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Reversible phosphorylation of proteins in proliferating and differentiating cells : cyclic variations and the effect of growth regulators / Gracinda Maria Nunes Ferreira.

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than one rhythm is present, and all the data here show that this is so.

It is clearly important to determine the true period of an observed oscillation, but one can only gain confidence in this respect if the same period is seen when sampling interval is decreased. For this reason we conclude, for example, that the LDH activity variations may be of shorter periods than the 1-5 minutes suggested by the present data (Lax 1972, Duffy 1971, Duffy and Sanderson 1971). The total duration of the experiments carried out are too short to be able to detect (with certainty) the occurrence of very slow rhythms (say of the order of 3-4 hours, eg. Gilbert 1968, 1971, 1984a), which could account for some of the discrepencies in reported frequencies. There are, however, indications of the existence of slower oscillations; these might be further studied using the sample averaging method (Gilbert, 1974b) as a means of smoothing out the high frequency periodicities discussed here. Similar considerations apply to all the results presented here. Otherwise one can use different smoothing methods (Visser et al, 1990).

THE AMPLITUDE OF OSCILLATIONS

It is evident from plots of the raw data that the amplitudes of none of the rhythms stay constant with time during the period of observation. Per-amp plots suggest that, in many instances, the amplitude is being modulated in periodic fashion and at a frequency comparable with the rhythms themselves (Visser et al 1992). Where this is not the case, the system could be in

a disturbed state. However, there can be no doubt about the existence of the rhythms as the amplitudes are so large. These determinations are made in cell extracts where the regulators have been diluted with the extraction buffer: in the intact cells the levels of the regulators should be considerably higher. Are the variations even more marked in the intact cells ?

THE WAVEFORMS OF OSCILLATIONS

The outline of an oscillation, as given by the line joining the peaks and that joining the troughs, is referred to as the waveform or waveshape of the rhythm concerned. If the latter is constant in characteristics (and, particularly, in amplitude) these lines will be linear and parallel but more generally they will change with time due to modulation of the oscillation. They can themselves vary in a periodic manner as in amplitude modulated radio waves. In many instances the variations in waveshape are not regular.

From the various diagrams it is obvious that the waveforms for the oscillations studied here are always far from simple (i.e., sinusoidal) irrespective of which oscillation is considered. The more complex behaviour can be due to any or all of a number of factors which may differ for individual rhythms. Generally the pattern of behaviour changes during the course of the experiment, presumably due to the lack of a steady state. However, very often (as seems to be the case with the P-T-Pase results in particular) there is a complex pattern which is repeated during the period of observation and, where long term studies have been

carried out (as with HMBA) essentially the same pattern is seen again at the later times. Thus (see Figs.9a,14b,c,15a,b) the waveform consists of a large amplitude cycle followed by a small amplitude one and this pattern recurs several times. In some cases several small amplitude cycles seem to be superimposed on a sawtooth rhythm of larger amplitude (see Fig. 56) and this pattern is repeated. Such behaviour has been reported for glycolytic oscillations (usually in the NADH level) and has been attributed to double or multiple periodicities (see, for example, articles in Chance et al, 1973) though the mechanism(s) of their origin are not clear. It seems to be accepted that the patterns are generated in the system responsible for the primary oscillation rather than due to the interaction between several oscillations. Some (such as the large/small amplitude patterns referred to above) could be due to periodic hysteretic switching affecting the amplitude (Gilbert, personal communication).

As indicated, the pattern of behaviour seen in these studies are generally even more complex giving rise to very irregular changes and it is important to consider other factors which may be partly or totally responsible. Attention is usually focused on the amplitude variations, but changes in the period and mean of a rhythm will influence ones interpretation of irregularity (see Ferreira et al, 1994a,b for further discussion).

REPRODUCIBILITY

There are a number of factors contributing to the

reproducibility of results. These include the reproducibility of the methods; the ability of the worker to reproduce identical culture conditions; the ability of the systems studied to be identically dynamic; the ability to sample cells at identical times in different systems and to add agents at particular times relative to the oscillatory process (e.g. at a peak or trough). Some aspects have been mentioned in the methods section, others are considered here.

The behaviour is essentially the same from one experiment to the next with regard to the periodic modulation aspects and the occurrence of several, apparently distinct, rhythms which contribute to the observed fluctuations. The results of parallel experiments can be very similar (data not shown) but differences do exist between such data, for example in the frequencies of the oscillations present. The question arises as to what degree of reproducibility should be obtained in such studies. There are two main aspects, agreement (i) between experiments, and, (ii) between duplicate (parallel) cultures.

(i) From experiment to experiment.

The behaviour is essentially the same from one experiment to the next with regard to, for example, the periodic modulation aspects and the occurrence of several, apparently distinct, rhythms which contribute to the observed fluctuations. However, there are differences in detailed behaviour, for example, with regard to the actual frequencies observed. The

involvement of a multiplicity of contributing oscillations can explain the lack of detailed reproducibility from experiment to experiment. Identical results could be expected only if the frequencies, amplitudes and phasings of the rhythms are identical in each instance and there is no reason why that should be so. Moreover the population of cells would have to be homogeneous and in a true steady state when examined and again that is unlikely to be the case here. Indeed, in periodograms we often see rhythms whose periods are gradually changing (see Figs. 21, 50, 52a,b,68) showing the lack of a steady state.

