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Most of us feel, from time to time, that other authors have not acknowledged the work of our own or other groups or have omitted to interpret important aspects of their own data. Perhaps we have observations that, although not sufficient to merit a full paper, add a further dimension to one published by others. In other instances we may have a useful piece of methodology that we would like to share.

The Editors hope that readers will take full advantage of this section and use it to raise matters that hitherto have been confined to a limited audience.

Christopher M. Thomas, Editor-in-chief

X-ray absorption spectroscopy of bacterial sulfur globules

Sulfur K-edge X-ray absorption spectroscopy is a powerful *in situ* probe of sulfur biochemistry in intact cells and tissues. Under favourable circumstances the technique can provide quantitative information on the chemical identity of the sulfur species that are present in a sample. Prange *et al.* (8, 9) have recently reported an X-ray absorption spectroscopic study of bacterial sulfur storage globules. Unfortunately there are substantial problems with the experimental technique employed that, we contend, lead to completely erroneous conclusions. In the more recent of their two papers Prange *et al.* (9) employed a curve-fitting method similar to that used by us (for more than 10 years) (2, 6, 7). In essence, the method employs simply fitting a linear combination of the spectra of standard compounds to that of the unknown (2), in this case cultures of bacterial cells. This type of analysis can provide quantitative estimates of the individual sulfur types in the sample, but is critically dependent upon the

choice of reference spectra. Prange *et al.* (9) deduce substantial differences between the chemical forms of sulfur stored in the globules of different organisms; they conclude that the globules of *Beggiatoa alba* and *Thiomargarita namibiensis* contain cyclo-octasulfur (S_8), while those of other organisms contain polythionates (*Acidithiobacillus ferrooxidans*) and polymeric sulfur (e.g. *Allochromatium vinosum*). This is in contradiction with our earlier study (7), in which we found that sulfur in all globule species examined resembled that expected for various sized spherical particles of S_8 . The discrepancy is due to an experimental artefact in the work of Prange *et al.* (8, 9) arising from their choice of transmittance detection, which we will now discuss.

X-ray absorption spectra of particulate samples collected in transmittance are known to be susceptible to 'leakage', 'pinhole' or 'transmittance thickness' effects (4). These arise from differences in absorption in different locations on the sample due to heterogeneity in sample thickness; distortions of an identical nature occur either with thin areas in the sample, or with actual holes. These effects distort spectra by attenuating intense features (4, 7). Thus, for transmittance measurements, particular care must be taken to prepare samples of uniform thickness. At the sulfur K-edge this requirement is particularly stringent due to the large X-ray absorption cross-section ($2200 \text{ cm}^2 \text{ g}^{-1}$ for elemental sulfur at 2480 eV). Indeed, a variation in sample thickness of only a few microns can result in significant distortion of the spectra. For example, approximating the variation in thickness as a Gaussian distribution, we calculate that a $100 \mu\text{m}$ thick sample of $\alpha\text{-S}_8$ with a distribution half-width of only $5 \mu\text{m}$ will have more than a twofold attenuation of the most intense feature of the spectrum, relative to the edge jump. Prange *et al.* (8, 9) prepared their samples as finely ground powders on adhesive tape. We have previously pointed out (7) that samples prepared this way are particularly susceptible to 'pinhole' artefacts, and that the resulting data are likely to be non-quantitative and difficult to reproduce experimentally.

Fortunately there is a technique that allows the collection of undistorted spectra of solid samples, using electron yield detection (e.g. 5).

The technique involves detecting electrons ejected from the sample by the X-ray photons, and because the electron path-length is much shorter than the photon path-length, sample thickness is unimportant and undistorted spectra result. As an example, Fig. 1 compares three datasets of $\alpha\text{-S}_8$, one collected using electron yield, and two collected in transmittance. The two transmittance datasets are from Prange *et al.* (8, 9), and are quite obviously different from each other and from the electron-yield spectrum. Both transmittance spectra are clearly distorted to different degrees in the manner expected for pinhole effects (7). The differences between the spectra reported by Prange *et al.* (8, 9) were not noted in the later paper, but nicely illustrate our point that such transmittance data should be hard to reproduce. We note in passing that the near-edge spectrum of $\alpha\text{-S}_8$ reported by others (e.g. 1) agrees with our data. A second major point, also ignored in the work of Prange *et al.* (8, 9), is that solids show quite different spectra from solutions (2). This is probably due to slight structural deformations and long-range order arising from crystal-packing effects, and the use of crystalline solids as standards for biological spectra is inappropriate (2). In the case of solutions the detection method of choice is by monitoring the X-ray fluorescence, although care must be taken that the solution is adequately dilute (3).

