Real-time models of morphogenetic processes

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Summary. Morphological transformations are often describable by simple series kinetic models, $A \rightarrow B$, $A \rightarrow B \rightarrow C$, etc., which allow assessment of the rates of interconversion of the distinguishable shapes or forms present and their probabilities of occurrence at various points in time, thus providing a means for kinetic comparisons with biochemical measurements of the molecular-level reactions that cause the transformations. When changes in cell morphology are followed turbidimetrically, the real-time progress curves can be simulated by fitting the data to a form of Beer's law for scattering by mixtures in which the species concentrations change with time in accordance with the chosen kinetic scheme. Because many even relatively large cells are mostly water, classical light scattering theory can be used to interpret the turbidimetric data in terms of simple geometrical models of *average* cell size and shape suggested by microscopic examination. Two examples are briefly considered, the stimulus-induced changes in blood platelet shape and apparent size and their correlation with cytosolic-free calcium, and apparent swimming motion exhibited by neutrophils in suspension.

Key words. Series kinetics; turbidimetry; light scattering; shape changes; blood platelets; neutrophils; actin; microtubules; free calcium.

"'What do you know about this business?' the King said to Alice.
'Nothing,' said Alice.
'Nothing whatever?' persisted the King.
'Nothing whatever,' said Alice.
'That's very important,' the King said."
Lewis Carroll (1865), in
Alice's Adventures in Wonderland

A fundamental challenge in cell biology is the reconstruction of whole-cell developmental and functional responses in terms of the biochemical and biomechanical events that cause them. The kinetics of many of the individual molecular-level processes that constitute the machinery for cell growth, maintenance and function have been examined in vitro and in vivo, thus opening the way for real-time comparisons of morphological cause and effect relationships. However, it is often difficult to express changes in morphology in the kind of quantitative language needed for meaningful kinetic comparisons. If the observed changes are essentially one-dimensional, as for example in the extension of the acrosomal process in sea urchin sperm[22] or the increase in the diameter of spherical cells due to growth, it is a relatively straightforward matter to evaluate the rate and extent of the change and to compare these parameters to the rates and extents of biochemical events such as actin polymerization into filaments (sea urchin sperm) or the synthesis of specific structural elements from ingested nutrients.

When the changes are more complicated, involving more than one step and/or complex shapes, it is no longer easy to distinguish between closely similar forms in a continuously changing population of cells. The populations themselves are usually not uniform, and different cells having somewhat different sizes, shapes and ages may also respond at somewhat different rates. These are familiar problems to those who utilize morphometry for evaluating successive changes in cellular form. An unbiased and sufficiently sophisticated scheme for classifying the various shapes and sizes present at different times during the transformation is hard to find, and it is very time-consuming to measure the dimensions of a large enough number of objects so as to ensure that the answers represent the whole population at each of the chosen time points, which must be numerous and closely spaced if a kinetic analysis is desired. Asymmetric objects represent a further complication in that they may present different aspects to the investigator at different times: front, top and side views, and all the gradations in between.

Here I discuss a turbidimetric approach to alterations in gross morphology that complements microscopy by attempting to simulate the real-time transformations. Application of the method results in a formal kinetic description of the cellular responses, which can often be interpreted in terms of simple geometrical models of *average* cell sizes and shapes suggested by microscopic examination. While such models contain none of the detail one is accustomed to seeing in micrographs, they do allow one to establish the rates of interconversion of the various forms present and the precise points in time at which particular forms have their maximum probability of occurrence, thus providing a means for a quantitative comparison with kinetic studies of biochemical mechanism[3].

Physical background

The alterations in morphology associated with cell growth and development or with the conversion of mature cells and organisms into other functional forms can be visualized as a continuous series of virtually infinite but real transient states generated by reactions occurring

at the molecular level within the cell. Stated mathematically,

$$X_1 \xrightarrow{\kappa_1} X_2 \xrightarrow{\kappa_2} \ldots \xrightarrow{\kappa_{i-1}} X_i \xrightarrow{\kappa_i} \ldots \xrightarrow{\kappa_{n-1}} X_n \qquad (1)$$

