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Water Relations in Single Cells [and Discussion]

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Water relations in single cells

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[Plate 1]

The intracellular water content is an important factor affecting the growth and survival of single cells of microorganisms under adverse environmental conditions. Certain types of bacteria, yeasts, filamentous fungi and algae are capable of growth in environments with water activities below 0.9 and even as low as 0.6, and are the most osmotolerant living organisms known. The two most important factors that determine such extreme osmotolerance are: (1) the resistance of the enzymes in a cell to the solutes present, and (2) the cell's ability to maintain within itself particular solutes, which are compatible with continued activity of intracellular enzymes, at levels sufficient to balance the external osmotic pressure and thus avoid dehydration. The levels of such compatible solutes are metabolically controlled and include polyols in yeasts, glutamic acid in the least osmotolerant bacteria, γ-aminobutyric acid and proline in the more osmotolerant bacteria and potassium in specifically halophilic bacteria.

In contrast, under certain conditions osmoregulatory mechanisms in microorganisms may reduce rather than maintain the water content of the cell. For example, during the morphogenic changes that accompany the formation of endospores by some bacteria, a special form of osmoregulation occurs in which a newly synthesized electronegative polymer ('peptidoglycan') in the outer region of the spore brings about and maintains, rather than avoids, dehydration of the central core. Indeed, spore heat resistance can be predictably modified experimentally by osmotic manipulation. The core dehydration mechanism is probably implicated in the enormous resistance of endospores to heat. It may also be involved in the exceptional dormancy and longevity of such cells, and suggests a principle that may operate in other dormant biological systems.

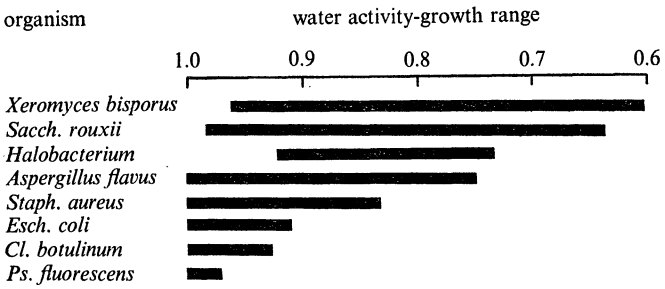
Introduction

The majority of living organisms are sensitive to the environment in which they live in that they are only able to maintain metabolic activity and viability within narrow limits of temperature, pressure, pH value, concentration of nutrients and water activity. However, among the microorganisms, including prokaryotes like the bacteria and the blue-green algae, and eukaryotes like the fungi, the yeasts and the green algae, exist organisms which have single cells that are capable of growth and multiplication under the most extreme of these environmental conditions. Extensive use has been made of such organisms in the study of water relations of living cells for three reasons. Firstly, they include the most osmotolerant organisms known, some of which are able to grow and multiply in environments at water activities as low as about 0.6. Secondly, including single-celled organisms, they are experimentally more versatile than many complex multicellular organisms, being directly in contact with the environment and having little opportunity for the development of specialized organs or organelles that influence the water status of the cell. Thirdly, they include the types of bacteria that produce extremely dormant spores, and these are the life forms that are most resistant to heat, and to

other physical stresses that normally kill living things, and it is becoming clear that control of endogenous water levels by spores contributes to their exceptional resistance.

In this paper we therefore review two contrasting aspects of the water relations of single cells: firstly, the mechanisms by which some microorganisms maintain a stable water content that remains compatible with viability and growth even in environments at very low water activities; and secondly, the mechanisms that allow the spore forms of microorganisms to reduce their water contents sufficiently to greatly increase the heat resistance of the cell, and possibly also to contribute to cell dormancy.

TABLE 1. WATER RELATIONSHIPS OF MICROORGANISMS



GROWTH AND VIABILITY

Water activity limits for growth

The effects of various environmental factors such as drying, or addition of solutes to the growth medium, on microbial physiology have, for a considerable period of time, been explained in terms of the effect of a single general factor, the thermodynamic water activity of the environment (see, for example, Scott 1957). In particular, growth limits of different species of microorganisms have been described in this way (table 1) so that for each species there is a value of the water activity below which growth will not occur.

Several points are worth emphasizing regarding the water relations of the single cells of microorganisms. First, the lowest water activities at which different microorganisms are capable of growth vary very widely. Most bacteria in the genus *Pseudomonas*, for example, are incapable of growth at a_w values below about 0.95, whereas some strains of *Staphylococcus aureus* can grow at 0.86. Amongst the so-called halophilic or 'salt-loving' bacteria are species that can grow in salt-saturated lakes at a_w values near 0.75. Certain of the so-called osmophilic yeasts and xerophilic fungi can grow, although slowly, at water activities as low as about 0.605. The absolute limit for microbial growth of any type seems to be around 0.60. It is remarkable that this value is not far above the value sometimes quoted for denaturation of DNA, about 0.55 (Pitt 1975).

Second, for some species of microorganisms there is an upper limit for growth. Thus, for example, the mould *Xeromyces bisporus* will not grow at water activities above about 0.97 (Scott 1957). Such organisms have an absolute requirement for a lowered water activity. In general this group, including several moulds, and obligately halophilic bacteria, are a minority. Most microorganisms have *optimal* water activities for growth which are above 0.99 (Corry 1973). Although growth occurs at lower water activities the growth rate is reduced as the water activity is reduced.

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The third point worth emphasizing is that although it has become popular to quote water activity limits for growth of microorganisms, the growth-limiting water activities depend, of course, to some extent on the nature of solutes which are present. For example, the water activity limits for growth of different bacteria in the presence of sodium chloride span a much wider range of values than the limits in the presence of glycerol (table 2). Despite such differences, however, it is still clear even from table 2 that although the absolute limits vary from solute to solute the relative order of resistance between the organisms is not substantially altered.

