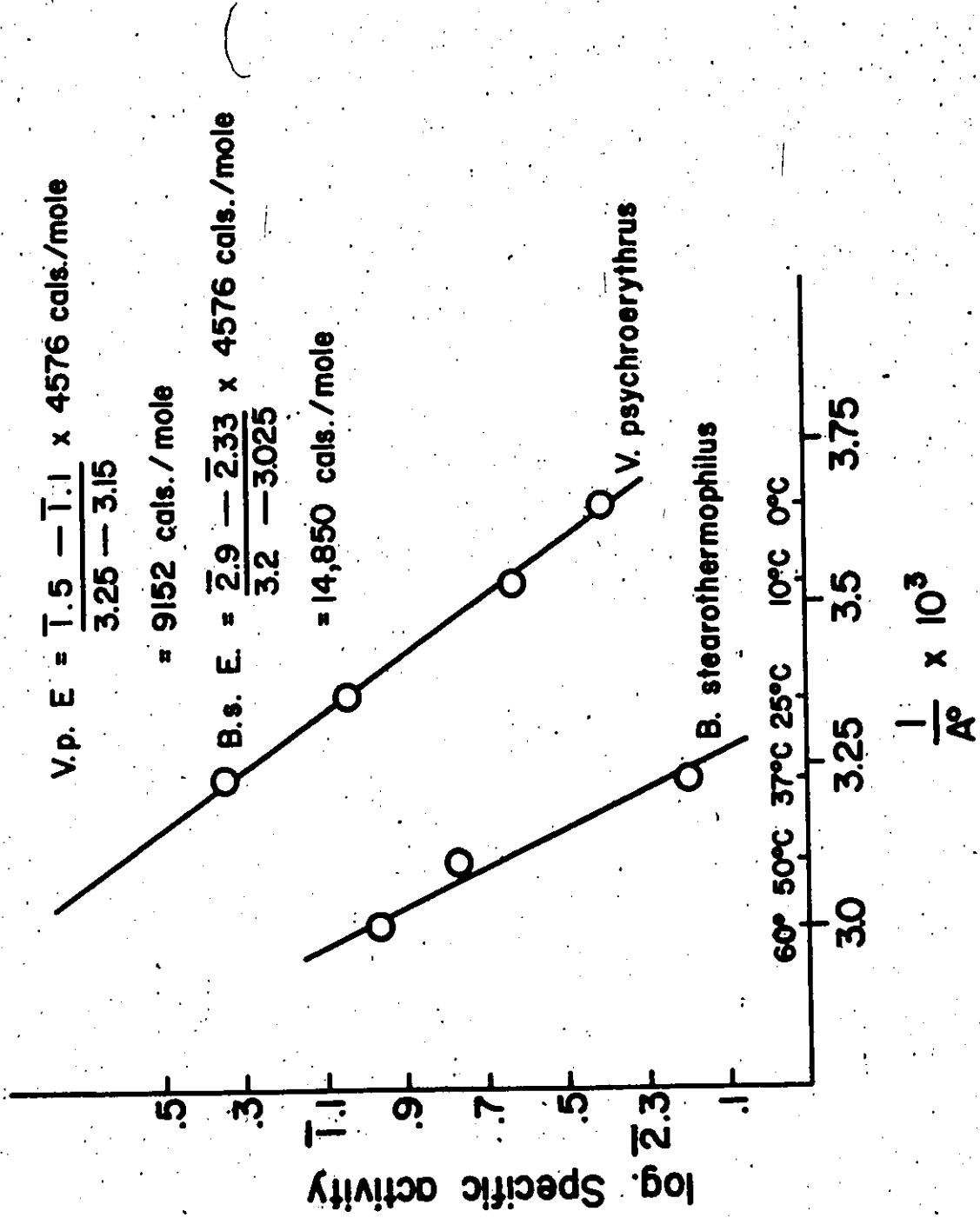
k_2 = specific activity at T_2

T_1 = lower temperature on absolute scale

T_2 = higher temperature on absolute scale

2.303 = conversion factor from natural to decalgorithms

$\frac{1}{A^\circ}$ = reciprocal of temperature on absolute scale



Stability of ATCase of *B. stearothermophilus* and *V. psychroerythrus* at different temperatures.

Stability during assay. Variation of the specific activity of the ATCase in crude extracts of *V. psychroerythrus* and *B. stearothermophilus* during incubation of the assay mixture at different temperatures, is shown in Figs. 18 and 20. From these it can be seen there is a reduction of 90% of the highest activity of the thermophilic enzyme at its optimal in vitro temperature of 60°C in 20 minutes, whereas the psychrophilic enzyme loses only 50% of its highest activity, in one hour at 37°C. Indeed at 37°C the activity increases by about 50% between 13 and 35 minutes incubation, though the increase is not linear. At 25°C which is above its growth range, it loses 20% activity in one hour. In the presence of substrate the enzyme of the psychrophile is stable for at least one hour at 10°C, a temperature within its growth range, whereas that of the thermophile is stable for only 3 minutes at 60°C, likewise a temperature within its growth range (Table 3). At 50°C, at which temperature *B. stearothermophilus* can grow, the enzyme is stable for up to 15 minutes though in some assays it was stable for no longer than 3 minutes.

Stability during storage at different temperatures

Crude extracts of *B. stearothermophilus* were stored at various temperatures for 24 hours. The residual ATCase activity was then measured at the optimum temperature (60°C).

FIGURE 20

Variation of the specific activity of ATCase in a crude extract of B. stearothermophilus with time of incubation of assay mixture, at different temperatures.

Specific activity = μ moles CAA/mg protein/minute.

X-axis - minutes of incubation of assay mixture.

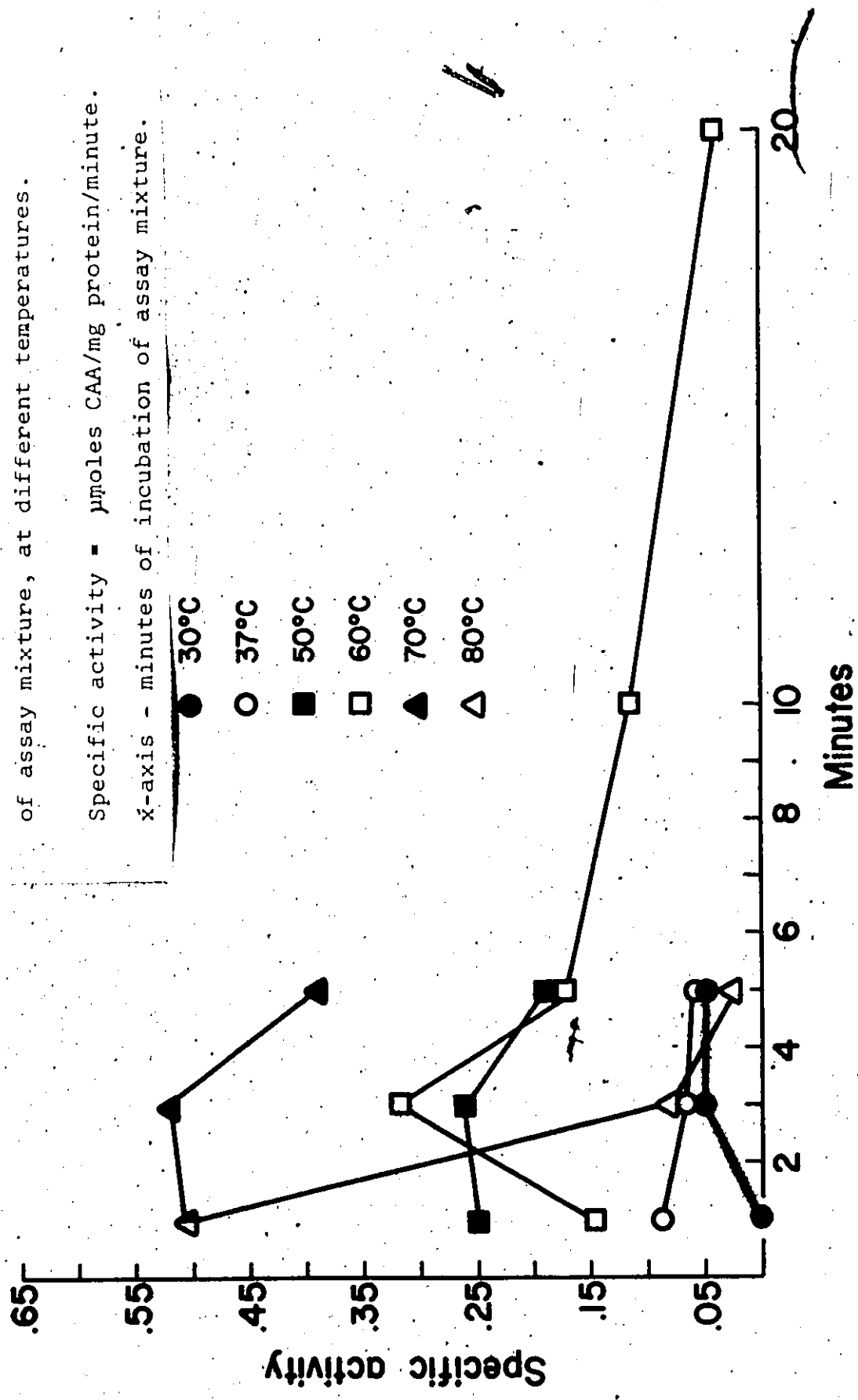


TABLE 3

Stability of ATCase of B. stearothermophilus and V. psychroerythrus in presence of substrate. Maximum time (mins) before decline in enzyme activity is observed at different temperatures of assay.

Temperature °C	10	25	37	50	60	70	80
<u>V. psychroerythrus</u>	>60	>65	36				
<u>B. stearothermophilus</u>			>30	<15	3	3	1

The results (Fig. 21) show that in absence of substrate the thermophilic enzyme retains 50% of its activity at 50° and 60°C for 24 hours. The shape of the curve suggests the possibility of the existence of two enzymes, one unstable at any temperature shown and the other stable below 60°C. The figure also shows 23% activity after storage at 40°C for 14 days.

Effect of CTP on ATCase of *B. stearothermophilus*

ATCase activity in *B. stearothermophilus* was measured in the presence and absence of 10^{-3} Molar CTP. At those temperatures in which ATCase activity was high enough to be measured accurately, CTP had no consistent inhibitory effect; but a stimulatory effect was observable in most cases (Table 4).

Because of the spontaneous reaction between aspartate and carbamyl phosphate, which is accelerated at high temperatures, further studies of possible allosteric properties of the *B. stearothermophilus* ATCase were not carried out.

FIGURE 21

Stability of the ATCase of B. stearothermophilus.

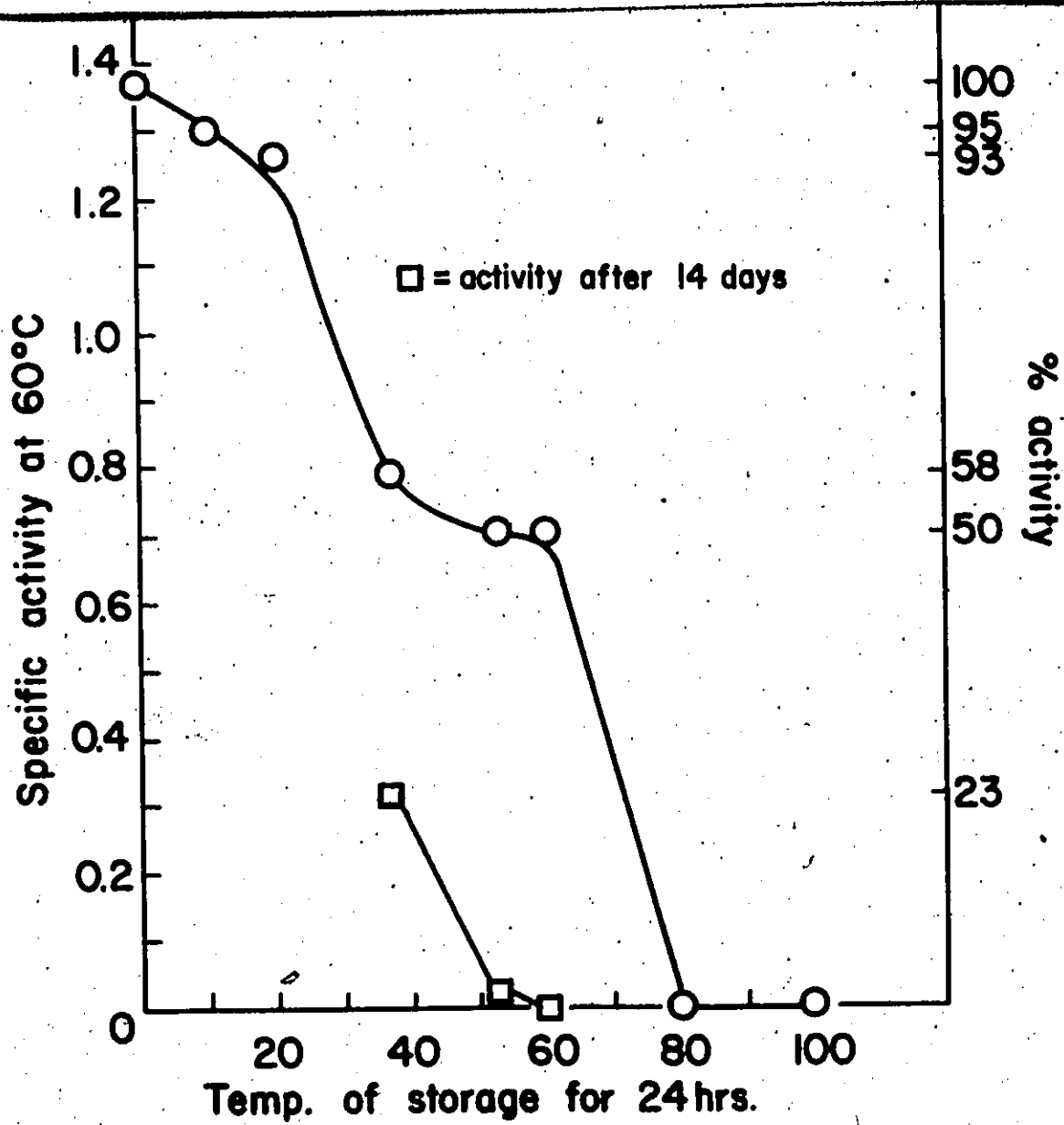


TABLE 4

Activity of ATCase of B. stearothermophilus with and without CTP

Temperature °C	37			50			60		
Duration of assay (mins)	1	3	5	1	3	5	1	3	5
Micro moles CAA produced	0	0	0.039	0.0375	0.125	0.145	0.039	0.245	0.212
Micro moles CAA produced in presence of 10 ⁻³ M CTP	0	0	0	0	0.15	0.20	0.15	0.214	0.25

