CHAPTER 3

MODERN LIFE AT HIGH TEMPERATURES

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1. Introduction

A variety of micro-organisms are now known which grow optimally above 65°C, and are defined as extreme thermophiles. As might be expected they are found in both natural and artificial hot environments. Until comparatively recently the upper optimum temperature for the growth of any living organism was about 85°C. Then in 1982 Stetter described an organism, isolated from the hot sea floor of a submarine solfatara field, which grew optimally at 105°C. Since then several other organisms have been found with optimum growth temperatures at 100°C or above and a few are capable of growth at 110°C (e.g. Huber *et al.*, 1987; Fiala and Stetter, 1986; Zillig *et al.*, 1987; Stetter *et al.*, 1990).

Work on extreme thermophiles is relatively recent. The pioneering work of Brock (1978), who first characterized micro-organisms isolated from hot springs, was carried out during the 1960's. Prior to that the conventional wisdom was that these high temperatures were inimical to life. Indeed one of the most interesting features of work on extreme thermophiles carried out over the last 30 years or so is the way it has challenged established thinking that heat is hostile to life. The use of autoclaves to sterilize solutions and boiled enzyme controls, are entrenched components of conventional scientific thinking.

All extreme thermophiles are eubacteria or archaebacteria, and no eucaryotes are known which can grow at these temperatures. Only archaebacteria are known to have temperature optima for growth above 85°C (Fig. 1).

2. Thermophily and Evolution - The Archaebacteria

Although archaebacteria are a quite recent taxonomic development (Woese and Fox, 1977; Fox *et al.*, 1980), their position as a kingdom separate from both eucaryotes and eubacteria is now well accepted. Recent advances in nucleic acid sequencing have enabled relatively precise genotypic data to be readily gathered for the purpose of determining taxonomic relationships (Wilson *et al.*, 1977; Woese, 1987). The most widely accepted unrooted taxonomic trees based on the sequence of Ribosomal 16S RNA place archaebacteria as a group closer to both the eubacteria and the eucaryotes than either of these are to one another (Figs. 2, 4), although variations on this have also been proposed

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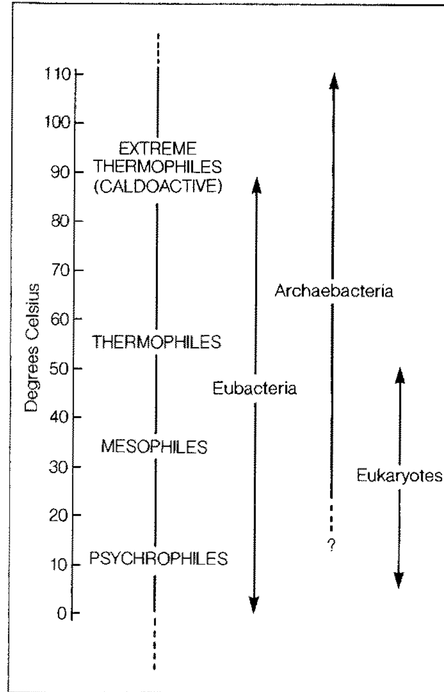

Fig. 1. Growth temperatures of organisms from the three kingdoms.

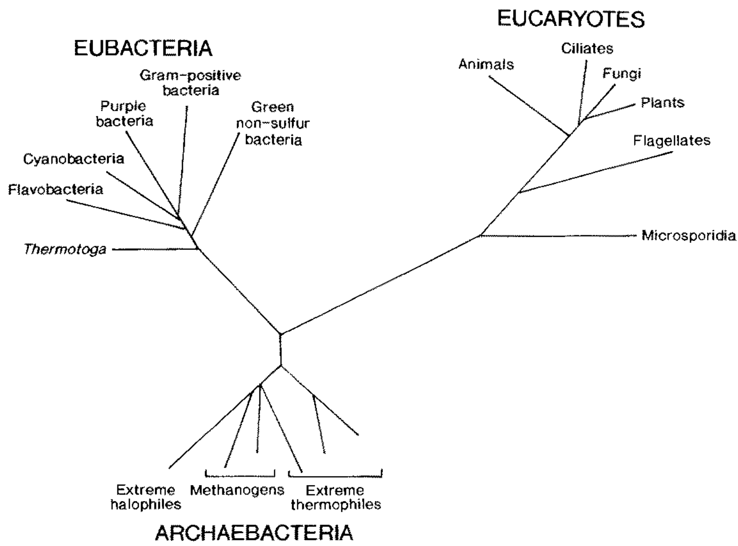

Fig. 2. Universal phylogenetic tree determined from rRNA sequences (Woese, 1987).

