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Oscillations in cell morphology and redox state

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Fluctuations in the intensity of light scattered and absorbed by cells in suspension have been analysed by smoothing, periodogram and power spectrum methods to reveal oscillations attributed to changes in cell morphology and the redox state of NADH and FAD (periods 10 s to 30 min). The rhythms are themselves periodically modulated in amplitude at a similar frequency and exhibit burst characteristics. The low frequency scatter dynamics are provisionally attributed to oscillations in gross morphology and the high frequency variation to changes at the cell surface. Agents, such as insulin and transferrin, affect the dynamics. The scatter results suggest that rhythmic changes in cell morphology associated with locomotion are largely inherent in the cell and not due to periodic attachment and detachment from a surface.

1. Introduction

It is with pleasure that we contribute to this special issue of the journal dedicated to Jeffries Wyman and we thank the editors for inviting us to do so. As our mark of respect for his contributions to our knowledge and understanding of periodic processes, we would like to take this opportunity to outline some of our recent studies on cellular oscillations. These we believe not only give us new insight into the dynamic operation of cellular control reactions but may also provide us with the means to observe the initial effects of a wide range of agents (such as growth factors, potential anti-metastatic compounds, tumour promoters, inducers of differentiation, anaesthetics and other membrane-active compounds) on cells. They may also eventually provide a rapid means of detecting and screening such compounds. At the same time the results also illustrate the generally unrecognised or unappreciated dynamic complexity exhibited by cell populations and the practical difficulties of analysing and interpreting such data.

Cells, which form the basis of life, are no exception to the general rule that all living entities are dynamic. Anyone doubting this need only watch a time lapse microscope film of cells (either in culture or tissue slices) in order to appreciate this point. Obvious dynamic features include cell locomotion, changes in morphology, ruffling of the plasma membrane, agitation of organelles within the cells (even rotation of nuclei) and cell division. Dynamic attributes imply that the underlying biochemistry is also dynamic. In turn, prolonged dynamic behaviour indicates the occurrence of either repeated perturbations of the system from an external source, or the oscillatory operation of one or more internal control processes. Only the latter behaviour is autonomous and likely to persist for the life time of the

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organism. We believe that the existence of periodic metabolic reactions is necessary for the above phenomena to occur. In addition, we have proposed (a) that intracellular signalling takes place through temporary modulation of the oscillations due to the action of external agents, and (b) that differentiation and cancer can result from permanent changes in the oscillation frequencies, amplitudes and phasings particularly as a result of disturbances in the coordination of cellular processes [1,2].

In a number of other articles, we have been concerned with rhythmic variations in the activities and effective isozyme patterns of cells and also in the amount of protein extractable from them (see ref. 2 for a list of references). In support of the concepts we have shown (a) that the phasing between two isozyme oscillations in a virally transformed cell line was different from that in the untransformed cells [1,2], and (b) that agents can affect the dynamics [2,3]. Another area of interest has been modelling of the cell cycle. An oscillatory basis was predicted for the phenomenon (now supported by experimental data) and it has since been shown (on theoretical grounds) that all major facets of replication can be interpreted in terms of a limit cycle oscillation [4,5]. Among the aspects which can be understood is the nature of the effect of the malignant transformation on cell proliferation. It is suggested that the altered replicative behaviour results from a shift in the bifurcation separating oscillatory and non-oscillatory states, producing an increase in the frequency of random triggering of cycles of the periodicity due to the control system exhibiting excitability [6,7].

More recently, using equipment which is available in the average laboratory, we have been attempting to monitor, in an essentially continuous manner, (a) morphological dynamics (because of their relevance to cell locomotion and hence tumour metastasis, wound healing, the immune response and embryonic development), and (b) the redox state of the cells (as reflected in the NADH and FAD levels), partly in an effort to determine any possible dependence of the morphological changes on these parameters but also to detect effects of agents on the redox state. In this article, we outline our approach and methods of analysis and then present a selection of our initial results together with provisional interpretations of some aspects of our data.

2. Methods

We have used variations in the intensity of light scattered by the cell population as a measure of morphological changes and both fluorescence and absorption to determine the redox state and levels of the flavin and nicotinamide coenzymes. Initially, we used a Beckman Acta III spectrophotometer for just scatter determinations and later a Hitachi 850 spectrofluorimeter for all three determinations. More recently, we have been able to monitor the cells with a Hewlett Packard 8425A diode array spectrophotometer. The latter has the major advantage of allowing us to follow scattering and the redox states simultaneously using the differences in absorption at 340 and 346 nm for the reduced nicotinamide level, between 450 and 460 nm for the flavin, and the sum of the absorptions at 620 and 630 nm for the scatter intensity. For the morphological studies the two spectrophotometers measure the forward scattering whereas the spectrofluorimeter determines the right angle scatter. The results for the two methods are thus not strictly comparable although we have so far been unable to attribute any aspect of our data to the difference. When using the Hitachi instrument for the scatter studies reported here, we set both excitation and emission wavelengths to the same value, 620 nm, while for the redox measurements these were set to, respectively, 340 and 450 nm for NADH determinations and to 450 and 550 nm for the FAD fluorescence. With the Acta we measured only the scatter at 620 nm. Phenol red, which absorbs in the regions of interest, is omitted from the culture media.

In addition, we have tried three experimental approaches in that the measurements were made on cells stirred in the cuvette, or circulated from an external stirred culture vessel through a flow cuvette. We are at present using the HP 8425A instrument to study cells grown as a monolayer on

one cuvette face: the results seem promising but other instruments may not have high enough sensitivity while not all cells may attach to the particular cuvettes used. On the other hand, most mammalian cells will not survive in suspension.