► GUIDELINES

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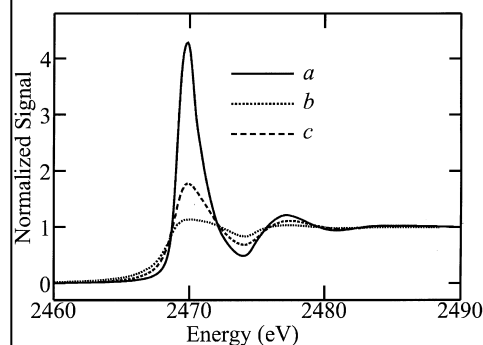


Fig. 1. Sulfur K-edge X-ray absorption spectra of solid α -S₈ measured by using electron yield (a) (data from ref. 7), and transmittance (b) and (c) for two different samples. The data shown in (b, c) were taken from references (8) and (9), respectively (digitized prior to replotting for comparison) with a -1.6 eV energy shift to account for the different methods of energy calibration. Charging artefacts in the electron yield data were eliminated by mixing with graphite.

Unfortunately, although Prange *et al.* (9) cite our sulfur globule paper (7) several times, they do not discuss our findings. Instead, they state that we ‘erroneously assumed that there is no difference between the S K-edge XANES spectra of carefully ground cyclo-octasulfur and polymeric sulfur, ...’ and that we ‘... assume that the reduced white line of cyclo-octasulfur is induced by pinhole effects’. In fact, we stated that no reliable spectrum of polymeric sulfur was available to us, and that the α -S₈ spectrum reported by Prange *et al.* (8) is distorted by pinhole effects – the actual spectrum has an intense peak (or white line), as shown in Fig. 1. In their most recent paper, Prange *et al.* (9) tested their methodology by fitting different mixtures of α -S₈ and polymeric sulfur with the spectra of the components. That accurate results were obtained merely proves that the particle sizes, experimental procedures and overall thickness of the samples were consistent within their test samples. It does not indicate that the data are free from experimental artefact.

In summary, X-ray absorption near-edge spectroscopy (XANES) is a powerful *in situ* probe of sulfur biochemistry, but care must be taken to avoid experimental artefacts, and to employ appropriate standards [e.g. solutions for aqueous biological samples (2)]. The basis of the quantitative analysis of sulfur globules presented by Prange *et al.* (9) resides not only in the spectra of the bacterial samples, but also in the model compound data. Because all of the model data presented by these workers show various degrees of pinhole-induced distortion, neither their quantitative analysis of bacterial spectra nor their conclusions are likely to be correct. This is important because their conclusion that different sulfur bacteria

have different forms of sulfur in their sulfur globules may mislead the field. Our work indicates that the globules contain a form similar to α -S₈ in a variety of photosynthetic and non-photosynthetic organisms (6). As we have previously pointed out (7), the presence of large-scale crystallites of α -S₈ can be excluded based on previous X-ray diffraction results (3). We have therefore suggested (7) a model consisting of a core of fragments with local structures resembling α -S₈ with a modified globule surface conferring hydrophobic properties. This might be due to the globule proteins or alternatively from modification of surface sulfur atoms to incorporate a polar group such as thionate, as previously proposed by Stuedel (10).

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Graham N. George,¹ Ingrid J. Pickering,¹ Eileen Y. Yu¹ and Roger C. Prince²

¹Stanford Synchrotron Radiation Laboratory, SLAC MS 69, 2575 Sand Hill Road, Menlo Park, California 94025, USA.

²ExxonMobil Research and Engineering Co., Annandale, New Jersey 08801, USA.

Author for correspondence:

Graham George. Tel: +1 650 926 4604.