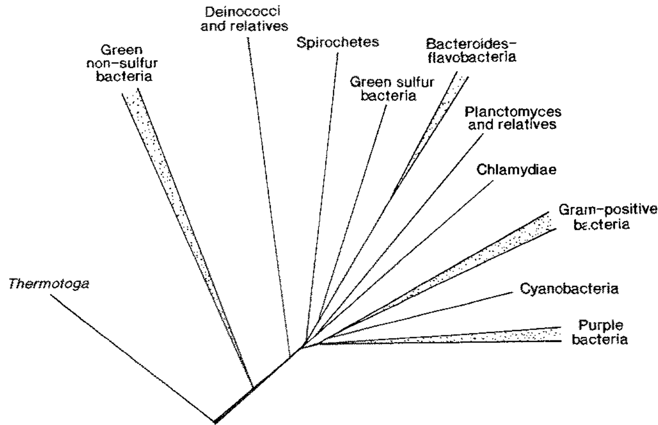

Fig. 3. Eubacteria phylogenetic tree based upon 165 rRNA sequence comparisons (Woese, 1987).

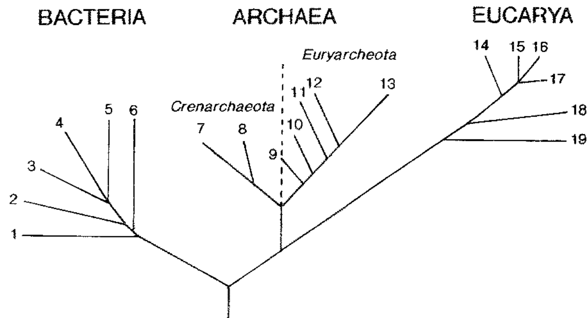

Fig. 4. Universal phylogenetic tree in rooted form, showing the three domains. Branching order and branch lengths are based upon rRNA sequence comparisons. The numbers on the branch tips correspond to the following groups of organisms. Bacteria: 1, the Thermotogales; 2, the flavobacteria and relatives; 3, the cyanobacteria; 4, the purple bacteria; 5, the Gram-positive bacteria; and 6, the green nonsulfur bacteria. Archae: the kingdom Crenarchaeota: 7, the genus *Pyrodictium*; and 8, the genus *Thermoproteus*; and the kingdom Euryarchaeota: 9, the Thermococcales; 10, the Methanococcales; 11, the Methanobacteriales; 12, the Methanomicrobiales; and 13, the extreme halophiles. Eucarya: 14, the animals; 15, the ciliates; 16, the green plants; 17, the fungi; 18, the flagellates; and 19, the microsporidia (Woese *et al*., 1990).

(see Lake, 1991). Furthermore, the common ancestor of the archaebacteria was also more primitive than the eubacterial common ancestor. Finally, the archaebacteria appear to be evolving more slowly than the eubacteria, so that if one accepts that no major change in the rate of evolution has occurred for these groups, the archaebacteria we see today are more primitive than the eubacteria (Woese, 1987).

On this basis the common ancestor of the archaebacteria is likely to be nearest to the earliest form of life. Woese (1987) has argued that the ancestral archaebacterium was an extremely thermophilic anaerobe dependent on sulfur reduction. Extreme thermophily is widespread among the archaebacteria and the most extreme thermophiles of the group appear also to be the slowest evolving. Extreme thermophily is also most strongly represented towards the root of the archaebacteria tree.

Additional evidence is available when we consider the eubacterial evolutionary tree (Fig. 3). The most deeply rooted branch in this tree is that leading to thermotogales (Achenbach-Richter *et al.*, 1987; Woese, 1987). These organisms are the most extremely thermophilic of all the eubacteria (Belkin *et al.*, 1986; Huber *et al.*, 1986; Huser *et al.*, 1986). The next most deeply rooted branch is that leading to the green non-sulfur bacteria containing a number of thermophiles such as *Chloroflexis* and *Thermomicrobium*. The third most deeply rooted group, the deinococci, includes one of the most widely occurring eubacterial extreme thermophiles, *Thermus*. Thermophiles are thus very strongly represented at the root of the eubacterial phylogenetic tree and all these groups are also relatively slowly evolving, strongly suggesting that eubacteria have risen from a thermophilic ancestor (Achenbach-Richter *et al.*, 1987).