in which the κ_i are the individual (microscopic) rate constants for the conversion of one morphological form (X_i) to the next (X_{i+1}) and n is an extremely large number. Adjacent steps in such a quasi-infinite series will be kinetically and morphologically indistinguishable, even though it may be possible to record the overall process by time-lapse photography and to trap (and isolate) any particular distribution of sizes and shapes by sudden freezing or treatment with reagents that arrest the progress of the reaction. Certain configurations of the system will be significantly different from others that are many kinetic steps removed, however, and these are the ones which are readily detected by eye or revealed by changes in some physical property, e.g., turbidimetry, which is used to examine the transformation. Thus if there is a single uniquely-recognizable morphological intermediate in scheme 1, say X_i, the reaction can be kinetically described as the transformation $X_1 \rightarrow X_i \rightarrow X_n$, or $A \rightarrow B \rightarrow C$. The observable or macroscopic first order rate constants (k) for the successive interconversion of the three forms are defined according to the probabilities of existence p_i of all the n indistinguishable forms by the statistical-mechanical averages [3]

$$\langle\kappa\rangle_{AB} = \sum_{i=1}^{j-1} \kappa_i p_i \Big/ \sum_{i=1}^{j-1} p_i = k_1$$
$$\langle\kappa\rangle_{BC} = \sum_{i=j}^{n-1} \kappa_i p_i \Big/ \sum_{i=j}^{n-1} p_i = k_2 \qquad (2)$$

When the changes in morphology can be expressed in terms of a single physical parameter, the progress curve for a series reaction can be fitted to an established kinetic model by means of nonlinear least squares, yielding a real-time mathematical description of the responses. In the present example of a single distinguishable intermediate or transient form, the time-dependent probabilities of existence of the three forms A, B and C are given by the well-known rate equations

$$A(t) = e^{-k_1 t}$$
$$B(t) = k_1(e^{-k_1 t} - e^{-k_2 t})/(k_2 - k_1) \qquad (3)$$
$$C(t) = 1 - A(t) - B(t)$$

Multiplying these expressions by the initial number of cells under observation or their concentration in suspension gives the numbers or concentrations of the individual recognizable forms at any chosen time t, in terms of the rate constants for their formation or disappearance. For a single cell, the probability of observing any one of the myriad of real transient species is 100% at the time when it appears, and not that which is indicated by the equations. What these and other classical kinetic equations do indicate is the probability that a real, non-uniform population of cells sampled at an intermediate time will have a morphology resembling the composite average of the kinetically-distinguishable species, weighted according to their respective probabilities. In other words, just the quantity which is needed for comparison with biochemical studies based on the same statistical distribution.

The parameter of choice for following morphological changes in real time is the turbidity of a suspension of the objects in question [12]. Turbidimetry is similar to microscopy, except that the scattered light is not reconstructed to form an image (inverse Fourier transform). Instead, the results are expressed in terms of a single number, the light scattering extinction coefficient or cross section ε, which is a characteristic constant for each particle at a given wavelength. Since cells are mostly water, their refractive indices relative to the surrounding medium (m) are close to one, and therefore classical scattering theory can be applied to surprisingly large objects. For Rayleigh-Debye scattering, for example, the (decadic) extinction coefficient is given by the expression [9]

$$\varepsilon = \frac{9\pi^3 V^2}{\lambda^4 \ln 10}\left(\frac{m^2-1}{m^2+2}\right)^2 \int_0^{\pi} P(\theta)(1+\cos^2\theta)\sin\theta\, d\theta \qquad (4)$$

where V is the effective scattering volume of the particle, $P(\theta)$ is the value of the intraparticle interference or form (shape) factor at a given observation angle θ, and λ is the wavelength of light in the medium. The scattering extinction coefficient is a sensitive measure of particle size and shape, capable of reporting changes in particle dimensions of the order resolved by scanning electron microscopy [12]. The scattering coefficients for simple geometrical models – homogeneous spheres, spheres with a thick or thin shell (membrane), cylindrical rods and plates, and ellipsoids of revolution – can be calculated by standard methods [9], thus providing a basis for interpreting turbidimetric data in terms of a wide variety of somewhat abstract but nevertheless quite useful models of cells undergoing morphological transformations.