Fax: +1 650 926 4100.

e-mail: g.george@stanford.edu

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X-ray absorption spectroscopy of bacterial sulfur globules: a detailed reply

(A) Biological part

Over the last decades the molecular nature and structure of sulfur globules has been discussed for various bacteria (e.g. 3, 4, 5, 6, 7, 13, 14, 17). It has become clear in all these investigations that the properties of the bacterial sulfur globules do not match those of pure elemental sulfur. Hageage *et al.* (5) obtained evidence using X-ray diffraction that the sulfur in sulfur globules of *Chromatium okenii* is liquid and amorphous, whereas all forms of elemental sulfur are solid and usually crystalline at ambient temperature. Furthermore, for the globules formed by *Allochromatium vinosum*, buoyant densities of only 1.22 g cm⁻³ (3) and 1.31 g cm⁻³ (6) have been determined. The density of liquid sulfur at 23 °C, however, is approximately 1.89 g cm⁻³ (18). The densities of all crystalline sulfur allotropes are in the range 1.9–2.2 g cm⁻³ (14). On the basis of these discrepancies, Stuedel *et al.* (18) pointed out that a large fraction of the sulfur globules must consist of a low density material and Guerrero *et al.* (3) have described the sulfur globules as ‘hydrated sulfur’. In addition, Raman-spectroscopic investigations yielded evidence that the sulfur globules of *A. vinosum*, *Halorhodospira abdelmalekii* and *Halorhodospira halochloris* do not contain significant amounts of cyclo-octasulfur (S₈) (12, 19). Furthermore, the sulfur globules of these bacteria are insoluble in non-polar solvents, which indicates the hydrophilic nature of the sulfur globules. The hydrophilic properties of the sulfur globules can not be related to the globule proteins as speculated by Pickering *et al.* (9) because the sulfur globule proteins of *A. vinosum* are extremely hydrophobic and insoluble in water (2, 8). Stuedel (12) proposed

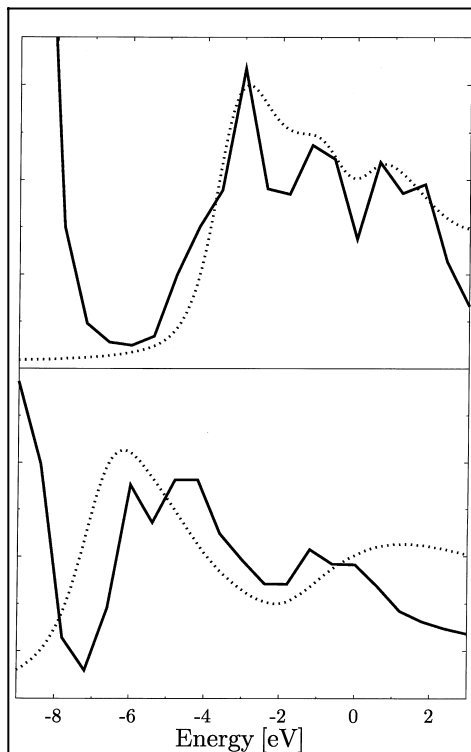


Fig. 1. Sulfur K-edge XANES spectra (dotted lines) of ZnS (top) and S₈ (bottom) compared to the pDOS (solid line) of these materials calculated on 190 atom clusters of different lengths using the FEFF8 code. The experimental spectra have been shifted to the energy scale of the calculation and rescaled.

mixtures of organyl sulfanes with hydrophilic end groups (e.g. cysteine or similar), which are insoluble in non-polar solvents, as the dominant form of sulfur in bacterial sulfur globules. Mixtures of bis-organyl sulfanes with sulfur chains of different lengths are pale-yellow or white-coloured oils with a high refraction index and have chemical properties similar to the S⁰ of the globules (12), which is also hydrophilic, of pale-yellow or white colour, of high refraction index and liquid-like (12, 15). Since chains of sulfur atoms are hydrophobic, the required hydrophilic nature can only be achieved by suitable end groups (R). To make a very hydrophobic molecule at least partly hydrophilic and to guarantee a certain solubility in water, which seems to be mandatory for the transport of the S⁰ material through membranes, cell walls etc., R has to carry an ionic charge or to contain polar groups (14).