Overall then, evidence is gathering that the view of thermophilic bacteria as specialist adaptions to enable colonization of a hostile environment is mistaken. Rather than marvel at life's ingenuity in surviving these harsh conditions, it may be more appropriate to wonder at the sacrifices and adaptions which have to be made by organisms evolving down-temperature into the slow frigidity of sub-80°C temperatures.

If all eubacteria have arisen from extreme thermophiles we might expect to find some evidence of this within a variety of eubacteria. It is tempting to assume that the ability of some thermophiles to grow over an extended temperature range, and the existence of cryptic extreme thermophiles, are manifestations of this (Wiegel, 1990).

Woese *et al.* (1990) have recently proposed that the archaea (archaebacteria) be divided into two groups (Fig. 4), the crenarchaeota, including the genus *Pyrodictium* and *Thermoproteus*, and the euryarchaeota, including the thermococcales, the methanococcales, the methanobacteriales and the methanomicrobiales as well as the extreme halophiles. The general phenotype of the crenarchaeota, it is argued, most closely resembles the ancestral phenotype of the archaea and so is the most primitive of living organisms.

3. Metabolism of the Most Extreme Thermophiles

A number of reviews are available dealing with archaebacterial metabolism (e.g. Danson, 1988), but given the relatively short duration of research on extremely thermophilic archaebacteria there is good reason to suppose that only a small proportion of the species available have been isolated, and that some major groups may yet be undiscovered. In addition, many of the isolated organisms have not been well enough investigated to be taxonomically placed or validly named with great confidence (and this also applies to extremely thermophilic eubacteria). More research is needed to even establish and confirm the main metabolic pathways in all known extremely thermophilic archaebacteria. We continue to find taxonomic and metabolic "missing links" such as *Archaeglobus* (Stetter, 1988) between known groups of organisms, and we may also expect to find outliers which may extend the taxonomic (and metabolic) framework provided by the achievements of Woese, Stetter, and others. Extrapolation based on current data must therefore be treated with caution.

Within the crenarchaeota, the thermoproteales (comprising *Thermoproteus*, *Pyrobaculum*, *Thermophilum*, *Desulfurococcus*, and *Staphylothermus*) grow in the laboratory at pH's between 2.5 and 8.5 and can be found in both marine and solfataric environments. Some members are heterotrophic and some facultatively autotrophic, but all grow anaerobically and are capable of using sulfur as an electron acceptor. All members of the group grow optimally between 85°C and 100°C. The second group of crenarchaeota, the *Pyrodictiales* has within it two genera, *Pyrodictium* and *Thermodiscus*. All grow optimally between 88°C and 105°C, between pH's of 5 and 7, and have been isolated from marine environments. Most members of the genera are capable of using carbon dioxide as their sole carbon source and of obtaining energy from the oxidation of hydrogen by sulfur, leading to the production of hydrogen sulfide (Fischer *et al.*, 1983). This capability is also found among some members of the thermoproteales. *Pyrodictium abyssum* and *Thermodiscus maritimus*, however, are not dependant on sulfur, but are capable of an unknown mode of fermentation or respiration (Stetter *et al.*, 1990). The relatively closely related *Pyrococcus furiosus*, from within the euryarchaeota, has recently been shown to ferment pyruvate to acetate, CO_2 and H_2 using only three enzymes (Fig. 5) (Schafer and Schonheit, 1991). The key enzyme, acetyl - CoA synthetase (ADP-forming) is also important in *Thermoplasma acidophilum* (Danson, 1988).

Work on archaebacterial metabolic pathways is relatively new, but as Danson (1988) has remarked, all three kingdoms possess variations on a small number of metabolic routes, which must have been established before their separation. The most interesting variation is perhaps the presence in some extremely thermophilic archaebacteria of a modified Entner-Doudoroff pathway with non-phosphorylated intermediates (Fig. 6) (De Rosa *et al.*, 1984; Budgen and Danson 1986; Wood *et al.*, 1987; Danson, 1988), and in the apparent absence of the Emden-Meyerhof pathway.

