POLYAMINE CONTENT AND CELL CYCLE IN A UNICELLULAR ALGA. DATA ARE CONSISTENT WITH POLYAMINES' FUNCTION OF PROTECTING DNA AGAINST OXIDATIVE DAMAGE BY SINGLET OXYGEN

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Three polyamines (putrescine, norspermidine and spermidine in peak concentrations of 6, 1, and 0.2 nmol/10^6 cells, respectively) were identified by HPLC in *Chlamydomonas reinhardtii*, a unicellular alga. The molar concentration of putrecsine, averaged over the cell volume is astonishingly high, 0.1 M. The amounts per cell of these polyamines and of chlorophyll were monitored at 2 hr interval during 24 hr periods (12:12 light/dark). As expected the chlorophyll content varies as a function of cell volume, thus of the time in the cell cycle; it serves as an indicator of the position of the cells along the cycle. Putrescine and norspermidine follow quite closely the time course of chlorophyll. In contrast, the level of spermidine both increases and decreases earlier, and is already high well before the synthesis phase of the cycle. This is consistent with our recent proposal that the function of polyamines is to protect the cells from oxidative damage, especially by singlet oxygen which spermidine quenches. The data on *Chlamydomonas* and two dinoflagellates are discussed in the framework of the present knowledge of polyamines, including evolutionary aspects.

Keywords: polyamines; cell-cycle; singlet-oxygen.

INTRODUCTION

Polyamines are ubiquitous in living cells and may protect them against oxidative damage.[1,2] Table I shows the structures of putrescine, spermidine and spermine, the most common polyamines in mammals and plants as well as in eukaryotes in general; prokaryotes contain no spermine, only putrescine and spermidine. Table I also includes norspermidine and norspermine, which have one methylene group less than their spermidine and spermine counterparts. These two "unusual" polyamines, first discovered in extreme thermophillic bacteria,[3] are important in our discussion.

Table I. Structures of some polyamines.

PUTRESCINE (PUT)	NH_2-$(CH_2)_3$-NH_2
SPERMIDINE (SPD)	NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH_2
SPERMINE (SPN)	NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2
NORSPERMIDINE (NSPD)	NH_2-$(CH_2)_3$-NH-$(CH_2)_3$-NH_2
NORSPERMINE (NSPN)	NH_2-$(CH_2)_3$-NH-$(CH_2)_3$-NH-$(CH_2)_3$-NH_2

Polyamines are the subject of an immense literature, going back four centuries;[4] many review articles, monographs and symposia focus on aspects of polyamine properties.[5] Yet most texts of organic chemistry, biochemistry or cell biology treat polyamines only briefly, if at all. The reason for this anomalous situation is that, in spite of so much effort, the biological function of polyamines is not definitely established; or perhaps it is because they have been attributed too many functions.

Very briefly, *in vitro* studies have established that spermine and spermidine bind and stabilize DNA and RNA, the positive charges carried by the protonated amino groups (at physiological pH) neutralizing the backbone phosphate groups.[6] Molecular biology recipes take advantage of this to selectively precipitate DNA from dilute solutions. Polyamines also induce B to Z conformation changes in DNA.[6] *In vivo*, mutant and enzyme inhibitor studies have demonstrated in many different organisms, from prokaryotes to mammalian cells, that polyamines are important for growth. An *E.coli* mutant lacking putrescine and spermidine grows albeit more slowly than wild-type,[7] but in mammalian cells spermidine and/or spermine are essential for growth. However, how these effects on growth regulation occur is still unclear, although many *in vitro* studies have demonstrated effects of polyamines on protein synthesis.[2]