The extinction of a suspension due to scattering (turbidity) is related to the scattering coefficients of all of the n forms present and their probabilities of existence by a form of Beer's law for mixtures. If the extinction is divided by the total number of cells or their concentration $c_0 = \Sigma_i c_i(t)$, the result is the statistical average extinction coefficient of the mixture [3]

$$\overline{\varepsilon(t)} = \sum_{i=1}^{n} \varepsilon_i c_i(t) \Big/ \sum_{i=1}^{n} c_i(t) = \sum_{i=1}^{n} \varepsilon_i p_i(t) \qquad (5)$$

which is a direct measure of cell size and shape at any particular time. For the simple $A \rightarrow B \rightarrow C$ reaction (three kinetically-distinguishable forms),

$$\overline{\varepsilon(t)} = aA(t) + bB(t) + cC(t) \qquad (6)$$

where a, b and c are the extinction coefficients and $A(t)$, $B(t)$, and $C(t)$ are the time-dependent probabilities defined in equation 3.

Although there are numerous reports using light scattering to evaluate the sizes and shapes of small biological particles such as bacteria, mitochondria and the like, few studies have addressed the problem of larger cells and almost no attempts have been made at kinetic modelling in the sense discussed here. Thus it will not be possible to give an in depth review of work in the field, and instead I will briefly discuss two examples of morphological changes that have been subjected to real-time kinetic analysis and subsequently examined in terms of light scattering theories.

Blood platelet shape changes

Platelets are small, ca. 3-μm diameter cells that change their shape and apparent size in order to perform their physiological functions of aggregation, hemostatic plug formation and eventual clot compaction. The real-time morphological changes associated with the activation of blood platelets have been studied by both microscopy[8, 16, 17] and by laser turbidimetry[2, 5, 6]. The response to stimulation is reasonably fast: the initially flat discoid cells round up into spheres within 6–10 s, and in the next 20–30 s they extrude five or six long, thin pseudopods or 'spines' that more than double the effective cell contact radius and markedly increase the chances for aggregation. The morphological alterations are accompanied by small changes in the turbidity of platelet suspensions, whose origins can be explained by light scattering theory[11, 14]. The salient point of the theoretical analysis is that fully extended pseudopods constitute only a fraction of the mass of the cell, and because of this and the fact that they are very thin, they scatter far less light than the body from which they are extruded and can be neglected. The total cell volume does not change during activation, so the extension of pseudopods can be treated as a simple decrease in the volume of the approximately spherical cell body[13].

It is well known that spheres scatter more total light than particles of any other shape having the same volume and refractive index (e.g., discs, in the case of platelets), and that small spheres scatter less light than large ones. The turbidimetric responses to platelet stimulation are biphasic, implying the presence of an intermediate that appears and disappears as a result of a continuous size and shape transformation, and the changes in sample extinction can be simulated by fitting the real-time (digitized) data to an $A \rightarrow B \rightarrow C$ series model of the kinetic process[6]. Scattering theory for an ellipsoid of revolution was used iteratively with different volumes and axial ratios until the extinction coefficients matched those obtained by curve fitting, leading to a predicted model of platelet shape changes

$$\text{disc} \xrightarrow{k} \text{equivalent sphere} \xrightarrow{k} \text{smaller spiny sphere} \qquad (7)$$

The important features of the model are the evidence for a more or less spherical transient intermediate, which was detected earlier in light microscope studies of chemically-fixed giant cells[16], the confirmation of pseudopod extension at the expense of body volume, and the establishment of the rates of change of one form into another. Significantly, the microscopic and turbidimetric views of platelet shape changes appear to agree with each other in all major aspects[2].

Results obtained with two different physiological stimulators of shape changes (ADP and thrombin) demonstrate that the scattering extinction coefficients of the transient and final forms are independent of the stimulator applied, and that the reaction is in each case a stochastic or random process (the rate constants k for the two halves of the reaction are identical). Thus, not only do the cells respond to different stimuli with a quantitatively invariant pattern of shape changes, but the entire process appears to be controlled by a single as yet unidentified rate-limiting step or series of reactions.

Since nature repeats itself endlessly in going from small to large scales, it would not be surprising to discover that – much as geographical features on a very large scale mimic features found on progressively smaller scales – a whole-cell stochastic response to stimulation might reflect the operation of stochastic reactions occurring on the molecular level. There are two such underlying reactions in platelets, microtubule disassembly, which is known to be a stochastic process[19], and linear actin polymerization, which is stochastic in principle. An influx of calcium into the cytosol apparently induces transient microtubule disassembly[20, 21], resulting in the breakdown of the microtubule ring that helps maintain the discoid shape of the resting platelet and allowing the cells to round up into spheres ($A \rightarrow B$ shape change). Pseudopod extension, the second or $B \rightarrow C$ shape change, appears to be due to the linear polymerization of actin into filaments and bundles arranged parallel to the axes of developing pseudopods and extending into the body of the cell. The time course for microtubule disassembly[21] seems to correspond with the turbidimetrically-detected disappearance of discoid platelets, which closely follows the change in axial ratio observed in the microscope, and the time course for the incorporation of actin into Triton-insoluble cytoskeletons[15] appears to be correlated with the appearance of pseudopods (fig. 1). The correlation between morphological changes and causative events is not perfect, but the data appear to provide starting points for future refinement.

