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Life behind cell walls: paradigm lost, paradigm regained

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Abstract. This review of the living cell wall [1] and its protein components is in two parts. The first is anecdotal. A personal account spanning over 40 years research may perhaps be an antidote to one stereotypical view of scientists as detached and humorless. The second part deals with the meaning of function, particularly as it applies to hydroxyproline-rich glycoproteins. Function is a difficult word to define objectively. However, with help from such luminaries as Humpty Dumpty: "A word means what I want it to mean, neither more nor less," and Wittgenstein: "Giving examples of usage … is the only way to talk about meaning," it is possible to construct a ziggurat representing increasingly complex levels of organization from molecular structure to ecology. Forty years ago I suggested that hydroxyproline-rich structural proteins played a key role in cell wall functioning. But because the bulk of the wall is carbohydrate, there has been an understandable resistance to paradigm change. Expansins, paradoxically, contribute greatly to this resistance because their modus operandi as cell-wall-loosening proteins is based on the idea that they break hydrogen bonds between polysaccharide chains allowing slippage. However, this view is not consistent with the recent discovery [Grobe et al. (1999) Eur. J. Biochem **263:** 33–40] that β-expansins may be proteases, as it implies that the extensin network is not a straightjacket but a substrate for expansin in muro. Such a direct role for extensins in both negative and positive regulation of cell expansion and elongation may constitute a major morphogenetic mechanism operating at all levels of plant growth and development.

Key words. Cell wall protein; extensin; HRGPs; hydroxyproline-rich glycoprotein; arabinogalactan protein; cell extension; expansin; hydroxyproline glycosylation.

Introduction

Always wanted to be a biochemist! And the South Downs[1] chalkland flora, a treasure trove of squinancywort, bee orchids and blue gentians, helped mold a future plant biochemist. This personal account of triumphs and vicissitudes during the journey describes aspects of the research game [2], sometimes described as 'living with failure' but rarely published [3]. Yet our understanding of science is immeasurably enhanced by understanding the humanity behind it, from the invention of the *bain-marie* [4] to Archimedes' cry of Eureka!

[1] Recently designated as a future National Park.

Early influences

Beginning in the early 1940s, around the age of 7 or 8, as an only child left to my own devices I discovered how to flush out earwigs (*Forficula* sp.) by pouring soapy water down worm holes[2]. Then came the breeding of mice, guinea pigs and rabbits[3]. Later, no doubt inspired by the WWII 'Brighton Blitz' I decided to make my contribution to the European Civil War. At 10 I was making fireworks guided by Sherwood Taylor's *The Young Chemist* a precious gift from my first science teacher, Henry Eastham of Blackburn where I had been evacuated, whereas Dad,

[2] Such use of the amphiphilic effect unwittingly predated Star Trek.
[3] How my mother put up with all this I do not know, but she was a truly remarkable woman, a veteran of one of the earliest 'hip replacement' surgery with chloroform anaesthetic and somehow survived simultaneous appendectomy and caesarian section.

who was in the infantry at the infamous Anzio Beachhead, helped win the war. We returned to Brighton in time for the fireworks of D-day and other depravities. My second chemistry teacher, the truly inspirational Harry Hobdell of Varndean School, Brighton, lamented that I had been 'bitten by the biology bug' but how did I get those chemicals to start a soilless garden?! Even the police were baffled by a little boy stealing shingle from Brighton beach[4]. The hydroponic scam was quite new to them. It was, of course, a perfect solution to gardening in a flat that didn't have a garden. And it became the slippery slope to cell culture and a lifelong addiction as a problem-generating, problem-solving scientific junkie.

Winning money in the local Dupree chess tournament[5] confirmed the habit, and at 17 I knew that I wanted to be a plant biochemist. Inspired by those immortal lines – 'The microbe is so very small you cannot make him out at all' – I used my chess winnings to buy a microscope. Now I could visit the invisible world of Antonie van Leeuwenhoek. But boyhood friends predestined for the priesthood sowed the seeds of doubt when they assured me that plants too had souls, albeit vegetable rather than animal souls. Despite Wohler's 1828 synthesis of urea [5], Pasteur, the Buchner brothers [6] and Darwin, vitalism was still very much alive then, as it is today. For a rebel both by nature and nurture it was a philosophical proposition that a reductionist biochemical approach could resolve.

Of course, there was only one place to resolve such questions.The day that letter arrived was surely the happiest day of my life – certainly the proudest day for Mum and Dad. Few parents 'in the hotel trade' (waiter and waitress to you) had sons or daughters accepted at Cambridge University. True this was postponed by a two-year hiatus (1953–55) at the RAF Radio School at Yatesbury as a National Serviceman:

> We are the Royal Air Force,
> We can do nothing right,
> We cannot fly or do P.T.
> We cannot even fight,
> But when it comes to Pay Day,
> We shout with all our might,
> *Per Ardua ad Astra,*
> Blow you Jack, I'm all right!

There I graduated as an instructor in radio theory. Those pretransistor days were an invaluable introduction to electronics and all those wonderful gadgets. My poor students could never understand why the copy of *Nature* on my desk minus the expected eye candy belied its title. Nor could they understand how a mere corporal fraternized daily with an attractive WRAF officer, ostensibly to learn Latin (a feudal remnant of the Cambridge entrance requirements) instead of the ritual end-of-day classroom cleaning. They did understand that learning with me was often fun – singing the jingle 'One over two pi root LC equals the resonant frequency' was their favorite. At times it became quite hilarious, especially when armed with a new-fangled tape recorder in 1954. Recorded kit inspections by 'the dimmest squadron leader in the RAF' and the drunken antics of a certain notorious sergeant helped to keep the lads amused.

The university, 1955

Literally within a few days the instructor reverted to student with amusing differences between the two cultures – service life and academia. The blunt no-nonsense orders-is-orders gave way to academic solipsistic periphrasis and unimpeachable manners combined with the occasional terminological inexactitude – academics *never* lie! Of course, the senior tutor of Pembroke College, W. A. Camps, did occasionally lay down the law: 'Gentlemen are reminded that women are not allowed in college before 8 o'clock in the morning' was one of his more severe strictures. Dean Meredith Dewey, a saint in saint's clothing, served divine cake from 'Fitzbillie's' (best cake shop in town) at his Sunday musical evenings, truly in loco parentis. Other 'Pemmas' of that era, destined for fame and fortune, included the comic Peter Cook (whom I never talked with) and Ray Dolby, who had a great hi-fi (well he would, wouldn't he?), served good coffee and had gadgets galore in his car.

I had been to a few exciting seminars before, but at Cambridge even normal lectures seemed like an unending feast. (I have since become more critical after attending too many really boring seminars that argue for renaming *Homo sapiens.*) And the range of personalities was enough to make an organization man blanch. I vividly recall being totally gobsmacked when J. C. Kernohan told me that the enigmatic Hill of the 'Hill reaction' [7] was in the department of biochemistry, and the fiercely critical David Keilin, who had (re)discovered the cytochromes[6] was in the Molteno Institute close by. Like Forrest Gump and his box of chocolates, I treasure personal contacts with other 'greats' like the physiologist E. D. Adrian[7]

[4] Erosion of British beaches by predatory gardeners is now a serious problem.

[5] No doubt spurred on by my headmaster who had described me, in the front of 750 other boys, as 'conceited'. Oh the inhumanity of man to boys! Nowadays we would just say that I had a good self-image.

[6] His lectures sounded like firsthand accounts of great battles of the past.

[7] Chancellor, vice-chancellor and sometime master of Trinity he reportedly said: 'The time may come when the colleges may become alms-houses for the old and cafeterias for the young.' How prophetic.

(brain waves) and R. A. Fisher (statistics). A few seminars were quite unforgettable. For instance J. C. Kendrew's seminar describing the structure of myoglobin via X-ray crystallography was remarkable. That crystal ball correctly predicted a trip to Stockholm in 1962 along with his colleague Max Perutz[8] [8]. Their use of EDSAC (electronic delay storage automatic calculator)[9], even with only 2000 valves (tubes) and miles of punched paper tape, also showed how useful computers would eventually become. There is a point to all this name dropping, surely one of life's lesser vices: Although we can't all be Fred Sangers and Francis Cricks, we sure can learn from them. But would I make the grade? I won a 'half blue' for chess, definitely a time-consuming sporting activity. In 1958, presiding over the final examinations in biochemistry (including a 3-day practical) were H. A. Krebs (TCA cycle) and F. G. Young, respective heads of biochemistry at Oxford and Cambridge. Somehow I survived yet another round of Darwinian selection for *Homo biochemicus* and was sent to plead for financial support from the Agricultural Research Council in London. The ARC committee was not too impressed with my presentation and my idea of studying the growing cell wall of tissue cultures. To the majority of the committee cell walls were dead, always were, always would be – paradigm[10] lost – apparently they were unaware of the 19th-century view of the growing cell wall as a living structure[11] permeated with cytoplasm. For example Wiessner (1888): 'Die Zellwande sind, zum mindesten so lange sie wachsen, eiweisshaltig, … das Wachstum der Zellhaut ist ein actives, und dieser überhaupt bis zu einer gewissen Grenze ihres daseins ein lebendes Gebilde' (cited in my 1965 review [9]). That might have been the end of the story had it not been for a minority of one, Sir Rudolph Peters [10], who like the proverbial deus ex machina, saved the day. He leaned forward saying 'I see you are interested in the *living* cell wall'. And that is how D. H. Northcote accepted a new research student into his laboratory – and the paradigm regained.