Figure 1 schematizes the biosynthesis of putrescine, spermidine and spermine, starting with ornithine. Of the participating enzymes, ornithine decarboxylase (ODC) is probably the most interesting and most studied.[5c,8] In mammals its turnover rate is the fastest of all enzymes. It is synthesised at the end of the G_1 phase of the cell cycle, before DNA and protein synthesis in which polyamines are considered to play an important (although still ill-defined) role. Various stimuli, such as growth hormones, can increase by orders of magnitude the activity (concentration) of ODC. A specific ODC inhibitor, 2-difluoromethylornithine (DFMO), whose mode of action is based on the concept of enzyme suicide (i.e. a "Trojan horse inhibitor")[9] was synthesised in 1978.[10] This led to an explosion of studies of potential medical importance for the treatment of cancer and parasitic diseases, where the control of fast growing cells is critical. Thus far, the greatest therapeutic success of this approach seems to be on *Trypanosoma brucei*,[11] the cause of African sleeping sickness: a low dose of DFMO has a greater effect on the ODC of the parasite because its protein turnover rate is slower than that of the host.

Polyamines have also been found to protect cells against oxidative damage by superoxide ion and free radicals, although this entirely different function has attracted far less attention. For example, an *E. coli* mutant, unable to synthesise spermidine but enjoying an apparently normal life thanks to its pool of putrescine, is killed much faster than wild-type when exposed to $O_2^{\cdot}$ from paraquat.[12] *In vitro*, polyamines were indeed

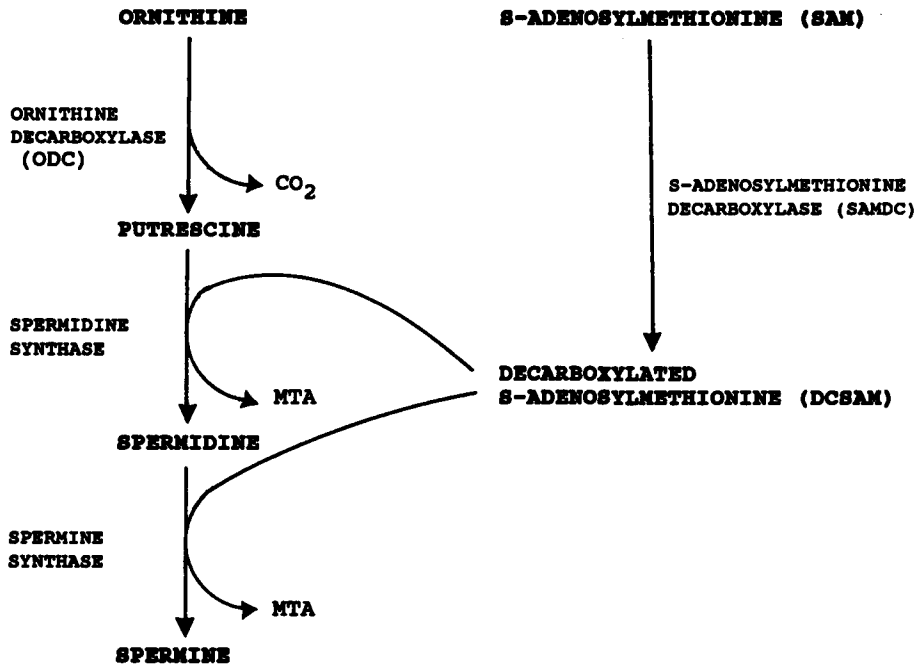

Figure 1. Biosynthesis of putrescine, spermidine and spermine. The diaminobutane groups of spermidine and spermine come from ornithine, whereas the aminopropyl groups come, ultimately, from methionine. MTA is methylthioadenosine.

shown to inhibit lipid peroxidation.[13]

It is this anti-oxidant property of polyamines that attracted our attention. Secondary and tertiary amines are efficient quenchers of singlet oxygen,[14] the long-lived and higly reactive excited state of molecular oxygen, $^1O_2$ ($^1\Delta_g$),[14c] which is generated by many enzymes and cellular processes.[15] It damages DNA as well as lipids and membranes.[14c,15,16] Thus we considered that polyamines might have an important role in protecting cell constituents against attack by singlet oxygen,[1] and that they would be singularly well placed to protect the nucleic acids with which they bind.