Thus, considerable evidence achieved by different methods indicates that the material the globules consist of clearly is not simply solid S₈ as claimed by Pickering *et al.* (9). It should be emphasized that the clear differences found in our work between the species of sulfur in the sulfur globules of different metabolic groups of sulfur oxidizing bacteria reflect the different ecological niches

and physiological properties of these bacteria. A clear correlation between sulfur speciation and occurrence of oxygen during growth of the bacteria is evident: sulfur chains occur in the anaerobically grown phototrophic sulfur bacteria, while sulfur rings (*Beggiatoa alba*, *Thiomargarita namibiensis*) and polythionates (*Acidithiobacillus ferrooxidans*) were found in aerobically grown organisms (10, 11). The influence of oxygen – which can lead to spontaneous ring formation – on the sulfur speciation can furthermore be clearly observed in the spectrum of isolated sulfur globules, which is quite different to that of intact cells of *A. vinosum* (11). It therefore seems plausible that in different bacteria different sulfur species are present, depending on the presence of oxygen in the environment. Our results are also in good agreement with previous findings by Hageage *et al.* (5), Guerrero *et al.* (3), Steudel (13), Steudel *et al.* (17), Mas & van Gemerden (6) and Steudel & Albertsen (15). Thus, beyond the successful verification on sample systems, we find independent support for our results in the literature and can provide a biological interpretation for our findings.

(B) Physical part

Contrary to the statements in the previous article by George *et al.* (12), all three modes of measuring X-ray absorption spectra, namely transmission, electron yield and fluorescence yield, imply pitfalls which may lead to distortions. The inherent problem connected to electron yield techniques is the accumulation of charge on the sample, which can lead to a distortion of the spectrum especially for nonconducting samples. As a remedy, graphite is mixed with the sample [compare with Fig. 1 legend of George *et al.* (12)]. This achieves an electronic contact between the sample and graphite, which is necessary to prevent charge accumulation. During the process of mixing, chemical and/or phase transformations can occur which may influence the measured spectrum. This argument is used in the paper by Pickering *et al.* (9) themselves to explain why none of the readily available other forms of elemental sulfur could be measured using electron yield techniques.

The key problem related to the fluorescence yield technique, however, is self-absorption, especially in samples with a high concentration of the target element. Pickering *et al.* (9) follow a theoretical scheme to correct for self-absorption effects in their paper to turn this weakness into a strength and propose a way to determine the size of the globules. They use a spectrum of α-S₈ measured in electron yield as the undistorted spectrum of α-S₈ and attribute deviations from it to self-absorption. To extract the particle radius

from this information, one needs to know the shape of the particle and its density. The method is not verified on a sample system with known particles and particle sizes. Until now, the procedure has only been applied to α-S₈ (9). None of the other solid forms of elemental sulfur is included in the basis set for the fit. Using their model, previously determined densities by Guerrero *et al.* (3) of the sulfur globules lead to unrealistic particle radii. These data are discarded and instead the ones of α-S₈ are used. Under these conditions, it turns out that of course α-S₈ contributes strongly to the best fit.

Measurements performed in transmission mode on powder samples may also yield distorted spectra. In fact, the risk of such artefacts is especially high for powder samples at soft X-ray energies due to pinhole effects. As a matter of fact, the discrepancy displayed between our measurements on cyclo-octasulfur (S₈) shown in Fig. 1 of the article by George *et al.* (12) might be due to a pinhole effect. Another possible reason, however, could be a different experimental resolution during the different sets of measurements, as this is dependent on the parameters of the electron beam, which considerably complicates a direct comparison between spectra measured at different synchrotron radiation sources. In addition to that, one should not forget that commercially obtained elemental sulfur as used for the last set of spectra presented in our recent paper, is not pure cyclo-octasulfur (S₈) but always contains varying minority contributions of cyclo-S₇ and in most cases certain amounts of polymeric sulfur (14).