To Northcote's immense credit – amazingly at that time no one was growing plant tissue cultures at Cambridge – he let me try out a really crazy idea: Gautheret's book on tissue culture depicted plant cells growing rather like fungal hyphae on the agar surface [11]. Inspired by E. F. Gale's eye-opening course on chemical microbiology, P. R. White's primer on plant tissue culture [12] and Nickell's seminal paper [13], I thought why not grow those plant cells in shake culture? I tried about 2 dozen different species (including *Rubia* sp.[12]). By far the best turned out to be the sycamore culture isolated from a tree (*Acer pseudoplatanus* L.) that I felled in Madingley Wood with help from J. P. Thornber, a classmate also in the Northcote lab. It grew phenomenally well in liquid shake culture, duly reported at the Annual Meeting of the Biochemical Society in 1960 at Cambridge [14], with figures presented there, and reproduced here for the first time (fig. 1). That meeting undoubtedly dates the first 'off-the-wall' statement – carried away by the moment, I described how plant cell suspension culture growth was similar to that of other microbes. 'For example for a few days they grow in log phase – and here is the original log', which I produced from under the podium. There was a shocked silence, followed by some sharp intakes of breath, while I continued to describe the first isolation of primary cell walls[13] from meristematic tissue[14]. However, the tortured analogy bothered Robin Hill not at all later on when, with culture flask in hand, I entered his lab next door to ours and very cheekily said, 'Look Dr Hill, I've turned a tree into an alga.' He replied, 'Wouldn't it be wonderful if you could turn an alga into a tree'. Touché!

To me, Hill[15], along with Joseph Needham, the biochemist-cum-sinologist[16], was the most amazing personality in the Cambridge galaxy. Peter Mitchell, the chemiosmotician (Nobel laureate 1978), had left just before I arrived – but I treasured his desiccator that I inherited as well as a lab bench where, legend had it, Joan Keilin had first crystallized myoglobin. Frederick Gowland Hopkins [15], one of the discoverers of vitamins (Nobel Prize 1929), had long left, but the legacy of his enlightened approach as the first professor of biochemistry at Cambridge remained palpable [16]. Sadly, I never met the botanical philosopher Agnes Arber [17] of the botany department headed by G. E. Briggs the formidable theo-

[8] This story [310] is probably inaccurate. I hope he forgave me.

[9] Judy Goldstein, one of EDSAC's 'baby sitters' on the evening shift in the Cavendish Laboratory, told me that her job was to replace burned-out valves.

[10] Used by scientists to imply a model based on shared rules and standards [27].

[11] This view was not entirely lost in the 20th century. For example, Preston [311] viewed the primary wall 'not as an enclosing sheath which reacts passively to stimuli from within the cell but as part of the whole growing organism'.

[12] I wanted to steal Robin Hill's line on showing visitors around his garden: 'Each year I grow a little Madder.' This has finally been achieved [312].

[13] As defined by Kerr and Bailey [313].

[14] At the same meeting E. C. Cocking described how to isolate protoplasts by enzymic degradation of the wall.

[15] Robin Hill anecdotes are the stuff of legend. My favorite is how he duped new colleagues to enter the biochemistry department building via the coal chute of an adjacent building, and then asked, 'Do you always come in this way?'

[16] I have to record my debt to 'one of Britain's greatest scholars' (as *The Sunday Times,* 26 March 1994 described him on his 94th birthday) for his kindness in offering me the use of his private library. And his charming habit of taking tea with the practical class when taught by his wife 'Doffy' gave us fascinating glimpses of work in progress, his heroic multi-volume magnum opus, *The history of science and civilization in China.*

retician who, with J. B. S. Haldane, developed the idea of the enzyme-substrate steady state. Lateral thinking [18] was, I suspect, Hill's only mode of thought. Like the oracle of Delphi, he would freely answer questions with even more difficult, abstruse yet ultimately revealing truths. His seminar questions were classic – 'Is the cell wall inside or outside the cell?' got to the heart of the matter 4 decades before the current revisionists. Hill was inevitably 'honored' at the biennial biochemistry department (a.k.a. the Sir William Dunn Institute of Biochemistry) Christmas pantomime, by some spoof or other. The year I helped the irrepressible Guy Greville (with his legendary insistence on clean glassware) to write 'Alice in Dunnderland', we cast the Cheshire cat as Robin Hill reciting a new version of 'Jabberwocky' appropriately renamed:

Photosynthy

'Twas brillig and the cytochromes
Did gyre and gimble in their chains
All blunty were the microtomes
And Robin Hill outbrains.
Beware the carbon path my child,
The hemes that howl, the plasts that claw
Beware the Thunberg tube
And shun the verdious chlorophyll
Long time the xanthophyll[17] he sought,
So quantised he by the grana tree
And stood awhile in thought.
And as in algal thought he stood,
The photosynth with eyes of flame
Came skiffling through the spectroscope
And ribulosed he came.

For some unaccountable reason, a sheik played by César Milstein appeared in our version of the pantomime. Somehow, he and his charming wife Cynthia survived both the pantomime and dinner that Michael Proctor and I cooked in the laboratory, and he went on to win a Nobel Prize (1984) for monoclonal antibodies.

Now it was possible to build further on earlier workers who had isolated bacterial cell walls [19] and algal cell walls [20]. Disruption becomes easier as cell size increases, so it was easy to isolate a relatively clean wall preparation[18]. And then it was at last possible to test a major hypothesis behind the tissue culture approach – the idea that in rapidly growing cells the wall contained enzymes required for its own synthesis. If true, it followed that these isolated primary cell walls should also contain protein. A surprisingly large amount, it turned out, but was it cytoplasmic contamination or wall protein per se? A yellow-brown ninhydrin positive spot unique to the wall hydrolysate was obviously not a normal protein amino acid, but what was it? It was not difficult with Fred Sanger's[19] lab right next door (directly opposite Robin Hill's lab). And so one of his coworkers, Leslie F. Smith, guided me to the specific isatin/Ehrlich's reagent test for hydroxyproline after high-voltage paper electrophoresis of the wall hydrolysate at pH 1.9. Working late at night in the lab (when all the best discoveries are made), Les and I watched the blue spots fade. 'No hydroxyproline', I thought. Then, slowly, a most beautiful vivid purple color developed. What a feeling! Like my whole life had been lived for that moment. But then angst set in – was it an artifact? Had a piece of my own collagenous skin nervously chewed off somehow dropped into my wall preparation? So the work had to be repeated, hastily, because an exciting trip behind the iron curtain to Poland was just days away. Angst turned into triumph. Walls prepared with scrupulous attention still tested positive. Guided by Elizabeth Cawlwell in Doffy Needham's lab, just down the hall, quantitative Hyp assays nailed down the point: the implication of a structural protein was clear by analogy with the animal protein that glues us together, i.e. collagen. Incidentally, it shows the advantage of a broadly based biochemistry course rather than a narrowly defined plants-only approach. It also shows what a crucial difference supportive colleagues and mentors make.

Like all good ideas, it had to run the merciless gauntlet of academic criticism. What seems so obvious and uncontroversial now was quite outré then. My research supervisor, D. H. Northcote[20], was healthily sceptical, demanding a wide range of isolation conditions, but he did, bless his heart, allow me to make my own mistakes[21]. My dear friend and fellow student Michael H. Proctor[22], was just as sceptical, suggesting an insoluble protein only loosely associated with the wall. Even after many hours of sonication, the Hyp content of the tiny wall fragments had not changed – the 'bug hut' sonicator was never the same again. Counterbalancing these local sceptics, J. E Varner, on sabbatical leave from Ohio State University, had the rare gift of critical encouragement combined with a wry

[17] Cf. [314].

[18] Of course others, notably Robert Bandurski, also saw the importance of isolating cell walls from plant tissues [315].

[19] Pick a good problem and stick with it was the lesson I learned from Fred Sanger.

[20] An earlier allusion [316] to an armchair referred to a lab practical joke. As homage to the hyperindustrious Don Northcote, who was always very active in the lab, we smuggled the objet d'art into the lab in the dead of night. Next morning the prominently displayed 'armchair of plant biochemistry' failed to amuse.