With circulated cultures there is the possibility of introducing periodicity due to the peristaltic pump used to pass the cells through the cuvette. There is also the problem that one is not looking at the same population of cells from moment to moment. Varying the stirring or flow rate is necessary to check such factors. The design of the cuvette could be important particularly for the flow studies. So far we have not been able to attribute any facet of the data to such factors. Preliminary results suggest that the monolayer studies could yield less noise. Ideally one requires continuous sampling or, at least, sampling at very short intervals. However, particularly where the duration of the experiment is long and the amount of computer memory available is very limited (as with our earlier studies), it is necessary to sample at relatively long intervals (for example, 10–20 s). This will have the effect of tending to eliminate very rapid changes from the data. Depending on the instrument available (e.g., the Hitachi and the diode array spectrophotometer), one can also achieve this effect by integrating the signal over this time interval to obtain an average reading. Alternatively, one can carry out such integration after logging the data on a computer.

On occasions, the data exhibit overt periodicities but in general the temporal fluctuations are noise-like. In order to study the oscillation content, we are thus forced to analyse the raw data in a number of ways. We first use the least-squares smoothing method of Savitzky and Golay [8] with different smoothing numbers from 5 to 25 points, this having the effect of eliminating the high-frequency components as determined by the smoothing number and the sampling time. It thus gives an indication of the changes in the mean level and the superimposed slower variations. The higher frequency components can be considered separately by examining the difference between the raw and smoothed values. In one experiment where this approach was adopted, we found that the addition of Sindbis virus resulted in a decrease in the amplitude of the high-frequency variations after a short delay.

In addition, we examine the data by both periodogram and power spectrum methods, in the

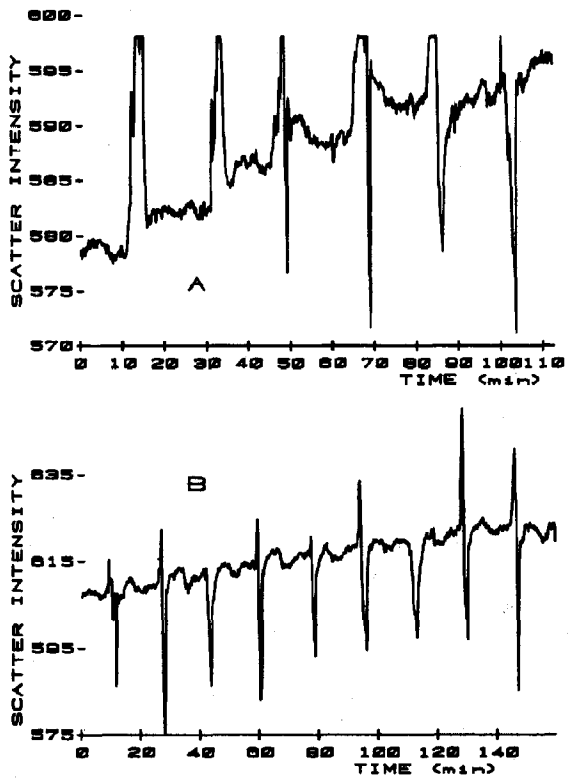

Fig. 1. Overt periodic fluctuations in scatter intensity. This diagram shows two sections of *atypical* raw data, obtained with murine erythroleukemia (MEL) cells which were circulated (using a Pharmacia peristaltic pump set at maximum flow rate) from a stirred external culture vessel through a fluorescence flow cuvette (Starna Limited, U.K.) in the Hitachi spectrofluorimeter. The trace in B was obtained shortly after that of curve A, the zero settings and sensitivity being adjusted in between. Note also the slightly different time scales for the two curves. The cells (in Eagle's MEM, 5% foetal calf serum, without phenol red) were allowed to proliferate and reach the late growth/early plateau phase of replication in the apparatus before data logging began. Sampling and integration times, both 10 s; scatter (excitation and emission) wavelengths, both 620 nm; response time, 0.5 s; bandwidths, 5 nm. All experiments reported here were carried out at 36.5°C. If the results are dependent on intercellular communication, one can expect them to be reflected in the cell density: at this time the level was approx. 1.5×10^6/ml. The reason for the spikes is not known but could involve diffusion of oxygen into the suspension.

latter case using the Hewlett Packard waveform analysis pac binary program, modified to permit the spectrum to be determined for sequential sections of the data in order to see how the contribution of different frequencies changes with time. We can then study the temporal variations in the total spectrum or for individual bands (frequencies) thereof. If band 1 is selected this gives a better measure of the mean value for that section of data, in relation to the smoothing value. In order to be able to compare results from different experiments, we standardize our parameter values as much as possible: thus, we use a fixed window (section of data) of 128 points for the power analyses: the frequencies extracted by this means are determined by the sampling interval which, so far, we fix at 5 or 10 s. According to the Nyquist principle, this limits the frequencies that can be studied from 10 s to 320 s for a 5 s sampling time and double these values for a 10 s logging interval. One can, of course, merely determine the dominant frequencies present by carrying out the analysis once on the whole set of data points or use different parameter values.

The power spectrum method fits a series of harmonic sine waves to the data but generally these cannot be selected. On the other hand, with periodogram methods one can examine particular frequencies and even interpolate as discussed by Enright [9]. An advantage of the power spectrum method is that it gives an overall picture for oscillations lying within the Nyquist range, since the frequencies are not linearly separated as with the periodogram method. The periodogram method we mainly use is that due to Enright [9], again adopting a moving window so as to show the temporal variations. We also use another version where, instead of looking at individual oscillation periods, we sum the contributions over a period range, again as a function of time. The reason for this will be discussed later. In addition, we have occasionally used the periodogram due to Lamprecht and Weber [10], but modified to linearise and invert the plot (Joosting and Gilbert, unpublished data). Again a window is moved along the set of data points in order to determine how the dynamics vary with time. However, we do not consider results obtained by this last method in the present article.