Consequently, we have no definite proof that distortions of the spectra do not occur in the data we show. But in contrast to George *et al.* (12), we feel reassured by the reliability of our analysis proven on various sample systems that these effects are sufficiently small. In this context, it is essential to bear in mind that the pinhole distortions in a spectrum are a non-linear function of the amount of radiation that leaks through the actual sample. To clarify what this implies, consider A) a distortion-free sample which reduces the intensity I₀ of the incoming beam to I₀e^{-μd}, and B) a sample with identical absorption which allows an additional leakage current of I₀c. From these two samples, we obtain a true spectrum μ_ad = -log(I/I₀) and a distorted spectrum μ_dd = -log[(I/I₀) - c]. For a sample C made of the left half of A and the right half of B, one determines an absorption μ_cd = -log[(I/I₀) - 0.5c]. The 'correct' decomposition of this spectrum is 0.5μ_ad + 0.5μ_dd = -{0.5log[I/I₀] + 0.5[log(I/I₀) - c]}. This is clearly different from the determined absorption, especially if c is big.

So, if our cyclo-octasulfur (S₈) spectrum is in fact heavily distorted and the polymeric sulfur spectrum is significantly less distorted, it should not be possible to fit the spectrum of

a mixture of both sulfur species using these reference spectra as demonstrated in the section 'verification of the quantitative analysis' (11).

What remains is an interesting physical question: why do the spectra of S_8 taken in transmission and electron yield mode look that different from each other? Presently, we cannot provide a final answer to this question. To us, it seems strange that such a drastic distortion effect should occur only when dealing with elemental sulfur. Our disbelief in this explanation is backed by theoretical calculations using the FEFF8 code (1). Fig. 1 shows a comparison between the calculated S pDOS (p-projected density of states) and the measured S K-XANES (X-ray absorption near edge structure) spectrum of ZnS. Evidently, the relative intensity of the structures in the white-line region of the measured spectrum below the ionization edge is directly reflected by the intensity of the corresponding structure in the pDOS. Also displayed is the pDOS calculated for S_8 at the S K-edge in comparison to an experimental spectrum. Although the energy scale of the calculation appears slightly contracted, the split structure between -5 and 0 eV in the pDOS is readily assigned to white line and first shape resonance of the spectrum. The relative intensity of these peaks is about 1.5:1. This is rather similar to the relative intensities of these structures in our measurement and relatively different from a value of 4:1 assumed to be true by George *et al.* (12), even if one considers the possibility of a varying transition matrix element. Still, due to the imperfect calculation we are not ready to discard the spectra obtained from electron yield data based only on the evidence at hand and think that the reason for the drastic differences between the spectra measured in different modes is an interesting question to be looked into.

Alexander Prange,¹ Christiane Dahl,¹
Hans G. Trüper,¹ Reinhold Chauvistré,²
Hartwig Modrow² and Josef Hormes²†

¹Institut für Mikrobiologie & Biotechnologie,
Rheinische Friedrich-Wilhelms-Universität
Bonn, Meckenheimer Allee 168, D-53115
Bonn, Germany

²Physikalisches Institut, Rheinische Friedrich-
Wilhelms-Universität Bonn, Nußallee 12,
D-53115 Bonn, Germany

†Present address: The John Bennett
Johnston, Sr. Center for Advanced
Microstructures & Devices (CAMD), Louisiana
State University, 6980 Jefferson Highway,
Baton Rouge, LA 70806, USA

Authors for correspondence (biological
aspects): Alexander Prange. Tel: +49 228
732119. Fax: +49 228 737576.

e-mail: A.Prange@gmx.de (physical aspects)
Hartwig Modrow. Tel: +49 228 733203.

Fax: +49 228 737869.

e-mail: Modrow@physik.uni-bonn.de

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Morphogenetic checkpoint in fission yeast? Yes!

Cylindrical-shaped fission yeast cells grow at their cell tips, where actin is polymerized and forms patches. Length growth has to continue until cells reach a critical length, when they enter mitosis; this mechanism is called cell-size (or mass) control. Very recently, Rupeš *et al.* (5) have carefully analysed the effect of the actin depolymerizing drug latrunculin A (Lat A) on cell growth and cell-cycle progression of fission yeast cells (5). As expected, the drug slows down (in a concentration-dependent manner) cell length extension by disrupting the actin cytoskeleton and it also causes a mitotic delay at lower concentrations, or a G2 block at higher concentrations of the drug. This delay of mitosis can be explained either by the activation of a morphogenetic checkpoint or by prevention of the cell reaching a critical length.