Indeed, both spermine and spermidine quench singlet oxygen, as anticipated. We first showed that they retard the photoperoxidation of rubrene, a process in which the intermediacy of $^1O_2$ is well established.[1] We then showed that spermidine and spermine prevent the formation of single-strand breaks in plasmid DNA exposed to singlet oxygen.[17] This is not surprising since $^1O_2$ is quenched 20 times faster by spermine or spermidine than it reacts with DNA.[1] Along the same lines, we hope to examine next the possible protective effect of polyamines *in vivo* on cells exposed to photodynamic killing.

The fact that, in mammalian cells, ODC activity peaks before the synthesis of DNA (end of G_1) is certainly consistent with our hypothesis that an important function of polyamines may be the protection of DNA against damage by singlet oxygen. Two studies of the unicellular alga *Euglena gracilis* reported fluctuations in polyamine concentration at specific times in the cell cycle, both in cells grown heterotrophically in the dark and in cells grown photosynthetically in light/dark cycles.[18] In the latter case, the highest level of polyamine coincided with the beginning of the dark period, when the cells begin to divide; it seems possible to us, therefore, that this peak could simply reflect the changes in cell number and volume. Older cultures, still synchronized but in stationary phase, showed very little fluctuation in their polyamine content.[18b]

We sought to repeat such experiments with two other unicellular algae, *Chlamydomonas* (a Chlorophyta) which is easy to synchronize, and *Gonyaulax* (a dinoflagellate) which exhibits circadian rhythmicity in many functions. We report here our results, some surprises and question marks along a research detour into very biological territory. We dedicate our tale of polyamines to Professor G. Cilento, who did so much to attract attention to the role of long-lived metastable electronically excited species in "dark" photobiological processes. If our hunch should prove correct, polyamines may be here to protect us against the smallest and most insidious of such species, singlet oxygen.

MATERIALS AND METHODS

Organisms, growth conditions and experimental protocols

Chlamydomonas reinhardtii (CC-124 from the Chlamydomonas Genetics Center at Duke University, Durham, NC) was grown at 31°C under a stream of 2-5% CO_2/air in high salt (HS) medium (Sucoka, N. Proc. Natl. Acad. Sci.USA **1960**, *46*, 83-91); the cultures were exposed to 12:12 light:dark cycles for 3 days in order to synchronize the cells prior to the start of an experiment.

Samples were then collected every 2 hours for 24 hours. The cells were counted (with a hemacytometer from Reichert Scientific Instrument), their chorophyll content determined photometrically at 645 and 663 nm (after addition of 2 ml acetone to .5 ml culture) and two 6-ml aliquots (A and B) centrifuged 2 min. The cell pellets from each of these two aliquots were immediately and separately cooled in liquid nitrogen, then kept at -70°C for later determination of their polyamines content.

Gonyaulax polyedra (strain 70) *and Pyrocystis lunula* Schutt (strain T37) were grown in F/2 medium in 12:12 light:dark cycles at 22°C. Because the cell cycle of *Gonyaulax* is long (ca. 3 days), the cells cannot be synchronized by this L:D cycle. A degree of synchronization can, however, be achieved by a double filtration procedure (using a Nytex filter, 41 μm pore diameter).[19] Large mother cells are first retained and resuspended in fresh medium at 8-10 hr in light

phase, then at 3-6 hr in the next light phase the small daughter cells passing through the filter are selected out, and constitute a synchronized culture. The effectiveness of this procedure was monitored in a cell counter (Model ZB1, Coulter Electronics, Hialeah, FL, with a 140 μm aperture diameter). Aliquots of culture under 12:12 L:D conditions were harvested at several hour intervals during two consecutive 24-hr cycles, separated on filter paper and frozen in liquid nitrogen prior to polyamine analysis. No attempt was made to synchronize *P. lunula*, which were grown in the same conditions as *G. polyedra*, and polyamine content was assayed at one time only.