[21] While the admirable Cambridge tradition of casting students into the lab to forge their own research destiny represented real academic freedom, the absence of lab meetings, surely an essential didactic tool, was unfortunate.

[22] From whom I learned: (i) the meaning of 'bucket biochemistry', (ii) the only substitute for data … better data and (iii) the antidote to passivity in scientific 'literature' [317].

sense of humor[23], an inspirational personality to countless students and sadly missed [21]. While Joe Varner regarded himself rather as a midwife to extensin, others were no less helpful. The noted protein chemist Kenneth Bailey donated technician, time and equipment for the first quantitative amino acid analysis of a primary cell wall. In those days a single quantitative amino acid analysis took about 2 days.

But there was trouble ahead. Shortly after we had submitted a short note to *Nature*, I was ensconced in one of my favorite haunts, the botany library[24], where Northcote tracked me down. He had a worried look on his face. 'Steward says it's in the cytoplasm.' I was not perturbed. Unlike F. C. Steward (one of the first to detect Hyp in cultured cells [22]), I had not discarded the cell wall fraction, yet his international reputation put Northcote in a predicament, a research supervisor possibly faced with an incompetent or perhaps even a dishonest student. (Northcote would not have had the chutzpah to publish a retraction in *Nature* and then list two *Nature* publications in his CV, as others have.) Yet it happens, sometimes undetected. At one of the Wednesday departmental seminars, quaintly referred to as 'tea clubs', a visiting professor, from Yale I believe, described very exciting results 'showing' that mitochondria from baby beef heart could synthesize cytochrome c in vitro. The specific activity data were anomalous and beyond my comprehension. Innocently, I asked which antibiotics were used to prevent the growth of bugs (so that I could then use them in my cell wall incubations) Francis Crick [23] turned in his seat, stabbed his finger in the air and said, 'I see what you're suggesting; he's making two kinds of cytochrome c, a bacterial one and a mitochondrial one.' (Methinks I did not protest too much at this astonishing hypothesis.) Later the sad truth emerged – a student had fabricated the cytochrome data. Thus, on publication of our note to *Nature* [24], other worldly-wise sceptics, possibly stung by similar problems[25], leaped into action. A curt note to Northcote from P. A. Roelofsen, author of a truly classic text [25], read: 'Permit me to put one question: Were the walls clean as examined by the electron microscope?' F. C. Steward was unrelenting in his criticism, both in public and private. A leading tissue culture expert and successful demonstrator of totipotency in plants, he had missed a revolutionary way of growing pipettable cell suspension cultures and got the location of the Hyp-rich component wrong. For a mere earnest student to have invaded his turf successfully, not once but twice, was, as Lady Bracknell would have said, quite unforgivable. Even more embarrassingly, although his labeling data showed that after ^{14}C-proline conversion to Hyp it did not turn over (i.e. the Hyp was not further metabolized), he rejected R. D. Preston's truly brilliant insight that such a component was therefore outside the cell, i.e. probably in the wall [R. D. Preston, private communication].

Peter Albersheim was less sceptical when he visited Cambridge in 1960. (We spent an afternoon punting on the River Cam, and neither of us fell in.) He took home a sycamore culture [26] (p6) to make discoveries like aceric acid, the xyloglucan-cellulose association, rhamnogalacturonans I and II and initiate another paradigm shift [27], discovery of the oligosaccharin elicitors, work for which he has not been properly recognized.

By happy coincidence another 'visitor' stayed somewhat longer–Sir Rudolph Peters had retired from Oxford to Cambridge, where his considerable erudition enlivened morning coffee[26]. His claim to fame was based, among many other things, on uncovering the first example of a 'lethal synthesis.' *Dichapetalum cymosum* contains fluoroacetate highly toxic to cattle but only after conversion by the condensing enzyme to fluorocitrate, a very effective inhibitor of animal aconitase. He wondered how the plant avoided the same fate. Naturally, I offered sycamore cell suspensions as a test. Judging from sycamore, plant aconitase is simply much more resistant to inhibition [28].

And so the work went on. E. F. Gale [29] introduced my dissertation seminar[27], remonstrating against the excessively lengthy title by complaining that I had only supplied him with the abstract (general laughter). Kenneth McQuillen, who organized these seminars, had warned me to give a straight performance. However, this bonhomie combined with the unconventional presentation that included a 'video'[28] of the cell suspension culture technique [30], including sterile explants from *that*

[23] He had a fine, almost English, sense of amusement at himself – at one meeting a colleague asked whether another 'Varner' at the same meeting was related: 'Debby, you're a wife behind!' he replied.

[24] Where I remember handling a first edition of Robert Hooke's *Micrographia* with awe.

[25] I cannot help but reflect that I am writing this in Lewes just a short stroll away from the former residence of Charles Dawson, a stone's throw from his Piltdown putdown [318]. On a more uplifting note, just a short hike over the South Downs past Bible Bottom takes one to the Glyndebourne Opera House. Literary and artistic inspiration from the Bloomsbury Group takes a somewhat longer hike either to Charleston Farmhouse or to Virginia Woolf's house by the River Ouse at Rodmell, but common sense and politics are real close: Thomas Paine lived just round the corner at the White Hart Inn (1768 – 1774) until forced into exile. Somewhat later, further up the Lewes High Street, Gideon Mantell was pursuing the Iguanodon and other dinosaurs [319].

[26] Morning coffee and afternoon tea were invaluable sources of information and ideas.

[27] At that time dissertation seminars were treated as part of the normal seminar (tea club) schedule, so the whole department was likely to turn up, at least for the free tea.

[28] In those pre-VCR days I used 8 mm film with a (poorly) synchronized tape recorder soundtrack.

sycamore log, was just too much for F. G. Young[29] (nothing was ever unofficial), who subsequently vetoed my application to work with the Nobel Laureate Luis Leloir in Argentina. I have always seemed to bring out the worst in authoritarians, who instinctively recognize a rebel and who believe that the nail that sticks out must be hammered down. It takes special skills to use authority and not be an authoritarian – people like Joe Varner and Charles Arntzen, who became the second Plant Research Laboratory (PRL) Director.

Bomber Merlin 1961

And so I ended up working for 3 very happy years in Varner's lab at the Martin Marietta basic research lab RIAS (Research Institute for Advanced Studies) in (say it real quick) Baltimore, Maryland. 'Now you're in America, think big', Joe said. My first significant experiment used $^{18}O_2$ gas at a cost equal to my entire year's budget at Cambridge. But it worked – the source of the oxygen in hydroxyproline was by the direct fixation of molecular oxygen. I lost my bet with Joe that it came from water by hydration of a putative dehydroproline intermediate, but published a good paper where the name 'extensin' first appears, for which Joe[30], very generously allowed me to take full credit [31].

The medieval Cambridge accounting system left a gap of several months between the final PhD. oral examination and formal (or quite shockingly, even informal) acceptance of one's dissertation. After several months in the United States it was a blow to find that both external examiners, Helen K. Porter, and M. J. R. Salton, in the finest traditions of academic pedantry had decided that it needed rewriting. True, it was not your average dull tome. It broke all the unwritten rules. I had opted for a light blue cover, divided it into two volumes (one exclusively for figures), used colored page inserts between sections and committed the most grievous sin of using nonstandard journal abbreviations (actually based on a system devised by Joseph Needham). The work described, isolation and characterization of the first pipettable cell suspensions [30] and discovery of structural wall proteins [14, 24] – enough for two dissertations – fell onto stony ground. The oral exam was strangely disappointing – they didn't recognize the paradigm shifts[31]. Of course, I had been a royal pain in the butt to Northcote, and at this stage I can only commiserate with any research supervisor who acquires a headstrong, know-it-all maverick. But creativity is protest against the status quo, and science is not absolute truth but a journey.

To the surprise of the photosynthetic wizard of RIAS, Bessel Kok (head of the bioscience division), who was rather more plugged in to the realities of the world than was I, they accepted the dull rewrite. More excitingly, R. D. Preston invited me to review the nascent field of cell wall proteins [9]. That review included much unpublished work from my dissertation, including the new paradigm of a dynamic cell wall as an organelle [32], replete with enzymes, and thus predated the current revisionist views [33–40] of a close interaction between cytoplasm and wall by 30 years. Electron microscopy of putative Hechtian strands (fig. 1B in [9]) and the idea of the wall as an organelle[32] were a tribute to Sir Rudolph Peters's prescience, and a blow to Steward's obduracy. He expressed disbelief about my work in a letter of complaint – not to me, but to the PRL director, Anton Lang. Naturally, I could not resist citing this letter (attributed to *** F.R.S. in reference [1]): 'When I talk about a cell wall I mean a cytological cell wall not a biochemical cell wall' for semiotic deconstruction and relation to other wall-related contexts. Humpty Dumpty's aphorism came to mind: 'When I use a word it means what I want it to mean, neither more nor less.' Of course publication of original data in reviews is definitely a no-no to the pedants who prefer the infallible process of surreptitious unsigned peer review rather than their own critical faculties. Science advances in spite of scientists. Why science is supposed to be so open yet remains so secretive beats me.