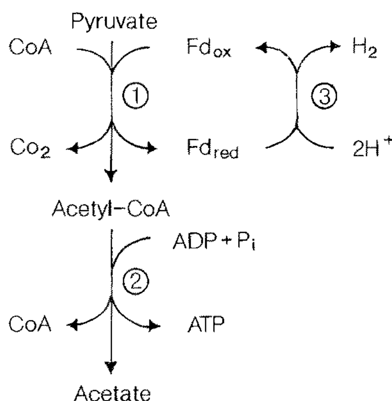

Fig. 5. Proposed scheme of pyruvate fermentation in *Pyrococcus furiosus*. 1: pyruvate: ferredoxin oxidoreductase; 2: acetyl-CoA synthetase (ADP forming), 3: hydrogenase; *CoA*, coenzyme A; *Fd*, ferredoxin (Schafer and Schonheit, 1991).

The study of both extreme thermophiles and of archaebacteria is relatively new and, because all of the most extreme thermophiles are archaebacteria, it is not clear that any of the features found in the most extreme thermophiles are due to their mode of life at high temperatures rather than to their archaebacterial nature. Enzymes whose function is to remove or utilize products of thermal decomposition might be expected, but have not been found.

There is, however, some evidence for the use of iron-sulfur proteins as cofactors in place of NAD or NADP (Mukund and Adams, 1991) in archaebacterial extreme thermophiles. The reduced forms of NAD and NADP have half lives of about 5 min at 80°C *in vitro* (Walsh *et al.*, 1983) and are thus often less stable than the oxido-reductases to which they are linked, with no evidence of stabilization by association with the enzyme (Hudson and Daniel, unpublished). Another feature of archaebacteria is the presence of unusual redox cofactors such as F_{420}, and a decreased specificity for NAD and NADP. While most non-archaebacterial enzymes have an absolute specificity for either NAD or NADP, many archaebacterial enzymes, and especially those from extreme thermophiles, will accept either. These observations can be explained if the use of NAD and NADP, at least as reductants, is a relatively late evolutionary development, possibly associated with evolution "down temperature". The role they now fill could have originally been carried out by non-haem iron proteins and cofactors such as F_{420}. While it could also be argued that the thermal instability of the reduced forms has led to their use having been "evolved out" of the most extreme (archaebacterial) thermophiles over time, if the extremely thermophilic archaebacteria most closely resemble the ancestral phenotype then this is a more complicated explanation. Furthermore, it does not explain the presence of a wider variety of redox cofactors, and decreased specificity between NAD and NADP, throughout the archaebacteria.