It is possible to enhance the 'sensitivity' of these studies by manipulating the analyses so as to eliminate minor components in the spectrum of frequencies by (a) varying the window width (the number of points used), and (b) ignoring those peaks with amplitudes less than a set value. Using this approach we have been able to show that

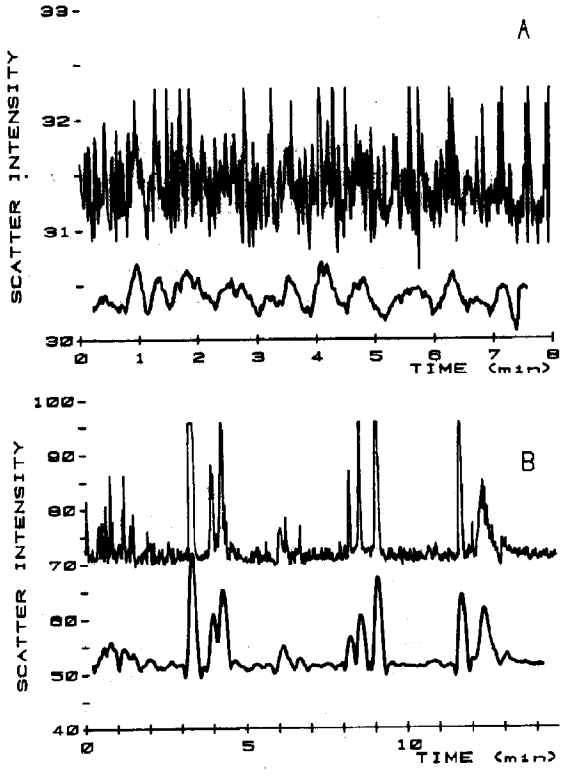

Fig. 2. Effect of smoothing. This diagram shows typical examples of raw data and the corresponding smoothed curves (offset for clarity) obtained by a running 25-point least-squares fit [8], MEL cells at densities of 0.12 and 0.5×10^6/ml for A and B, respectively, were suspended in Eagles MEM with Earle's salts supplemented, in all cases reported here, with Hepes buffer (10 mM) and antibiotics. Foetal calf serum was 10% in A and 1% in B. The scatter wavelengths were 478 nm in A and 620 nm in B. Sampling, integration and response times were 1, 1 and 0.5 s, respectively, in both cases. The bandwidths were both 5 nm in A and 1 nm (excitation) and 5 nm (emission) in B.

insulin influences the high-frequency scatter dynamics (results to be published).

3. Results

Fig. 1 gives atypical results from a long-term experiment wherein the cells were allowed to proliferate and to reach the late growth/early plateau phase before monitoring began. The increasing mean is presumably due to the residual increase in cell numbers but the origin of the overt periodic spikes is not evident, particularly in view of their low frequency. Fig. 2, on the other hand, shows typical results from short-term studies and the effect of smoothing the data. In one case (fig. 2B),