TABLE 2. GROWTH LIMITS OF BACTERIA AT LOW WATER ACTIVITIES

organism	limiting a_w in media adjusted with	
	sodium chloride	glycerol
Pseudomonas fluorescens	0.97	0.95
Salmonella oranienburg	0.95	0.935
Escherichia coli	0.95	0.935
Clostridium botulinum	0.945	0.93
Bacillus megaterium	0.945	0.925
Micrococcus lysodeikticus	0.93	0.93
Bacillus cereus	0.92	0.92
Bacillus subtilis	0.90	0.92
Staphylococcus aureus	0.85	0.89

And further, the growth limits for most yeasts and moulds are in general lower than those for most bacteria regardless of how the water activity is adjusted. The mould *Xeromyces bisporus* grows at water activities below 0.70 irrespective of how that value is reached (Pitt 1975). This in itself raises interesting questions since solutes are known to exhibit different behaviour with regard to permeability into cells. For convenience the solutes used to lower water activity are crudely placed in two groups. The first, typified by sodium chloride, include those to which the cell is largely impermeable in gross quantities, and the second, typified by glycerol, includes those to which the cell is rapidly permeable (Alemohammad & Knowles 1974). As we shall see, the first group induce specific metabolic changes in the microbial cell. The second group does not induce such changes. Yet growth limits in solutes from the two groups are remarkably similar. The reasons for this are not yet clear, but it may indicate that while water activity is not the sole arbiter of microbial growth under these circumstances, it may still have an important rôle.

Two questions then that are suggested by examination of the data summarized in these two tables are: first, by what means does a microorganism maintain efficient growth at very low water activities? Alternatively: why do some microorganisms, like the *Pseudomonas*, *not* have the same ability to grow at low water activity as do others like the halophiles and osmophiles? Second, what is the explanation of the effect of different solutes on growth-limiting water activity?

Partial answers to both questions are given by an examination of what happens to a single-celled microorganism when the water activity is suddenly shifted down from a high value to a new value which is not quite low enough to completely stop growth.

Reaction of bacteria to lowered water activity

Figure 1 summarizes some of the changes that occur when the water activity of a culture of the bacterium *Bacillus subtilis* growing at approximately 0.995 is suddenly shifted down to

about 0.97 by the addition of 1 M sodium chloride. The immediate response is that the optical extinction of the culture rises slightly accompanying shrinkage of the cells as they become osmotically dehydrated, because sodium chloride does not readily pass across the membrane of those cells. Growth ceases abruptly, presumably because of loss of water, and the effect of this loss on many of the cells' enzymic activities. Simply drying cells, for instance, will also reduce their overall activity (for instance, as measured by respiration) to near zero at a water content commonly of about 20 % or so, corresponding to a water activity of about 0.96 (Koga, Echigo & Nunomura 1966).

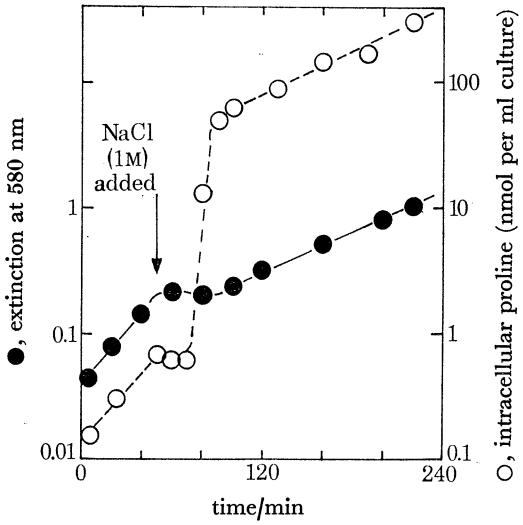

FIGURE 1. Osmoregulation by *Bacillus subtilis* growing in a medium to which sodium chloride is suddenly added at the point indicated.

However, some minutes after this osmotic dehydration and cessation of growth, the cells rehydrate and growth resumes, though at a slower rate than before. Comprehensive analysis shows that one of the greatest changes that takes place during the adaptation period prior to the recommencement of growth is in the soluble amino acid pool of the organism which, in *B. subtilis*, becomes particularly rich in proline. The proline may be transported into the organism if proline is present in the environment, whereas if the environment is devoid of free proline, then the organism synthesizes it. The concentration of intracellular proline achieved is identical per mole of extracellular solute whichever mechanism is used, and the concentration of proline in the cell rises until the cytoplasm is slightly hyperosmotic with respect to the environment; during this time rehydration of the cell is occurring, and then growth starts.

The changes in the concentration of proline that occur in this sort of experiment can be enormous. *Prior* to the addition of salt the intracellular proline concentration is usually about 15 mM if proline is provided in the environment, 2–5 mM if it is not. About 1 h *after* the addition of sodium chloride, the concentration of proline in the adapted cell may be a few molar, at a ratio of about 1.6 M intracellular proline per mole of extracellular sodium chloride.

Although most of the cells' metabolism is arrested by the salt-induced dehydration, the reactions involved in adaptation must, of course, be resistant to the low water condition. For instance, for such adaptation to occur, some protein synthesis seems to be necessary since adaptation, for example, is prevented by rifampicin, puromycin or chloramphenicol; and this

synthesis must take place within the dehydrated cell, when the measured level of total protein synthesis is very low.

Examination of the amino acid pools in a variety of bacteria reveals that an increase in the concentration of particular pool amino acids is a common response to a reduction in water activity, or to an increase in osmolality in the medium around the cell (Measures 1975).

In table 3 the major pool amino acid changes are listed for typical examples of a number of bacteria along with the limiting water activities for growth. A clear pattern is seen, in which those organisms that can grow at the lowest water activities are able to accumulate high concentrations of proline; those organisms that are moderately resistant to lowered water activity are able to accumulate γ-aminobutyric acid and either proline or glutamic acid; those organisms which are only able to grow at high water activities are able to accumulate only glutamic acid.

TABLE 3. OSMOREGULATORY AMINO ACIDS IN BACTERIA

organism	minimum a_w for growth in NaCl	amino acids showing major pool increases (mmol per mol of NaCl)
Ps. aeruginosa	0.97	Glu (900)
S. oranienburg	0.95	Glu (520)
		Pro (500)
Cl. sporogenes	0.945	Glu (280)
		GABA (500)
		Pro (420)
Strep. faecalis	0.94	GABA (750)
		Pro (750)
B. subtilis	0.90	Pro (1650)

Examination of the biochemistry and metabolism of these amino acids gives some indication of the reasons for the difference in osmotolerance observed among the bacteria. Glutamate will normally carry a net negative charge at physiological pH values and will be accompanied by a molecule carrying a positive charge, in order to maintain electrical neutrality. In most cells this will be a cation, normally K^+, but in some organisms the accumulation of K^+ is partially spared by accumulation of a positively charged organic molecule, for example, lysine or betaine. In any case there is an osmotic contribution from both the glutamate and its balancing counter-ion.