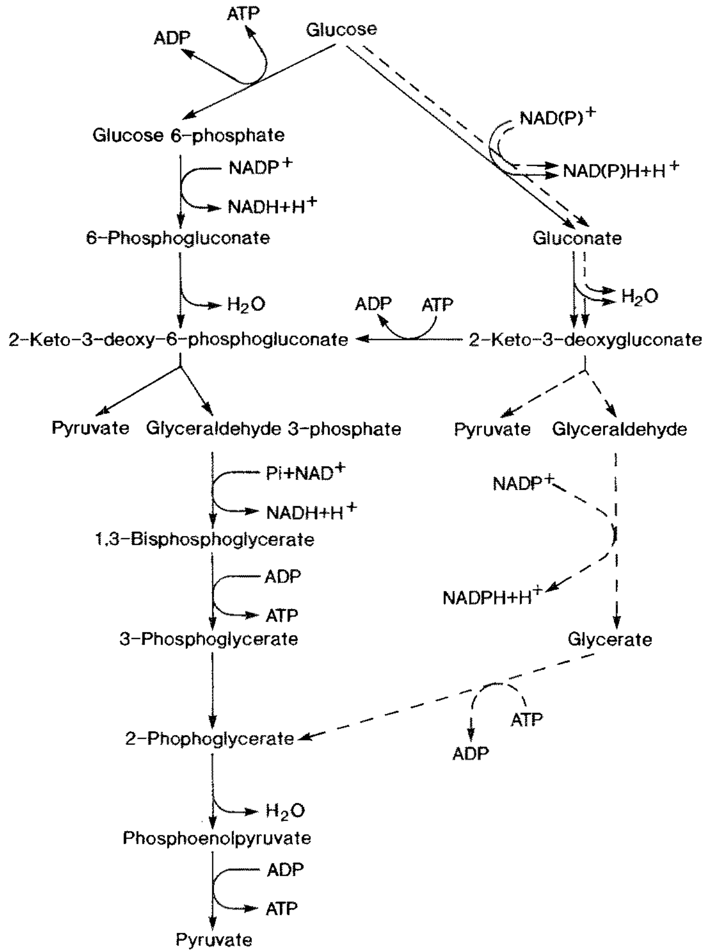

Fig. 6. Pathways of glucose catabolism in halophilic and thermoacidophilic archaebacteria. The modified Entner-Doudoroff pathway of halophiles (—→) and the non-phosphorylated Entner-Doudoroff pathway of *Sulfolobus solfataricus* and *Thermoplasma acidophilum* (- - —→) are shown alongside the classical Entner-Doudoroff pathway of *eubacteria* (—→). Conversion of glyceraldehyde into pyruvate via glycerate (· · —→) has been demonstrated only in *Thermoplasma acidophilum* (Danson, 1988).

4. Thermal Stability in Thermophiles

Among the macromolecules and supramolecular structures found in extreme thermophiles, the stability of lipids and membranes above 100°C is not surprising. There seems no particular reason why the various homeoviscous adaptions which permit survival both of psychrophiles and moderate thermophiles should cease at 65°C. The presence of ester-linked lipids (rather than ether linkages) outside the archaebacteria may be a

response to low temperature rather than an advanced characteristic, but the evidence is weak. There is no good evidence that the membrane-spanning tetra-ether lipids found in some archaebacterial extreme thermophiles are required for membrane stability at high temperatures rather than for homeoviscous adaption. (See Russel and Fukunaga (1990) for a general review.)

DNA is apparently stabilized *in vivo* at high temperatures by DNA-binding proteins (Reddy and Suryanarayana, 1988), so that the melting of the double helix (separation of the two strands) takes place at temperatures well above those that might otherwise be expected. There is no evidence for systematic stabilization of DNA by increasing the proportion of the (stronger) G - C interactions compared with A - T interactions, since the percentage of G - C interactions ranges at least from 38 to 62 mole per cent among organisms capable of growth above 100°C (Stetter *et al.*, 1990).

Studies on the nature of the forces stabilizing protein conformation show no obvious upper temperature limit for the maintenance of protein conformation. It has been known for some time (e.g. Brandts, 1967) that the net free energy of stabilization of proteins is of the order of 25 KJ/mol, and is the result of a delicate balance between large stabilizing and large destabilizing forces, both of the order of 1,000 KJ/mol. Individual intramolecular stabilizing interactions can contribute in the region of 10 KJ/mol, so a small number of these can make a considerable difference to the stability of a protein. This is supported by the findings of Langridge (1968), and others (Matthews, 1987; Grutter *et al.*, 1979; Albers, 1989) that single point mutations within a protein can dramatically alter thermostability without significantly affecting the structure. A number of enzymes have now been characterized from extremely thermophilic bacteria which are both stable and functional for tens of minutes above 110°C (Bragger *et al.*, 1989; Simpson *et al.*, 1991; Ruttersmith and Daniel, 1991). It is possible to calculate that four or five additional stabilizing interactions, for example hydrogen bonds or hydrophobic interactions, are all that would be needed to confer conformational stability for useful periods of time to such enzymes at temperatures in the region of 150°C. There is no obvious or clearly delineated upper temperature limit to the conformational stability of proteins.

This leaves unexplained why, if mesophiles have evolved from thermophiles, enzymes from mesophiles are so unstable, with half lives usually of the order of minutes about 25°C above the optimum growth temperature. The answer seems to be that enzymes must be flexible to function effectively (e.g. Daniel, 1986; Vihinen, 1987; Daniel *et al.*, 1990) at the appropriate temperature for the organism. The intramolecular interactions needed to confer adequate stability in thermophiles are likely to render the structure undesirably rigid at lower temperatures in mesophiles. The high thermal stability of enzymes from extreme thermophiles also confers resistance to proteolysis at mesophilic temperatures (Daniel *et al.*, 1982), so high stability may hinder protein turnover. Thus, enzymes from mesophiles evolved to have the necessary degree of instability to maintain molecular flexibility, and protein turnover, at lower temperatures.