Plasmodium falciparum was grown in 50% red blood cells in RPMI 1640 medium (Sigma, supplemented with, per liter, 2 g glucose and 3 g NaTES, 1.1% (w/v) pyruvate, 0.05% hypoxanthine, 3% (w/v) glutamine, 500 μl 50 mg/ml gentamicin, 100 ml plasma), at 38 °C under an atmosphere of 7% CO_2 and low oxygen (1-5%) for several days, until ca. 15% RBC was infected with *P. falciparum*. 2.5 ml culture was resuspended in 10 ml unsupplemented RPMI 1640 medium and centrifuged at 500 g for 5 min. The pellet was resuspended and centrifuged again. 1:1000 (v/v) 15% saponin solution and 1:100 (v/v) 0.5 M EDTA were added to the resulting pellet, and the mixture shaken for several minutes to break the red blood cell membrane. After centrifuging at 14,000 g for 10 min, the resulting pellet (of *P. falciparum*) was separated and treated with 10% sarkosyl (1:100, v/v) for 0.5 min.

Polyamine analysis[20]

10^{-8} mole and $5x10^{-8}$ mole cadaverine was added as internal standard to the pellets of *C. reinhardtii* obtained from aliquots A and B, respectively. The cells were broken by sonication in water, extracted with 3% trichloroacetic acid and centrifuged at 8,000 g at *ca.* 4 °C for 20 min. The pellets were resuspended in NaOH and recentrifuged in 3% trichloroacetic acid. 1.5 ml 2 N NaOH and 10 μl benzoyl chloride was added to the pooled supernatants (about 6 ml) from each of the 6-ml aliquots collected at given times, and the mixture magnetically stirred for 20 min for complete reaction. A saturated NaCl solution (2 ml) was added, the solution extracted twice with 1 ml ether and ether evaporated under a stream of nitrogen. The residue, containing the benzoylated polyamines, was dissolved in 42% acetonitrile/water.

Separation and quantification of the polyamine derivatives was performed by HPLC (Beckman, RP-C8 column 4.6 mm x 25 cm, mobile phase 42% acetonitrile/water, flow rate 1.2 ml/min, room temperature, detection wavelength 198 nm).

The same procedures of cell treatment and polyamine analysis were essentially followed in the case of *G. polyedra*, with the difference that hexanediamine was used as the HPLC internal standard instead of cadaverine and the cells were broken with a minibeadbeater (Biospec Products).

The polyamine content of *P. falciparum* was obtained by following the same protocol as for *Chlamydomonas*. It was checked that the concentration of sarkosyl used to break the cells did not interfere with the derivatization and HPLC analysis.

Chemicals

1,3-diaminopropane, 1,4-diaminobutane (putrescine) and 3,3'-iminobispropylamine (norspermidine) were from Sigma. 1,5-diaminopentane (cadaverine), 1,6-hexanediamine, N-[3-aminopropyl]-1,4-butanediamine (spermidine) and N,N'-bis[3-aminopropyl]-1,4-butanediamine were from Aldrich. N,N'-bis[3-aminopropyl]-1,3-propanediamine (norspermine) was a gift from Dr. S. Marvin Friedman. All other chemicals were of the best commercial grade and used as received.

RESULTS

1. *Chlamydomonas*

Only three polyamines, putrescine (PUT), norspermidine (NSPD) and spermidine (SPD), were found to be present in significant amounts in *C. reinhardtii*, in our growth conditions. The chromatographic separation was excellent (retention times: 4.6 min for PUT, 6.0 for NSPD, 6.8 for SPD and 5.3 for cadaverine, the standard). By repeating the HPLC assays at each time point with two different concentrations of the standard, the most and the least abundant polyamines (PUT and SPD, respectively) could be accurately assayed.

As expected, the concentration of chlorophyll per cell varies considerably during the 12:12 light dark cycle. Figure 2A presents the results averaged over two independent 24-hr experiments. As the cells grow during the light phase (subjective day), their chlorophyll content increases proportionally to cell volume. It remains constant during most of the dark period, until cell division occurs at the end of the subjective night. The sharp drop in Chl/cell at LD time 20-22 hr is congruent with visual observation of cell division and separation during this period, and in accord with the literature. Thus Chl/cell provides a way to monitor the progression of the cells through the cell cycle.