Another university: December 1964

And so to the PRL at Michigan State University, rather on Joe Varner's coat tails, I suspect [21]. Joe arrived at the newly founded lab a year or so later but soon enough to see multiple purple spots after paper electrophoresis of cell wall alkaline hydrolysates, hence the first evidence of the alkali-stable Hyp arabinosides, a glycopeptide linkage unique to plants [41] and therefore studiously ignored by modern textbooks of general biochemistry.

The first director, Anton Lang, was very definitely old school – 'born in St. Petersburg at the age of five' wrote one wag. Folke Skoog summed him up thusly: 'Anton,

[29] Later he remarked: 'You should work in a large laboratory where your personality can be absorbed', no doubt anticipating the Borg – not bad for the fifties.

[30] From whom I also learnt how to keep up to date using the Biochemistry Sections of the Chemical Abstracts, hand-searching the literature is still the best way, according to Allison Mitchell writing recently in *Nature 396*:723.

[31] I suspect that the cell suspension paradigm has never been fully accepted. Some consider them 'wounded' cells despite their healthy appearance, ability to respond to elicitors and expansin [178]. But as Kuhn notes: "Professionalization leads, on the one hand, to an immense restriction of the scientist's vision and to a considerable resistance to paradigm change" p. 64 in [27].

[32] This idea is nowadays not so controversial. For example it is 'the organelle that ultimately controls the shape of the cell' [38], cf. [157].

when you're in Russia you look Prussian, when you're in China you look Russian, and when you're in Germany you look Chinese!' A gifted lecturer, he spent his research life hunting the Snark, alias florigen[33], that eventually became a Boojum – antiflorigen. In between complaining about the addition of irrelevant books like '*Herbal Remedies*' to the PRL library (guilty, sir!), he performed useful public service at a high level as a member of President Nixon's committee to investigate the effects of agent orange in Vietnam:

> Anton's not in his lab anymore
> He's gone to help wind up the war
> Out among the Vietnamese
> He's planting lots of plastic trees
> Immune to defoliation and disease.

No doubt these contributions will be objectively discussed at subsequent Anton Lang Memorial Lectures[34], except that as he himself would have said, 'Is that appropriate for a hagiography?' Nevertheless, the stable funding base of the PRL Atomic Energy Commission/Department of Energy permitted 'high-risk' work that theoretically did not have to show an immediate payoff, although this was partially negated by constant internal and external reviews (Dean Byerrum once remarked that we were 'reviewed to death') with occasionally quite vicious undertones. However, the equipment budget was superb and could be accessed in short order given reasonable justification. Thus, when we needed an expensive Edman Sequenator costing, I noted, about the same as a Shrike antimissile missile, Anton was persuaded that, unlike a one-shot missile, a sequencer could be fired repeatedly, hence sequencing costs could be amortized over many runs! And when our incursion into HF solvolysis[35] required an expensive apparatus constructed entirely of Kel-F, it was acquired pronto!

How we, that is, Andrew Mort and I, both arrived at the idea of using anhydrous HF to deglycosylate glycoproteins [42] is difficult to describe because we both have somewhat different recollections. Modesty forbids describing this method as a great breakthrough, although its crucial use in isolating genes for glycoproteins by getting to the core of highly glycosylated glycoproteins is undeniable [43–51], though unappreciated by some [52]. My reconstruction of events (or is it deconstruction?) revolves around the course at the Beckman lab in Palo Alto, where I went to learn how to run the sequencer along with Jim Ross, a postdoctoral colleague on whom I perpetrated a hilarious practical joke that cannot even be described here. There, of course, they also wanted to sell me a peptide synthesizer. From this interaction I learned about peptide synthesis and the need to protect the reactive side groups during chain elongation. More important, I learned that anhydrous HF was often the reagent of choice for final cleavage of the protecting groups. If one viewed glycoproteins as polypeptides with saccharide protecting groups, then deglycosylation was simply a question of selecting the most effective deprotecting reagent, in this instance, anhydrous HF substantially enhanced by Andrew's bravery – anhydrous HF has a very bad reputation and aged cylinders have even been known to explode due to the slow accumulation of hydrogen gas. (One researcher remarked that he didn't want to work in a lab that used HF, or for that matter even in the same state![36]) Thinking that extensin was cross-linked by wall polysaccharide, we both hoped that its removal would solubilize extensin, as that would help us to elucidate the structure of the polypeptide backbone. However, the protein-rich insoluble residue simply mirrored the results of enzymic digestion reported earlier [9] and led to the idea of interpenetrating protein-polysaccharide networks [53] ('warp-weft' [54]) rather than one supercolossal cross-linked cell wall molecule. However, this latter view remains tenable in the light of recent evidence of a covalent linkage between pectin and extensin [55, 56].

Sabbatical, 1976

The Guggenheim Foundation generously funded a sabbatical year of renewal with the stimulating and multitalented Keith Roberts[37] at the John Innes Institute to learn more about *Chlamydomonas* [57]. This time to read and think and play cricket and introduce my five children to the British Isles was also an invaluable opportunity for recharging the batteries drained by lab politics.

Extensin precursors, AGPs and Hyp-polysaccharide

On my return to the MSU-DOE PRL we renewed the search for soluble precursors to the extensin network. These had been on the verge of discovery even in the early sixties [9], when I introduced the 'intact cell elution' technique, variously described by others as 'leaching' 'washing' and so on. I called it elution because the

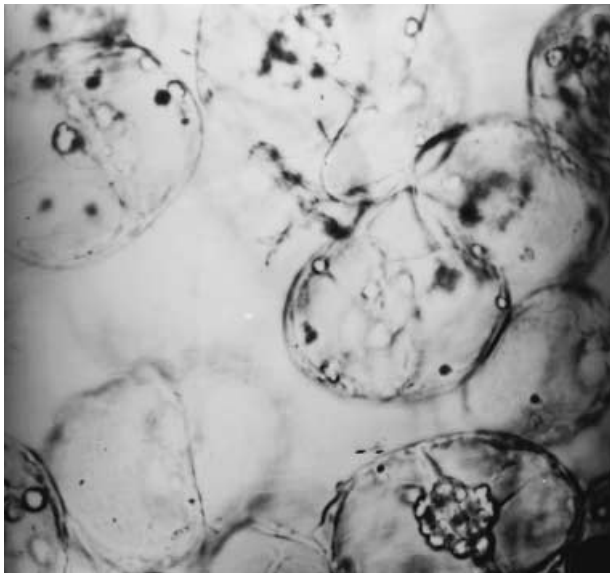
[33] Was it a substance or a state of mind? The difficulty of isolating a specific flower-inducing hormone has effectively relegated florigen to the status of phlogiston [320].

[34] I was a soft thorn in a very tough hide.

[35] This assumed that extensin was a glycoprotein, judging from our earlier work on the Hyp arabs [41] and galactosylserine [79], very ably supported by Sandy Roerig and Laura Katona, who was the first of over 25 High School Honors Summer Student contributors to my lab. The last was Cynthia Fong [117, 321], who enjoyed it so much that she returned a second year despite the 'local difficulties'.

[36] 'A man who is afraid is a liability and may someday be responsible for injury to himself or others because of his fear' [322].

[37] Another protégé of Don Northcote's.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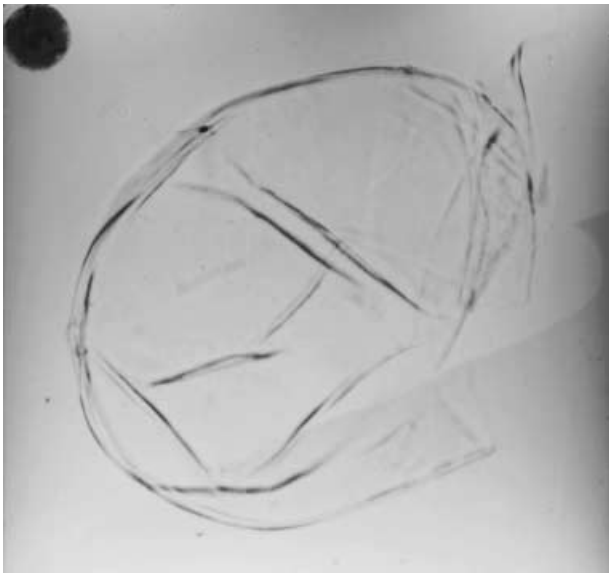
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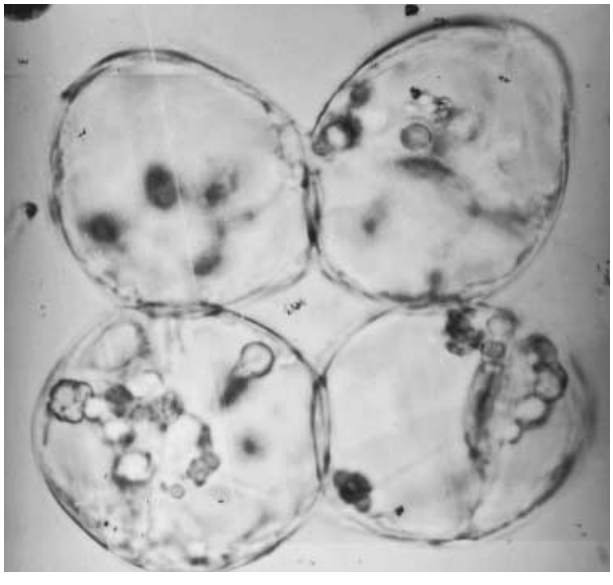
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E