However, it is unlikely in fact that the upper limit for the stability of proteins will be governed by conformational stability. There is good evidence to suggest that degradative reactions including hydrolysis and deamidation will become the dominating factor in determining the useful lifetime of proteins above 100°C (Ahern and Klibanov, 1985). It is not clear at what temperatures these degradative reactions will render the lifetime of enzymes too short for them to function *in vivo,* although "modern" proteins will not survive 250°C under the conditions used by White (1984). The work of Klibanov and his colleagues (Zale and Klibanov, 1984; Ahern and Klibanov, 1985, 1986) suggests that the limit may be in the region of 105-110°C, but this work was done on mesophilic proteins at about 100°C and, although relatively little data are available on enzyme stability above 100°C, the stability of some enzymes from archaebacterial extreme thermophiles suggests that the limit may be somewhat higher. The proteinase from *Desulfurococus* has a half life of 8 minutes at 105°C (Cowan *et al.*, 1987) and the immobilized xylanase from *Thermotoga* (Simpson *et al.*, 1991) has a half life of 5 minutes at 115°C in the presence of xylan. This could be raised to at least 10 minutes at 130°C in molten sorbitol, indicating the importance of hydrolytic reactions in the loss of activity. (It has been shown that even mesophilic proteins can display considerable high-temperature stability in anhydrous conditions, e.g. Ahern and Klibanov, 1986.)

There is evidence that some of the reactions, for example deamidation, are strongly dependent on the flexibility of the protein backbone and on the nature of the adjacent amino acid (Aswad, 1990; Stephenson and Clarke, 1989). Deamidation can thus be prevented or slowed, either by substituting different amino acids for either the asparagine (for example) or its neighbor but also by maintaining the protein in a stable (less flexible) conformation. More work of this type needs to be done at 100-150°C on proteins which are stable conformationally at these temperatures before we can make decisions regarding the temperature at which degradation will limit protein stability. It may be, for example, that the combinations of redox and pH buffering, possible surface adsorption, and high pressure, found in hydrothermal systems, can stabilize some types of proteins against degradation. The water activity of the environment will play an important role.

There is no particularly good evidence that macromolecular instability (i.e. the instability of proteins, membranes, DNA, etc.) will set the upper temperature limits for life. Equally good candidates are the stabilities of essential small molecules such as amino acids and enzyme cofactors (Daniel, 1985). Although their stability is likely to be dependent on their particular environment, in aqueous solution some are known to be unstable even around 100°C. The half life of glutamine at 120°C is about 10 minutes (Ratcliffe and Drozd, 1978) and the half life of reduced nicotinamide adenine dinucleotide at 80°C is about 5 minutes (Walsh *et al.*, 1983). There is no evidence to suggest under what conditions, if any, such molecules can be significantly stabilized.

5. Growth Temperature of the Ancestral Organism

On taxonomic grounds there is good support for the ancestral organism being thermophilic (e.g. Woese *et al.*, 1990). On a biochemical basis there would seem to be no objections other than our entrenched psychrocentrism, and some obvious advantages. The question of how thermophilic is more difficult. The temperature range for the growth of the proposed kingdom crenarchaeota (including the genera *Pyrodictium* and *Thermoproteus*), together with the most closely related euryarchaeote genus (*Thermococcus*), is 65°-110°C, with optimum temperatures for growth in the range 85°-105°C, under laboratory conditions. It has to be borne in mind that, at least for the more thoroughly investigated eubacterial thermophiles, there is evidence that organisms growing optimally in the laboratory at 75°C dominate and thrive in some ecosystems at 100°C. Growth experiments under laboratory conditions, with their emphasis on rich media and relatively high growth rates and yields, may underestimate the environmental temperature at which extreme thermophiles thrive. Furthermore, sampling of stable ecosystems containing liquid water above 120°C is difficult and of recent origin, and the techniques for enriching and growing microorganisms above 100°C are poorly developed.

If we could assume that the ancestral organism originated at atmospheric pressure, then 100°C would represent an attractive optimum growth temperature. Given its widespread utilization and the dramatic reduction in surface area which accompanies the melting of sulfur, it has been suggested that its melting point (~113°C) may set an upper temperature limit for the growth of some thermophiles (Stetter, 1982).

If we assume that proteins stable in an aqueous environment are a prerequisite, then, although more research is required, 150°C may be an upper limit, conferred by the susceptibility of proteins to irreversible degradation. It is, however, very much an open question whether a protein with the composition, sequence, and structure to resist degradation at this or higher temperatures and still function as a catalyst, can be found from the enormous variety of possible proteins. Given what we already know of the stability, or potential stability, of most biological macromolecules and metabolites, then 130°C is certainly not an unreasonable upper growth temperature to expect of the ancestral organism. There is currently no evidence to support a higher growth temperature; but 10 years ago there was no convincing evidence for life above 100°C.