FIGURE 22

V. psychroerythrus. Growth curve.

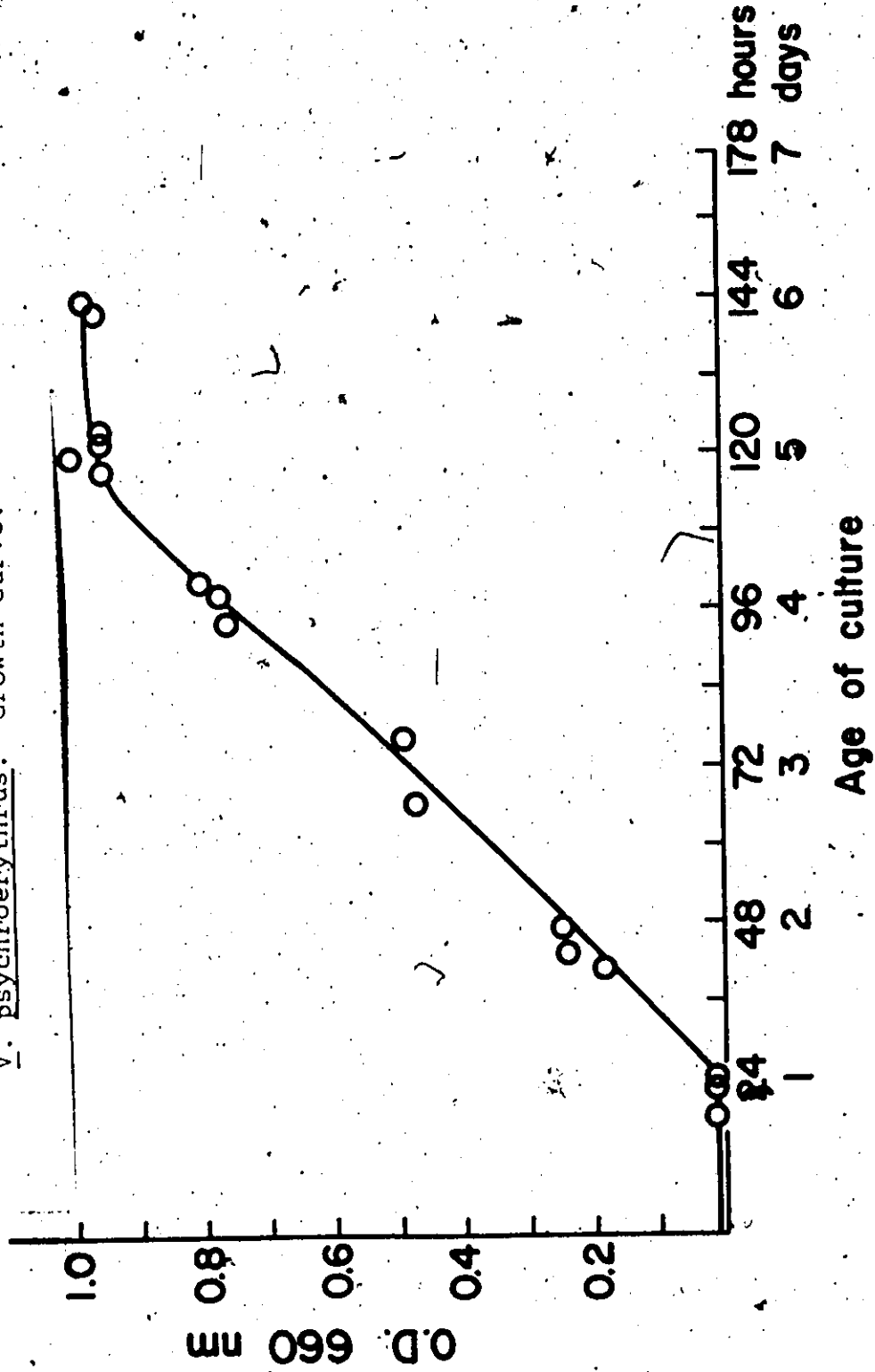
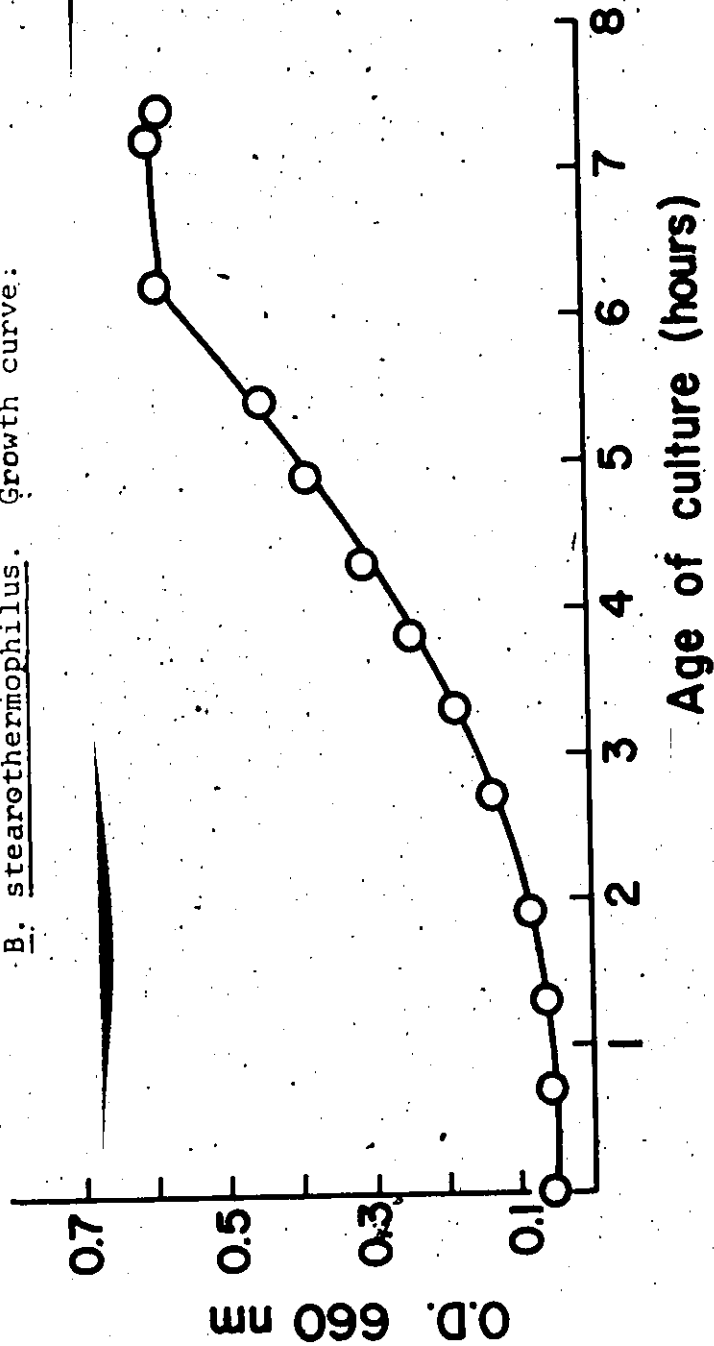


FIGURE 23
B. stearothermophilus. Growth curve:



DISCUSSION OF ATCase INVESTIGATIONS

The optimum in vitro temperature of the ATCase of B. stearothermophilus, 60°-70°C is within its growth range (45°-70°C). In contrast the enzyme of the psychrophile V. psychroerythrus is not at optimal activity in vitro within its growth range (0°-20°C).

As the generation time of B. stearothermophilus is given as 10.8 minutes at 60°C (Stanier et al., 1971, p. 310), and ATCase is an anabolic enzyme, a high activity at optimal growth temperature is to be expected.

However Brock (1969) concluded that the growth rates of thermophiles, although greater than those of mesophiles, are not as high as is theoretically possible from a consideration of their growth temperatures. In fact we found the doubling time of B. stearothermophilus to vary from 24 minutes at 62°C to 1½ hours at 55°C which, in fact, was a slower growth rate than for E. coli whose doubling time is 20 minutes at 37°C, (Stanier et al., 1971).

We found the doubling time of V. psychroerythrus to be about 4 hours at 10°C. We also found the ATCase to be very stable (60 minutes at 10°C in the presence of substrate). This correlates with the slow growth rate in that one can say that this stable enzyme, even when not optimally active maintains the slow rate of metabolic activities at this low temperature.

It is tempting to regard the correlation between optimum in vitro ATCase activity and optimum growth temperature of B. stearothermophilus as evidence for adaptation by natural selection. The ATCases of the mesophiles E. coli, S. cerevisiae and Halobacterium cutirubrum have been assayed at temperatures of 25-30°C, up to 40°C and 25° or 30°C, respectively (e.g. Gerhart and Holoubek, 1967; Kaplan and Messmer, 1969; Liebl et al., 1969). The authors do not state that these are the optimum temperatures. However it seems reasonable to conclude that the optimum in vitro temperature of the B. stearothermophilus enzyme is much higher than those of these mesophiles. Whether this difference is due to a more rigid conformation of the molecule of the thermophilic enzyme it is impossible to say. It is not possible to know whether, if any differences in conformation do exist, they resulted from mutations in a primordial thermophile which were selected for at lower temperatures, or the reverse process. The high in vitro temperature optimum of the thermophilic enzyme may be simply a physical characteristic of this enzyme in any organism. At physiological substrate concentrations, which are not saturating, enzyme activity regulation is achieved by the enzyme substrate affinity, which may change with temperature and by the substrate concentration. If organisms are not completely at the mercy of outside temperature conditions it is because they have both thermodynamic and kinetic means at their disposal to allow cellular reactions to continue (Hochachka, 1974).

The energies of activation of the ATCase of the psychrophile (V. psychroerythrus) the mesophile (S. cerevisiae) and the thermophile (B. stearothermophilus) are in ascending order. That is, over the temperature range of increasing enzyme activity the thermophile shows the greatest response to increasing temperature, which can be correlated with its short generation time. A higher energy of activation of an enzyme is no disadvantage to a thermophile, because of the greater kinetic energy of molecules at higher temperatures. The energies of activation of the psychrophilic and mesophilic ATCases are nevertheless quite high and because of the lower growth temperatures of the organisms may be correlated with slow growth rates. In considering the possible inferences to be drawn from the differences in energies of activation between the three organisms it is relevant to refer to current work on enzymes of multicellular eukaryotic organisms living at different temperatures.

Comparison of ΔG^* and turn-over numbers of substrate molecules in, for example, the glyceraldehyde-3-phosphate dehydrogenase and phosphorylase-b of rabbit and lobster show a small reduction in ΔG^* between the rabbit and lobster enzymes but a large increase in turn-over number (Hochachka and Somero, 1973, p. 224). Furthermore the change in entropy ΔS^* is high and positive for the rabbit enzymes but negative or a little above zero for the lobster enzyme. The enthalpy change, ΔH^* , for the lobster enzyme is about 75% that for the rabbit enzyme (Low et al., 1973).

*However, at lower temperatures activity falls rapidly; this could correlate with the comparatively high lower temperature limit for growth.

The authors explain this by reference to the intracellular environments. The endothermic (homeothermic) mammal has more thermal energy available and the enzyme has a high ΔH^* to overcome the energy barrier, whereas the ectothermic (poikilothermic) lobster has a low heat content and entropy in its cells and it is thermodynamically more feasible to increase order (ΔS^* is negative). They thus demonstrate that the contribution of enthalpy and entropy changes to the free energy of activation (ΔG^*) of these and other enzymic reactions differs between the two classes of organisms. They believe that amino acid differences outside the active site of the enzyme molecules of the two classes give the ectothermic enzymes a more rigid molecule, which can account for the thermodynamic differences.

It is possible that similar differences in entropy, ΔS^* , for ATCase exist between psychrophilic, mesophilic and thermophilic microorganisms, as those between the enzymes of ectothermic and endothermic organisms noted above. The temperatures at which the ATCase of B. stearothermophilus is active are above those optimal for mammalian enzymes. It would be thermodynamically feasible to increase ΔH^* but not to increase order. Therefore the contribution made to ΔG^* by ΔH^* would be great because the ΔS^* might be high and positive. The psychrophile which may have a lower ΔH^* may have a greater relative contribution to ΔG^* made by ΔS^* . Entropy at temperatures from 0-20°C could be reduced.

Because of the thermal energy available the higher ΔH^* of the thermophilic enzyme is not a disadvantage. Indeed it may even be an advantage, though this is highly conjectural. If ΔS^* is high and positive ΔG^* may be relatively small and the actual free energy of activation high. Therefore the rate of catalysis of the enzyme would be low. The organism would not benefit from an overproduction of metabolites, using nutrients unnecessarily.

The higher the energy of activation the greater is the sensitivity to temperature of a reaction. Consequently the thermophilic ATCase activity would drop more rapidly with temperature than would that of the mesophile, and that of the psychrophile would be least reduced. This may help to explain why B. stearothermophilus cannot grow below 40°C , and V. psychroerythrus can grow, albeit slowly, below 20°C .

It is necessary to interpret figures for the energy of activation cautiously. Those for catalase showed a reversal in order between E. coli and B. stearothermophilus when they were measured in crude extracts and whole cells (Table 8, p. 128). It is not possible to measure ATCase activity in whole cells. Furthermore other metabolic controls are likely to be operative. The affinity between enzyme and substrate, dependent as it is upon weak bonds, is altered by temperature.

At physiological substrate concentrations (non-saturating) a positive thermal modulation is known to exist, whereby the weak bonds of the E-S complex are strengthened at low temperatures. The opposite phenomenon occurs if the weak bonds are

hydrophobic ones, the affinity of the enzyme and substrate are greater at higher temperatures. Allosteric properties too are known to be altered by temperature (Hochachka and Somero, 1973; Hochachka, 1974).

From Fig. 8 it can be seen that 50% of the ATCase activity of B. stearothermophilus remains after storage at 60°C for 24 hours, (in comparison with storage at 0°C for 24 hours). This is only 8% less than after storage at 37°C for the same time. Whilst the rate of inactivation increases above 20°C, 20% activity was still present after 14 days at 37°C. There was even a trace (<1%) after 14 days at 53°C. As no substrate was present in the crude extract it seems that this enzyme is remarkably stable in the absence of substrate. Indeed from Table 1 it would seem that the presence of substrate at 50°C and 60°C hastens inactivation. It was ascertained that decline in activity was not due to substrate limitation. Such rapid inactivation of the enzyme in the presence of its substrate supports the theory that thermophiles survive in part by a high rate of synthesis of macromolecules which are rapidly inactivated by heat. However, as it is not possible to measure ATCase activity in whole cells, its stability in vivo, in the presence of substrate cannot be determined.

Fig. 8 indicates the possible existence of two enzymes having differing heat stabilities. The more heat labile one is greatly or completely inactivated by storage at temperatures above 20°C and the heat stable enzyme shows activity after storage at 50° and 60°C even after 24 hours.

It seems that one of the ATCases of B. stearothermophilus

is stable at the growth temperature of the organism so long as its substrates are not present. Once these are available it has a short life and must be rapidly replaced so long as ATCase activity is required for growth.