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Figure 1. These archival pictures are from the original lantern slides used at the Biochemical Society Meeting held at Cambridge in 1959. *(A)* Sycamore cell suspensions in logarithmic growth phase. Cells are 50–60 μm in diameter with large vacuoles and lots of starch grains. *(B)* Less rapidly growing sycamore cells. *(C)* Sycamore cells plasmolysed by sucrose. *(D)* A clean cell wall isolated from a sycamore cell suspension culture. *(E)* An electron micrograph of a Pt/Au shadowed wall fragment after extraction with strong alkali to yield an α-cellulose fraction with cellulose microfibrils characteristic of primary cell walls. Note that the Hyp assays presented at this meeting were published as table 1 in [14].

sycamore cell suspensions packed into a column looked just like a Dowex resin and could be eluted like one[38]. It was just one of those quirks of nature that whereas sycamore cells were superlative suspensions, they yielded only very small amounts of elutable extensin monomers. I had the right technique but the wrong culture. However, it was not a complete waste of time, because when we tried to isolate a soluble cytosolic extensin precursor, we purified the first arabinogalactan protein[39] (conservatively described as a protein-polysaccharide complex [9]) but put this work 'on the back burner'[40] when analysis showed that it was alanine rich and therefore not a precursor to extensin [9]. Interestingly, alkaline hydrolysis of the protein-polysaccharide complex yielded a range of hydroxyproline arabinosides, but in addition a much higher molecular weight 'free Hyp' (i.e. nonpeptidyl Hyp) component that for a time we called 'Big Hyp-X'. In David Pope's hands this turned out to be an arabinogalactan polysaccharide involving an O-galactosyl-Hyp linkage [58]. How did that paper miss the precursor status of the salt-extractable soluble Hyp-rich fraction?

The answer to that question and our quest for a bona fide extensin precursor came, as it so often does, with persistence and a little help from our friends. Sadly, we misinterpreted an earlier report of soluble extensin [59] because the amino acid composition was even less extensin-like than the elusive 'precursor extensin' that had already burnt our fingers. However, sometimes one can be too critical of colleagues' work that is basically on the right lines! Joe Varner, who by this time had left the PRL for the University of Missouri, confirmed the report from Maarten Chrispeels' lab, but this time with a convincing amino acid composition [60]. He also mentioned [private communication] that cell walls isolated in the presence of ascorbate enhanced the salt-elution of extensin. So it was once again into the breaches. Yet again we salt-eluted intact cells, this time in the presence of ascorbate. Nathan Krupp, an undergraduate working as a summer student led the charge. Unhindered by preconceived ideas, he discovered large amounts of elutable Hyp-rich material not in sycamore but in tomato (surprisingly not dependent on added ascorbate). Why this was not discovered earlier is an object lesson in choosing an experimental system. The 'best' (i.e. rapidly growing pipettable) cell suspension culture, sycamore, was actually the worst producer of elutable extensin and with the toughest walls most resistant to enzymic degradation. This last factor had prompted our choice of tomato cultures ('Bonnie Best', a generous gift from Harry Murakishi in the MSU Botany and Plant Pathology Department) for cell wall degradation.

The oil crisis of the seventies plus the fact that the US Department of Energy paid the bills for basic plant research made us all a bit more politically aware. Working with anhydrous HF, we realized the possibilities of 'cracking' cellulose via 'HF-saccharification'. Thus began a productive and most enjoyable collaboration [61–71] with Martin Hawley in the department of chemical engineering. His group of bright young lads and lasses added greatly to the cosmopolitan mix. I only wish the oil crisis had gone on longer.

Lignin was a byproduct of HF saccharification, and a new graduate student began a comparison of HF lignin with milled wood lignin (MWL) only to conclude that HF-treated lignin was even more intractable to chemical analysis than MWL. This conclusion coincided with Krupp's startling results. And that is why this bright young graduate student, Jim J. Smith, confirmed Krupp's Last Experiment and joined the fray. Using pulse-chase labeling with ^{14}C-proline, he demonstrated that the turnover kinetics of the eluted soluble extensin (i.e. specific activities as a function of time) were consistent with its status as a precursor to a product, wall-bound extensin [72]. However, the precursor half-life of ~48 h was much greater than Pope's earlier time course[41]. This may explain why monomeric extensin precursors to network extensin were not identified earlier in sycamore[42].

With a groundbreaking paper under his belt Jim could be forgiven for thinking that he had earned his PhD. Of course he had, so when told he had completed half a PhD, understandably he was a bit miffed. By chemically characterizing the eluted extensins he might have a once-in-a-lifetime chance. Or was he just being pushed by an overly demanding mentor?! The wisdom of the American-style graduate student committee as a buffer between student and research supervisor was the proof of the pudding. The eponymous Norman Good[43] had a deservedly great reputation – and he was also scrupulously fair and objective [73]. He and other committee members thought that Jim could and should do it. And he did in great style, resulting in another milestone paper. It demonstrated multiple extensins possessing an amazingly highly periodic structure comprised of only two or three repetitive peptides [74]. These data explained why the early assumption that a highly biased amino acid composition of the wall represented only 'one protein' was paradoxically both correct and incorrect. Collagen is the major extracellular

[38] And because the elite elute.

[39] But as so often happens, others were working along similar lines. Aspinall's extracellular arabinogalactan polysaccharide from the sycamore culture medium [323] was undoubtedly an AGP, as was most probably Pusztai's glycoprotein mixture isolated from broad bean leaves [324].

[40] Rediscovered as LeAGP1 by Allan Showalter [325].

[41] Moral: Not all popes are infallible.

[42] But see fig. 19 of [9].

[43] His invention of 'Good' buffers [326] was in progress on my arrival at MSU in 1964 when he generously shared his lab with me until other space became available.

structural protein of metazoans, but there are 20 different collagens [75] (at the last count), compositionally very similar. Likewise extensins, the major extracellular structural protein of metaphytes, originally viewed as monolithic have begun to proliferate, starting with the isolation of two (P1 and P2) from tomato and a third (P3) inferred from the isolated peptide fragments (later confirmed by the isolation of the genomic clone J-10 [76]). Nowadays, of course, we classify all hydroxyproline-rich glycoproteins (HRGPs) as an extensin superfamily [77], analogous to the collagen superfamily.

Jim Smith's sequencing was important: it demonstrated that extensins were highly periodic, and confirmed Ser-Hyp_4 [78, 79] as a diagnostic motif [74]. This was crucial for identification of the first extensin complementary DNA (cDNA) (isolated from carrot by Chen and Varner[44] [80]). And it led to Marcia Kieliszewski's brilliantly innovative use of synthetic genes to create new HRGPs de novo for the elucidation of glycosylation codes and the functional analysis of HRGPs [81, 82]. To think that all this began with Sanger protein sequencing itself based on Martin and Synge's paper chromatography in the fifties – in one short lifetime we've come a long way.

The isolation of soluble precursors to the putative extensin network took us one step closer to the vexed problem of extensin crosslinkage. It had been clear for many years [9] that extensin was crosslinked either to itself or some other wall component – perhaps both. It was now possible to ask which agent was responsible and what crosslinks were formed. If extensin formed a crosslinked network in muro[45], then it should be possible to isolate the enzymes responsible from the cell surface and use them to generate crosslinks in vitro for subsequent identification, a simpler approach than attempting to identify those produced in muro.