The other two amino acids will be mostly uncharged. The total osmotic contribution from these amino acids, plus the smaller contributions from *other* pool amino acids notably alanine, isoleucine, leucine, valine and aspartate, adds up to just exceed the environmental osmotic pressure, and maintains the cell in a slightly hypertonic and water-rich state. The growth-limiting water activities for such bacteria thus seem to reflect mainly the effectiveness with which the organism can balance the osmotic pressure of the medium by increasing its pool amino acid concentration, and the order of increasing effectiveness is glutamate, γ-amino-butyrate, proline. Significantly, reduction of the water activity of a culture by the addition of sucrose instead of sodium chloride also brings about these same amino acid pool changes, but addition of glycerol does not. Glycerol, unlike sodium chloride and sucrose, is known to pass rapidly through bacterial membranes, so that osmotic dehydration of the cell does not occur. Evidently, the metabolic reaction of the cell is not to water activity *per se*, nor to the particular solute molecules which are present, but to loss of water, or of course, to the consequent rises in the concentrations of cytoplasmic constituents.

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It is interesting that the three major 'osmoregulatory' amino acids are closely related metabolically (figure 2). The trigger that initiates the rise in these pool acids is not certainly known, but probably involves, in some organisms in situations where *de novo* amino acid synthesis is occurring, the stimulation by potassium ions of the synthesis of glutamate from α-ketoglutarate, which is catalysed by the enzyme glutamate dehydrogenase, and of other K^+-dependent systems. The concentration of K^+ ions in the cytoplasm will be raised during dehydration since the cell is relatively impermeable to such ions, and they will not therefore flow out of the cell with the water.

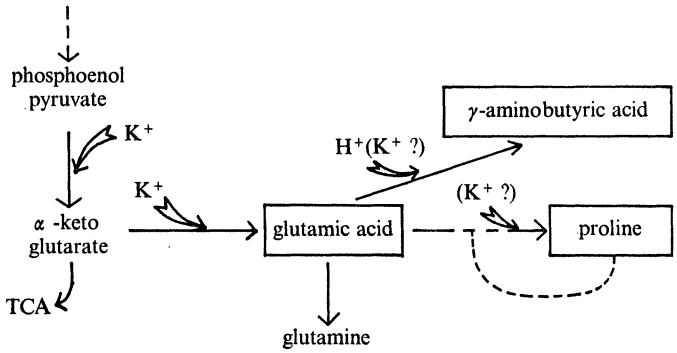

FIGURE 2. Interrelationships of the major osmoregulating amino acids (boxed) in bacteria and the reactions accelerated by potassium.

Further, as the glutamate concentration increases, an increasing concentration of balancing cation is necessary to maintain electrical neutrality. This cation level helps to maintain the α-ketoglutarate/glutamate equilibrium heavily weighted to the side of glutamate. In organisms in which glutamate accumulates, the intracellular K^+ level can be observed to increase simultaneously.

Conversion of glutamate to proline or GABA metabolically, has the advantage of reducing the concentration of charged species in the cell, using two readily available metabolic pathways. The third pathway available which would also neutralize the molecule, conversion of glutamate to glutamine, seems not to be used. Having accumulated or synthesized a high concentration of pool glutamate, γ-aminobutyrate or proline, the organism must maintain the pool constantly at its new high level in order to avoid dehydration and to continue growth. This is partly achieved by increased efficiency of transport of the amino acid into the cell, across the cell membrane, but there is no evidence for the synthesis of novel 'transport proteins' for this rôle. Further, there seems to be no evidence of a change in cell permeability to maintain the intracellular pool. The increased level of amino acid is free to exchange with environmental amino acid, and there is no evidence for gross changes in membrane protein or lipid composition.

If the energy-yielding reactions of the cell are inhibited, for example, by use of the uncoupling agent TCS, then the proline in the cytoplasm leaks out into the environment again very rapidly, that is within seconds.

Rapid leakage of the increased amino acid content is also induced when cells are returned to higher water activities, so that normal pool levels are re-established. It is worth reiterating at this stage the somewhat obvious fact that because optimum growth of these non-halophilic bacteria occurs at water activities above 0.99, it is these conditions which are normal to the cells.

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Adaptation to growth at lower water activities, while a necessity, is an abnormal condition. The cell must therefore be able to rapidly reverse the adaptation mechanism. Thus cells which are returned to high water activity start growth, without a lag, in the new medium at the growth rate normal to that medium. Rapid loss of the amino acid pool seems to achieve this rapid reversibility, and it is perhaps not surprising therefore that in the adapted cell there are no gross macromolecular compositional changes which could take some time to reverse.

Not all microorganisms that can grow at low water activities react exactly in the manner described above, but for each group of organisms there is some solute which accumulates intracellularly to balance the external osmotic pressure. Among the halophilic bacteria this internal build-up of solutes in response to the external osmotic pressure is well known and indeed well documented. Such bacteria contain internal salt concentrations as high as those found outside the cells. To the extent to which they have been studied, the main internal cation is potassium; extreme halophiles growing in saturated sodium chloride contain more than enough potassium ions to saturate their cell water.

Over 20 enzymes of extreme halophiles have now been studied. All can function in high salt concentrations, up to saturated sodium chloride or potassium chloride, and most are inactivated reversibly, or irreversibly in the absence of salts. Some of the most recently studied enzymes include catalase (Lanyi & Stevenson 1969), the $NADH_2$ oxidase complex (Lanyi 1968), isocitrate dehydrogenase (Hubbard & Miller 1969) and aspartate transcarbamylase (Liebl, Kaplan & Kushner 1969), which requires salt not only for activity but also for retro-inhibition by cytidine triphosphate.

The ribosomes of *Halobacterium cutirubrum* need high salt concentrations for stability and dissociate as the salt concentration is lowered. The proteins of these ribosomes are acidic, in contrast to the basic ribosomal proteins of all other microbial species studies. It seems likely that a screen of cations can prevent interaction between negative charges from disrupting the nucleic acid–protein association. A cell-free system capable of protein synthesis has also been prepared from this organism. As might be expected, high salt concentrations were needed for protein synthesis; the system specifically required magnesium ions, potassium ions and ammonium ions in addition to sodium chloride (Bayley & Griffiths 1968*a*, *b*).