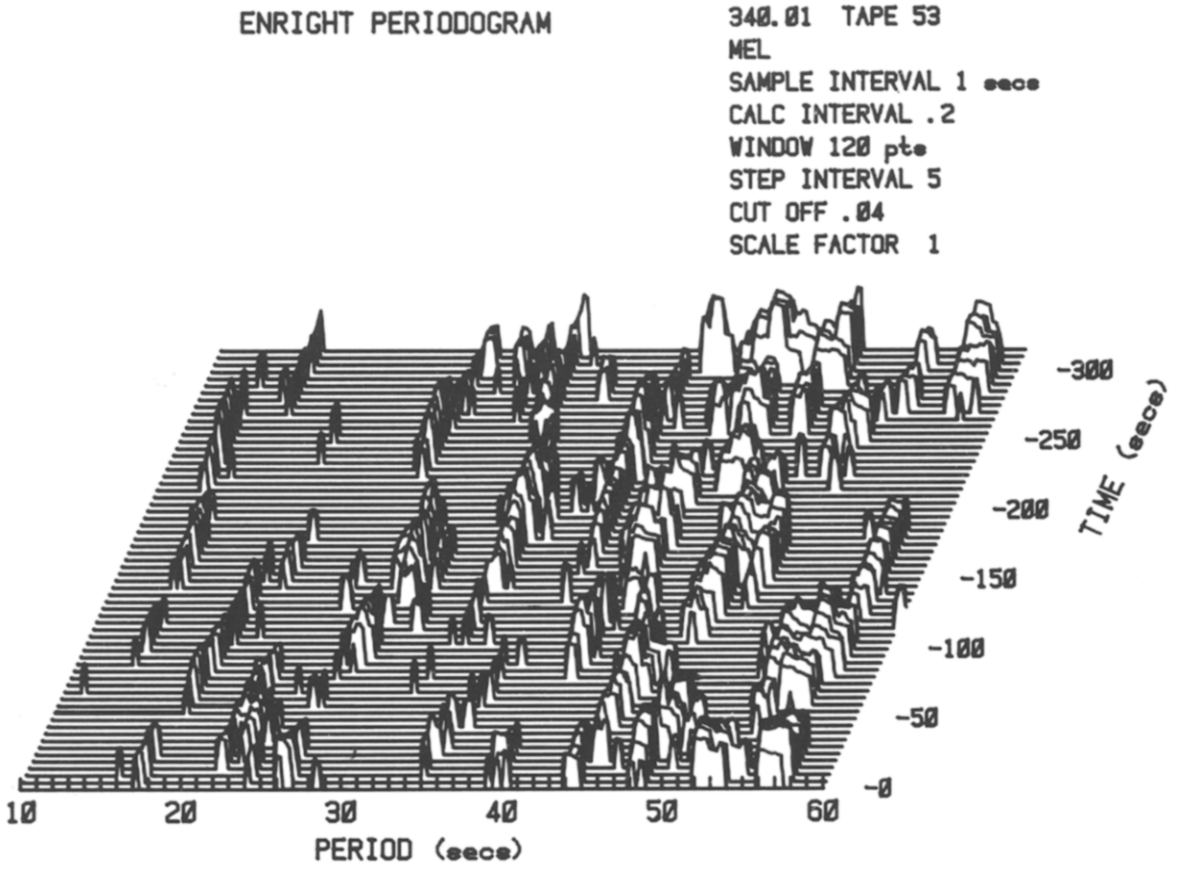

Fig. 3. Periodogram analysis. This shows a typical result of applying the Enright method directly (without smoothing or detrending) using a window of constant size, in this case, 120 data points, which is moved along the whole sequence of values (in steps of 5 data points) in order to see how contributing components vary with time. Here, MEL cells were circulated through a flow cuvette in the Hitachi spectrofluorimeter. Their density was 0.12×10^6/ml. The general procedure is to passage cells at least 12 h before the experiment and to set up the system several hours beforehand in an effort to allow cells to reach a steady state. A much longer time may be necessary. For this experiment cells were suspended in Dulbecco's MEM, with phenol red, containing 5% foetal calf serum. Scatter wavelengths, 478 nm; bandwidths, both 5 nm; response time, 0.5 s. As described by Enright, the calculation interval can be less than the sampling interval, in this example 0.2 s compared with 1 s. A cut-off value of 0.04 for the variance was used to eliminate minor peaks. Certain oscillations are present fairly consistently with essentially constant period but others show frequency drift or occur in pulses (see fig. 4). The choice of values for the analyses is generally arbitrary: the picture obtained can depend to some extent on the values selected (irrespective of which method is used), for example, longer windows will tend to decrease the contribution from minor components.

the amplitude of the high-frequency variations is low enough that the trend in the mean and the longer period changes are obvious but in the other (fig. 2A) the low-frequency fluctuations are obscured by the faster ones but the smoothing process reveals them. These two pairs of curves illustrate the basic patterns of behaviour generally seen: (a) a continuous periodicity as in 'A' and (b) burst-like characteristics in 'B'. While the high frequency changes seen in 'A' appear noise-like further analysis of such data indicates that it has a high rhythmic content. The fact that the amplitude of these rapid fluctuations can be modified by agents supports this view.

Fig. 3, which gives the results of periodogram analysis of data, adds further credence to the view that the high-frequency component is not pure noise. As can be seen, specific frequencies appear in the data. Observations on the temporal variation of a single period are complicated by the fact that frequency drift and frequency modulation can occur. Hence, as indicated earlier, the average contribution of a band of adjacent frequencies is also computed. Fig. 4 shows results of this kind from which it may be deduced (a) that the temporal patterns for certain adjacent bands can be very similar yet quite distinct from others (both with regard to the magnitudes and/or timings), and (b) that oscillatory behaviour frequently occurs in pseudo-periodic bursts: this is so for both high- and low-frequency oscillations. The latter characteristic is not always clear from an examination of the time course for a single period, presumably because of the drift and modulation aspects just mentioned: by averaging over a range of periods the effect of these factors is minimised.

Power spectral analysis confirms this general picture which is essentially the same for all three parameters though there are occasional exceptions. In figs 5 and 6 the results of such analyses are depicted: the former shows the burst-like characteristics over the whole spectral range while the latter examines the mean level and individual frequencies observed in another experiment. Fig. 6 indicates more clearly the periodic modulation in the contributions of different frequencies: in most cases it is quite evident that the amplitude of the modulating rhythms (their envelope) is itself mod-