While Jim Smith was working on soluble extensins, Steven Fry in Edinburgh isolated isodityrosine from cell wall hydrolysates [83]. This diphenyl ether-linked dityrosine seemed to be the Holy Grail, since it fit the bill perfectly for an intermolecular crosslink. I promptly wrote to Stephen Fry congratulating him on a perspicacious discovery and searched through the lab deep freeze for peptides isolated from enzymic cell wall hydrolysates made some years earlier. These peptides contained an unknown tyrosine derivative [84] corresponding to the newly identified isodityrosine (IDT). We assumed that it would be easy to demonstrate peptide dimers with IDT as the intermolecular crosslink. Fortunately, I persuaded Lynn Epstein that this postdoctoral topic was in an area of plant pathology relevant to her research interests. However, none of the experiments seemed to 'turn out right' until we remembered Rutherford's dictum that 'no experimental result is ever wrong'. In the crucial experiment Lynn counted the number of N-termini in a putative homodimer, but found only one [85, 86], hence the inescapable conclusion that IDT exists as a very short intramolecular link not an intermolecular link, amply confirmed by all the subsequent gene jockeys and the recognition of the IDT peptide motif Tyr-Xaa-Tyr [77].

With help from Joseph Hsung in Alfred Haug's laboratory, CD (circular dichroism) spectra had shown polyproline II character even in quite small extensin peptides [87]. This corresponds to a collagen-like three-residue-per-turn helix with a pitch of about 9.4 Å, generating an extended rodlike molecule. Rather naively, we and others [88] assumed that IDT would generate kinks in the molecular rod. How could we test that idea? Molecular modeling experiments 'in computero' were beyond our reach at that time. Anton Lang came to the rescue, despite complaining about the cost, and promptly coughed up the dough for a nice set of CPK molecular models – there were some advantages of having a 'program director' sitting in an office downstairs! Once again 'Lucky Jim' did his stuff [86] – no kinks.

Extensin peroxidase

With extensin chromatographically purified 'precursors' (i.e. monomeric precursors to a presumed extensin network) now readily available, it was possible to think about discovering the identity of the enzymes involved in crosslinking them. Curiously, apart from brief forays into cell wall enzymes [9, 14, 28, 89], my Cambridge training had left me relatively unprepared for this. With the notable exception of Marjorie Stephenson [90], no one ever seemed to discover new enzymes at Cambridge despite the presence of the great gurus Malcolm Dixon and Edwin Webb, who taught the fundamentals with a rigor that stood the test of time [91].

Occam's razor [92] decrees that one start with the simplest hypothesis, provided one realized that Occam is not a direct line to absolute truth, merely 'an instruction in an operating manual' [93]. Thus, if extensin were crosslinked to itself, what enzymes might be responsible?

As an undergraduate I had been both bemused and amused by the organic chemist Bernard C. Saunders's infectious enthusiasm, not just for teaching and radical statements[46] but for peroxidase, an enzyme that seemed

[44] This was Joe Varner's greatest contribution to the field as, aided by his less contentious and inclusive term HRGPs, it finally legitimized extensin and made it respectable.

[45] This term, introduced in 1970 [1] cf. [85], is intended to convey the idea of an active living cell wall or organelle.

[46] Bernard Saunders was the great showman of organic chemistry [327] and always impeccably dressed. Halfway through his lecture, 'And that reminds me…' inevitably prefaced an amusing story: 'I am standing on the very spot where the great Fenton [328, 329] lectured … of course, in those days they wore the academic gown to hide their shabby suits.' Riotous applause and stamping of feet.

(at that time) to do everything, yet nothing. Years later David Plass, a high school summer student did some of our first experiments with peroxidase. He incubated extensin monomers with hydrogen peroxide in the presence of crude cell eluates and detected the first extensin oligomers formed in vitro. Fortunately, the newly available high-resolution gel filtration column Superose-6 permitted excellent resolution and quantitative assay of extensin monomers and oligomers in a size range larger than that possible using SDS-polyacrylamide gel electrophoreses (PAGE) [94]. This direct demonstration of peroxide-dependent extensin crosslinking led to the isolation of a pI 4.6 isozyme that shows high crosslinking activity [95], and therefore fulfills the criteria for a bona fide extensin peroxidase, rather than other isozymes whose specific activity under comparable conditions is very much less [96]. Thus subsequent indirect evidence for extensin crosslinking in vivo [97] supports the idea that extensin may actually turn out to have something to do with cell extension, albeit as a negative regulator. However, some take a dystopian view of the name extensin, which was coined at Joe Varner's lab at RIAS [31]. It is provocative [98]. And the DOE/PRL footed the bill for a laboratory so well equipped that Hans Kende quipped that perhaps I should rename it expensin.

The idea of a dynamic cell wall replete with enzymes (cytoplasm in the ancient literature) was hardly new, but the techniques to demonstrate it were. Cell suspension cultures yield a 'generic' primary cell wall faithfully reflecting that of the parent species. This brings us to the question of function.

Sometimes a ziggurat is just a ziggurat

Like beauty, function is in the mind of the beholder, difficult to define objectively [99] yet with a physical basis rooted in evolutionary history [100] rather than evanescent and subjective cultural values [101]. The text *Plant Form and Function* beguiled me plantwards many years ago. Later, Agnes Arber enlightened me with a philosophical approach to plant form [102], in particular her chapter on 'The mechanism of plant morphology', summarized by the aphorism 'Form follows function' ascribed to the architect Louis Sullivan[47]. If structure is function [103, 104], then to equate function purely with physiological function is myopic[48]. On the other hand, used as a holistic term like growth, it lacks the reductionist precision of a title like 'Structure and mechanism in protein science' [105]. There is a third way of looking at this [106]:

We can view 'function' as different levels of complexity [107]. A ziggurat of cause and effect extends from subatomic physics[49] to ecology and beyond.

At level 1 a signpost points to the structural analysis of molecules, starting with the primary structure and proceeding to the secondary and tertiary structures. For HRGPs this means decrypting the codes of an enigma, since most HRGPs with their highly hydrophilic overcoat are designed not to fold. The molecular shape of HRGPs is a product of the proline/hydroxyproline content and its glycosylation [87, 108–110], particularly the Hyp arabinosides, that is presumed to hydrogen-bond with the polypeptide backbone [111, 112], although not yet confirmed rigorously. An extended polyproline-II helix stabilized by extensive glycosylation confers a flexible extended rodlike shape to extensins[50]; hardly surprising, since protein folding is largely driven by hydrophobic interactions [113]. However, the lightly glycosylated proline-rich proteins (PRPs), having less contiguous Hyp and hence lacking the stabilizing saccharides, probably do fold into a series of reverse β turns [114]. The shape of the hyperglycosylated AGPs is surprisingly contentious. Are they 'wattle blossoms' [115, 116] or 'twisted hairy ropes' [117, 118]? Varki's aphorism comes to mind [119]: All of the hypotheses are right! AGPs, in sensu lato, include a very wide range of macromolecules that defy neat and tidy definitions so beloved by the academic mind. There are long skinny ones, short fat ones and ones that 'Frankly, my dear, I don't understand' – strange chimeras like the solanaceous lectins [120], which show true partial or fractional homology [121] since they are hybrids of HRGPs that contain the P3-extensin motif and other quite unrelated protein domains [122]. Other chimeric extensins [123] and chimeric PRPs [124] also exist.

Posttranslational proline hydroxylation and subsequent glycosylation of HRGPs in the ER/Golgi is not random, but controlled [77]. Thus, Kieliszewski and her collaborators using synthetic genes confirm the Hyp Contiguity Hypothesis for arabinosylation. They have also dramatically confirmed the corollary that clustered noncontiguous Hyp motifs direct polysaccharide addition. Their de novo design of a new AGP [125] is a molecular tour de force that will surely breathe fresh life into the living cell wall! It certainly throws down the gauntlet for us to design ingenious new experiments to test 'off-the-wall' ideas with rational exuberance.

Such progress allows us to ascend with confidence to level 2 of the ziggurat, functional analysis of structural macromolecules. This involves identifying and mapping HRGP functional domains and subdomains, functional motifs, glycomodule functional residues and elucidating

[47] For a modern treatment, see [330].

[48] Nevertheless, some journal editors will not put their stamp of approval on a paper unless it addresses function narrowly circumscribed at this level.

[49] The first subatomic particle to be discovered was the electron.