Rupeš *et al.* (5) distinguish between these two possibilities by using oversized cells made by a temporal cell cycle block in a *cdc25^{ts}* (temperature sensitive) mutant. Since oversized cells were not blocked in G2 by even a high concentration (10 μ M) Lat A treatment, the authors rule out the possibility of a morphogenetic checkpoint (5). Although all of these conclusions seem to be straightforward and logical, they are based on the following paradigm: any checkpoint mechanism should work equally well in cells irrespective of their size. However, this assumption is not necessarily valid and actually it contradicts the present models of cell-cycle controls (8).

The measured parameters in former size control studies were either protein contents (2) or cell lengths of fission yeast cells (1, 4, 6), and both ways were correct, since in exponentially growing cultures these two parameters are closely correlated during the division cycle of a cell. The ultimate question is how cells measure their size (or mass) and how the size-control checkpoint operates. Despite the long history of these control mechanisms, an absolutely satisfactory answer cannot be given. However, it seems more likely that cells do not measure their size (e.g. length) *per se* but rather the concentration of a molecule that is proportional to their total cytoplasmic mass. That cell length is not the critical factor for entering mitosis is also obvious from the data in Fig. 1B of Rupeš *et al.* (5): by estimating cell length at the time when 50% of the cells entered mitosis, we find that cell length progressively decreases with Lat A concentration (Fig. 1). If it is not cell length that is measured before mitotic onset, then what is it?

The master regulator of the fission yeast cell cycle is the Cdc2/Cdc13 protein kinase. Cdc2/Cdc13 activity can have three characteristically different values during the cycle: a

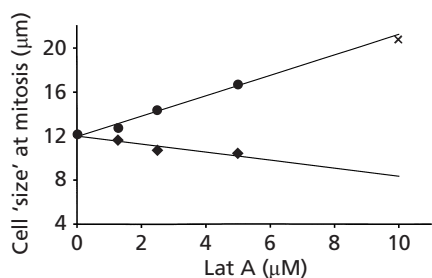


Fig. 1. Effects of Lat A on mitotic size of wild-type fission yeast cells. On one hand, increasing Lat A dose increases the mass (protein) requirement of mitotic onset (●), causing an increasing delay in G₂. On the other hand, increasing Lat A decreases growth rate in cell length, leading to an apparent decrease in cell length at mitosis (◆). Diverging cell mass from cell length results in a density increase, which at a critical dose (somewhere between 5 and 10 µM) prevents mitotic onset and this morphogenetic checkpoint blocks the cells in G₂. Cells having an abnormally large length (oversized *cdc25^{ts}*), may override this checkpoint (×). The calculations were made using the data from Rupeš *et al.* (5).

very low value in G₁ phase, an intermediate value in S and G₂ phases, and a high value in mitosis (8). Which activity of the Cdc2/Cdc13 kinase is taken depends on total cellular protein content. As a consequence, to get to the higher Cdc2/Cdc13 activity stage and to the next cell-cycle phase, the cell has to reach a critical cytoplasmic mass (protein content). In this picture the (nuclear) concentration of the Cdc2/Cdc13 kinase is dependent on cytoplasmic mass. For the present discussion the G₂/M transition that is important is inhibited by the Wee1 tyrosine kinase and promoted by the Cdc25 phosphatase; the enzymes that are phosphorylating and dephosphorylating the Tyr15 residue of the catalytic Cdc2 subunit, respectively.

Imagine that Lat A, by disrupting the actin microfilament system, activates a morphogenetic checkpoint in fission yeast as it does in budding yeast (3). The morphogenetic checkpoint in budding yeast blocks initiation of mitosis via tyrosine phosphorylation of Cdc28/Clb complexes (the budding yeast homologues of Cdc2/Cdc13), which is not characteristic of the normal cell cycle [One major difference between the two yeasts is that fission yeast tyrosine-phosphorylates Cdc2/Cdc13 dimers even in the absence of actin disruption (i.e. in the absence of a morphogenetic checkpoint)]. If Lat A treatment increases the ratio of Wee1:Cdc25 activity in a concentration-dependent manner, either by up-regulating Wee1 or down-regulating Cdc25, then the threshold cell mass at which cells can enter mitosis, will be increased (but not to infinity!). This increase in the threshold mass could be an alternative explanation for the mitotic delay observed in

wild-type cells after Lat A treatment by Rupeš *et al.* (5).