These extremely halphilic organisms can be considered as an extreme case in the study of osmoregulation. They are unable to grow at high water activity, that is in the absence of external ions at a high concentration. Their enzymes are specifically designed to work in high ionic concentration and will not function in the absence of high intracellular ion concentrations. This kind of protein adaptation is not found amongst other groups of organisms as far as has been discovered.

In other microorganisms the mechanism depends more on the ability of the cells to concentrate a solute which does not greatly disrupt the cell function, exemplified by the amino acid accumulation by non-halophilic bacteria described previously.

A further good example of this is the osmotic regulation demonstrated amongst the osmophilic yeasts. These are yeasts able to grow in extremely high concentrations of sugars, although tolerance of salts is also sometimes encountered. Onishi (1963) found that high external osmotic pressure in the growth medium altered the fermentation pathway of many of these osmotolerant yeasts, resulting in higher proportions of extracellular and intracellular polyalcohols. Brown & Simpson (1972) studied the differences between sugar tolerant yeasts and non-tolerant yeasts. Two basic differences were found. One of these was permeability to

sucrose, this being affected by, among other things, the presence in the non-tolerant strains, but not the tolerant strains, of invertase. The other property, the accumulation of polyhydric alcohols, is of direct relevance to the water relations of these yeasts. All the sugar-tolerant strains contain one or more polyhydric alcohols as a major intracellular component. These alcohols were not detected in any of the non-tolerant yeasts. In most of the tolerant yeasts the major polyhydric alcohol has been identified as arabitol but there are exceptions. A yeast which was less tolerant than others contained a hexitol but no arabitol. One strain which was atypical contained arabitol, and in addition glycerol and traces of a hexitol (Brown 1974).

The intracellular polyols were present when the organisms were grown at high water activity. For example, one strain grown at a water activity of 0.997 contained arabitol equivalent to 18.5% of the dry mass of the organism. The total arabitol production varied greatly with growth conditions, and a high extracellular solute concentration, that is a low external water activity, was associated with a high intracellular polyol concentration. The arabitol is retained within the cell against the high concentration gradient.

Compatible solutes

Brown & Simpson (1972) have, on the basis of this information, proposed a theory of compatible solutes. For each organism there is a solute which is built-up intracellularly, firstly to compensate the external osmotic environment, and secondly to allow normal enzyme function at the lower water activity. In the case of the halophilic bacteria, it is potassium, and the enzymes are adapted to cope with the high ion concentration. For the osmotolerant yeasts, the polyhydric alcohol molecule fulfils this function. We have now shown that for non-halophilic bacteria amino acids are the most important osmoregulatory factor.

TABLE 4. INHIBITION OF CELL-FREE ENZYME ACTIVITY BY SOLUTES

		activity of isocitrate dehydrogenase (% max) at a_w:				
organism	solute	*0.98*	*0.96*	*0.94*	*0.92*	
B. subtilis	NaCl	78	20	0	0	
	proline	90	75	52	50	
Sacch. rouxii	sucrose	60	30	15	7	
	glycerol	90	85	80	75	
		0.98	*0.96*	*0.96*	*0.87*	*0.80*
Halobact. salinarium	NaCl	90	100	65	52	40
	KCl	80	82	70	64	62

'Compatibility' refers to the fact that the cells' cytoplasmic enzymes are able to operate in the presence of high concentrations of such solutes, whereas the enzymes are much more sensitive to environmental solutes like salts, that are normally excluded from the cell. For example, the different sensitivities of a cytoplasmic enzyme to sodium chloride, proline, glycerol and KCl are illustrated in table 4.

The table shows the relative activity of the cell-free enzyme isocitrate dehydrogenase from three different organisms at a range of water activities. The activities are maintained by either environmental solutes, like sodium chloride or sucrose, or by the particular cells compatible solute, like the proline, glycerol or K^+.

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The maintenance of high activity in the presence of the compatible solute compared to that in the presence of the environmental solute is clear.

As has been pointed out halophilic bacteria are a special case with specially adapted enzymes but for the other species then a metabolic choice of compatible solute has been made. One might well ask: 'why proline?', or 'why polyols?'. A trivial answer might in fact be that these substances have very high solubility. If a cell needs to maintain internal solute concentrations of a few molar, then the variety of low molecular mass substances that are readily available metabolically must be rather limited. Evolution would dictate selection of the most soluble materials and the cells enzymes would evolve to be operative in the presence of high concentrations of them.

Whether compatible solutes show a low degree of interference with *all* enzymes, or only with enzymes that have been modified through evolution towards halotolerance or osmotolerance has not been established in many instances. Many enzymes of organisms which are not salt-tolerant however are certainly stabilized to some degree by high concentrations of proline and glycerol.

It is interesting that these same solutes are also involved in osmoregulation in other microorganisms, higher plants and animals. For example, the marine alga *Dunaliella* contains high levels of glycerol (Borowitzka & Brown 1974) as do some terrestrial lichens that live in dry conditions (Lewis & Smith 1967). Coastal plants like the thrift or sea pink contain high levels of proline (Stewart & Lee 1974). High intracellular proline levels are associated with drought resistance of plants like barley (Singh, Aspinall & Paleg 1972) and with resistance of some brassicas to frost damage (Le Saint 1966) where the high solute concentration depresses the freezing point sufficiently to avoid the formation of ice crystals that otherwise disrupt cell structure. Many invertebrate estuarine animals compensate for increased or decreased salinity in their environments by changing intracellular amino acid levels also (Gillies 1974).