In other experiments[10], cytosolic free calcium in platelets loaded with a fluorescent calcium indicator (indo-1) was examined in combination with shape changes, using an optical multichannel analyzer[7] to simultaneously acquire the necessary data. Like shape changes, the free calcium response is biphasic and can be described by an $A \rightarrow B \rightarrow C$ series model. The calcium response is not stochastic, however, consisting of a fast transient increase in free calcium which reflects the release of storage pool calcium into the cytosol, followed by a slower calci-

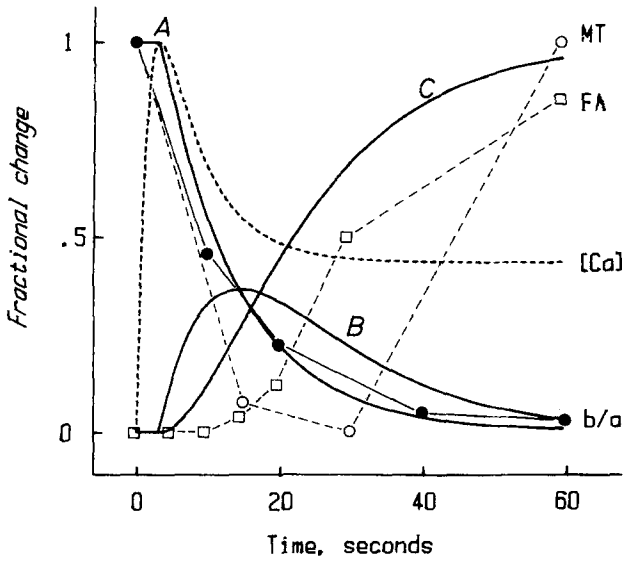

Figure 1. Comparison of turbidmetrically-detected changes in blood platelet shape and size with selected morphometric and biochemical measurements. Shape changes (solid curves) are expressed as the time-dependent species probabilities in a disc (*A*) to sphere (*B*) to smaller spiny sphere (*C*) reaction having equal rate constants [10] and the other data as a fraction of the observed change. Solid line, morphometrically-measured change in axial ratio *b/a* (long to short axis, filled circles) [17]; broken curve, real-time cytosolic free calcium ([Ca]) [10]; broken lines, microtubule disassembly (MT, open circles) [21] and Triton-insoluble filamentous actin (FA, open squares) [15].

um-consuming reaction that reduces the excess free calcium to a level about halfway between the initial and maximum values. Shape changes begin to occur only when free calcium reaches its maximum 3 s after stimulation, and the disc to sphere shape change appears to be associated with the disappearance or re-uptake of released calcium (fig. 1). By contrast, the bulk of the supposedly actin polymerization-dependent extension of pseudopods occurs at a time when calcium re-uptake is practically over, suggesting that the incorporation of developing filaments into cytoskeletal elements may not require significant amounts of cytosolic calcium.

The rate of change of free calcium consumption is an approximate measure of the combined activities of the calcium-binding enzymes and regulatory substances that control calcium utilization. As an added bonus to the kinetic analysis, the rates of change at any time can be obtained analytically by taking the derivative of the real-time progress curve, or of the individual state response curves. Such data show that the apparent activity of the calcium re-uptake machinery is itself transient, rising rapidly to a maximum 2 s following the onset of the shape change and decreasing slowly to zero as the shape change proceeds. This further indicates a connection between calcium consumption and the disc to sphere shape change, and gives a more detailed idea as to how the two processes might be correlated in time than that implied by the rate constants alone.