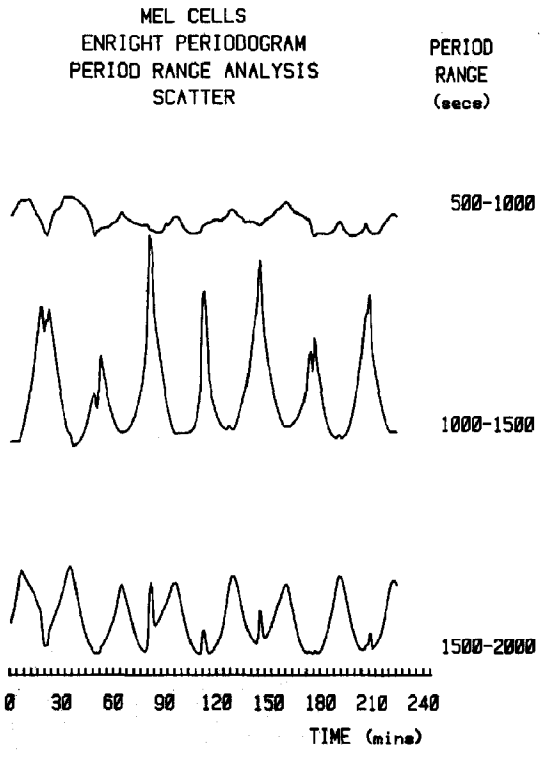

Fig. 4. Periodogram range summation. This diagram, in essence, shows a cross-section along the time axis of a plot of the kind shown in the fig. 3 except that the contributions of oscillations with periods in the ranges shown were summed in order to obtain an 'average' pattern of behaviour, in this case, for scatter curves. This minimises the effects on the interpretation of frequency drift or modulation. These diagrams confirm that the various rhythms are generally not constant in amplitude but occur in quasi-periodic bursts although the pulses are rarely as regular as shown in this diagram, perhaps due to the lack of a steady state. As can be seen, the patterns for adjacent bands can be similar (but may be distinct in phase and magnitude), while differing considerably from other nearby period ranges. We attribute the similarities to the existence of subpopulations of cells in which essentially the same processes are occurring but in which there is heterogeneity with regard to frequency. The distinct patterns of behaviour are consistent with the view that different reactions are being followed simultaneously, for example, the lower frequencies may be due to gross changes in cell morphology whereas the faster rhythms may be due to membrane movements. The complexity within any band may thus depend on the range limits chosen. In this example MEL cells (0.25×10^6/ml) (in Dulbecco's MEM medium containing 1% foetal calf serum and no phenol red) were circulated through the Hitachi instrument using scatter wavelengths of 620 nm and bandwidths of 5 nm, and a response time of 0.5 s. The sampling and integration times were 5 s. The calculation used a window of 100 points stepping through the data at intervals of 2 points.

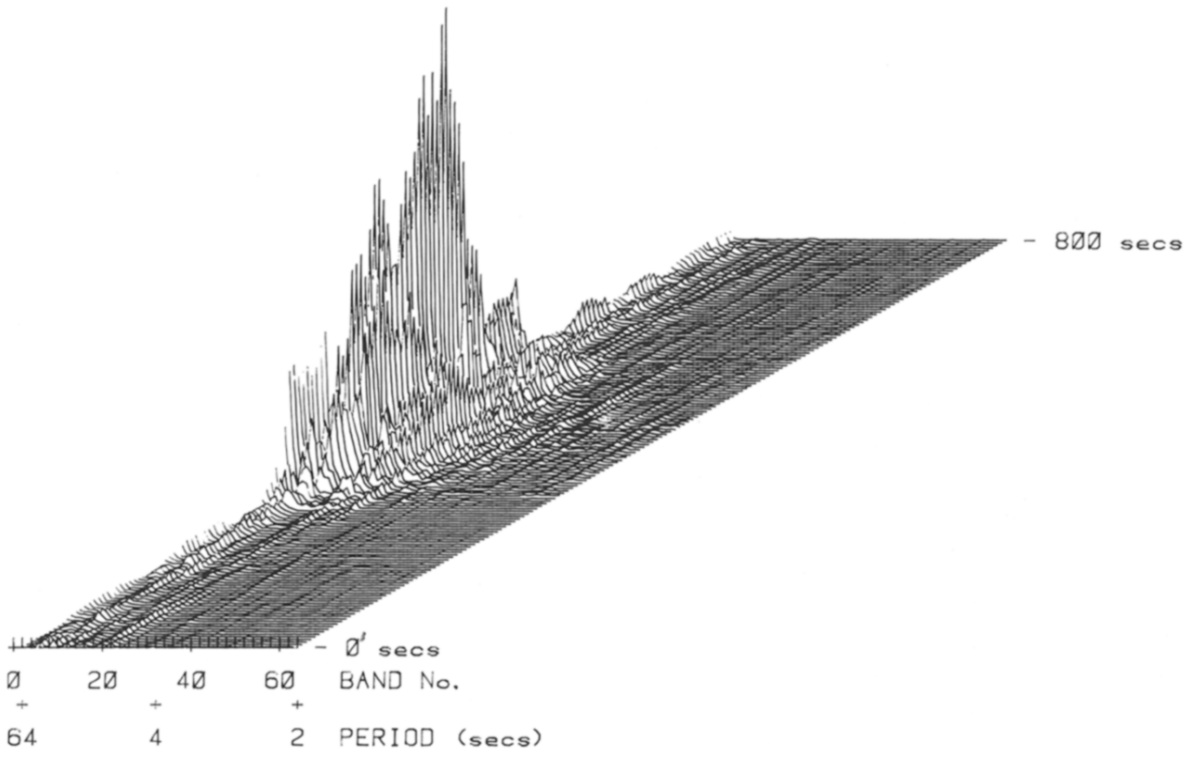

Fig. 5. Burst activity in scatter dynamics. This diagram gives the result of power spectrum analysis of a broad-frequency-range burst of morphological activity seen in freshly prepared human lymphocytes suspended initially in saline but diluted with Dulbecco's MEM without serum for the observations. The pulses occurred a number of times at intervals over several hours although the detailed picture varied. The cells were circulated through the Hitachi at 0.6×10^6/ml using scatter wavelengths of 478 nm, bandwidths of 5 nm and a 1 s sampling interval being integrated over 5 s at the computer. The sensitivity was 5 and the zero offset 23 units.

ulated, often in an overtly periodic manner. The temporal curves of the power spectrum band 1 (mean) values for all parameters generally exhibit step characteristics (cf figs 7 and 8). These also often occur at pseudo-periodic intervals although in many instances the length of data is not sufficient to prove that this is the case. As can be seen in the former diagram, agents (in this case transferrin) can affect the pattern of behaviour of the band 1 values, including the step frequencies, and do so in a differential manner with regard to the three parameters. In some instances, there is a clear correlation between the variations in the mean values for the different parameters indicating that the changes in, for example, the NADH and FAD levels are in phase with one another. However, this is not always the case: the patterns of variations can be quite distinct. In different experiments the mean levels may be increasing, decreasing or fairly constant, possibly reflecting the existence of a very long periodicity which is being sampled in different experiments when in distinct phases.