The existence of two ATCases in C. freundii and P. vulgaris has been demonstrated by Bethel and Jones (1969). One enzyme shows feedback inhibition by CTP and the other, which has a smaller molecular weight shows no such inhibition. Indeed in P. vulgaris there is stimulation of ATCase activity by CTP. They speculate that the enzyme lacking feedback inhibition is catabolic and the other enzyme is anabolic. Table 4 shows stimulation by CTP of the ATCase of B. stearothermophilus at higher temperatures. It is possible that the enzyme of this organism which is stimulated by CTP is catabolic and is the more heat stable one. However stimulation by CTP of the anabolic enzyme might partially counteract its rapid decline in activity at high temperatures in the presence of substrate.

For an enzyme to be functional at high temperatures it must have sufficient rigidity of structure to withstand the disruptive effect of heat. For this it may ipso facto sacrifice ability to make the conformational changes associated with allostery. Furthermore studies on the ATCase of other bacterial species (B. subtilis, S. faecalis, O'Donovan and Neuhard, 1970) demonstrate that they lack this type of regulation. B. subtilis ATCase has no regulatory sub-unit (O'Donovan and Neuhard, 1970) and it is possible that the genus as a whole lacks it.

The ATCase of B. stearothermophilus is compared with
and contrasted to its catalase in the final discussion.

RESULTS OF THE CATALASE INVESTIGATIONS

The catalase activity of *B. stearothermophilus* during the life of a culture.

Samples of a 10 litre culture of *B. stearothermophilus* were harvested at intervals and assayed for catalase activity after overnight storage in the refrigerator.

Reference to Figs. 24 and 25 shows that the catalase activity of unbroken cells dropped rapidly during the lag phase, and the beginning of the logarithmic phase, rose gradually during the remainder of the logarithmic phase and rose rapidly after the end of the logarithmic phase and throughout the linear phase of growth, reaching a peak when about half the total cell mass was produced. It then dropped rapidly as the stationary phase was approached to reach its minimum before the commencement of the stationary phase.

FIGURE 24

Cells stored overnight in refrigerator.

Catalase activity of a cell suspension measured by the
titanium sulphate method.

Substrate = 0.02 M H_2O_2

Temperature = 40°C

FIGURE 24

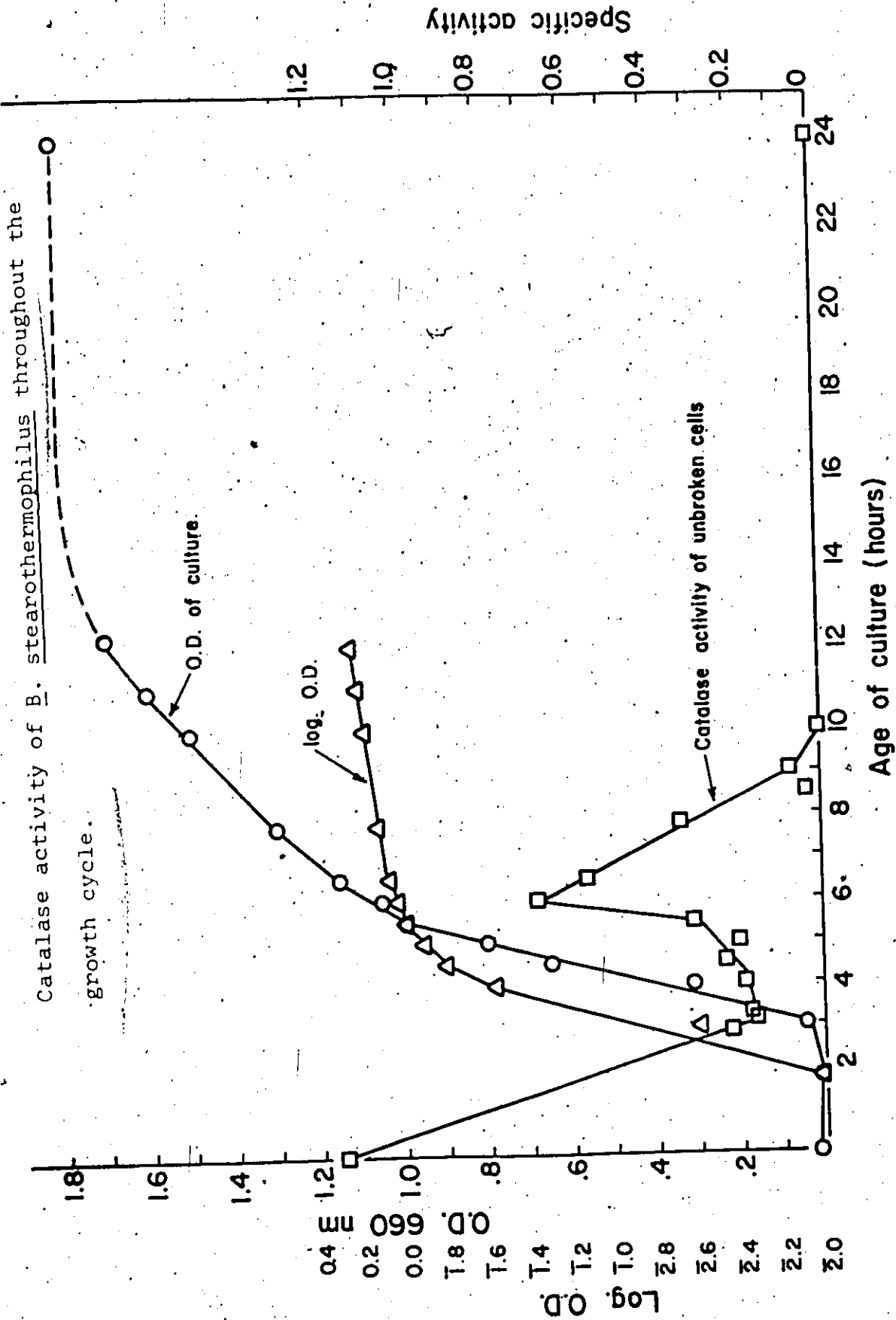
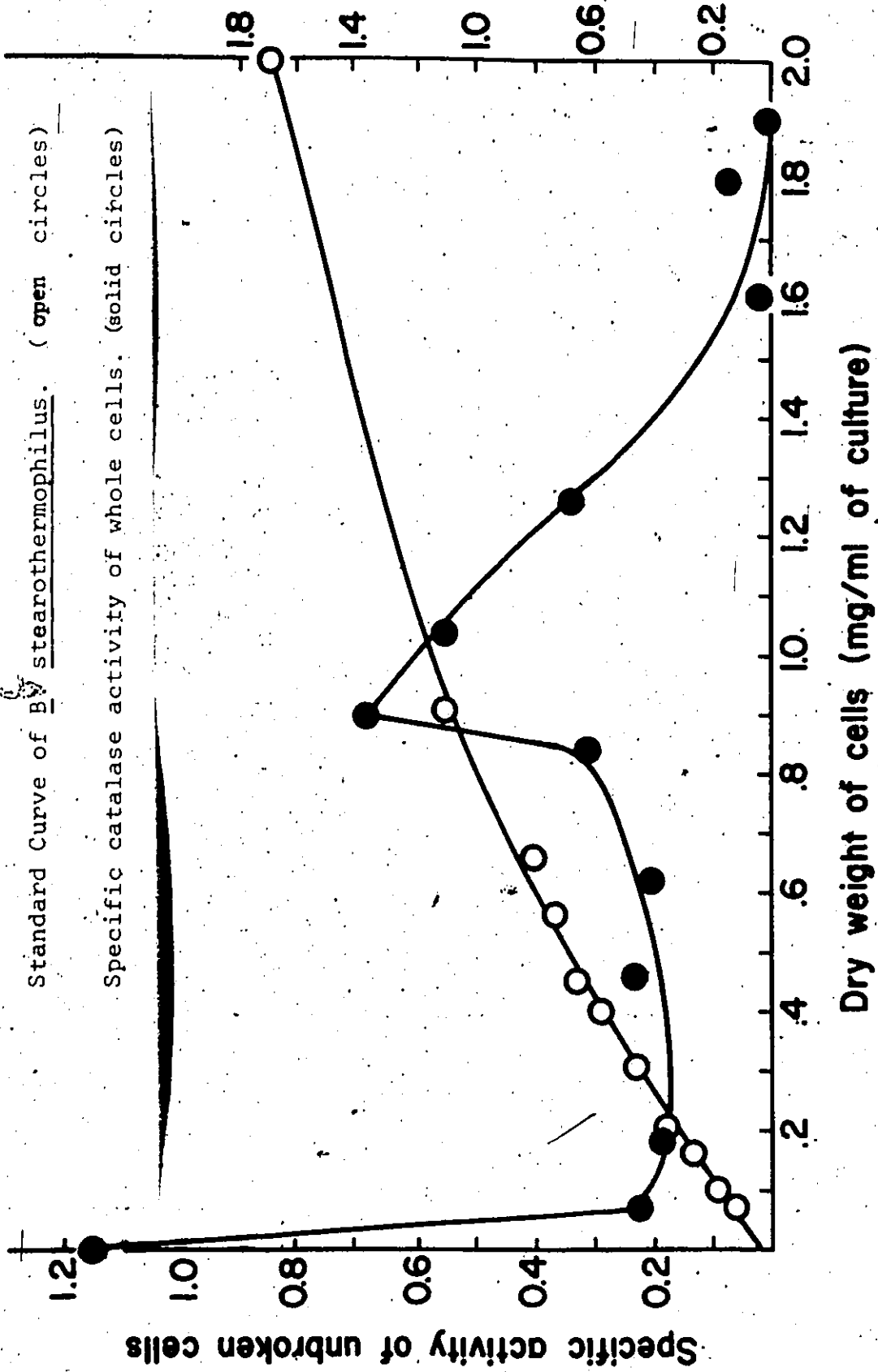


FIGURE 25



Localization of catalase in *B. stearothermophilus*

Results

The activity of catalase of *B. stearothermophilus* was found to be present only in the cells and not in the culture fluid, showing that it was not an exo-enzyme. Table 5 shows that activity was demonstrable in cell suspension and crude extracts, and that there was 50% loss of activity after freezing cells for 6 days. Activity after breaking the cells, but not centrifuging them, was lowered. After low speed (500 g or 3,000 g) centrifugation, the supernatant had more activity than the broken cells. Upon high speed centrifugation there was some loss of activity but all remained in the supernatant and no activity was measured in the pellet. A more detailed study was made of enzyme location during growth. Because it was necessary to take a number of small samples, total activities were too low for valid measurements but there seemed to be no major change in location at different growth phases.

TABLE 5

Percent total catalase activity of whole cells and crude extracts of B. stearothermophilus

Age of culture (hours) when cells harvested	4½	7½
Cell suspension before freezing	100	100
Cell suspension frozen 6 days	-	50
Broken cells not centrifuged	-	39
Supernatant after centrifuging at 500 g for 10 minutes	58	-
Supernatant after centrifuging at 3,000 g for 10 minutes	-	76
Supernatant after centrifuging at 100,000 g for 60 minutes	40	43
Pellet after centrifugation at 100,000 g.	0	0

Titaneum sulphate method

Substrate 0.02 M H₂O₂

Temperature of assay 40°C

Catalase activity of B. stearothermophilus and E. coli at different temperatures.

Optimal temperatures

As can be seen from Figs. 26 and 27 the optimal temperatures for the catalase of the two organisms are the same for cell suspensions and not greatly different for crude extracts. This is in contrast with results for the ATCase, where the temperature optima for the mesophile and thermophile were quite different.

The above results were obtained by calculating "zero-time velocities". There are certain difficulties in interpreting temperature optima in such assays. The higher the temperature the greater is the velocity, but the greater, also, the speed of heat inactivation. Velocity falls more quickly with time. It is therefore questionable to speak of optimal temperatures as including those higher temperatures at which the initial high velocity is rapidly reduced. This reduction in velocity is attributable both to denaturation of the enzyme and to formation of inactive complexes with hydrogen peroxide. The temperature coefficient for this process is higher than that for the enzymatic reaction (Maehly and Chance, 1954). In fact, however, very little difference in the temperature optima was noted when velocities were measured over longer periods. Table 6 shows the zero time, one minute and two minute velocities of catalase of E. coli at different temperatures of assay. The 'optimal' temperature

FIGURE 26

Titanium sulphate method

Substrate 0.02 M H_2O_2

One experiment only for crude extract is shown. See text
for discussion of others.

FIGURE 26

Catalase activity of B. stearothermophilus in cell suspension and crude extract, at different temperatures.

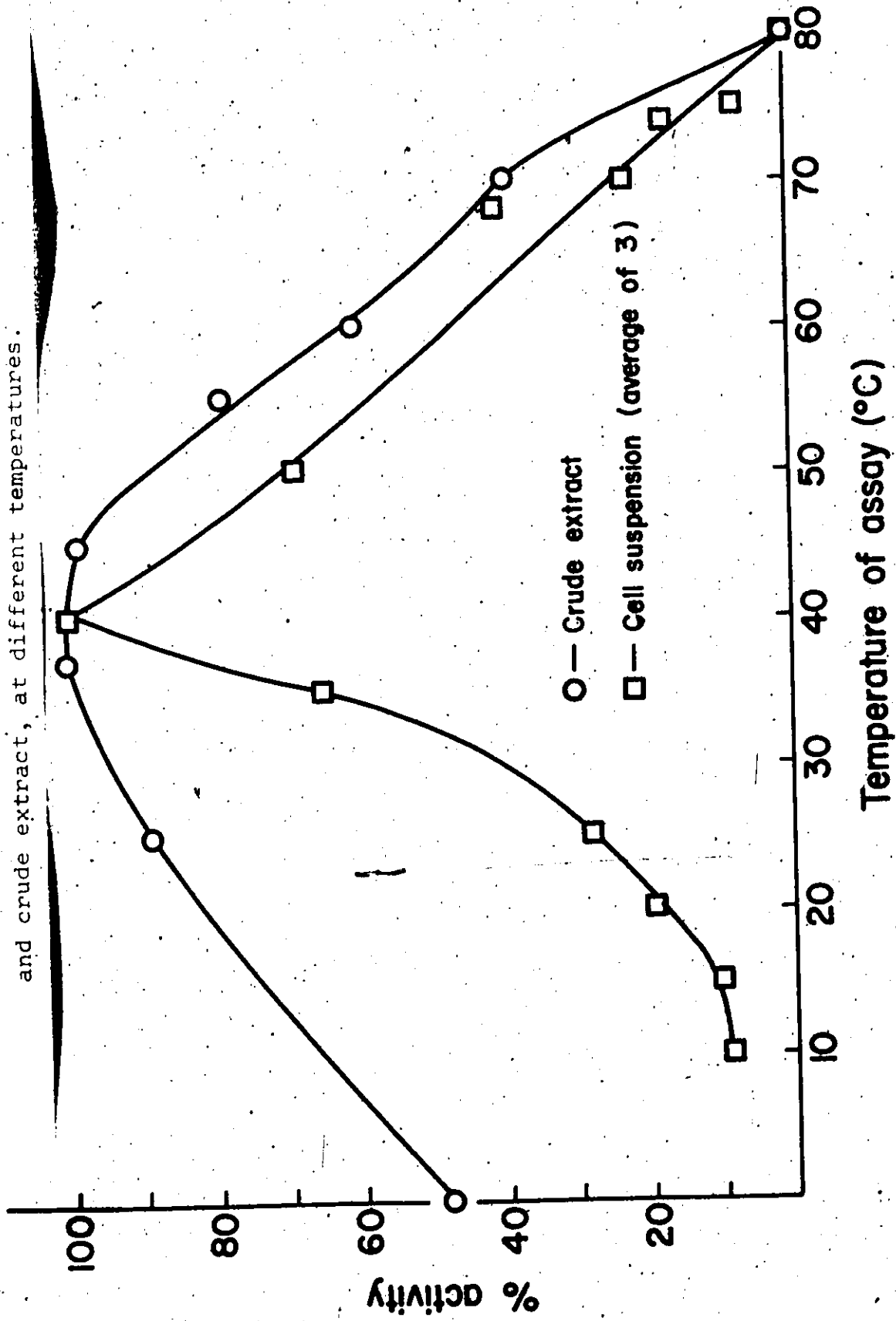


FIGURE 27

Titanium sulphate method

Substrate 0.02 M H₂O₂

One experiment only for crude extract is shown. See text
for discussion of others performed.