Analysis of the data of Rupeš *et al.* (5) suggests that this is indeed the case (Fig. 1). Let's assume that Lat A has no effect on overall protein synthesis. Therefore after Lat A treatment the balanced growth between cell length and protein content is dissociated: cells are increasing their cytoplasmic mass at a normal rate (at least for some time) while their length and volume extension is slowed down. From the data given in Fig. 1B of Rupeš *et al.* (5) we evaluated the (linear) length growth rate, and found that it is sharply decreasing with increasing Lat A dose (data not shown). With the assumption that mass (protein) growth rate is not influenced, we can calculate the relative cell mass at mitosis in arbitrary cell-length units (cell mass is expressed in length units, which means how long the cell with this mass would be if balanced growth were not perturbed by Lat A treatment), and this quantity increases linearly with Lat A dose (Fig. 1). This correlation between Lat A concentration and calculated cell mass at mitosis can be observed up to 5 µM Lat A. The only analysis with higher concentrations was at 10 µM when wild-type cells could never enter mitosis. The extrapolation of the regression line (estimated between 0 and 5 µM Lat A) to 10 µM Lat A gives a threshold mass value for mitosis of 21 µm units (Fig. 1). However, this value cannot be reached by wild type cells starting with small mass (and length) values and inhibited in length growth, because their density should increase more than 2.5-fold above the normal value. As a consequence, small wild type cells treated with 10 µM Lat A will not enter mitosis.

However, as Rupeš *et al.* (5) established, the oversized cells (produced by *cdc25^{ts}* temperature block and release) can enter mitosis in the presence of this drug concentration, if they are longer than 20.7 µm (5). Observe that this data point is situated almost exactly on the extrapolated regression line (Fig. 1). Oversized cells can enter mitosis because their balanced growth is perturbed by the Lat A treatment at higher mass value and their density increase does not become effective before reaching the threshold mass value. This result supports the notion that there is a mass (protein) requirement of mitotic onset in fission yeast rather than a length requirement, and this threshold mass is elevated by the disruption of the actin cytoskeleton (morphogenetic checkpoint).

By analysing the response of different cell-cycle mutants to a treatment with 10 µM Lat A, Rupeš *et al.* (5) established that Cdc25 is a target of the mitotic size-control mechanism rather than Wee1. This is in sharp contrast to former experimental results, which all proved the opposite; namely that *wee1* is an essential gene operating in this process (2, 6), but *cdc25* is not (7). This contradiction can be simply

solved now, after showing that the experimental protocol used by Rupeš *et al.* (5) is not suitable to study size control. Instead, they have discovered that the morphogenetic checkpoint acts through *cdc25*.

Based on this analysis of the data of Rupeš *et al.* (5), we propose the existence of a morphogenetic checkpoint in fission yeast, which operates as it does in budding yeast (3). The only difference between the two yeasts in this respect is that the checkpoint down-regulates the Cdc25 activity in fission yeast rather than stabilizes Wee1 (the budding yeast homologue of Wee1).

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Akos Sveiczler, Attila Csikasz-Nagy and Bela Novak

Department of Agricultural Chemical Technology, Budapest University of Technology and Economics, H-1111 Budapest, Szt. Gellert ter 4, Hungary

Author for correspondence: Akos Sveiczler.
Tel: +36 1 4632349. Fax: +36 1 4632598.
e-mail: ASveiczler@mail.bme.hu

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Morphogenetic checkpoint in fission yeast? No!

Fission yeast do not have a ruler. They cannot use their length as a direct measure of their

size, and we agree with Sveiczzer *et al.* (6) that it would be difficult to suggest otherwise. Although simple experiments clearly point to a sizing mechanism, we do not yet understand what biochemical correlate of size is actually measured by cells. Concentration of a particular protein, total protein mass, the density of ribosomes in the cytoplasm, cell volume and various other structurally based determinants may ultimately all be found to contribute to an integrated measure of cell size. For the moment, simple measures of length, volume or mass must serve as proxies for the underlying mechanism.