Available evidence again indicates that the dynamic characteristics can be modified by agents: in fig. 7 is shown the effect of transferrin on the mean (power spectrum band 1) values. Although insulin produces a general increase in the amplitude of the high-frequency scatter oscillations in MEL cells (to be published), if one examines particular individual frequencies one can see the opposite effect (fig. 8). It is not yet clear whether

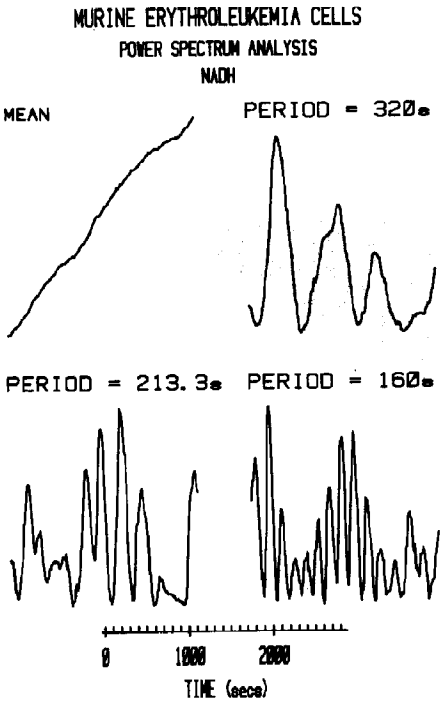

Fig. 6. Temporal modulation of the power values. Shown here are the variations in the magnitudes of the powers for four components contributing to NADH fluctuations in one experiment, namely, the 'mean' (band 1) and three harmonics of the periods indicated. The step changes in the mean are just distinguishable but it is evident that the power for each rhythm is modulated (cf. fig. 4) periodically. Moreover, the amplitude of the modulating rhythms also varies, and does so in pseudo-periodic fashion although in this example the record is too short to show this for the 320 s primary oscillation. It is thought that this kind of behaviour may stem from self-modulation (see text). These characteristics are observed for all three parameters but are not normally as regular as seen here, which result could be due to the fact that one is observing a smaller population of cells than when circulated from an external vessel, or, because of the high cell density allowing synchronisation to occur. As can be seen from fig. 8, the pattern of behaviour can be affected by agents implying that such curves are not due to artifacts introduced by the methods of analysis. MEL cells were diluted 1:1 with fresh Dulbecco's MEM (10% serum) 16 h prior to the experiment for which 2 ml were transferred to a sealed cuvette in the HP 8452A diode array spectrophotometer at a density of 0.5×10^6/ml. The time scale applies to all diagrams.

this is due to an effect on the frequency of the oscillation or if the hormone dampens the pre-existing rhythms: such problems show why it is necessary to analyse the data in several ways.

4. Discussion

As indicated, time lapse microcinematography of cells clearly reveals relative slow fluctuations in

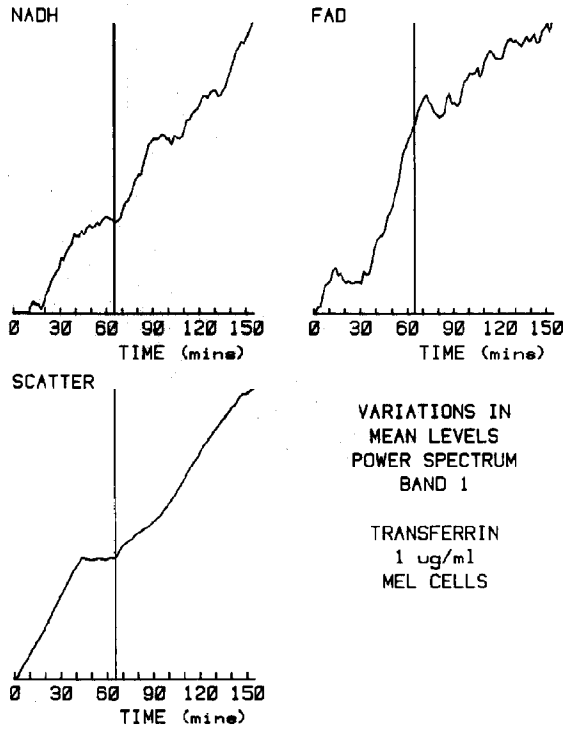

Fig. 7. Power spectrum mean value variations. This diagram shows the variations in the value for the power spectrum band 1 (the 'mean') for all three parameters from the same experiment and the effect thereon of the addition of transferrin at the time indicated by the vertical line. The frequently seen step changes in the mean values are evident and the agent obviously affects the step frequency of the FAD curve and also that for NADH, though to a lesser extent: there also appears to be a differential effect on the slope of the FAD curve. In this example the values for the three parameters, NADH, FAD and scatter, all increase in unison but this is not always the situation. It is presumed that the cells here were in a pseudo-steady state but that this is not the case where no particular correlation exists. Not discussed here are results of phase plane plots for the NADH, FAD and scatter values (cf. ref. 3). In this experiment MEL cells were stirred in a cuvette in the Hewlett Packard diode array spectrophotometer using Dulbecco's MEM medium without phenol red but with 5% foetal calf serum. In the last three figures the values for each curve have been scaled from the minimum to maximum values for the data concerned: thus, direct comparisons of magnitudes are not possible. These transferrin experiments were carried out in collaboration with Dr. E. Bey of Highveld Biologicals, who supplied the agent.