FIGURE 27

Catalase activity of *E. coli* in cell suspension and crude extract at different temperatures.

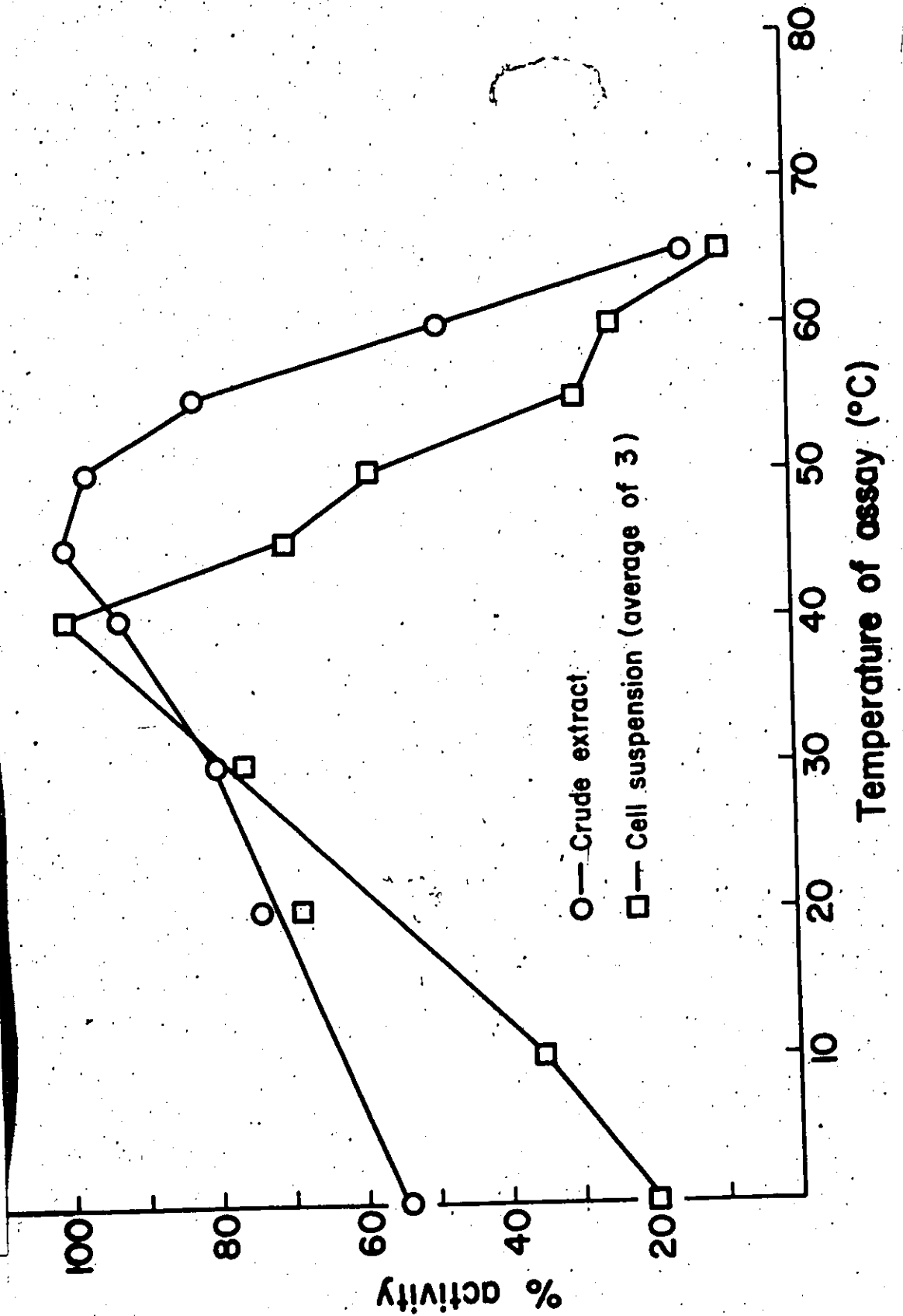


TABLE 6

Velocity of catalase of E. coli at different temperatures and times of assay

Temp. °C	Crude Extract			Cell suspension		
	ZT	1'	2'	ZT	1'	2'
0	0.028	0.019	0.008	0.028	0.024	0.030
10	-	-	-	0.052	0.064	0.070
20	-	-	-	0.099	0.099	0.095
25	0.033	0.022	0.018	-	-	-
30	-	-	-	0.110	0.110	0.123
35	-	-	-	0.109	0.107	0.093
37	0.038	0.019	0.027	-	-	-
40	-	-	-	0.145*	0.115*	0.098*
45	0.042	0.026	0.027	0.100	0.070	0.073
50	-	-	-	0.084	0.102	0.047
55	0.051	0.033*	0.031*	0.042	0.051	0.021
60	0.052*	0.029	0.021	0.035	0.026	0.013
65	0.038	0.024	0.021	0.013	0.010	0.005

Titanium sulphate method

Substrate = 0.02 M H₂O₂

ZT = zero time

* = optimum temperature

Velocity = $\frac{\log \text{O.D. at ZT} - \log \text{O.D. at 1' or 2'}}{\text{minutes}}$

ZT velocity calculated by extrapolation.

appears at 60°C for the crude extract for zero time velocity and at 55°C for one minute and two minute velocities. This difference is not found in cell suspensions. Indeed at 50°C and 55°C the velocities at 1 minute are greater than those at zero time. This phenomenon will be discussed later. Table 7 shows velocities at different times for suspensions and extracts of B. stearothermophilus. Here the optimal temperature for the cell suspension appears as 40°C at zero time and one minute, but as 30°C at two minutes. This difference is not found in the crude extracts. These results do not contradict the evidence, to be presented and discussed later that B. stearothermophilus has a highly heat-sensitive catalytic component measurable in whole cells. Such a component was not apparent in E. coli cells, in which short periods of heat treatment of cells actually produced a rise in activity.

TABLE 7

Velocity of catalase of B. stearotherophilus at different temperatures and times during assays

Temp. °C	Crude extract.			Cell suspension		
	ZT	1'	2'	ZT	1'	2'
0	0.146	0.139	0.172			
15				0.011	0.014	0.022
20				0.022	0.029	0.032
25	0.269	0.240	0.238	0.039	0.049	0.057
30				0.034	0.051	0.073*
35				0.072	0.069	0.066
37	0.302*	0.311*	0.296*			
40				0.12*	0.089*	0.044
45	0.299	0.294	0.225	0.055	0.062	0.043
50				0.081	0.06	0.035
55	0.238	0.178	0.188	0.013	0.028	0.033
60	0.182	0.219	0.104			
70	0.116	0.066	0.027			

ZT = zero time

* Optimum temperature

Titanium sulphate method

Substrate = 0.02 H₂O₂

Velocity = $\frac{\text{Log O.D. at ZT} - \text{log O.D. at 1' or 2'}}{\text{minutes}}$

ZT velocity calculated by extrapolation

The energy of activation and the energy of activation of heat inactivation

Sample Arrhenius plots for the catalase of E. coli and B. stearothermophilus are shown in Figs. 28 and 29 respectively. Values obtained for the energies of activation are given in Table 8.

It must be remembered that activity of previously unheated preparations measured at high temperatures is a measure of increased activity due to the effect of temperature upon a chemical reaction less the decrease in activity due to the inactivation of the enzyme by heat. This decrease in activity increases during the time of the assay. Extrapolations back to zero time velocity, in theory, measures the velocity before heat inactivation has begun. Therefore the energy of activation of heat inactivation is best measured by the residual activity of enzyme preparations after heat treatment.

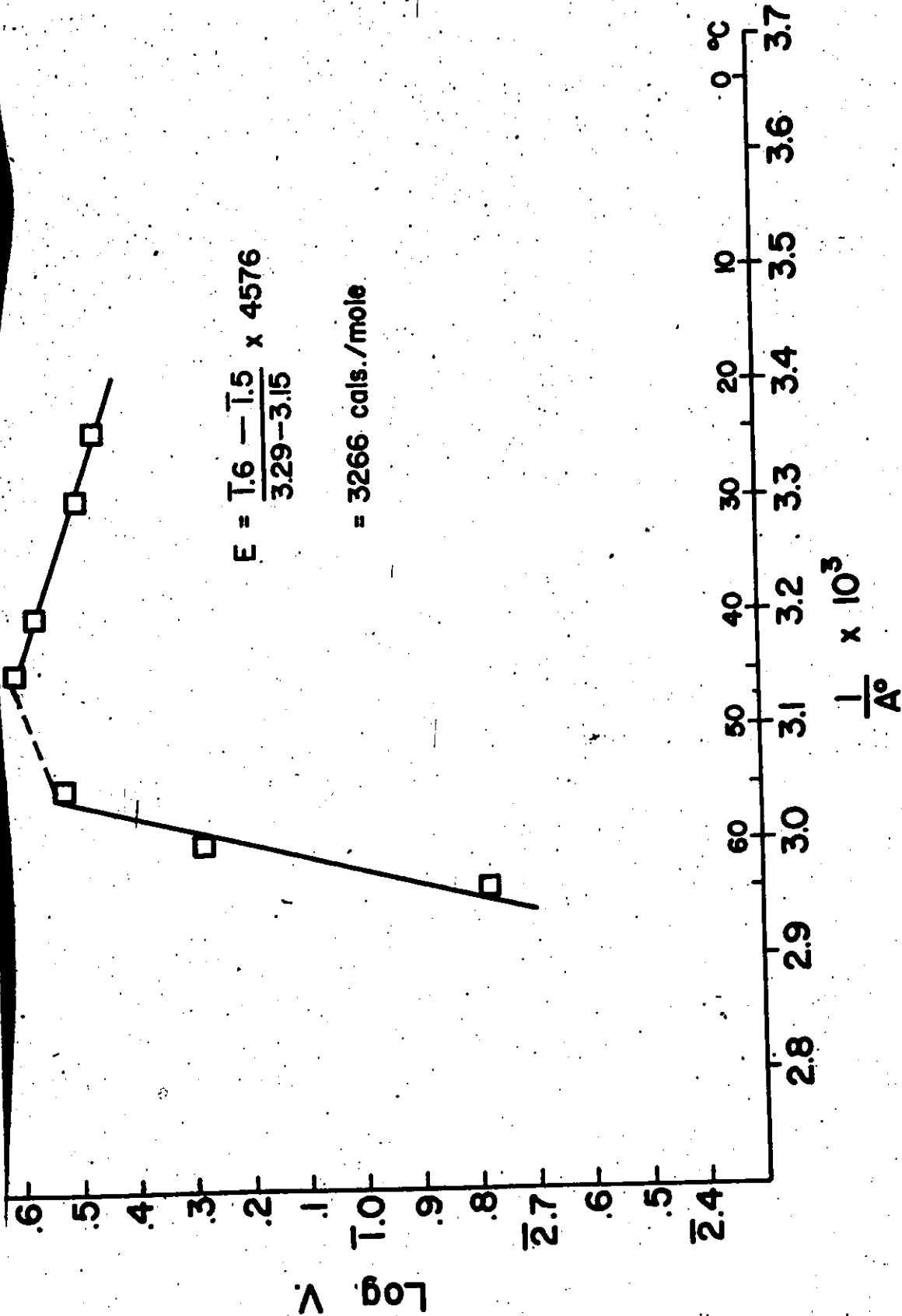
By first subjecting the enzyme preparation to heat for different lengths of time and then measuring residual activity at optimal temperature for enzyme activity, one can calculate the rate of heat inactivation at that temperature. The logarithms of these rates at various temperatures when plotted against the reciprocal of the absolute temperature give an Arrhenius plot from which the energy of activation of heat inactivation can be derived (Figs. 30 and 31).

Theoretically it is possible to calculate the combined

FIGURE 28

Arrhenius plot of catalase activity in a crude extract of

E. coli.



Arrhenius plot of catalase activity in a crude extract of B. stearothermophilus.