At high stimulus concentrations, shape changes are accompanied by the secretion of substances from storage granule contents and the synthesis and release of others, which separately or together trigger activity in nearby platelets, initiate blood coagulation and aid in blood vessel constriction. Relatively high doses of thrombin generate an additional decrease in turbidity, over and above that due to shape changes alone, which scattering theory quantitatively accounts for as an additional decrease in platelet body volume [5] caused by the actomyosin contractile complex-dependent [1, 18] squeezing out of (alpha) granule contents. The complete physiological reaction including shape changes and secretion (but not aggregation) follows stochastic $A \rightarrow B \rightarrow C$ reaction kinetics, suggesting that the mechanisms which are responsible for shape changes and secretion of granule contents are coordinated in a similar fashion.

Neutrophil motilty

Via a series of amoeboid-like movements, neutrophils leave the blood stream and migrate through the tissues in search of bacteria or parasites, following a gradient of chemotactic substances released by the invaders. When neutrophils arrive at the infection site, they ingest the foreign organisms and kill and dismantle them. Locomotion on a substrate or in a three-dimensional collagen matrix is characterized by the periodic extension and retraction of a broad, thin and often highly convoluted lamellipod, and neutrophils treated with chemoattractants in suspension exhibit a rapid and transient decrease in turbidity [23, 24]. The volume of the cells does not change during this time, and as in the case of pseudopod formation in platelets, scattering theory indicates that the decrease in turbidity is mainly due to a decrease in body size associated with lamellipod extension.

Under certain conditions, the suspension turbidity oscillates in a regular manner following stimulation with any of a number of chemoattractants [23]. Both Rayleigh-Debye theory and the anomalous diffraction theory of van der Hulst indicate that the oscillations are due to lamellipod extension and retraction cycles, which are reflected by complementary decreases and increases in the volume of the cell body. The real-time data can be simulated using an $A \rightarrow B \rightarrow C$ model modified by the inclusion of a periodic function in the kinetic description of the transient species *B*, represented analytically by the oscillating *ABC* model [4]

$$A \xrightarrow{k} \underset{\nu \;\; \nu}{B' \;\; B''} \xrightarrow{k} C \qquad (8)$$

B

Here *A* is the initial sphere, *B* is the putative transient which oscillates between spheres with large (*B'*) and small (*B''*) lamellipodia, and *C* is the quiescent final sphere with an intermediate-size lamellipodia. The mean reaction is

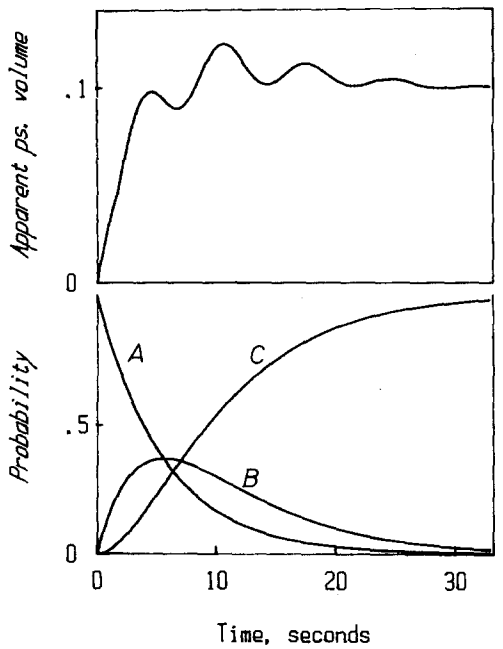

Figure 2. Pseudopod extension/retraction cycles exhibited by human neutrophils in suspension (*top*, expressed as the apparent fraction of the total cell volume calculated from the experimental extinction coefficients by means of equations 4 and 8), and the predicted *mean* probabilities of the three putative species in the $A \rightarrow B \rightarrow C$ (mean) shape transformation (*bottom;* see text).

$A \rightarrow B \rightarrow C$, and the amplitude of the oscillations depends on the probability of existence of the putative transient (fig. 2). As in the case of platelets, the rate constants for the $A \rightarrow B$ and $B \rightarrow C$ parts of the reaction are equal, showing that here too, a stochastic mechanism controls the transformation. The frequency of the oscillations (ν) is similar to the frequency of interconversion of the basic forms (rate constant k), indicating that the two phenomena are closely related. Additional calculations demonstrate that the frictional drag of the lamellipod is large enough in comparison to that of the body so as to provide a theoretical basis for a type of swimming motion in suspension. Since tissues contain spaces largely filled with water, this may be a significant new result related to the migratory ability of the neutrophil. Again, the real-time kinetics of the motion are available for comparison with biochemical studies of their causes.