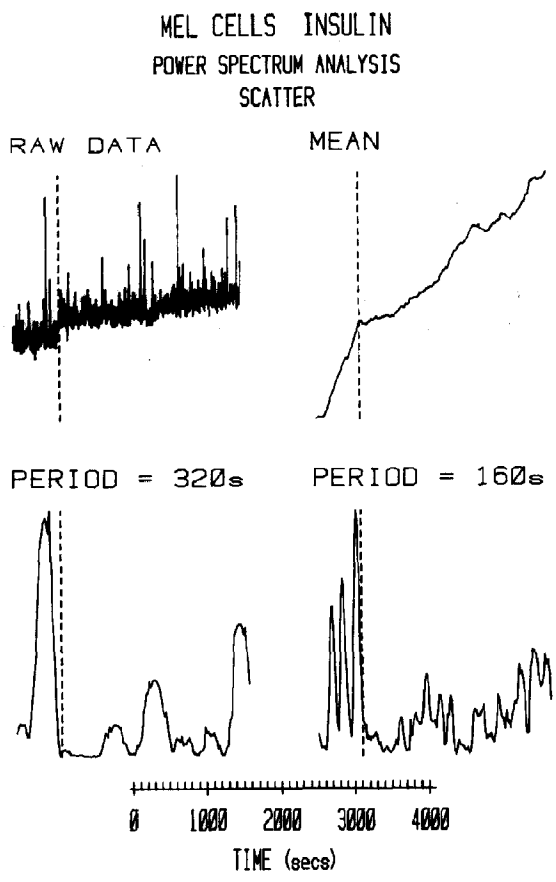

Fig. 8. Effect of insulin on scatter dynamics. The dominant effect of insulin (added at the point shown by the vertical broken line) on MEL cells would seem to be primarily that of increasing the amplitude of the high-frequency components (membrane ruffling?) but as can be seen here, if one analyses the data in terms of particular frequencies various other effects can be seen. In this diagram (which shows the variations in the raw scatter intensity data, the power spectrum band 1 value and two low-frequency components) the hormone apparently dampens the periodicities and changes the modulation pattern. However, the real effect could be that of changing the periods of the rhythms. It would need more frequent sampling to be able to see the trend in the time course for the individual oscillations over the immediate post-perturbation interval. Even then the large number of rhythms could still complicate the interpretation. In this experiment MEL cells were circulated through the Hitachi at a concentration of about 0.4×10^6/ml in Dulbecco's MEM with 5% foetal calf serum and no phenol red. Scatter wavelengths, 620 nm; bandwidths, 5 nm; response time, 0.5 s. Bovine insulin was added to approx. 1 μM. To minimise disturbances, agents are dissolved in supernatant medium taken from a sample of the culture immediately before the start of monitoring. The time scale applies to all diagrams.

the overall morphology of cells and more rapid changes in the number, kinds, sizes and distribution of cytoplasmic protrusions and localised undulations of their surface membrane (ruffling, flickering, see refs 11 and 12 for example). There is evidence to suggest that the detailed behaviour may be characteristic of the cell [13,14] and that it can be modified by contact with other cells, depending on their particular properties [11]. Changes in the morphology normally accompany replication with especially marked alterations in shape occurring at the time of mitosis and division. Theoretical considerations indicate that continuous dynamic behaviour is likely to have an oscillatory or quasi-oscillatory basis and rhythmic variations in cell morphology have been noted in several relevant studies. Apart from in vivo situations, where behaviour could be determined solely by external forces, spontaneous oscillatory contractions of isolated heart muscle cells are probably the most obvious example [16] (contractile elements have also been found in non-muscle cells and could contribute to morphological effects discussed here [11,12]). Gentle squashing of amoebae between coverslips induces them to oscillate visibly in shape [17] while periodic pulsations have been detected in amphibian eggs after fertilisation: surface waves have also been observed in these latter and other cells [18,19]. Cyclic protoplasmic streaming occurs in strands of the myxomycete *Physarum* [20] and the period of the local rhythm has been shown to be diversely affected by nutrients and toxic agents in a manner which suggests that the relative frequencies of streaming at opposite ends of the cell determine in which direction the slug moves [21]. Both radial and longitudinal contractions have been reported [21] (it is probably pertinent to point out that the leading edge of moving mammalian cells is that which ruffles at high frequency [11]). Rhythmic extensions of pseudopodia by fibroblasts have also been noted [14]. Time lapse video observations involving direct measurements of the area and periphery of cells have shown that the movement of amoebae and guinea-pig macrophages over glass surfaces, is accompanied, if not determined, by oscillations in the shapes and the complexity of the cells (as defined by the ratio of periphery ^{2}/

area) [22]. The movement of such cells depends on alternate attachment and detachment of regions of their surface to the substratum [11,12]. The questions arose: (i) do morphological changes occur only when cells are attached to, or are in contact with, some surface, and (ii) do reactions taking place at the plasma membrane affect the morphological dynamics? It seemed that if the answers to these questions were in the affirmative then continuous monitoring of the dynamics could provide a general way of studying the interactions of a wide range of agents (e.g., hormones, viruses) (a) with the cell surface, and (b) with the cytoskeleton. The observations could also yield information about the processes themselves. On the other hand, there is the problem of the source of energy for the dynamics, this question providing reason for examining the redox state of the cells.

Apart from requiring special equipment, time lapse visual studies on individual cells in free suspension are difficult to perform because of the need to keep the cell in focus and in the field of view. Light scattering [23] has been used as a sensitive means of detecting gross morphological changes due to, for example, osmotic effects and the action of chemotactic agents. Thus, the method has been used to study the response of suspensions of the amoebae, *Dictyostelium discoideum*, to cAMP; it was found that pulsed treatment resulted in periodic variations in their shape, changes which are considered relevant to their ability to aggregate when plated on a surface [21]. This indicates that attachment is not required for continuous morphological changes to occur but this example could be considered atypical and irrelevant with respect to, for example, cancer metastasis. In this instance, the coherence necessary for such changes to be observed in a population was imposed by the cAMP pulses, though the periodicity continued after the signal was stopped.