[50] I am indebted to Joe Varner for this rationalization of extensin nomenclature [331].

their role. There is gratifying progress at this level, too:
The N-terminal domain targets HRGPs [126] to the appropriate subdomain [127] of the endoplasmic reticulum for posttranslational modification and subsequent secretion but 'is not just a greasy peptide' since it encrypts further information [128].
In plants [129], a C-terminal domain directs the glypiation of many AGPs, resulting in their attachment to the outer surface of the plasma membrane by a GPI anchor [130, 131].
The role of other HRGP motifs and secondary structure is also coming into focus:
Intermolecular crosslinking of extensins probably involves the VYK motif [95].
The highly arabinosylated Ser-Hyp_4 motif is hypothetically a site of homophilic self-association that aligns rodlike extensins for peroxidatic cross-linking [77].
The IDT motif, Xaa-Tyr-Xaa-Tyr, as an intramolecular linkage locks the local conformation and thus helps rigidify extensins [86], whereas the alternating Ser-Pro_3 and Ser-Pro_4 motifs of an *Arabidopsis* extensin [132] probably reflect exquisite control of molecular flexibility.
Chemical and physical properties of HRGPs contribute to level 3 of the ziggurat where self-assembly of a molecular fabric 'woven' from six polysaccharides [133] creates a primary cell wall at the cell surface. Arguably, this involves extensin HRGP scaffolds [134] crosslinked by extensin peroxidase [95]. True or false? Consider the *Chlamydomonas* paradigm. This primitive protist's cell wall lacks polysaccharides and is built almost entirely as a self-assembling [135] noncovalent lattice of HRGPs [136] homologous with the HRGPs that form covalent cross-linked networks in higher plants [137, 138] and containing similar motifs [139] and O-linked Hyp glycosides [140]. These even include short arabinose-galactose oligosaccharides suggestive of an arabinogalactan [135, 140, 141]. Scaffolds keep things in place. This implies that HRGPs interact with other wall components and organize them. One infers this because the prolyl hydroxylase inhibitor dehydroproline inhibits wall assembly in protoplasts [134] and also inhibits root hair growth [142].
But how do HRGPs organize wall assembly? In muro there is a straightforward ionic interaction between basic extensins and acidic pectin because monomeric extensins are salt-elutable [72, 143]. However, the three-dimensional details of how different extensin monomers slot into place and polymerize remain conjectural. The existence of multiple elutable extensins such as tomato P1 and P2 is noteworthy. The existence of the nonelutable P3 is particularly intriguing. It has never been isolated as an intact monomer, but inferred from palindromic peptide fragments in wall digests [74]. Later cDNAs show that the P3 type characterized by the Ser-Hyp_4-Ser-Hyp-Ser-Hyp_4-Tyr_3-Lys motif and variants [144, 145] is widespread, perhaps even universally present in higher plants [122, 144, 146-151]. It is especially tempting to ascribe a self-assembly role to the symmetrical peptide palindromes of P3 [77, 152, 153]. The ~80-nm length [154, 155] of the elutable extensin monomers roughly coincides with the width of a primary cell wall, suggesting their transmural insertion. Elution kinetics [72] are consistent with the rapid ionic desorption of extensin monomers. However, one suspects that HRGPs and the chimeric AGPs in particular also bind other ligands, again work for the future.
Ascending the ziggurat to level 4, cell wall properties, we see that self-assembly [156] creates 'the cell wall … the largest organelle' [157], a supramolecular microcomposite with the seemingly antithetical properties, of high tensile strength[51] and extensibility. Extensin contributes to these wall properties profoundly influencing physiological function, therefore best discussed at a higher level (7) of the ziggurat.
Other cell wall properties such as porosity depend on the crosslink density and therefore include other intermolecular crosslinks such as pectin-extensin [55] and diisodityrosine [158]. These crosslinks will also affect cell wall elasticity, an important property, rarely discussed [159].
At the levels 5 and 6 of the ziggurat, morphogenesis and morphology suggest that HRGP-dependent cell wall properties contribute to the architecture of cells, tissues and organs [160]. The cell wall itself is a morphogenetic substrate par excellence – without it there is no morphology, hence the growing appreciation of the fundamental role that the cell wall plays in morphogenesis [161]. But how does it do it? As the complexity of an organism increases, so does the number of distinct cell types with diverse cell walls. How does the cell generate diversity from just a few polysaccharides like cellulose, pectin and xyloglucan [162, 163] that seem to be highly structurally conserved with only minor variation [164]? The answer may lie in the profusion of cell surface HRGPs (AGPs, extensins, PRPs), their tissue-specific location [165] and regulation of their spatiotemporal expression. It is worth reminding ourselves that land plants are descendants of Hyp-rich chlorophycean algae [166–169]. The first land plants with an upright habit, like the fossil psilophyte *Cooksonia,* relied on cortical turgor for support rather than secondary thickening, lignification and xylem rigidity [170]. Indeed, turgor regulation itself [171] may well involve extensins. Morphogenesis per se – 'the war of the whorls' [172] – depends on meristems; it also depends crucially on the spatiotemporal regulation of cell enlargement [173, 174]. This involves the cell-wall-loosening expansins, for example, an upregulated expansin gene predicts the site of leaf formation in tomato stem meristem [175, 176]; expansins occur in cell suspension cul-

[51] However the name 'tensin' has been given to the protein that anchors actin filaments at the focal adhesion [332, 333].

tures, too [177, 178]. Expansins may be proteases[52] [179]. Thus in algae [180] plants [181] and animals [182, 183], crucial interaction between proteases and hydroxyproline-rich glycoproteins of the extracellular matrix may constitute a major morphogenetic mechanism.

AGPs, including chimeras, seem especially widespread and variable. They exhibit remarkable patterns of tissue specificity [184–186] and are often attached by a glycosylphosphatidyliositol (GPI) anchor to the outer face of the plasma membrane where their relatively high level [187] may provide a protective hydrophilic periplasmic cushion between membrane and wall. AGPs associate on addition of the Yariv reagent in vitro, whereas in vivo addition inhibits the growth of rose cells [188] and pollen tubes [189], implying a perturbation of wall assembly [190]. Nothnagel (p. 209 of [39]) suggests the possibility of a naturally occurring Yariv analog. Flavonoids [191] are contenders. They are implicated in signaling between symbionts [192, 193]. They have a great affinity for cell walls [194], they influence the growth habit [195] and they are required for pollen tube growth in *Petunia* [196]. AGPs are notable cell wall components of *Pinus* pollen tubes [197], but also detected in those of *Nicotiana* [198], *Lilium* [199] and *Brassica* [200]. AGPs oriented on the plasma membrane surface may direct the orderly self-assembly of other polymers. Hence, the type of AGP and the type of wall under construction may be related, probably including the secondary cell wall.

Finally we reach level 7 of the ziggurat, physiological function, where one views mechanism from a biological rather than a purely chemical perspective, particularly for tissues and organs. Questions about function often start at this level, but it will take the persistence of Piers the Ploughman to achieve final enlightenment. The prospects for HRGP functional elucidation here are daunting, involving the entire corpus of plant physiology: vegetative growth, reproduction and stress, including, of course, the contentious issues of cell extension, cell expansion [201] and acid growth, both pro [202, 203] and con [204–207]. Control theory demands feedback loops, but how many describe the interplay of hormones, enzymes, wall macromolecules, organic and inorganic ions? 'Growth is far too important for plants to rely on any single control point' [34].

Like the seven blind men inspecting the elephant[53], each murologist feels the essential features within his grasp! If extensin is involved in cell extension, how and to what extent? Monomeric extensins can be rapidly crosslinked in muro via extensin peroxidase [95, 208]. Because the resultant extensin network stiffens the wall [209–212], extensin, particularly in the epidermis [213–216], functions as a negative regulator of extension growth by slowing it down. Positive regulation by cleaving the network is a logical corollary. Extensin is not a molecular straightjacket! There are indeed reports of cell wall proteases [217–220], with the most recent surprising and dramatic addition of a character masquerading as a polysaccharide-binding protein but finally unmasked as a putative protease–expansin itself. The evidence [179] that β-expansin is a protease with specificity for arginyl and lysyl residues, and possibly homologous with cysteine proteases [179], has profound implications for future work: Do expansins cleave peptide bonds of extensin in vitro and in muro? If so, are site-specific bonds cleaved? For example, cleavage of the VKPYHP 'inserts' in tomato P1 extensin would allow an orderly relaxation of the cellulose-extensin 'warp-weft' [54]. Is the number of different expansins related to the number of different extensins? Are there different target sequences in tissue-specific extensins? Do expansins cleave other HRGPs and other wall proteins? When did expansins originate and what was their original function?

AGPs may [189, 221, 222] or may not [223] enhance cell extension, although not enzymically like expansins [224, 225], but perhaps as plasticizers released into the wall via phospholipase cleavage of the GPI anchor [131]. In muro the highly acidic mobile AGPs would compete with immobile pectin to bind extensin. Thus, wall-loosening processes [226, 227] compete with wall-stiffening processes [98, 228]. These include, pectin deesterification [229], hemicellulose-ferulate crosslinking [230] and quite possibly PRP-dependent lignification [231, 232] involving stereoselective coupling of lignin monomers by dirigent proteins [233].

That bare bones description does not yet explain the presence of multiple (cleavable and noncleavable?) extensins in a tissue (e.g. tomato P1, P2 and P3). Nor does it explain the presence of variants of these extensins in different tissues, such as root hairs [142, 149] or in adventitious root formation [234, 235] or xylogenesis [236]. Nor does it explain the presence of the closely related PRPs. Despite the presence of the Val-Tyr-Lys (VYK) putative crosslink motif in some, but not all PRPs, evidence for their cross-linking in muro is indirect [97, 237] and remains to be demonstrated in vitro. Indeed PRPs are suspiciously soluble. Perhaps they behave as analogs of the minor collagen 'shape modules' [238]. These act as organizers of the extracellular matrix during morphogenesis by regulating or stabilizing the structural properties of the major collagens [239] by 'subtle modifications in intermolecular interactions'. (A recent report that some PRPs bind specifically to an 80-kDa plasma membrane protein seems relevant.) Certainly, the result of collagen disruption [240] – fragile skin – can be physiologically disastrous. In animals targeted disruption, alteration or re-

[52] The 'expansin activity' of snail gut extracts [334] may be due to their proteases [335].