Concluding remarks

There are probably many examples of morphological transformations that might benefit from the kind of kinetic analysis discussed here. The connection between cell size and shape implied by the experimental extinction coefficients must be worked out in each case, however, using for comparison the extinction coefficients predicted by light scattering theory for applicable geometric models of the cellular forms. Classical light scattering was once a method of choice for investigating the size and shape of proteins in solution, and although it partly faded from view when crystallographic studies yielded structural details at the atomic level, it has not been superseded as a method of following transformations in real time. A parallel situation seems to exist with cells: microscopy gives structural details not available by any other method so far, but is not the ideal choice for examining the *average* real-time changes in size, shape and reactivity that we hope to compare with classical biochemical studies of mechanism. Although scattering methods rely upon theory for their interpretation, it may be useful to recall a statement made a hundred years after Lewis Carroll's *Alice*

"It is not true that we can pursue science completely by using only those concepts which are directly subject to experimentation"
Richard Feynman (1965), in
The Feynman Lectures on Physics

Much of the future development of morphological cause and effect relationships seems to lie with imaging methods for simultaneously recording the necessary parameters, particularly the latest ones that use a laser to scan successive layers of the sample. Until such systems become better adapted for broad spectrum single-cell biochemistry, however, techniques like that briefly described here will perhaps find a useful temporary niche in cell biology.

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Physical fields and cellular organisation: field-dependent mechanisms of morphogenesis

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The articles in this review series have considered several very different aspects of morphogenesis. As much as it was desirable to do so, however, we have not included a paper which deals specifically with the role of the gene during this process. To some extent this heinous crime is mitigated because reviews have recently appeared which comprehensively (and exclusively) cover the subject [1, 19]. Some of the other contributors to this volume have made reference to the genome as an important component of the developmental process and for the sake of completeness I shall also say a few words. Without any prior prompting of the other contributing authors, however, the prevailing theme which has emerged is that in order to solve problems in morphogenesis one must draw inspiration from physics and chemistry to complement biological intuition. And, indeed, this review synthesis was conceived principally with that aim in mind.

For the main part of my contribution, I would like to emphasise the likely influences of various types of physical fields on cellular organisation and to morphogenesis in particular. Thus in reply to Schatz's edict it is held that some of the hitherto obscure structures within eucaryotic cells may be chemical, dielectric, electrical, mechanical or osmotic, scalar or vector fields. The nature of physical fields (vector or scalar), however, is not readily appreciated within conventional cell biology and so to begin I shall briefly describe their nature and origin.

Scalar and vector fields

A physical field may be said to exist if a discrete value of a parameter may be identified over a given spatial region (unit) within the total area designated by the field. The parameter may be any quantity that can be measured. A scalar field represents a field of scalar quantities such as temperature or concentration (e.g. Ca^{2+} or electric charge). Or it may be a vector field whereby a field of vector quantities exist such as electric (or ionic) currents. A good example of both occurring together is illustrated by a standard weather map. The discrete values of temperature at each position on the map represent a scalar field whereas discrete wind velocities represent a vector field.

Similarly, in a living cell there may be concentration gradients of ions within the cell which would represent a scalar field of electrochemical potential or the individual currents which generate the concentration gradient may represent a vector field of currents. Another way of expressing this state of affairs which is more in line with the jargon of developmental biologists is by describing the fields as patterns of molecular species within cells. Pattern analysis is a major preoccupation of developmental biologists and there are some elements of this work which I shall now discuss.

The formation of spatial patterns within cells

Dynamic, apparently spontaneous, pattern formation is a characteristic of almost all eucaryotic cells. A rather nice introduction to the subject may be found in *Pattern Formation* [26]. This phenomenon is not exclusive to living systems, however, as even 'simple' chemical systems may exhibit it, of which the most celebrated example is the Belousov-Zhabotinski (B-Z) reaction. Although the reaction consists of rather simple component parts, such as an organic reductant (such as bromate) which is oxidised by a redox couple (such as Mn^{2+}/Mn^{3+}), nevertheless, it exhibits a very rich phenomenology [8].

In an effort to describe these systems Winfree [60] has elaborated the concept of an oxidising 'trigger wave' moving through an excitable reducing medium. The 'wave' and its effects are visualised by colour changes of an appropriate redox indicator or from the redox potential measured around a platinum electrode. The 'trigger wave'