We considered whether rhythmic changes occur in the morphology of mammalian cells and, if so, whether they could be monitored by the same means, bearing in mind that, to be observed, the oscillations would need to be (partially) synchronised. From the preliminary results presented here, we conclude that they do occur and that they can be so followed. Moreover, similar dynamics are seen with regard to the NADH and FAD levels. The oscillation periods are of the same order as those seen in the other studies referred to, including our enzyme experiments. However, the behaviour is complex for all parameters with both high- and low-amplitude variations being detected over a range of frequencies. In addition, the oscillations are modulated, apparently by other rhythms, to the extent that the populations often exhibit (pseudo) periodic bursts of morphological activity and redox state. Both periodogram and power spectral analyses yield the same conclusion on this point. It is pertinent to point out that in some situations at least, cell movement occurs in bursts [14] and with a period of the order seen here, namely 2–10 min. The fact that bursts occur in all parameters might be taken to indicate a close dependence of shape and size on the oxidation reactions but the fact that distinct phasings are often observed argues against this although published data suggest that the morphology is governed by the FAD state [22]. It is possible, of course, that the requirements of other cellular processes for these redox components dominate and hence overshadow those of the morphological reactions.

Although overt periodicities are often seen, this is not the usual situation and hence the need for statistical methods of analysis. Referring to the periodogram band studies (fig. 4) it can be seen that the pattern of changes for different, adjacent bands can be very similar yet the phasings can be different. We interpret this last point as indicating that essentially the same processes are occurring in different subpopulations of cells but that the frequencies and timings are somewhat different, i.e., that there is heterogeneity with regard to frequencies. Thus, the 1000–1500 and 1500–2000 s bands are very similar to each other but distinct from that of the 500–1000 s band. On the other hand, the former two curves are out of phase. This might reflect the ability of the cells within each of the two subpopulations to synchronise but the inability of the two populations to synchronise with each other, at least in the short term. The overt rhythm of fig. 1 could, perhaps, indicate that a greater degree of overall synchrony is possible if the system is left undisturbed for a longer period.

It is unclear whether or not the anti-phase relationship between the two lower curves of fig. 4 is due to co-incidence. The minor, sharp peaks seen in the two bottom curves may be due to the slight overlap in the frequency distributions for the two subpopulations: the range limits are arbitrarily chosen. On the other hand, these peaks appear to shift from one band to the other; are they due to a minor subpopulation attempting to synchronise with one or other of the major populations?

That rhythms can be detected implies that a degree of synchrony exists in a population. A paramount question arising from all these studies (and also from the enzyme experiments) is how such synchronisation is achieved when no deliberate attempt has been made to evoke such behaviour (one might note that in time lapse studies, Satoh et al. [22] reported that adjacent cells were seen to oscillate in phase though this could have been co-incidence). One possibility is that the cells are inadvertently disturbed during the manipulations and this provides the timing clue. However, the oscillatory behaviour is always observed and it persists even under adverse conditions: it can be seen even if one uses (in the enzyme studies) a series of individual minicultures. At no time have we observed a spontaneous dampening effect leading to no periodicities being detectable. In view of the results of studies of electrical and electromagnetic fields on cells [24,25], we wonder whether intercellular signalling by such processes could bring about the synchronisation which must be rapid and long ranging.

Another point arising from our results is that the modulating frequencies are generally similar to the primary frequencies. This could indicate some kind of artifact but one would not expect two totally different analytical methods to yield the same conclusions. Then again this pattern of behaviour is not always seen and the behaviour can apparently be modified by agents. A possible alternative explanation is that self-modulation occurs: movement of the surface membrane, whether due to ruffling or to gross changes in morphology, can be expected to alter the effective thickness of the surrounding diffusion layer and hence the uptake of nutrients, thereby affecting the primary oscillation. The latter mechanism might also account for the burst-like characteristics if there is some time lag in the response of the primary oscillation to the secondary, diffusion effects.

The complexity of the variations is suggestive of chaotic behaviour (see, for example, the study of Di Cera and Wyman [26]) but a preliminary analysis of some of our data has so far provided no evidence that this is a prime factor. Thus, three-dimensional plots of successive triplet values (A_n, A_{n+1}, A_{n+2}, cf. next amplitude plots [27,28]) are surprisingly very linear, especially if one uses smoothed values thereby eliminating the very high frequency components (what behaviour can give rise to such plots is not clear). Instead the complexity in the observed dynamics is certainly due (in part at least) to (a) heterogeneity within the populations with regard to frequencies and amplitudes (possibly due to genetic factors, non-uniformity in cell size and the number of mitochondria per cell, differences in the degree of membrane fluidity, etc.), (b) the number of interacting rhythms, and (c) the cells not being in a steady state in most situations (despite attempts to give them time to be so before starting data logging). Unfortunately, like the enzyme oscillations [3], it is not possible beforehand to determine if the cells are in a steady state. Therefore, one cannot choose a suitable time to start monitoring. Such factors presumably contribute to the lack of detailed reproducibility even though the general pattern of behaviour is usually the same. Regarding the heterogeneity aspect, we note that cells within a population need not move with the same mobilities [14] and that adjacent areas of the cell surface can move independently [14].

At this stage we tentatively attribute the very rapid scatter fluctuations to local membrane movements, such as ruffling, and the slower rhythms to gross changes in cell morphology though contributions from scatter by internal components cannot be ruled out.

Changes in scatter dynamics produced by agents can be observed in published studies (e.g., ref. 29) but the effects have apparently been overlooked. There seems reason to examine other data, including observations on Ca^{2+} oscillations (e.g., ref. 30) by the methods discussed here in case additional information is being lost.

The situation is such that the power spectrum and periodogram time course curves are themselves periodic. Like the raw data they are thus subject to the same limitations, such as aliasing (see, for example, ref. 3) and they too need to be analysed in a similar fashion. *And so on ad infinitum?*

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