Dormancy and resistance

Heat resistance of bacterial endospores

While some vegetative microorganisms have evolved these very effective measures for maintaining a high and stable water content over very wide ranges of environmental water activity, other forms of microorgansms seem to have evolved procedures for doing the opposite; that is, for drastically reducing their water contents, even in the presence of pure water. These forms are the endospores. Endospores are formed by some, but not all types of bacteria, usually when the nutrient supply runs out, and they serve as efficient survival and dissemination mechanisms. Typically, endospores are extremely dormant, having almost undetectable endogenous metabolism (Desser & Broda 1969). They are very long-lived and resistant to killing by heat, pressure, ultraviolet and ionizing radiation and chemical agents: for example, the resistance of spores of some species is such that wet heat at temperatures as high as 120 °C for 5 min is necessary to inactivate 90 % of a population (see Roberts & Hitchins 1969). Resistance to dry heat is even more pronounced, a temperature of 150 °C for 2.5 h being necessary to inactivate 90 % of the spores of a *Bacillus* species described recently (Bond & Favero 1975): and yet, many of the enzymes (Gardner & Kornberg 1967; Tono & Kornberg 1967), the DNA, ribosomes (Chambon, Deutscher & Kornberg 1968) and other constituents of spores, once extracted from the cells, are no more heat-resistant when tested *in vitro* than those from their vegetative counterparts. How then does the spore impose such enormous resistance on its contents?

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Spore structure, composition and water content

There is evidence that suggests that the water content of spores is low. For instance, the refractive index of spores as measured by interference microscopy is near 1.5, suggesting, if a few assumptions are made (Leman 1973), a water content in the region of about 10 %: the apparent specific gravity of spores in equilibrium gradient centrifugation experiments using low molecular mass solutes is sometimes as high as 1.4 (Lewis, Snell & Alderton 1965), again suggesting a low spore water content. In contrast, measurement of the total water content of spores in centrifuged pellets, using high molecular mass solutes that do not penetrate the spore to measure interspore space, give values of 65–75 %, which are only slightly below the water contents of vegetative cells (Black & Gerhardt 1962). The two sets of results can only be satisfactorily reconciled by assuming some compartmentalization of water within the cell, and indeed, this is compatible with the structure of the spore (figure 3, plate 1), which contains a central core or protoplast which occupies about 30–40 % of the cell volume, with surrounding cortex and coat layers accounting for the remainder. The central core contains the cytoplasm, DNA, ribosomes and probably most of the spore's enzymes, as well as about 10–15 % by dry mass of dipicolinic (2,6-pyridine-dicarboxylic) acid and probably an approximately equimolar amount of calcium.

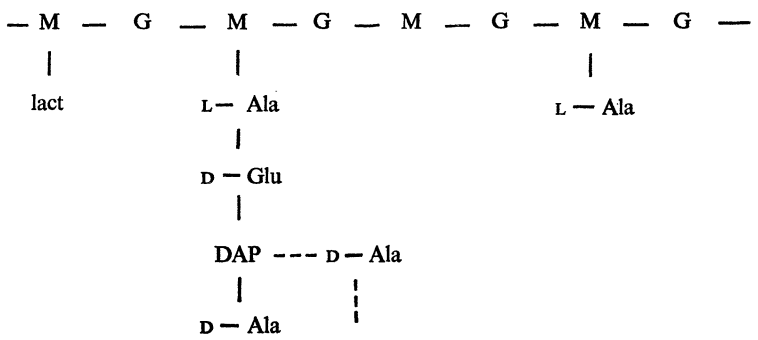

FIGURE 4. Generalized structure of peptidoglycan. The polymer backbone consists of *N*-acetyl muramic acid (M) β–(1 → 4) linked to *N*-acetylglucosamine (G). Some of the muramic acid residues are unsubstituted, some contain lactam groups (lact) or a single L-alanine substitution (L-Ala), some are linked to a tetrapeptide consisting of L-alanine, D-glutamic acid (D-Glu), meso-diaminopimelic acid (DAP), D-alanine (D-Ala). Some of the tetrapeptide side chains cross-link adjacent amino sugar chains via DAP and the terminal D-Ala (dotted lines).

Raman spectroscopy studies indicated that dipicolinic acid is not present in spores as the simple calcium salt, but may be involved in a ring stacking interaction in the core (Woodruff, Spiro & Gilvarg 1974). The high dipicolinic acid content had been considered for many years to be essential for the maintenance of heat resistance of spores, but the recent isolation of dipicolinic acid-negative, and yet heat-resistant, spore mutants has made its direct involvement now seem unlikely (Hanson, Curry, Garner & Halvorson 1972).

The cortex, surrounding the core, is composed largely of an electronegative polymer, peptidoglycan (figure 4). Compared with vegetative cell peptidoglycan, the spores type is less extensively cross-linked and probably more elastic, and has a large negative charge (Warth & Strominger 1972). The outer coats are composed principally of protein.

Examination of the quantities of peptidoglycan components in spores and the volume occupied by this polymer in the cortex led Gould & Dring (1974) to suggest that the polymer was