The concentration of putrescine follows fairly closely that of chlorophyll (Figure 2B). In contrast, the level of spermidine rises much earlier during the light phase and starts to decrease ca. 6 hrs before that of chlorophyll (Figure 2C). The same trend is also visible in the time course of the norspermidine concentration, although to a lesser extent (data not shown).

At peak time (hr 12-14), the absolute concentrations of putrescine, norspermidine and spermidine were 6, 0.9 and 0.2 nmol/10^6 cells. The levels of chlorophyll and polyamines all fell to their lowest values at the very end of the dark phase, averaging a 5-fold drop.

2. Dinoflagellates

In our growth conditions, only one polyamine, norspermine, was found to be present in any significant amount in the two species of dinoflagellates we looked at, *G. polyedra* and *P. lunula*. The identification of this "unusual" polyamine was based on its HPLC retention time and co-chromatography with an authentic sample of norspermine (benzoylated together with the unknown) in a different mobile phase, as well as on the mass spectrum of the isolated derivative, which is identical to that of the authentic sample.

Even in fairly well synchronized *Gonyaulax* cells, our analysis reveals no consistent trend in the level of NSPN as function of either the circadian time or the expected position of the cells relative to the cell cycle. But these results are too preliminary to draw strong conclusions. The concentration of NSPN is ca. 0.065 μmol/g wet weight for *Gonuaulax* or, on the average, 5 μmol/10^6 cells, and *ca.* 6 nmol/g wet weight for *P. lunula*.

3. *Plasmodium falciparum*

Three polyamines were found in these cells, putrescine, spermidine and spermine; their respective concentrations were ca. 0.01, 0.03 and 0.005 nmol/10^6 cells, based on an average of 15% of red blood cells infected.

DISCUSSION

Three aspects of our data are of interest: 1) the nature of the polyamines present in the three species of unicellular algae studied, two dinoflagellates (or Pyrrophta) and *Chlamydomonas*, a Chlorophyta; 2) the absolute concentration of these compounds in the cells; 3) the time course of polyamine con-