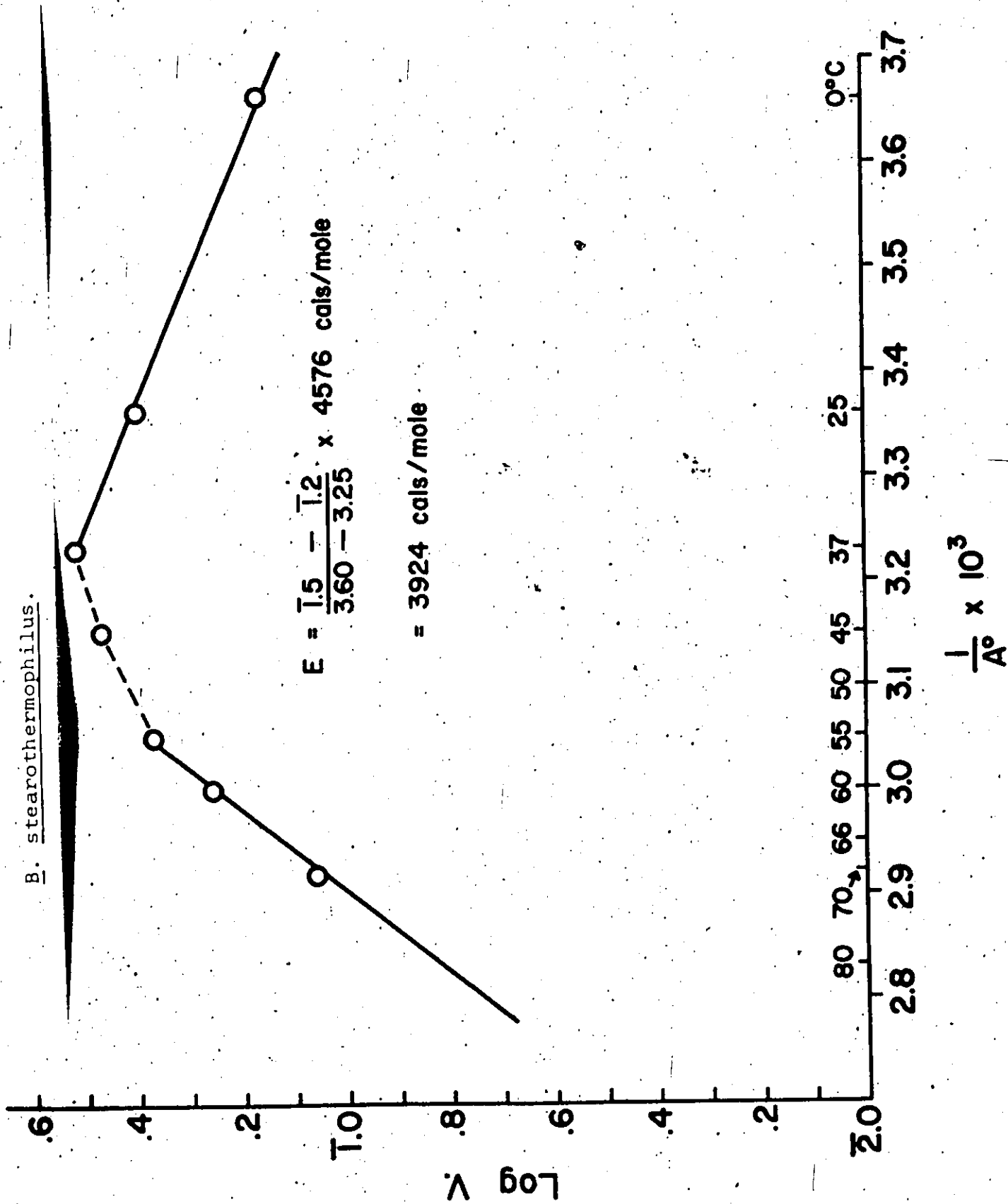


TABLE 8

Energy of activation of catalase of E. coli and B. stearothermophilus in crude extracts and cell suspensions.

Crude extract	<u>B. stearothermophilus</u>	5,156 cal/mole ± 1,419	(6 expts.)
	<u>E. coli</u>	2,378 " " ± 248	(2 expts.)
Cell suspension	<u>B. stearothermophilus</u>	5,073 " " ± 1,630	(3 expts.)
	<u>E. coli</u>	6,630 " " ± 230	(2 expts.)

FIGURE 30

Heat stability of catalase in a cell suspension of

B. stearothermophilus.

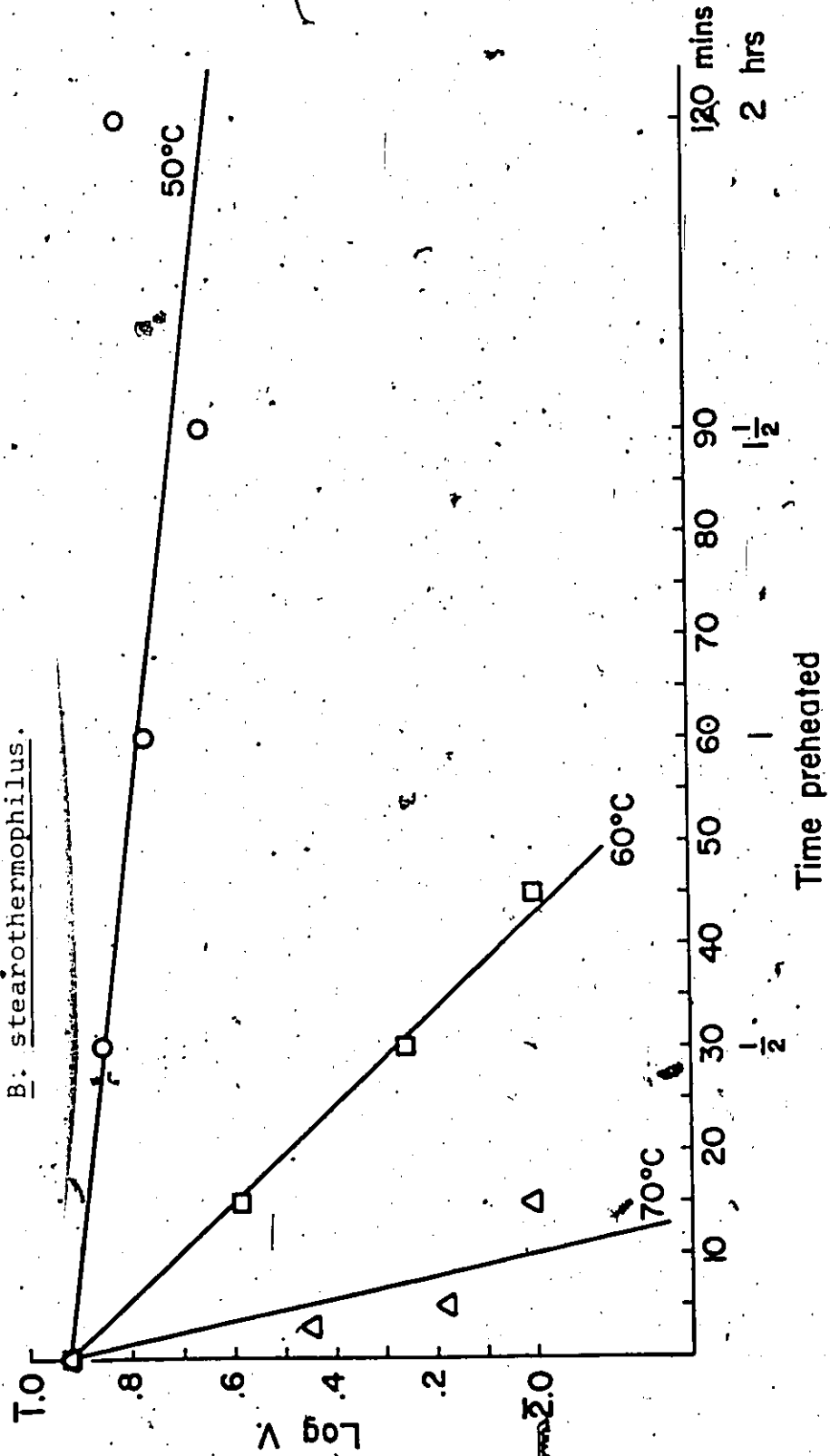
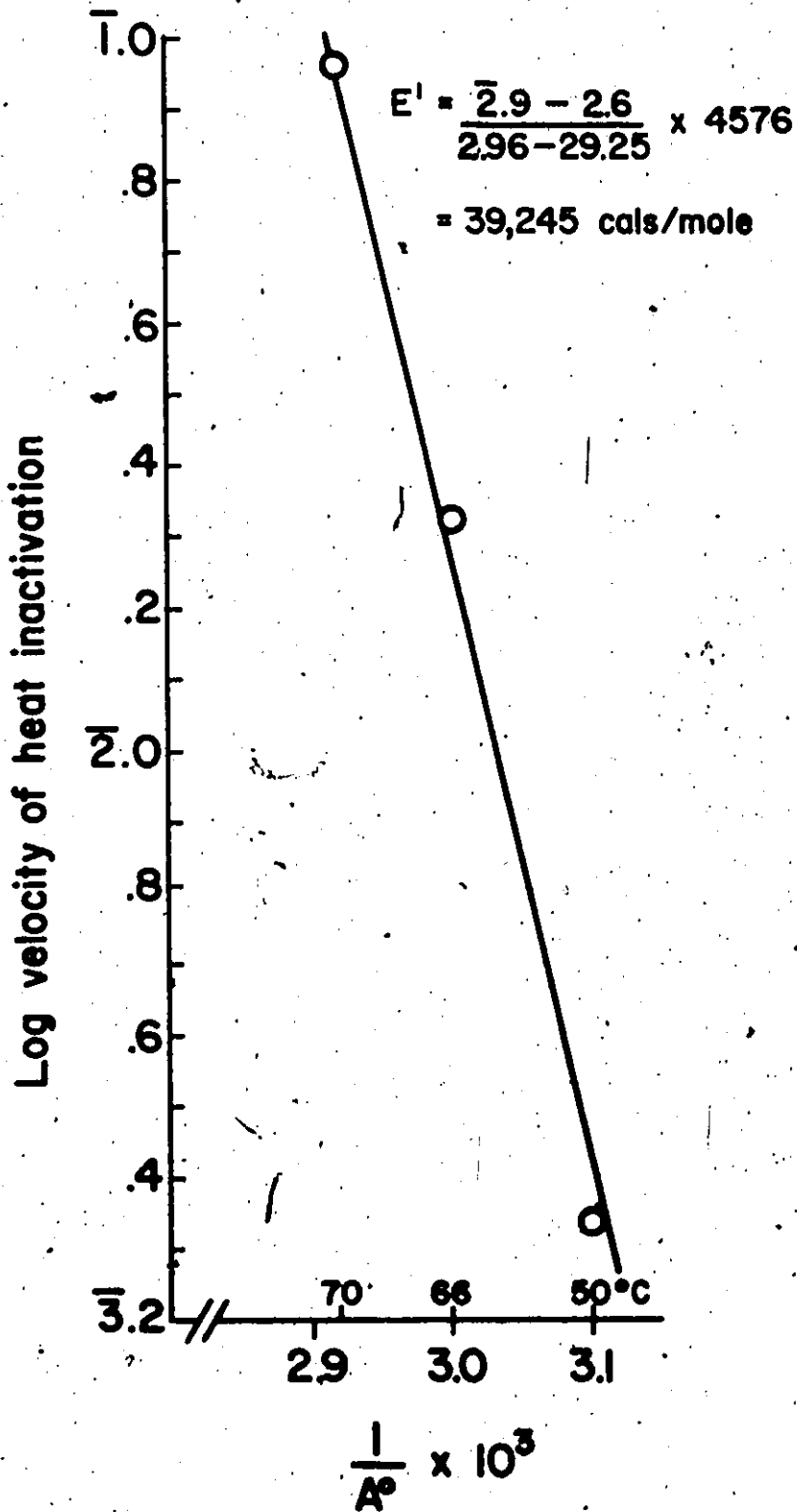


FIGURE 31

Arrhenius plot for heat inactivation of the catalase of a cell suspension of B. stearothermophilus.



energy of activation and energy of activation of heat inactivation from the Arrhenius plot of activities of previously unheated enzyme preparations at temperatures above the optimum. Then the energy of activation, calculated from the slope of the line below the optimum temperature, is deducted. Values of the energy of activation of heat inactivation so calculated were always much lower than those derived from stability experiments, because the slope of the line after optimal temperature is less than it would be if more time were allowed for heat inactivation.

In two experiments made using cell suspensions of B. stearothermophilus there were indications that the rate of inactivation was not uniform. Activity after long periods of heat treatment was too high, and the slope of the line of best fit did not always extrapolate back to the activity of the unheated extract (Fig. 32). This suggested the existence of two catalytic components, one very heat labile and one heat stable. This possibility was further investigated (see below). As the overall rate of heat inactivation in whole cells represents the effective result of heat in vivo the energy of activation of heat inactivation was calculated from this. Moreover, if the time course graphs of heat inactivation were drawn with a break (Fig. 33) two energies of activation of heat inactivation could be calculated. That from the first part of the graph was lower, and that for the second part of the graph was higher than the overall rate. Even the higher values were not as great as that for the E. coli enzyme (Table 9).

FIGURE 32

Heat stability of catalase in a cell suspension of B. stearothermophilus.

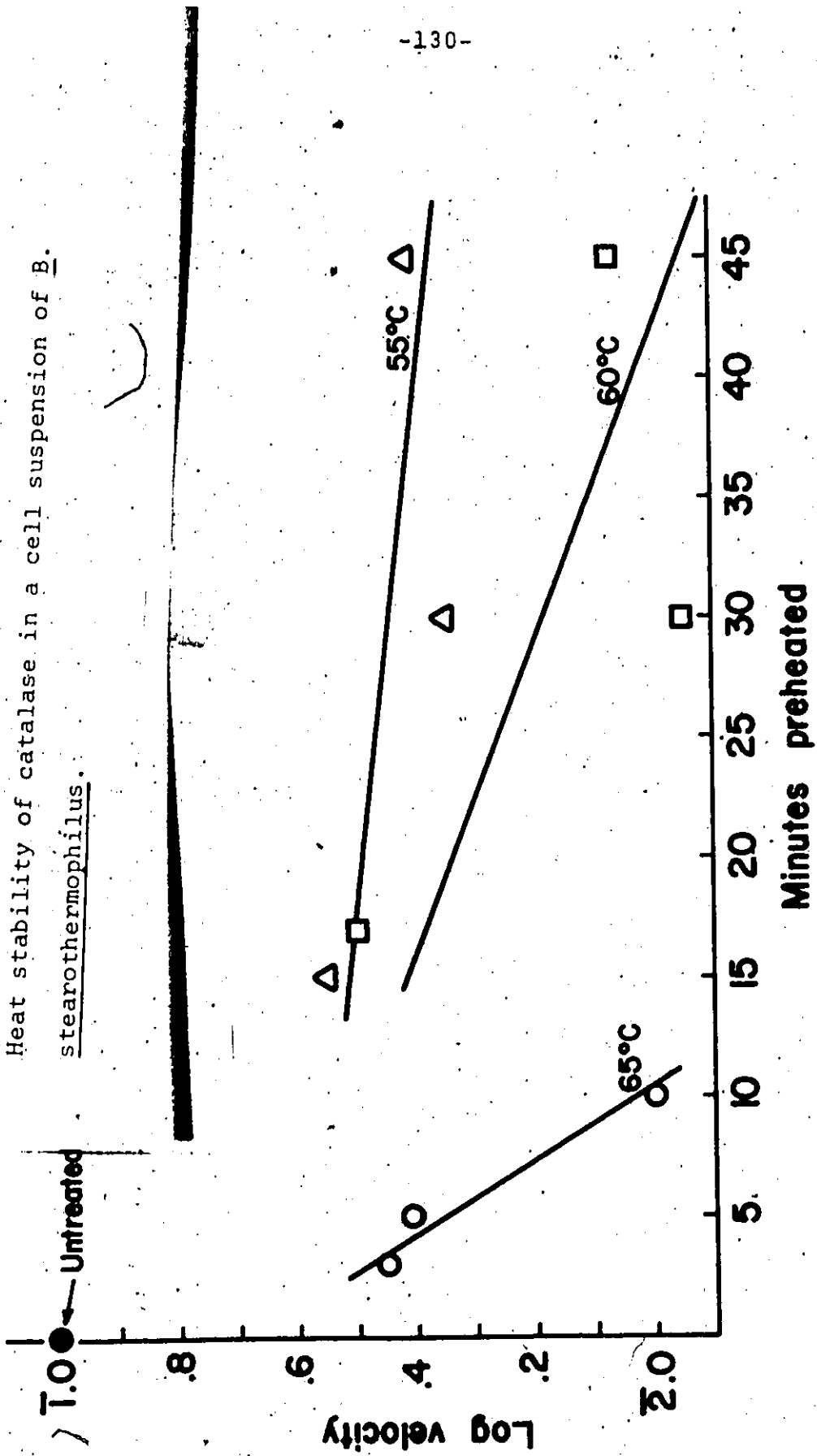


TABLE 9

Energy of activation of heat inactivation of catalase of E. coli and B. stearothermophilus in crude extracts and cell suspensions

Crude extract	<u>B. stearothermophilus</u>	43,000 cal/mole*
	<u>E. coli</u>	15,425 " "
Cell suspension	<u>B. stearothermophilus</u> [†]	41,700 " "
	<u>E. coli</u>	70,000

* All figures are average values from two experiments

† In one of the two experiments the figures were as follows: -

Overall value = 37,800 cal/mole

Value for first period = 33,400 cal/mole
(period of rapid heat inactivation)

Value for second period = 57,200 cal/mole
(period of slow heat inactivation)

(See text for discussion)

The stability of catalase of *B. stearothermophilus* and *E. coli* over very short periods of heat treatments. Evidence for two catalatic components in *B. stearothermophilus*.