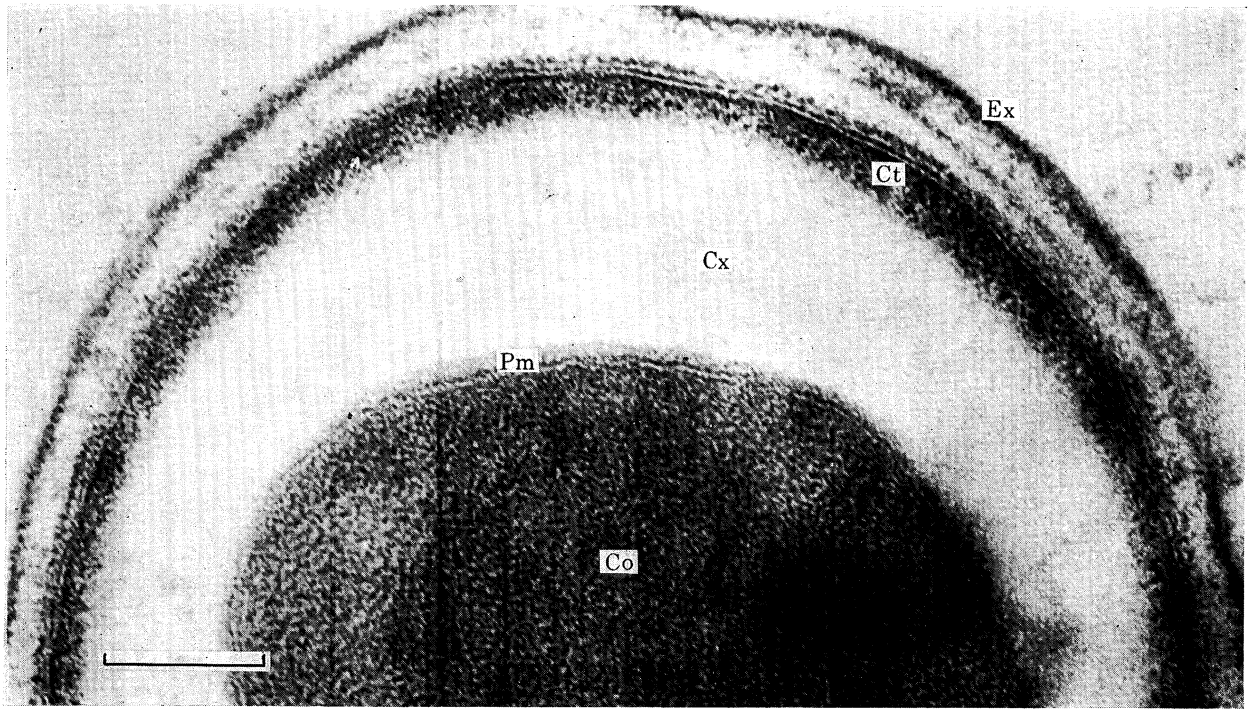

FIGURE 3. Electron micrograph of a section through part of a spore of *Bacillus cereus*, showing the main structures: ex, exosporium; ct, coats; cx, cortex; pm, plasma membrane; co, core. The scale mark represents 0.1 μm.

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normally expanded and relatively water filled in spores, and yet surrounded a relatively dehydrated core. Such an arrangement would be compatible with the high measured total water content of spores, and yet would not conflict with the observed high refractive indices and the apparent high specific gravities of these cells.

Mechanism of heat resistance

The arrangement also begins to suggest a mechanism for the enormous heat protection of components in the spore. Could the core be sufficiently dehydrated to increase the resistance of the various macromolecules it contains to denaturation by heat?

A low core water content is thought to be attained through two activities that occur during spore formation. Firstly, the soluble low molecular mass pool components in the core of the developing spore decrease dramatically, unlike those in the osmotically shocked vegetative cells considered above. The fully-formed spore, for instance, contains dipicolinic acid, glutamic acid, phosphoglyceric acid and sometimes sulpholactic acid (Nelson *et al.* 1969) but also such a high level of calcium that these weak acids are likely to be mostly insolubilized. Secondly, the loosely cross-linked electronegative peptidoglycan polymer (figure 4) is laid down around the core at this time to form the cortex layer (figure 3). This shell of polymer, and the counter-ions that will be associated with it, then bring about dehydration of the core and maintain it in a dehydrated condition by osmosis (Gould & Dring 1975). The model of the heat-resistant, dormant spore is then of a shrunken dehydrated core, containing only low levels of low molecular mass substances in solution, but in osmotic equilibrium with the surrounding water-rich cortex containing the expanded cation exchange polymer.

The magnitude of the osmotic pressure that may operate in this system can be inferred from an examination of the effects of solutes on the heat resistance of germinated spores. Spores can be made to germinate within time periods of less than a minute by the additon of the correct chemicals, which include, for spores of different species, 'metabolizable germinants' like L-alanine, adenosine and inosine or 'non-metabolizable germinants' like long chain alkyl amines and calcium dipicolinate. When germination is initiated, the characteristic properties of spores are rapidly lost (Gould 1969). Resistance to heat, irradiation and chemical agents falls, the previously stain-resistant cells become readily stainable and the refractibility of individual spores and the extinction of spore suspensions falls. Accompanying these changes, calcium and dipicolinic acid are released from the spores, and respiration, ion and nutrient transport, and other enzymic activities that were inoperative in the dormant spore become detectable. Within the next few minutes, synthesis of RNA, protein and DNA commence and growth resumes, leading to the formation of a new vegetative cell.

One important change that occurs during germination is that the peptidoglycan polymer in the cortex is hydrolysed by a lysozyme-like enzyme which becomes active within the spore (Gould & King 1969). Depolymerized peptidoglycan fragments leak out of the spore and the cortex region becomes thinner at the expense of a large swelling of the enclosed core. This swelling reflects mostly redistribution of water within the spore, from the cortex space into the core, as well as an additional net uptake of water from the environment.

Such germinated spores have lost the extreme protection against inactivation by heat, and are consequently much more heat-sensitive than the ungerminated spores, i.e. commonly by factors of 10^4–10^5-fold. However, if these germinated spores, which are heat-sensitive if heated in water or in dilute solutions, are heated instead in sufficiently concentrated solutions of a

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solute like sucrose, the enormous heat-sensitization is reversed. Indeed, table 5 summarizes the results of such an experiment in which germinated spores in sucrose became even more heat resistant than the ungerminated spores at the start of the experiment (Dring & Gould 1975). Examination of the effect of different sucrose concentrations in experiments like this indicated that concentrations of about 1.5–2 M or above were necessary to confer such protection against heat. The simplest inference is that this represents the approximate osmotic pressure, i.e. about 30 atmospheres (3 MPa) normally exerted by the cortex in the resistant spore. The osmotic dehydration is lost during germination, as the cortex peptidoglycan is hydrolysed enzymically, and the core hydrates, but can be completely reimposed by the sucrose solutions.

TABLE 5. REIMPOSITION OF HEAT RESISTANCE ON GERMINATED SPORES OF *B. cereus*

	D-value (min.) at 90 °C of cells heated in:	
organisms	water	sucrose(2 M)
ungerminated spores	7.5	31
germinated spores	0.001	37

Interestingly, if the germinated spores are left to develop further and grow to become vegetative bacteria, then the protection against heat afforded by a constant concentration of sucrose in the environment becomes less and less. This probably parallels the increase in low molecular mass pool components inside the cell which decreases the osmotic pressure difference across the cell membrane so that the exogenous sucrose becomes less and less effective in withdrawing water from the cell and therefore no longer able to protect it from inactivation by heat.