In their Comment, Sveiczzer *et al.* (6) reinterpret aspects of our work in favour of an alternative hypothesis unrelated to size control. They make two assumptions. First, from a theoretical model summarized by Tyson *et al.* (7), they assume that cells measure their size through accumulation of the Cdc13/Cdc2 complex in the nucleus in a way proportional to the total cell mass. Arguably, accumulation of Cdc13/Cdc2 during G2 depends on cell growth. The role of Cdc13/Cdc2 accumulation as a sole measure of cell size, however, is not well supported by the evidence, since its accumulation in the nucleus levels off well before cells enter mitosis (1). This does not challenge the conceptual framework for understanding the dynamic behaviour of the cell-cycle regulatory network as outlined by Tyson *et al.* (1), but rather encourages us to look for other components that may communicate the information about cell size to the network. We propose that Cdc25 is one such component.

Sveiczzer *et al.* (6) believe that we misinterpreted our data and that Cdc25 is in fact a downstream effector of a morphogenesis checkpoint. This checkpoint is proposed to monitor the integrity of the actin cytoskeleton as originally described by McMillan *et al.* (4) in budding yeast. To support their argument, in drawing their protein mass line Sveiczzer *et al.* (6) make a second assumption. They assume that the rate of protein synthesis in cells remains unaffected after actin has been depolymerized by a drug such as Lat A. This is quite apart from the inability of the cell to grow in length under these conditions. In their interpretation this leads to continuing mass accumulation and an increase in protein

density. This is an *ad hoc* premise that has not been tested and has no support in the literature. Indeed, there is evidence to the contrary, showing that the rate of protein synthesis gradually decreases after cells have been exposed to actin-depolymerizing drugs (3). In addition, there is increasing recognition that cells may tolerate only limited macromolecular overcrowding (2). However, even if we assume that overall protein synthesis continues unaffected for some time after the exposure of cells to Lat A, problems remain with the rest of the argument. Most importantly, the value of 20.7 μm taken from our data at 10 μM Lat A, which the authors equate with a minimum cell mass at which cells are able to enter mitosis under these conditions, is in fact an arbitrary value. It depends entirely on the duration of the *cdc25-22* conditional arrest used to generate oversized cells. In the experiment in question (Fig. 2 in ref. 5), the 20.7 μm refers to a cell length at the second mitosis after release from a 3 hour arrest. A longer arrest would produce larger cells at mitosis and *vice versa*. Keeping in mind that their projection of the rate of cell mass increase would be independent of duration of *cdc25-22* arrest, the fact that 20.7 μm falls on their line is due to chance alone. Presumably, any *cdc25-22* cells larger than $\sim 17 \mu\text{m}$ (mitotic size for this strain at permissive temperatures) will have little trouble initiating mitosis in Lat A.

The actin checkpoint model does not explain other experiments reported in our paper. The model predicts that perturbation of actin will arrest progression into mitosis even after a change of critical size such as that induced by nutritional shift. Furthermore, if Cdc25 is not involved in cell size control then Cdc25 deletion strains made viable by heterologous tyrosine phosphatase activity would accelerate mitosis after a nutritional shift with kinetics similar to wild type. Instead, as we see in Fig. 8 of ref. 5, exponentially growing wild-type cells accelerate mitosis whether Lat A is present or not, and in a strain lacking Cdc25 this acceleration is delayed.

Two other points are important to emphasize. By no means are our results in contradiction with earlier results identifying Wee1 as a component of the mitotic cell-size control. Our results are a step towards

defining distinct roles for both Wee1 and Cdc25 in this process. Critical cell size is set by a Wee1-dependent process and mitosis is released by Cdc25 activation (see our model in Fig. 9 in ref. 5). Finally, the idea of a cell-size checkpoint does not contradict the view that conventional checkpoints operate by raising the barrier and with it the apparent critical size that has to be overcome to allow transition from one stable dynamic state to another (7). The difference is that the cell-size checkpoint through its molecular mechanism simply prevents reaching that barrier if cell growth is perturbed.

We believe Cdc25 responds to a correlate of cell size and not to the state of actin itself. We also continue to believe that the data in our paper are best interpreted as in support of this model.

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Ivan Rupeš and Paul G. Young

Dept. of Biology, Queen's University,
Kingston, ON K7L 3N6, Canada

Author for correspondence: Paul Young.
Tel: +1 613 533 6148. Fax: +1 613 533 6617.

e-mail: youngpg@biology.queensu.ca

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