To further investigate the possible existence of two catalatic components in *B. stearothermophilus* the following investigations were carried out.

- 1- The stability of the catalase of a cell suspension of *B. stearothermophilus* was measured at very short intervals over 10 minutes at 55°C and 60°C (Fig. 33).
- 2- That of *E. coli* was measured at very short intervals over 10 minutes and also after 30 and 60 minutes at 40°C, 50°C and 55°C (Fig. 34).
- 3- The stability at 55°C of catalase in cell suspensions of *B. stearothermophilus* and *E. coli* was measured simultaneously. The heat treatment was carried out in the same water bath at the same time. The cell suspensions were adjusted to the same optical density at 660 nm and their protein content was similar. Before estimations of residual activity were made the preheated suspensions were washed with butanol (BuOH), to eliminate the effect of any permeability factor (Fig. 35).
- 4- Cell suspensions of *B. stearothermophilus* and *E. coli* of identical optical density at 660 nm and close protein content, were subjected to simultaneous heat treatment at 55°C in the same water bath. One set of tubes for each species was toluene washed and one was not. Both sets were

shaken for 30 minutes. Toluene was chosen because it frequently increases the activity of unheated cells; whereas butanol had reduced it in Expt. 3 (Fig. 36).

FIGURE 33

The stability of catalase in cell suspensions of B. stearo-
thermophilus at 55°C and 60°C.

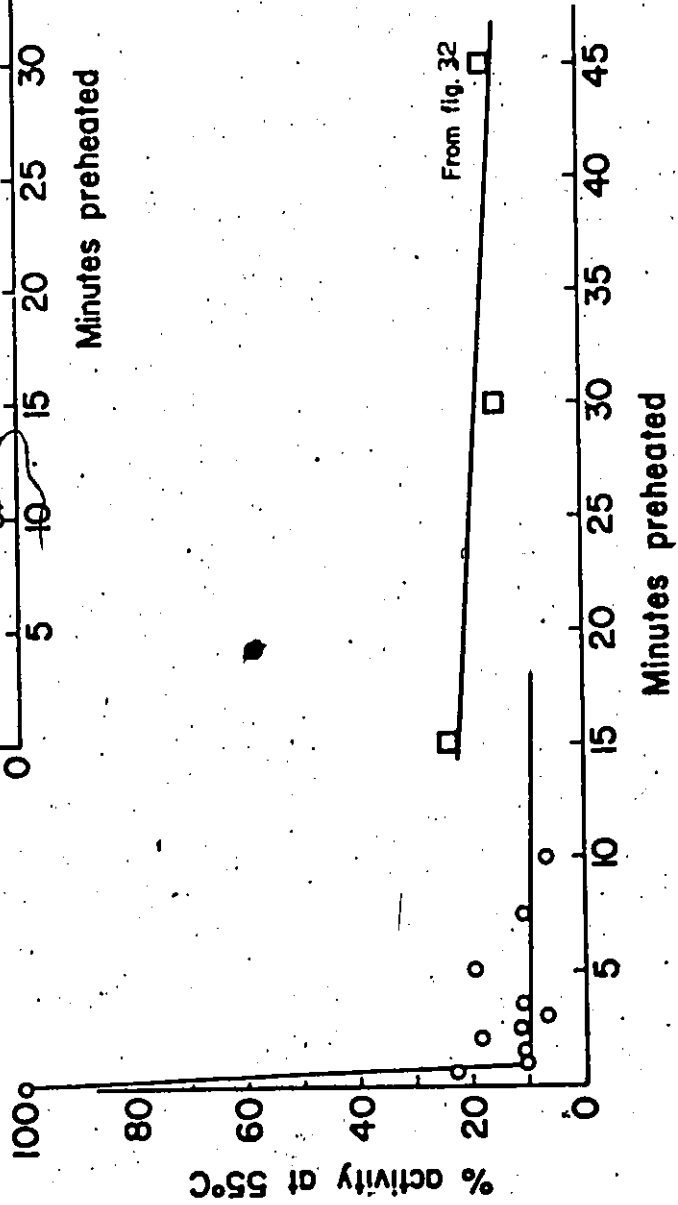
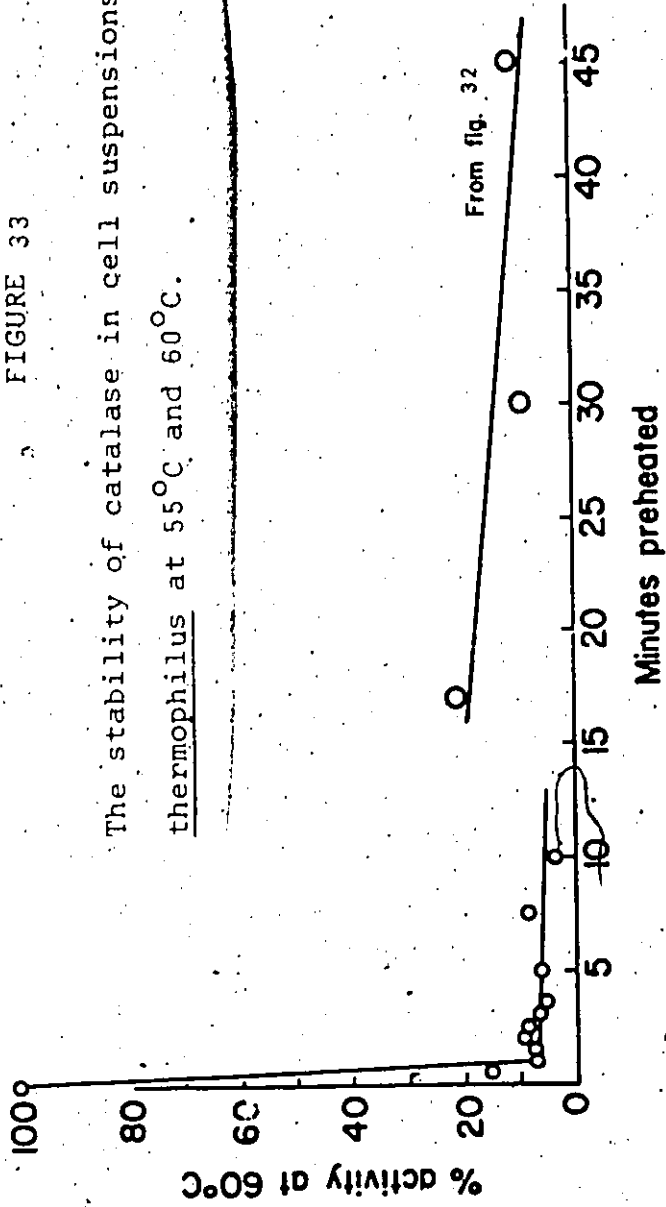


FIGURE 34

Stability of catalase in a cell suspension of *E. coli* at 40°C, 50°C and 55°C.

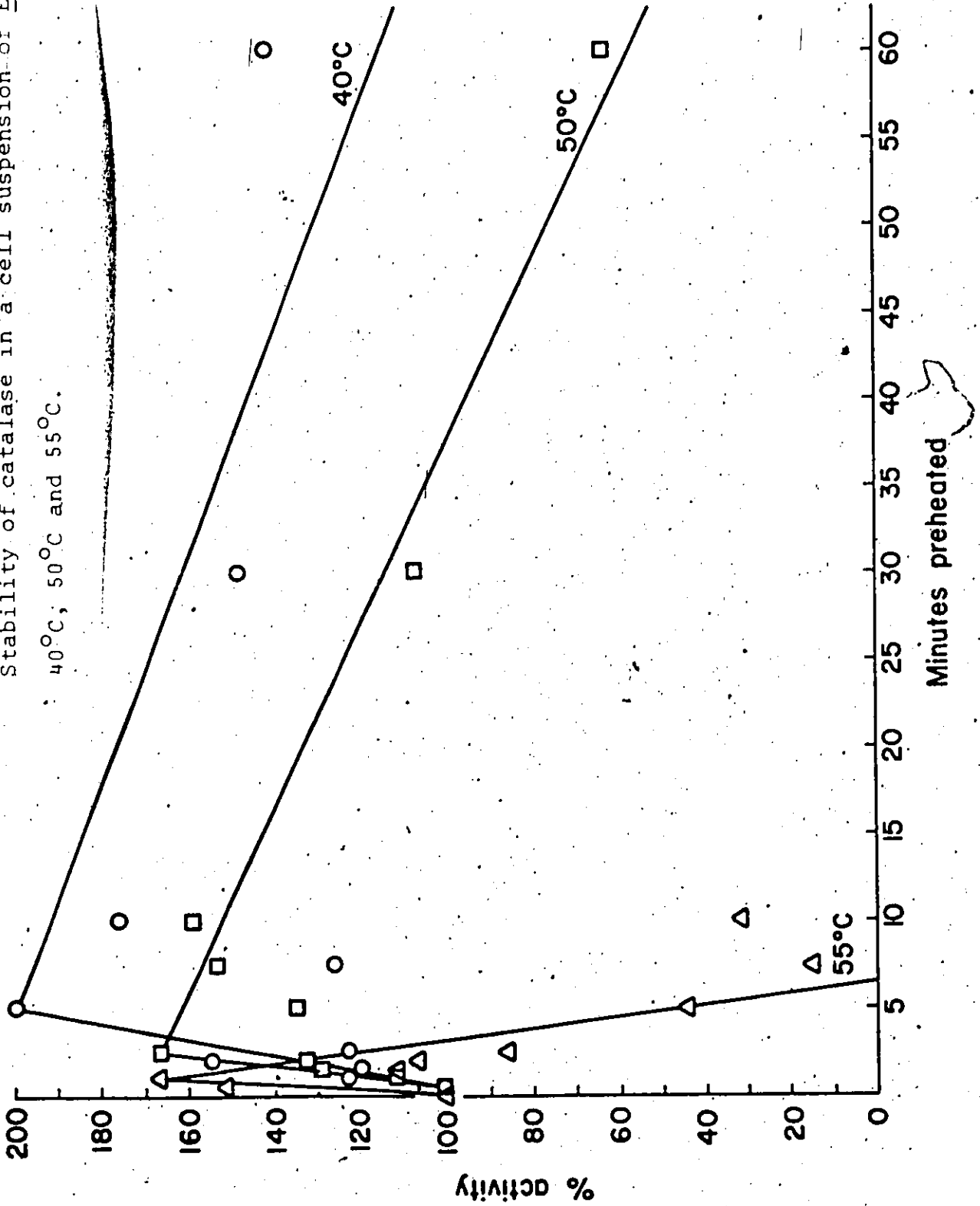


FIGURE 35
Stability of catalase in cell suspensions of B. stearothermophilus and E. coli at 55°C.