In addition, however, certain spore enzymes do differ from their vegetative counterparts. Spore aldolase, for example, seems to be modified by partial proteolysis during sporulation (Sadoff, Celikkol & Engelbrecht 1970) and it is possible that dehydration imposes resistance on these modified enzymes more effectively than on the vegetative forms. Heat resistance of some spore enzymes (e.g. glucose dehydrogenase; Sadoff 1970) can certainly be increased *in vitro* by raising the concentration of group IA cations to promote disaggregation of the enzymes into subunits. *In vitro* heat resistance may then approach that of enzyme in the intact spore. Nevertheless, Corry (1974) has shown that suspension in solutions of solutes like sucrose that do not easily penetrate bacterial cells, can cause increases in the heat resistance of even some non-spore forming vegetative bacteria by factors of up to 700-fold, presumably through osmotic dehydration.

Glycerol, as one might again expect, since it easily penetrates bacterial cell membranes and therefore will not set up an osmotic gradient, does not confer resistance on germinated spores at concentrations equiosmolal with the sucrose shown in table 5.

The concept of an osmoregulatory cortex controlling the water content and heat resistance of the spore fits well with a number of previous observations. Alderton & Snell (1963) noticed that equilibration of spores at acid pH values reduced their heat resistance, and re-equilibration at higher pH values led to reimposition of resistance. It is now thought that this observation reflects the protonation of the peptidolycan polymer at low pH, causing its collapse; the replacement of counter-ions from the cortex compartment, and a fall in osmotic pressure with consequent partial rehydration of the enclosed core. At high pH values, ionization of the peptidoglycan leads to expansion, a rise in osmotic pressure, movement of water from the core,

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and a consequent increase in heat resistance. Supporting this idea, the suspension of spores in sucrose solutions at low pH values results in a large protection against the heat sensitizing effect of acid.

Another procedure by which the heat resistance of spores can be altered utilizes spores in which the outer protein coat is modified. This can be achieved by chemical treatment to make the coats 'leaky', or simply by use of mutant spores which have defective coats. These spores can be made heat-sensitive by suspension in solutions of salts of multivalent cations, but not univalent ones or anions like thiocyanate that are known to destabilize proteins to heat (Von Hippel & Wong 1965) (table 6). It is thought that the multivalent cations replace the normal univalent ions in the cortex and bind weakly to carboxyl groups on the cortex peptidoglycan causing its collapse, and a fall in osmotic pressure and consequent partial rehydration of the core. Again, the suspension of such sensitized spores in sufficiently osmotically active solutions (e.g. sucrose, last line of table 6) will reimpose resistance.

TABLE 6. *B. CEREUS* SPORES WITH LEAKY COATS: HEAT-SENSITIZATION BY CATIONS AND PROTECTION BY SUCROSE

	survivors(%) following heating at 70 °C for 30 min	
suspending solution(4 M)	untreated spores	mercaptoethanol-urea-treated spores
water(control)	85	83
NaCl	89	90
KCl		64
$CaCl_2$	98	0.85
$MgCl_2$		0.1
$La_2(NO_3)_3$		0.01
KNCS (5 M)	100	100
sucrose (3.6 M)	100	120
$CaCl_2$ + sucrose		84

The already high heat resistance of spores can normally be further increased by equilibration with atmospheres at low equilibrium relative humidities (Murrell & Scott 1966) and also to some extent by suspension in various solutions at low water activities (Harnulv & Snygg 1972), and these effects are particularly marked with those spores that are normally the most heat-sensitive at high water activities. Generally spores have maximum heat resistance in such strong solutions or when equilibrated in atmospheres at water activities of about 0.2–0.3.

Such observations again strongly suggest that the equilibrium water status of spores can be readily changed, and that the heat resistance then changes greatly in consequence.

Finally, the new model of the heat-resistant bacterial endospore is summarized in figure 5. The central core is considered to be low in water (exactly how low is not known) but surrounded by, and in osmotic equilibrium with, the water-rich peptidoglycan in the cortex. The cortex, in turn, is surrounded by the coat, which is normally relatively impermeable to multivalent cations.

The new model suggests that the water content of the core is amongst the most important contributory factors to the enormous heat resistance of the spore. One must therefore now ask: How does control of water content so greatly influence the heat resistance of such a mixture of components as those that make up a living cell, including the enzymes, nucleic acids, and larger structures like the ribosomes and the cell membrane? At the molecular level, steric

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hindrance is likely to be an important factor that could increase thermal stability of diverse macromolecules within the spore, and which would depend on the water content of the core but, at this time, the detailed mechanism is quite unknown. Experiments to measure the heat resistance of osmotically dehydrated enzymes and other components from spores as well as from other cells in model cell-free systems are really needed to investigate this further.

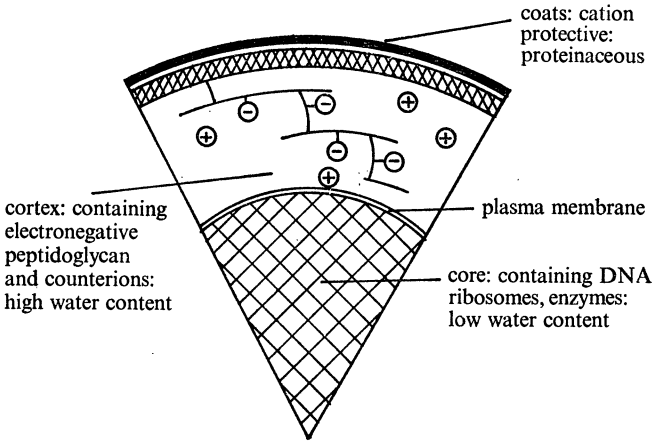

FIGURE 5. Osmoregulatory expanded cortex model of a heat-resistant bacterial endospore.

In conclusion, it seems that even the single cells of microorganisms have sometimes evolved mechanisms that allow very effective control of cell water content, whatever the conditions in the environment. At the one extreme, some single cells can so manipulate their internal levels of particular solutes as to avoid water loss even in saturated brine and in other environments at water activities well below 0.7. And the cells' cytoplasmic enzymes have evolved to be able to remain operative in the presence of high concentrations of these internal or 'compatible' solutes. At the other extreme, the single celled spore becomes compartmentalized during the morphogenic changes accompanying sporulation in such a way that the water content of the cytoplasm is not maintained, but the reverse; loss of water from the core is in fact ensured. One of the effects of this water loss is to make such cells the most heat-resistant life-forms on earth: another, not unexpected, result may be to impose on them the extreme dormancy that is typical of spores, for a low water content is characteristic of dormant biological structures of all types, including not only those of microorganisms, but those of higher plants and animals as well (Sussman 1969).