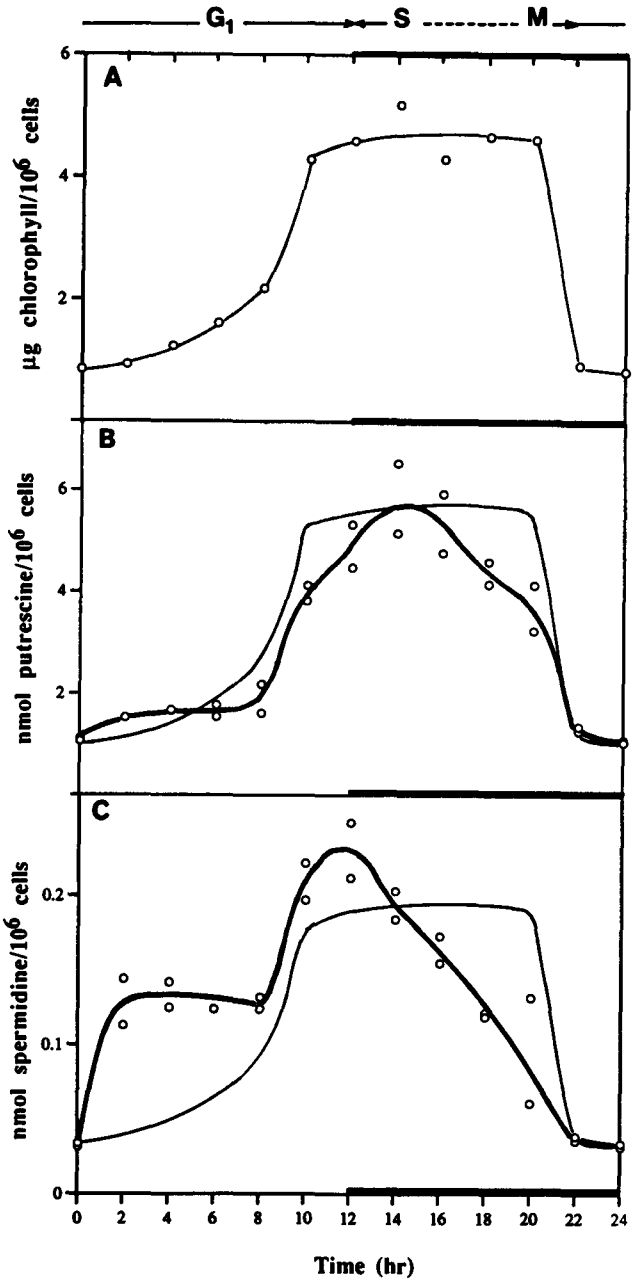

Figure 2. Levels of chlorophyll, putrescine and spermidine in Chlamydomonas reinhardtii cells as a function of time, during a 12:12 light:dark cycle. A Chlorophyll. B Putrescine. C Spermidine. In panels B and C the heavy lines represent the time courses of the polyamines, drawn through two time points (two different 24 hr experiments); the thin lines repeat the chlorophyll data from panel A. The curves in B and C were normalized to the chlorophyll curve at hr 14. Approximative timing of DNA synthesis (S) and cell division (M) is indicated at the top of the figure.[33]

centration in synchronized *Chlamydomonas* cells. We will discuss the last point first.

Whether the primary biological function of polyamines is to stabilize the nucleic acids (and derived functions) or, in addition, to protect them against oxidative damage, the role of polyamines at the time of DNA synthesis is suggested in mammalian cells by the timing of the burst of ornithine decarboxylase synthesis and its rapid degradation at the end of G_1, before the S phase of the cycle. It is likely that ornithine is also the precursor of putrescine in *Chlamydomonas*, although the turnover rate of its ornithine decarboxylase is probably slower than in higher organisms. This may be why the level of putrescine in *Chlamydomonas* seems to follow closely that of chlorophyll, which in turn simply reflects cell growth and division. In contrast, the intracellular concentration of spermidine follows a markedly different course. It reaches a high level earlier than that of chlorophyll, and starts to decrease before cell separation, probably at the beginning of mitosis. The precursor of spermidine can be assumed to be putrescine, whose intracellular concentration in *Chlamydomonas* is always high (even at its lowest level, it is 4 times higher than that of spermidine at its peak). Since spermidine is both a better stabilizer of DNA and a better quencher of singlet oxygen than putrescine, its regulation is more critical to the cell than that of putrescine. It is probably controlled by the activities of either a S-adenosylmethionine decarboxylase (resulting in S-adenosylmethionine) or of a spermidine synthase (which catalyses the transfer of the aminopropyl group to putrescine). Interestingly, in *Euglena*[21] as well as in the thermophyllic bacteria *Calderia acidophila*,[22] isotopic labelling experiments have shown that norspermidine is not derived from 2,4-diaminobutyric acid (the analog of ornithine), but from 1,3-diaminopropane (DAP) via transfer of two aminopropyl groups. DAP, in turn, results from spermidine degradation. If DAP is an intermediate in the biosynthesis of norspermidine, it is intriguing that no detectable amounts of DAP were found either in *Chlamydomonas* or in *Calderia* and only traces of it in *Euglena*.[21]

Over the years, *Gonyaulax* has become one of the guinea pigs of chronobiologists. It exhibits very striking and well studied circadian rhythms in several physiological functions, notably bioluminescence emission and concentration of the enzyme, substrate and binding protein responsible for the emission.[23] We had hoped to observe time-of-day dependence of norspermine concentration. This was not observed, either because the level of norspermine remains indeed constant with circadian time, or because our cultures were not well enough synchronised to allow us to detect fluctuations associated with the position of the cells along their long cycle (ca. 2-3 days). At this junction, our results have therefore to be regarded as inconclusive.