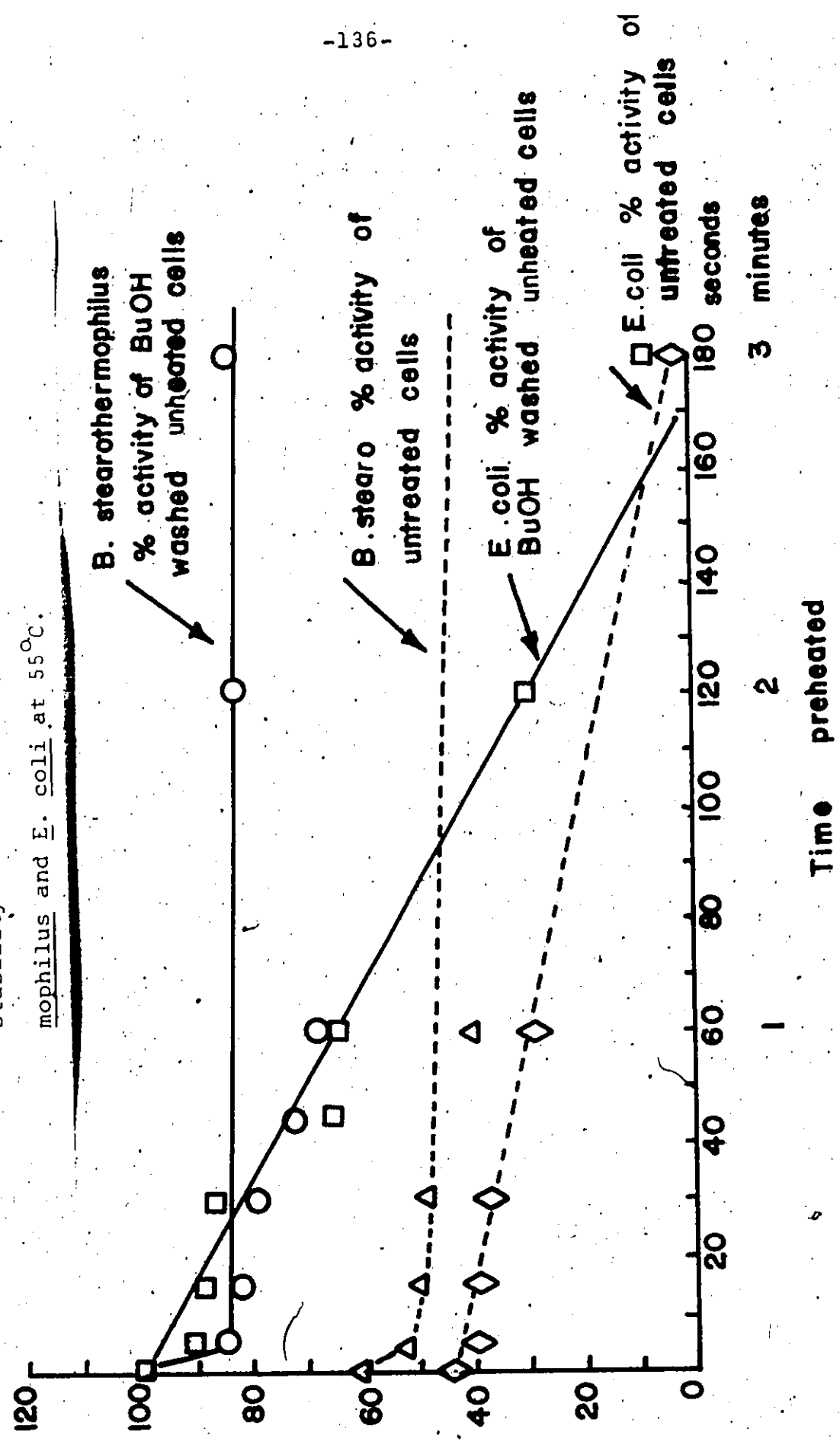


TABLE 10

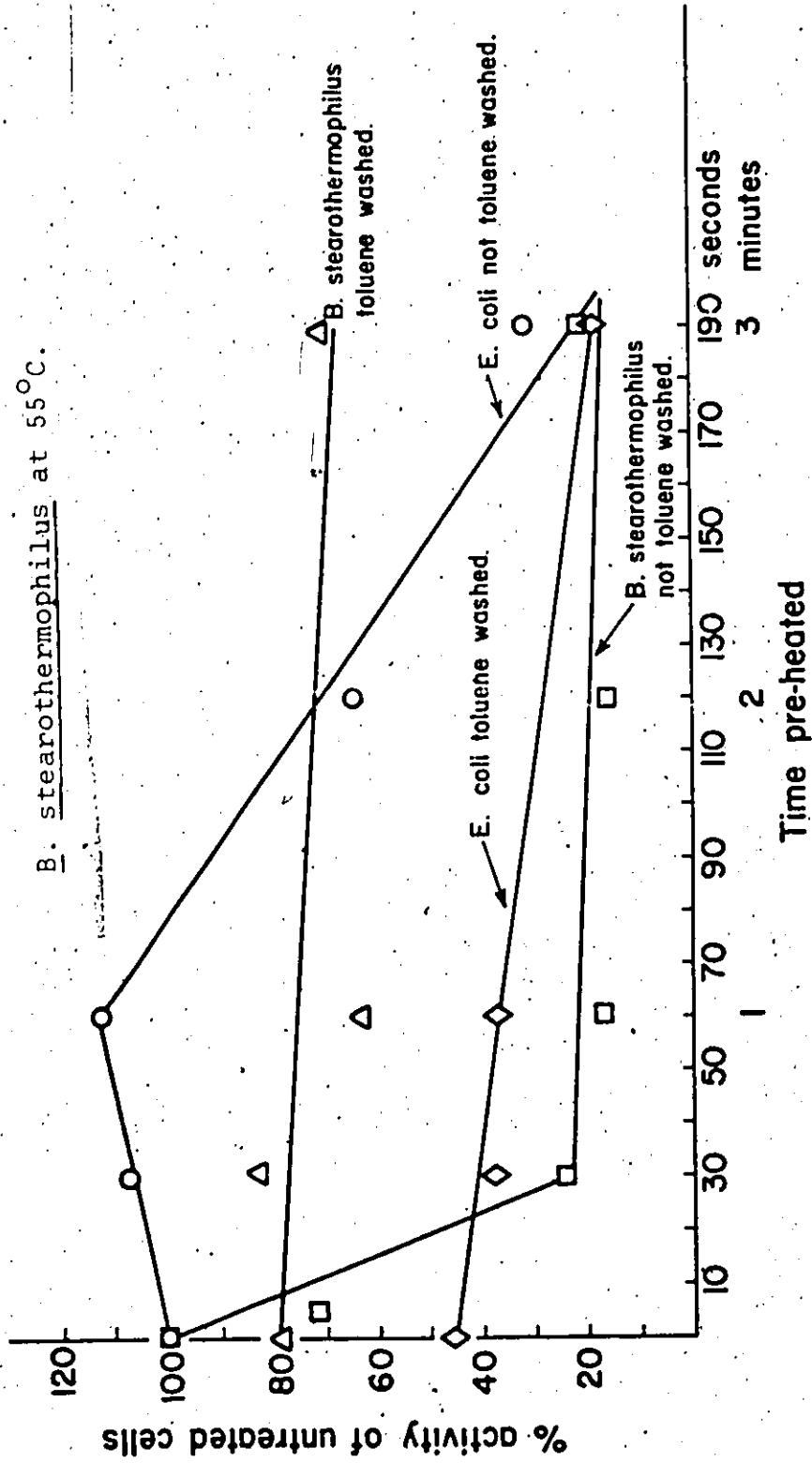
Effect of heating at 55°C for varying lengths of time (followed by BuOH or toluene treatment), upon the catalase activity of cell suspension of B. stearothermophilus and E. coli

Treatments	Residual activity (% of activity of unheated untreated cells)										
	E.C.	B.S.									
None	100	100									
BuOH	44	61									
Toluene	46	79									
Heated 55°C for	5 secs	Ex2	Ex4	Ex1	Ex4	72	40	50	40	50	
	15 secs						39				
	30 secs	151	107	25	24		38	48	38	84	
	1 min	166	112	10	17		29	40	38	63	
	3 mins		30	6	20		3.5	50	16	69	
	5 mins	44									
	8 mins	15									
	10 mins										

E.C. = E. coli. B.S. = B. stearothermophilus. BuOH = butanol.

FIGURE 36

Stability of catalase in cell suspensions of *E. coli* and *B. stearothermophilus* at 55°C.



Results

Fig. 33 shows that at 55°C and 60°C an initial, rapid reduction in percent activity of unheated cells of B. stearothermophilus to 10% or less in 1 minute is followed by a slow reduction of activity in the next 9 minutes.

In contrast Fig. 34 shows, in E. coli, an initial rapid and temporary rise in activity of 50-100%. This occurs even at 55°C though activity is rapidly reduced after the first minute to about 20% in 10 minutes. At 40°C and 50°C reduction of activity after the initial rise is slower. Even an hour later, activity at 40°C is still above that of unheated cells, and that at 50°C is 60% of unheated cells.

Butanol treatment of unheated cells reduced the catalase activity to 44% for E. coli and 61% for B. stearothermophilus. Heat treatment followed by butanol washing showed no transient rise in the catalase activity of E. coli and a reduced amount of rapid reduction in that of B. stearothermophilus (fig. 35, Table 10).

Three-minutes heat treatment followed by butanol washing caused a further 11% reduction in activity of B. stearothermophilus catalase and a further 40% reduction in E. coli catalase.

Toluene treatment had the same overall effect as butanol treatment. Activity was reduced, contrary to expectation, by toluene treatment to 46% of untreated cells for E. coli and to 79% for B. stearothermophilus. Three minutes heat treatment followed by toluene washing caused a 10% reduction in activity of B. stearothermophilus catalase and a 30% reduction

in E. coli catalase compared to toluene washed unheated cells (Fig. 36 and Table 10). Clayton (1959) found catalase poor cells showed inactivation by toluene, or a slight decrease in activity.

It is of considerable interest that whereas the catalase activity of the E. coli cells was further reduced by toluene washing after heat treatment, the activity of the B. stearothermophilus cells was actually increased about $3\frac{1}{2}$ times by the toluene treatment (Table 10 and Fig. 36). The same phenomenon is observable after butanol treatment, but comparison can be made only with cells heat treated but not butanol washed in different experiments.

Discussion of catalase investigations

A cycle of catalase activity throughout the growth cycle of a culture of B. stearothermophilus is similar to that observed in Aerobacter aerogenes, formerly Bacterium lactis aerogenes, (Cole and Hinshelwood, 1947; McCarthy and Hinshelwood, 1958), Haemophilus parainfluenzae (White, 1962), four species of Pseudomonas (Frank et al., 1963), and Saccharomyces cerevisiae (Seah et al., 1973). McCarthy and Hinshelwood noted that increase in catalase activity coincided with the drop in pH of the medium which occurs when total growth rate is falling, and related this to an indirect effect upon cell metabolism such that more hydrogen peroxide was produced. White showed that catalase activity was greatest where cytochrome content was lowest and considered it possible that catalase potentiates cytochrome oxidases in the presence of oxygen in H. parainfluenzae, which is more susceptible to hydrogen peroxide than other bacteria. Seah et al. suggested that the drastic drop in activity during the lag phase could be due to a degradative process or to the formation of an inactive catalase complex.

In our experiment the inoculum was from a young culture in the exponential phase of growth. That it had higher catalase specific activity than the maximum reached by the larger culture is attributable to the oxygenation conditions. Catalase is known to be inducible by air in yeast (Chantrenne and Courtois, 1954), Micrococcus lysodeikticus (Chance, 1952)

and Rhodospirillum rubrum (Clayton, 1960). It is therefore probably inducible in B. stearothermophilus. Aeration in the 10 litre culture could not, for practical reasons, be as copious as in the 25 ml culture in a 250 ml flask, which was used as an inoculum.

The increase in activity in crude extracts over cell suspensions has been observed in catalase from other organisms. Few et al. (1957) observed increased activity when cells of Micrococcus lysodeikticus were broken at pH 6.5, and attributed it to a change in pH at the site of enzyme activity when cells were broken. Their experiments, they say, could be interpreted by assuming that the pH within the cell was 4.7. The catalase activity of lysate increased rapidly between pH 4.7 and pH 6, whereas that of cell suspensions was constant between pH 4.4 and pH 9. This was attributable, in their opinion, to a permeability barrier in the cell membrane, such that the internal pH did not vary with the external pH. That the permeability barrier was the cell membrane and not the cell wall was indicated by the similarity of the pH activity curve for intact cells and protoplasts.

Clayton (1960) observed increased catalase activity when cells of Rhodospirillum rubrum were broken and attributed it to a permeability barrier limiting the rate at which hydrogen peroxide enters the cells. Hydrogen peroxide is a small molecule and may penetrate easily. However, hydrogen ions may be actively excluded by cell membranes (Few et al., 1957; Brock, 1969). Although the pKa of hydrogen peroxide is 11.6 at 20°C in aqueous solution (Nicholls and Schonbaum, 1966) and therefore hydrogen peroxide is unionized at pH values used in the assays of catalase activity, it is still possible that there is some mechanism affecting the penetration of hydrogen peroxide through the cell membrane, other than a diffusion gradient.

Kaplan (1965) suggested that in yeast 'patent' catalase (measurable in whole cells) was membrane bound, and 'cryptic' catalase (measurable after cell breakage) occurred within the cell membrane and was inaccessible to substrate. He demonstrated (1965) that the uranyl ion, UO_2^{+} , which does not penetrate the cell membrane could completely inhibit the patent catalase. The mercuric ion, at concentrations below those at which it could penetrate the cell membrane, he showed could significantly inhibit patent catalase activity. Subsequent washing with butanol revealed that the cryptic catalase was fully active after such mercuric ion treatment of whole cells. The cryptic catalase revealed on butanol washing of untreated cells was much more sensitive to uranyl ions than the patent catalase. Kaplan suggested that the enzyme was stabilised by its supposed binding to the membrane.

The decrease in catalase activity upon breaking the cells but before centrifugation is possibly due to the release of an inhibitor upon cell breakage. Subsequent low speed centrifugation, presumably by removing the inhibitor, left more activity than was present before. Nakamura (1960) believed he had evidence for a charcoal removable factor which lowered activity in crude extracts of a gram positive thermophilic bacterium. Other, naturally occurring catalase inhibitors have been detected by Miyazaki et al. (1973) in rats. They concluded that a histone protein and a non-histone acidic nuclear protein are responsible for depressing catalase activity in Rhodamine sarcoma, brain, spleen, kidney and liver. The histone protein had less activity than the non-histone protein.

The effect of temperature upon the catalase activity of both E. coli and B. stearothermophilus differs between

cell suspensions and crude extracts. In the latter there is a broad optimum and in cells suspensions there is a peak. This could be a permeability effect such that at low temperatures substrate does not penetrate so easily. Above 40°C the rapid reduction in catalase activity in cell suspensions of B. stearothermophilus can be explained by the heat labile catalatic component to be discussed later. However catalase activity, due to the heat stable catalatic component is measurable even at 75°C. The cell suspension of E. coli decreases rapidly in catalase activity over 40°C and is not measurable above 65°C.

The effect of temperature on the catalase activity of crude extracts shows the effect of heat on the enzyme substrate complex. In both crude extracts activity at low temperatures is a higher percentage of maximum activity than is that of cell suspensions. This supports the permeability barrier theory. At temperatures above the optimum the less rapid decline in activity may also argue for the removal of a permeability effect. As will be discussed subsequently this is much greater for E. coli.

The catalase of E. coli, whether in crude extract, or cell suspension, does not have an optimum temperature significantly different from that of B. stearothermophilus. Furthermore in 2 of the 3 E. coli extracts tested there was measurable activity at 65°C, in one case this represented over 70% of the maximum activity and was higher than that at 25°C. However activity was never measurable above 65°C and

fell more rapidly after optimal temperature than did that of B. stearothermophilus.

It is to be noted that the catalase of E. coli in cell suspensions has near-optimal activity at its normal growth temperature of 37°C. This contrasts with the situation of B. stearothermophilus in some cell suspensions. The growth range of B. stearothermophilus is 45°-70°C. The data concerning cell suspensions suggest that the catalase may not operate in vivo at maximum capacity. Nevertheless it is capable of some activity at temperatures at which mesophiles would not grow. It must be remembered that B. stearothermophilus in nature grows in rotting vegetation, compost and manure heaps, where oxygen tension would be comparatively low, and peroxides as by-products of metabolism probably not present in high concentration. Furthermore catalase has the highest turnover number of any known enzyme. Therefore a low specific activity does not preclude an adequate total velocity. In other words, there was little adaptational value in the possession of a catalase having optimum activity at optimum growth temperature for this organism.

From Tables 9 and 10 it can be seen that the energies of activation of heat inactivation are 5-10 times the energies of activation. This, because we are observing heat denaturation of protein, which has a high ΔH , and ΔS but a low ΔG . The free energy change is small, but the rate of denaturation is high. In the denaturation of a protein a large number of hydrogen bonds of small energy (about 5000 cal/mol), which

maintain the conformation of the molecule, are broken.

This leads to a great increase in entropy and results from the increase in kinetic energy of all molecules as the temperature rises. As ΔG is equal to $\Delta H - T\Delta S$, it is small because ΔS is high and positive. According to both the collision and the absolute reaction rate (transition state) theories the reaction rate varies inversely and as an exponential function of ΔG . Therefore the rate at which protein denaturation takes place is high. The resulting change in conformation of the enzyme molecule leads to loss of catalytic activity. The higher the temperature the greater is the number of enzyme molecules inactivated. The Arrhenius plot for heat inactivation is steep and therefore the energy of activation is high.

There is a difference between the values of both the energy of activation and of heat inactivation between the two species. The energy of activation of B. stearothermophilus catalase is over twice that of the E. coli in the crude extracts, yet in the cell suspension the average is 0.75 that of the E. coli average. Also the energy of activation of heat inactivation of B. stearothermophilus catalase is nearly three times that of the E. coli in the crude extracts, yet in the cell suspensions it is .5-.8 that of the E. coli.