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Discussion

D. R. Wilkie (*Department Physiology, University College London*). In those organisms whose normal free-living form is thermophilic (for example those that live in hot springs) are the enzymes protected in a similar way to that found in spores, that is, by surroundings of high osmolarity?

G. W. Gould. No. About 20 proteins have been isolated from thermophilic microorganisms and, with few exceptions, they seem to be inherently thermostable, i.e. even when extracted and, in some instances, crystallized. Thermostability may rely on increased metal binding by proteins from thermophiles compared with those from mesophiles, on increased hydrophobicity, or on the maintenance of active site conformation (of enzymes) during 'melting' of the hydrophobic cluster. However, the evidence in favour of any of these hypotheses is not particularly strong (see review by Singleton & Amelunxen 1973 *Bact. Revs.* **37**, 320–342).

P. Meares (*Chemistry Department, University of Aberdeen, Meston Walk, Old Aberdeen, AB9 2UE*). You have told us that cells which have adapted to low external water activity by the generation of compatible solutes such as proline lose these solutes very rapidly by outward permeation when the external water activity is restored to almost unity. When these solutes are providing

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protection against high external osmotic pressure the cell membrane must be almost impermeable to them. It would be interesting to know whether the sudden increase in permeability which accompanies elevation of the external water activity is brought about mechanically by a rapid transient stretching of the membrane due to an osmotic in-flux of water or whether some more complex biochemical mechanism is operative.

G. W. Gould. The sudden release of proline if brought about by a decrease in external water activity, but also by addition of metabolic inhibitors like TCS (tetrachlorosalicylanilide) or CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone), i.e. at constant external water activity. In the latter case, extensive membrane stretching is less likely to occur than in the former, suggesting that more complex biochemical mechanisms are operating, in particular those concerned with energy coupling within the cell.

Further, isotope exchange experiments show that the intracellular proline accumulated at low water activity is rapidly exchangeable with extracellular proline. It is therefore unlikely that the increased pool is achieved by an increased impermeability of the cell membrane to proline.

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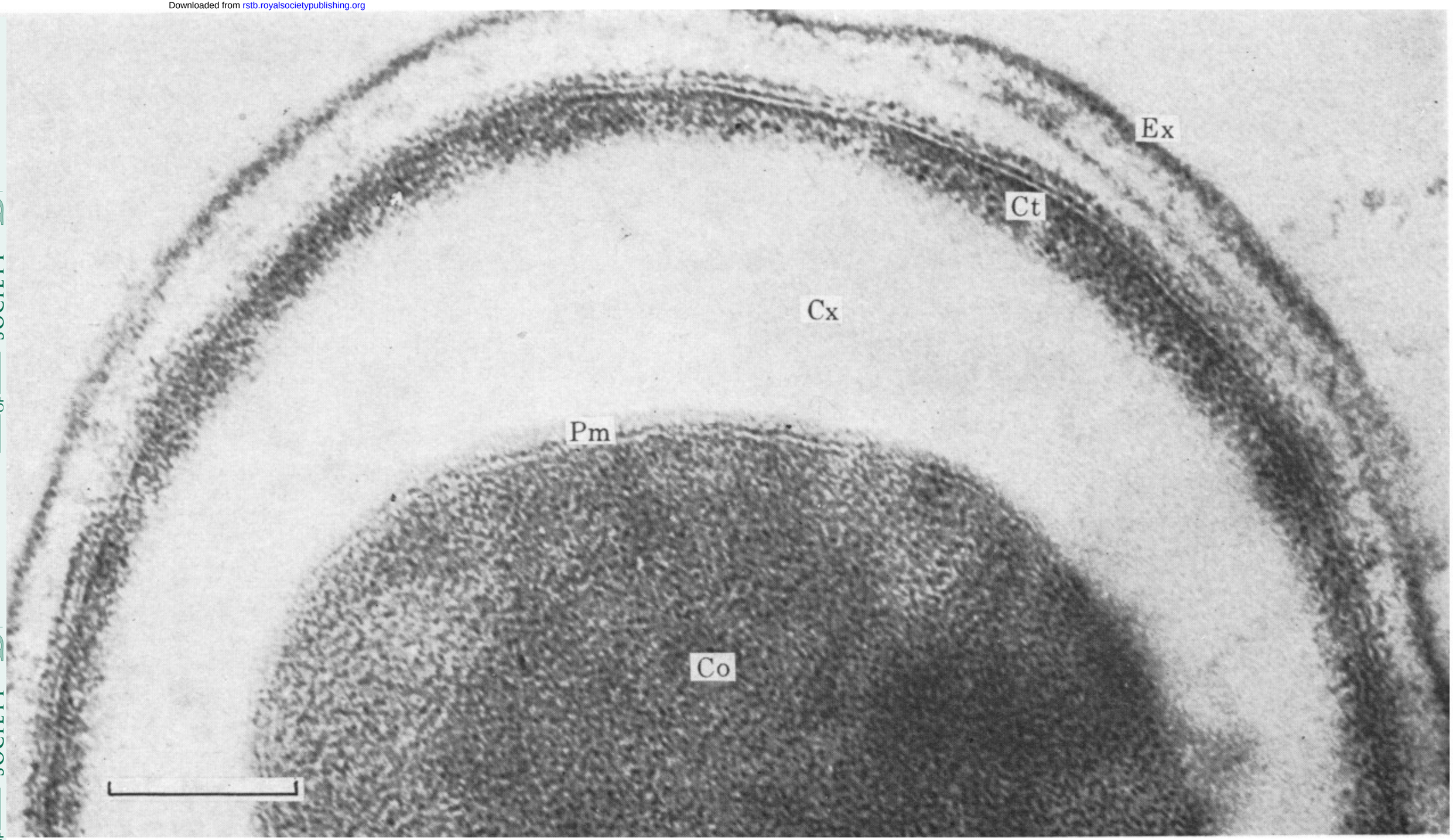

FIGURE 3. Electron micrograph of a section through part of a spore of *Bacillus cereus*, showing the main structures: ex, exosporium; ct, coats; cx, cortex; pm, plasma membrane; co, core. The scale mark represents 0.1 μm.