[53] Jacques Monod's aphorism about the similarity of *E. coli* and *E. lephant* does not apply to the extracellular matrix.

design of specific matrix components is helping to elucidate their physiological role. Hence, the de novo design and expression of simple HRGP glycomodules [82, 125, 241] (see Kieliszewski in this issue) is a powerful paradigm that promises to demystify HRGP function at all levels of the ziggurat.

Consider HRGPs and reproductive physiology. AGPs are involved in microspore nutrition, pollen capture, pollen tube nutrition, growth, guidance to the ovule and embryogenesis itself – but the details are generally unclear except in *Nicotiana tabacum.* Here the TTS HRGP acts as a pollen attractant and guidance mechanism [242], and may be deglycosylated during the process [243]. However, the reported specificity of pollen β-expansin and the abundance of Lys-Pro also suggests cleavage of the TTS polypeptide backbone, since Lys-Pro is not always immune to cleavage [244]. TTS degradation corroborates the classical work of Labarca and Loewus [245, 246], who identified AGP-like material in the *Lilium* stigma exudate as a carbon source for the growing pollen tube. Nevertheless, AGP-dependent chemotropism was not confirmed in the sister species, *N. alata,* which expresses a related HRGP galactose-rich stigma glycoprotein, NaPRP4, with an almost (97%) identical sequence [247]. Others, however, do confirm a role for hydrotropism (chemotropism involving water) in pollen tube guidance [248]. Perhaps AGPs help form the directional water gradient.

After fertilization there is notable HRGP involvement in forming a tough seed coat [249] and in ballistic seed dispersal mechanisms that rely on cell wall elasticity [250]. Finally, consider fruit maturation. In tomato, the epidermis regulates fruit growth; there are specific PRP HRGPs [251, 252] and an ethylene-regulated fruit-specific expansin [253] associated with fruit softening [254], reminiscent of the classical cysteine proteases like papain and ficin, both derived from fruit.

Germination, and the livin's not easy! Welcome to stress physiology. Life is precarious for seedlings, vulnerable to stress from all directions, abiotic, biotic, osmotic. PRPs are involved [255]. Extensin HRGPs are hugely involved: tensile stress increases HRGP levels, indicating a role in strengthening the support tissues [174, 256–258]. There are also quite specific responses to wounding [76, 124, 144, 145, 148, 213, 257–264], including gummosis [265, 266], pathogen elicitors [124, 148, 267], nematodes [268], insect herbivory, and to mycorrhizal [269, 270] and rhizobial symbionts. The common theme first noted by Esquerre-Tugaye [271] is the formation of a 'barrier' that excludes pathogens [272].

Barriers also restrict symbionts ('domesticated parasites') to a designated tissue or even an organ like the root nodule dedicated to nitrogen fixation. Here lenticels, endodermis, cell wall chemistry and leghemoglobin ensure a delicate balance between providing just enough oxygen for respiration yet not enough to poison the oxygen-sensitive nitrogenase. What resolves this physiological paradox? Some suggest that barriers of nodule-specific glycoproteins and HRGPs (ENODs) occlude the intercellular spaces [273], but the messenger RNA (mRNA) levels did not change with the oxygen tension [274]. Others suggest a more dynamic control of intercellular porosity via osmocontractile cells [275, 276], whose cell wall elasticity no doubt depends on other HRGPs, notably extensins, as these appear to be the major insoluble HRGPs present in nodule cell walls [277–279] rather than nodulin PRPs.

Drought resistance is a battle to maintain turgor. Water conservation is the first line of defense. When that fails, plants compensate for water loss by changes in cell wall elasticity [280] through the deposition of cell wall proteins [281, 282] that decrease the cell volume and maintain turgor. In resurrection plants [283] tolerant to desiccation, the final line of defense involves preadaptations that allow almost complete loss of water corresponding to ~30% relative humidity [284]. Osmolytes play a role in stabilizing proteins [285], but one suspects that if GPI-anchored AGPs protect the plasma membrane, there may be something rather special about resurrection AGPs.

Close to the ziggurat summit, levels 8 and 9 deal with the whole plant and the contribution of HRGPs to their ecology and evolution. At this lofty level the clouds of speculation almost obscure the gems of fact. Yet ecosensitivity is paramount if we are to avoid ecocide:

> What would the world be, once bereft
> Of wet and of wilderness? Let them be left,
> O let them be left, wildness and wet,
> Long live the weeds and the wilderness yet.
> ['Inversnaid' – Gerard Manley-Hopkins]

Mother Nature does not recognize victimless crimes. Algal blooms at the base of the food chain are indicators. These often form gelatinous colonies [286] at the surface or extensive sheets under sea ice [287]. And they probably consist of HRGPs as archetypal adhesives [199, 288–290] and surface-patterning agents from diatoms [291] to pollen [292]. Further speculation about the ecological role of HRGPs may be a useful didactic exercise [293], particularly in the area of plant-microbe interactions.

Here one cannot do justice to the inevitable questions of origin and evolution. Nevertheless, general principles are emerging based on the idea that many proteins, particularly those forming the extracellular matrix, consist of modules and glycomodules, defined as functional subunits: 'The extracellular matrix is perhaps the largest biological system composed of modular mosaic proteins, and its astonishing complexity and diversity are based on them' [294]. This also applies to the HRGPs and is exemplified by the Volvocales where 'there is a combinatorial

advantage of shuffling [HRGP] modules' [295]. Amazingly (if we have not lost our sense of wonder), these algae invented sex [296] using HRGPs as sexual agglutinins for mating recognition [297]. Thus, small changes in their adhesion specificity resulted in sexual isolation and speciation represented by the 450 known species of *Chlamydomonas* [298].

The surprising conclusion [299] that the volvocine radiation from *Chlamydomonas* to *Volvox* is a 'recent event' [295] that occurred within the past 75 million years seems asking too much even from the versatile HRGPs. The occurrence of exceedingly ancient fossil algae like *Eovolvox* [300], erratic molecular clocks [301, 302] and the structure of Volvocalean histone genes intermediate between plants and animals [303, 304] suggests a reappraisal here. Indeed, evidence of a close affinity between 'living fossils'[54] like the glass sponges (Hexactinellida) and *Volvox* also suggests an ancient origin, because hexactinellids were abundant in the early Cambrian seas ~590 MYA [305]. Furthermore, some phylogenetic trees place sponges and plants in the same clade [306]. It would be tempting to go further by suggesting that Volvox is not an evolutionary cul-de-sac but the relic of a prototype metazoan like *Eovolvox* [87]. However, the changeover from a Hyp-rich HRGP matrix to a glycine-rich collagenous matrix [307] raises formidable problems that are currently unanswerable.

Schwanengesang: from metaphytes to metaphysics

Is expansin the Rosetta stone of the cell wall or just a stumbling block for preconceived ideas? Will it help us translate the hieroglyphics of the wall to a more demotic lingua franca? And if so, will it validate the essential premise of the extensin hypothesis that postulates its pivotal role in growth and development? Is the paradigm regained? Is there life behind cell walls, or am I just a prisoner of my own paradigm[55]? Possibly we are at last beginning to understand a major mechanism of morphology at the molecular level. It may not be the discovery of π, but it is a piece of the pie and pretty damn satisfying! Scientifically, the future looks more interesting than ever, with amazing tools to escape superstition and solve any question that our imagination can dream up, including our human limitations and our personal identity as 'thinking machines'. The evolution of a few 'self-awareness neurons' [308] in the anterior cingulate cortex[56] makes introspection, hence science, ethics [3] and a 'theory of everything' [309] possible. The ability to simulate increasingly complex phenomena in computero will make Nostradamus roll over in envy. And perhaps, just perhaps, one day we may truly begin to 'consider the lilies of the field' and understand how they grow.

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[55] To some extent we are all prisoners of the paradigm: 'There is no such thing as research in the absence of any paradigm' p. 79 of [27], while the *Oxford English dictionary* [337] cites Gale in 1669, 'The Universe… was made exactly conformable to its Paradigme, or Universal Exemplar' and Jowett in 1875, 'Socrates makes one more attempt to defend the Platonic ideas by representing them as paradigms'.

[56] 'I had a brain scan and there is nothing in the frontal cortex where the blood is supposed to rush when you concentrate.' A country singer in *The Times* magazine, 23 Oct 1999.

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