In B. stearothermophilus there is little difference between values obtained in cell suspensions and in crude extracts for the energy of activation, and for the energy of activation of heat inactivation.

In contrast, in E. coli the energy of activation is about three times as great in the cell suspensions as in the crude extract. The energy of activation of heat inactivation is 4-5 times as great in the suspension.

Consideration of the values of the energy of activation obtained from cell suspensions suggests that in vivo the activity of the B. stearothermophilus enzyme is, if anything, only very slightly less sensitive to temperature than the E. coli enzyme, over the same temperature range (see Arrhenius plots, Figs. 28 and 29). This correlates with the fact that the high turnover number of catalase allows its activity at high temperatures to be sufficient under the microaerophilic conditions usual for B. stearothermophilus.

The possibility exists that the contribution of enthalpy change to the lowering of the energy barrier, ΔG^* , is greater for the thermophilic enzyme than for the mesophilic enzyme. This follows from analogy with enzymes of ectothermic and endothermic animals as was discussed for the ATCase of B. stearothermophilus, S. cerevisiae and V. psychroerythrus. The entropy change may be smaller for the E. coli catalase than for that of B. stearothermophilus because the temperature at which the mesophilic enzyme is active is lower, and there is greater thermodynamic possibility of restraining disorder. In whole cells the ΔG^* may therefore be higher and the reaction rate lower for the mesophilic enzyme.

In crude extracts the B. stearothermophilus enzyme has a higher energy of activation than the E. coli enzyme whereas

in cell suspensions the thermophilic enzyme has an energy of activation a little less than that of the mesophile. This could be interpreted as an effect of the removal of a permeability barrier and/or the stabilizing effect of a membrane, in E. coli. Catalase is not an exo-enzyme but it can be measured in cell suspensions. These findings suggest some catalase may be membrane-bound. It is possible that the stronger but more flexible membrane of B. stearothermophilus (Friedman, 1958; Brock, 1969) is less affected by heat than is the membrane of E. coli. In the latter organism the energy of activation is three times greater in the cell suspension than in the crude extract. If there is some mechanism in the cell membrane restricting the access of the substrate to the enzyme this would be lost in crude extracts and the effects of heat upon the enzyme substrate complex would be all that is measured. It is possible that in whole cells of E. coli but not in B. stearothermophilus increasing temperature affects this mechanism such that at higher temperatures it ceases to function. The observed rapid increase of activity upon preheating E. coli cells at 40°C correlates with the higher energy of activation of cells compared with crude extracts.

In cell suspensions of B. stearothermophilus the inactivation of the catalase is far less sensitive to heat than the inactivation of that of E. coli. The difference in rate of inactivation at two temperatures being smaller in B. stearothermophilus than in E. coli does not preclude the unlikely possibility that the rate of inactivation of B. stearo-

thermophilus is higher, as can be argued from the presumption that a lower ΔH may indicate a lower ΔG and therefore a higher rate. As we are dealing with a protein denaturation this presumption is allowable as ΔG is known to be low, and ΔS high and positive. In fact at 55°C the highest rate of inactivation was measured in B. stearothermophilus.

Table 11 compares the rates of heat inactivation of catalase of B. stearothermophilus and E. coli in several experiments. It is of great interest that the highest rate measured ^{was} for B. stearothermophilus during 30 seconds at 55°C (Expt. G), and was not reversed after 5 seconds by toluene treatment. Over the first 3 minutes the overall rate was almost the same for the enzymes of E. coli and B. stearothermophilus, in this same experiment. However this does not take into account the initial rise in catalase activity of E. coli. After this the rate of inactivation is greater for the E. coli enzyme (Fig. 36).

Heat inactivation takes place between 40°-55°C for E. coli and between 55°-65°C for B. stearothermophilus. Thus over its normal growth range of temperature the E. coli enzyme is not inactivated, but that of B. stearothermophilus is. In this respect B. stearothermophilus would be less well adapted to its environment than is E. coli, but for the high turnover number of catalase.

In crude extracts the energy of activation of heat inactivation of B. stearothermophilus is greater than that of

TABLE 11

Rates of heat inactivation of the catalase of B. stearothermophilus and E. coli at different temperatures and for differing time periods

Expt.	Organism	Protein mg/ml	Rate of heat inactivation				Duration of Expt.
			40°C	50°C	55°C	60°C	
A	B. stearo	7	.002	.02	.09	.09	Temp. 120'
							Temp. 50°
							Temp. 60'
B	B. stearo	5.8	.01	.01	.04	.04	Temp. 15'
							Temp. 55° & 60°
							Temp. 65°
	1st part		.015	.015	.06	.06	Temp. 45'
	2nd part		.002	.002	.04	.04	Temp. 10'
C	B. stearo	5.8	.22	.3	.0007	.003	Temp. 58° & 60°
							Temp. 65°
D	E. coli	13.5	.002	.04	.73	.04	Temp. 15'-45'
							Temp. 55° & 60°
E	E. coli	28.75	.01	.04	.5	.04	Temp. 5'-10'
							Temp. 60'
F	E. coli BuOH	21	.19	.11	.01	.01	Temp. 3'
							Temp. 1'
G	B. stearo BuOH	27	.25	.05	.13	.13	Temp. 3'
							Temp. 1'
	E. coli toluene	29	1.4	.49	.91	1.52	Temp. 5"
							Temp. 3'
	B. stearo not toluene	30	.53	.53	.53	.53	Temp. 3'
							Temp. 1'
	E. Coli	"	"	"	"	"	Temp. 30"
							Temp. 3'

Rate of heat inactivation expressed as the ratio: -

reduction of the log O.D. of zero time. velocity
minutes pre-heated

for 1 ml of a 100 mg/ml cell suspension.

E. coli. Thus the B. stearothermophilus catalase in crude extracts is more sensitive to heat than the E. coli catalase. Once again a low rate of heat inactivation may be postulated. There being little difference between the crude extracts and cell suspensions of B. stearothermophilus, in the energy of activation of heat inactivation, also suggests that the membrane has little stabilising effect in contrast to that of E. coli.

There is evidence for the presence of a highly heat sensitive catalytic substance in B. stearothermophilus, and also a very heat stable one. The heat stable enzyme is much more heat resistant than the E. coli enzyme at 55°C. The transient increase in the activity of the E. coli enzyme may be a permeability effect upon the membrane or be due to a conformational change in the enzyme, or indeed to some effect upon an inhibitor or an activator. The phenomenon also occurs in Yeast (Fraser and Kaplan, 1955).

Butanol or toluene treatment of previously heated cells negates the effect of the heat treatment in E. coli. The presumed increase in membrane permeability is reversed, possibly by a dehydration effect, or a conformational change in membrane proteins and the enzyme is seen to be progressively and rapidly inactivated. As this inactivation has already taken place before butanol or toluene treatment, the presumed permeability effect of heat is seen to be very great.

Adding butanol or toluene to heated B. stearothermophilus cells reverses the destructive effects of heat on catalase activity. This reversal could be a membrane effect or an ef-

fect upon the actual enzyme conformation. It is not certain that both the heat sensitive and heat stable components are increased in activity. The heat labile enzyme is clearly the one that is most affected by the toluene or butanol. The high rate of heat inactivation is maintained for only 5 seconds after which the catalase activity drops exceedingly slowly and is higher than that in cells not washed with butanol or toluene. This could be explained by supposing that the heat stable component has been increased in activity by the butanol or toluene treatment.

In baker's yeast (Kaplan, 1962) the residual catalase activity after heat treatment at 60°C drops gradually with time of preheat treatment. If the cells are butanol washed after heat treatment the residual catalase activity is higher but drops more rapidly with heat treatment. It drops very rapidly indeed if the cells are first butanol washed and then heat treated. These results were interpreted by the existence of two catalatic factors, one called the patent form, was measurable in whole cells and was relatively heat stable, and the other, called the cryptic form, was much more heat labile. This was revealed when the cells were treated with butanol either before or after heat treatment.

In B. stearothermophilus the heat stability of the catalase is greater if the cells are subsequently washed with butanol or toluene. This suggests that the cryptic factor in this organism is the more stable one, and the patent factor heat labile. It is difficult to postulate the functions of

the two forms of catalase. Clearly the heat stable one provides the catalatic activity required for survival in the natural habitat as temperatures rise above 55°C. Perhaps at temperatures below that the heat labile enzyme provides additional catalatic activity of value before conditions become microaerophilic from the activities of mesophilic aerobic organisms which decrease the oxygen tension.

The two possible enzymes in B. stearrowthermophilus are not necessarily both catalase. Lactic dehydrogenase, of yeast, a flavo-heme-protein is known to have catalatic properties and there is evidence of the existence of two other catalatic proteins in yeast in addition to catalase of molecular weight 250,000. One, a heme protein, has high specific activity and a molecular weight of 170,000. The other, a non-heme, iron containing ribonucleo-protein has low specific activity and a molecular weight of 90,000: About 20% of catalatic activity in crude extracts of wild type yeast is due to these proteins described above (Seah, Bhatti and Kaplan, 1973a and 1973b; Seah and Kaplan, 1973).

Some extracts of B. stearrowthermophilus harvested in stationary phase had exceedingly low catalatic activity. It is possible that this was brought about by lactic dehydrogenase or some other protein having low catalatic activity. It would be necessary to carry out purification of the enzyme(s) involved to decide this point.

In B. cereus the presence of two catalases has been demonstrated by immunological differences and starch gel electro-

phoresis (Norris and Bailey, 1964). One, found in young vegetative cells was inactivated at 60°C in 5 minutes. The other present in older cells and in spores was resistant to 80°C for 30 minutes. Lawrence and Halvorson (1954) found it resistant to 100°C for 5 minutes. B. stearothermophilus did not readily form spores under our culture conditions. In order to obtain them it was necessary to keep a culture in stationary phase for several days. Even if B. stearothermophilus also produces a different catalase in spores it is unlikely that such an enzyme was present in our cells harvested in late linear phase.

Summary of the catalase investigations and
comparison with the ATCase of *B. stearothermophilus*

The pattern of catalase activity throughout the growth of a culture of *B. stearothermophilus* follows that already found in other organisms, it is at its highest when cell growth has passed its maximum.

An increase of activity occurs when cells are broken. No activity was found in the pellet after broken cells were centrifuged at low or high speed. Therefore if any enzyme is membrane bound in the cells it is released when they are broken. The broad temperature optima in crude extracts contrasted with a well defined one at 40°C in whole cells. This suggests that substrate permeability may be adversely affected by temperatures a little lower or a little higher than the optimum. It is also possible that the conformation of the enzyme in the intact cells is altered more by temperature than in crude extracts.

There is no simple relation between the energy of activation of the catalase of *B. stearothermophilus* and growth temperature, for, in whole cells, it is a little lower than that of *E. coli*. In crude extracts it is, however, twice as high. Thus in vivo the thermophilic enzyme is, if anything, less responsive to increasing temperature. Likewise the stability of the catalase of *B. stearothermophilus* does not show a simple correlation with growth temperature, there being two catalytic components, one of which is more heat labile than is the catalase of *E. coli*. However the other catalytic component of the

thermophile is more heat stable than the catalase of the mesophile.

Values of both the energy of activation and the energy of activation of heat inactivation show little difference between crude extracts and whole cells of B. stearothermophilus. This situation is in contrast to that in E. coli and suggests that the membrane effect is more marked in the mesophile. In the latter brief periods of heat treatment stimulate enzyme activity in whole cells, whereas the activity of B. stearothermophilus is rapidly and greatly reduced.

The heat inactivation of the catalase in whole cells of B. stearothermophilus is partly reversed by toluene and butanol treatment. This may be due to an effect upon membrane permeability or the conformation of the enzyme. The effect is quite different when E. coli cells are butanol and toluene washed after heat treatment. The activity is reduced relative to cells merely heat treated. Once again a difference in membrane constitution is indicated, though the possibility of a difference in the molecular conformation of the enzymes in the two species is not impossible.

The heat inactivation of the catalase of B. stearothermophilus takes place within its growth range of temperature, contrary to E. coli. This may limit its growth under natural conditions, unless temperatures only rise high enough to completely inactivate the enzyme when conditions become anaerobic or microaerophilic.

By comparing and contrasting the ATCase and the catalase

of B. stearothermophilus one can say that only the ATCase shows some attributes that one might expect of an enzyme of a thermophile. It has an optimum temperature of 60°C which is within the growth range of the organism and its energy of activation, 13,900 cal/mole, is more than twice that of the catalase, 5,000 cal/mole. The catalase has an optimum temperature of 40°C which is below the growth range of the organism, but, as has been stated, the microaerophilic conditions of the situations in which it is found (piles of rotting vegetation, canned food) do not make the possession of high catalase activity, whether constitutive or induced, a characteristic for which there would be strong selective pressure.

The possible existence of two catalases and two ATCases of differing thermostability is interesting in the context of Zuber's evidence (1973) for thermostable and thermolabile varieties of various enzymes in the same organism. Our cultures of B. stearothermophilus were grown at 55°-60°C and it is possible that, as the temperature range of the organism is 45°-70°C both kinds of enzymes were produced over this temperature range.

The ATCase of B. stearothermophilus (that is, the presumed more thermostable one) belongs to the class of enzymes which are stable without substrate protection at the temperature of production, but not at higher temperatures. The thermostable catalase is also stable at the temperature of production but not above it. There can be no question of

substrate protection here as hydrogen peroxide inactivates catalase rapidly at all temperatures. Thus we have not found either of the enzymes investigated to be intrinsically heat stable; the ATCase functions optimally at the temperature of growth of B. stearothermophilus but is rapidly inactivated in the presence of substrate and the overall catalase activity at this temperature though sufficient for microaerophilic conditions, is reduced very rapidly indeed to a small fraction. The optimum temperature of the catalase of 40°C may be explained by the very heat labile catalatic component which has been demonstrated.

The work reported in this thesis has demonstrated that in B. stearothermophilus the ATCase shows adaptation to thermophily in having an optimum temperature within the growth range of the organism. It is also stable at the temperature of production until substrates are present, in vitro. Its instability in the presence of substrate in vitro may not necessarily reflect the in vivo situation. The same reservation applies to its estimated energy of activation, which would seem to be higher than that of baker's yeast which is above that of the psychrophile. Although catalase activity might not be of great importance in the natural habitat of B. stearothermophilus, nevertheless there is a catalatic component which is capable of some activity for at least 45 minutes at 60°C in whole cells. This, in view of the high turnover number of the enzyme, is likely to

be sufficient in microaerophilic conditions, and, at lower temperatures, before oxygen tension is reduced by the activities of other organisms and temperature raised the much more active heat labile (patent) component protects the organism.

The investigations described did not reveal that enzymes of thermophilic organisms are necessarily performing optimally under natural conditions. If there was any predilection to such a prejudice it vanished during the course of the work. Organisms in high temperature environments must be successful because the sum total of their metabolic activities, each one of them not necessarily perfect from our point of view, allows them to succeed in those thermally extreme situations. This particular conclusion is part of a more general realisation that many, if not all, organisms succeed because of some characteristics and in spite of others.

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