Since most algae contain putrescine and spermidine, the finding of norspermine as the **only** polyamine in *G. polyedra* as well as in *P. lunula* was a surprise, especially since no clue emerges regarding its biosynthesis. One could speculate that, as in *Euglena* and *Calderia*, DAP and spermidine are precursors, but neither of these amines (nor putrescine) were found in *Gonyaulax*, which is puzzling. Up to 1975, norspermine and norspermidine had been found only in extreme thermophillic bacteria.[3] It was thought that these polyamines could be responsible for the thermal resistance of the cells as a result of specific stabilizing interations with DNA. A correlation between optimal growth temperature and pattern of polyamines in bacteria seemed to support this possibility.[3,22,24] However, the finding of norspermine in *Euglena gracilis*[25] destroyed this theory, and no new hypothesis has been offered to relate a particular type of tri/ or tetramine to function.

Contrary to the situation in *Gonyaulax*, norspermine is not the only polyamine present in *Euglena*, but it is the dominant one. Hamana and Matsuzaki have analyzed the diamine and polyamine content of many eukaryotic algae from seven phyla.[26] No pattern is easy to discern, although significant amounts of norspermidine and norspermine were found only in Euglenophyta, in two species of Chlorophyta (notably *Chlamydomonas*) and in *Peridinium* (like *Gonyaulax*, a Pyrrophyta or dinoflagellate). Hamana and Matsuzaki remark that the distribution patterns of polyamines differ from alga to alga within the same phylum, suggesting changes in biosynthetic pathways after the branching out of a phylum. Norsper-

mine and norspermidine have also been found in higher organisms (cricket, cockroach, etc.).[27] However, possible contamination by parasites should be considered. Norspermine has been reported in a shrimp,[28] but since dinoflagellates were a likely part of its diet, and the whole shrimp (including its gut) was analyzed, this result may not be significant.

Why, when and where from did the "unusual" polyamines appear -and disappear- in evolution? Morphological and molecular (rRNA) evidence indicates that dinoflagellates are phylogenetically related to the protist phylum Apicomplexa (to which *Plasmodium* belongs).[29a] This is what lead us to look for norspermine in *Plasmodium*. Somewhat disappointingly, we found only the very banal putrescine, spermidine and spermine mix, previously reported.[29b]

None of these considerations bear directly on the value of our hypothesis that polyamines' function is to protect cells against oxidative damage, particularly by singlet oxygen. In order to be effective in that role, especially in the protection of nucleic acids to which they bind, polyamines should be available at replication time. Our results in the case of *Chlamydomonas* indicate that spermidine is indeed poised to protect DNA at synthesis time. This is consistent with our hypothesis.

Finally, Table II lists the amounts of polyamines per cell and the volumes of the different cells; included are literature data for *Euglena*.[18] It can be calculated that spermidine is present in millimolar concentrations in the three organisms which contain it. But **in *Chlamydomonas* the concentration of putrescine, averaged over a whole cell, is 0.1M**, an astonishingly high value for a cell constituent of still unclear function![30] And since the localization of the polyamines within the cell is not established, they could be present in extremely high local concentration in, for example, the nucleus or some organelles.

Table II. Concentration of Polyamine (in nmol per 10^6 cells)

	cell vol.(μm^3)[a]	PUT	SPD	SPN	NSPD	NSPN
P. falciparum	4	.01	.03	.005	-	-
C. reinhardtii[b]	50	6	0.2	-	1	-
E. gracilis[c]	2500	1	3.5	-	2	5.5
G. polyedra	30000	-	-	-	-	5

[a] 1 $\mu m^3 = 10^{-15}$ l. [b] Listed are the peak concentrations. [c] See ref. 18.

In contrast, the sole polyamine of *Gonyaulax* is present only at the level of ca. 0.2 mM. Dinoflagellates are unusual among eukaryotes in having a very large amount of DNA (30 times more than in the human nucleus, with no histones nor nucleosomes).[31] One of its genes has been reported to be present in many copies.[32] If the function of norspermine in *Gonyaulax* depended on its association with DNA, one might have expected this polyamine to be present in high concentration. However, it may be that by having multiple copies of each gene, the survival of the genetic information is sufficiently insured by redundancy and less protection is required against oxidative damage than in other cells.

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