Moreover, identical time curves from experiment to experiment require identical degrees of synchronisation in the cell populations (even ignoring factors such as cell and cell-cycle heterogeneity). This, in turn is dependent on cell-cell communication (and hence, for example on cell density, stirring and shaking characteristics etc) which are likely to be distinct for the different contributing oscillations.

(ii) In parallel experiments.

Neither is it unexpected for such a complex system to show poor agreement between parallel experiments. Only if each rhythm involved was the same in all aspects with regard to frequency, mean, amplitude and phasing relative to others could agreement be perfect. Many factors argue against the existence of such rigid processes in cells; one is that it can imply constant rates and kinetics for all of the system in a dynamic steady state, this could then mean that each culture

would have to be subject to sequences of disturbances which are identical in all respects (eg handling of the cultures, feeding, magnitude, timing). With all of this in mind, the agreement observed between parallel cultures may be considered very good. Slow deviations are likely to occur in the long term especially if agents cannot be added to each culture at exactly the same moment. The timing of a perturbation relative to the phase of an oscillation can markedly affect the response: with high frequency rhythms (as here) the requisite timing becomes more critical. Then again, small frequency differences between two rhythms will result in a gradually increasing difference in their phasing and hence marked distinctions in the observed waveforms as the experiment proceeds. If the processes are chaotic then no detailed agreement seems possible yet it is often good.

THE IRREGULARITY OF THE OSCILLATIONS

As I and others (Brodsky et al, 1992) have noted, the observed periodicities are generally very irregular and there are a number of possible reasons. Before considering some of these it is noted that the period and amplitude plots (e.g. Figs.9a,14b,15b,18) are usually disimilar but where the cells had been left standing for some time after the addition of an agent (as in the HMBA studies), it was found that the two curves could be very similar (see Fig.9a, 14c, 51, 62, 64). This suggests the involvement of distinct rhythms affecting the two parameters which are coupled and synchronised in the steady state but which can become desynchronised by

disturbances. In the present experiments we wished to obtain some idea of the number of oscillations affecting the parameters of interest. Therefore no attempt was made to achieve a steady state situation prior to the commencement of sampling. It was hoped that the non-steady state circumstances might dissociate rhythms as appears to be the case. However, such approach does complicate the interpretations.

As discussed in articles in the press, (Ferreira et al, 1994 a,b), the irregularity could be due to (i) the **aliasing** problem, (ii) **chaotic** behaviour, (iii) **heterogeneity** in the population of cells, (iv) the involvement of **mutiple rhythms**. These four possibilities will now be considered.

An explanation for the irregularity is that the system(s) examined are oscillating in regular fashion but at higher frequency than our data would seem to suggest. This would mean that the apparent periodicity is incorrect because of **aliasing** (Gilbert, 1974b, 1984a), i.e. the sampling interval is too long compared with the true period(s) of the rhythm(s). It is still not possible to eliminate aliasing as at least a contributory factor. As the sampling time has been reduced it has been found that the LDH, extractable protein and P-T-Pase activity oscillations have a higher frequency than originally believed. Hence aliasing must have been present in the earlier data and could have caused irregularity. However, the irregularity is still

seen in the latest results. This suggests that the true period is even shorter than appears to be the case even now (see later comments). But is this explanation adequate?

We also note that both the periods and amplitudes appear to be rhythmically modulated but apparently by different processes (see above). Moreover, the periodograms appear to indicate the presence of several distinct periodicities. Although chaotic data can give rise to the presence of a range of rhythm frequencies (Olsen and ~~Dane~~ Dean 1985), one would not expect particular oscillations to be present continuously even in short data sets. Nor might one expect such behaviour to give rise to periodic modulation characteristics on each and every occasion. In the absence of firm contradictory evidence we therefore believe that we are dealing with several interacting oscillations. It is noted that the morphological and redox studies of Visser et al (1992) revealed the existence of periodic modulation (and therefore irregularity) in the oscillations of those parameters.

The existence of heterogeneity of characteristics within the population of cells examined (Visser et al, 1990) could also contribute to irregularity. We have no independent evidence to show that this exists although the cultures presumably contain both replicating and non-proliferating cells which may well exhibit distinct behaviour according to the stage of the cycle they are in when examined or disturbed (an aspect which rarely receives due attention). Attempts were made to study cells at about the same stage of the growth curve but it

would be difficult to adjust results to take into account different proportions of cells in such states. However, future studies should include observations on non-proliferating cells. Unfortunately, there are other causes of heterogeity, in fact, most cultures contain cells with, for example, different chromosome abnormalities even if initially cloned.

Visser et al (1992) have considered this possibility in an effort to explain some of their results and presented some evidence in support. Again the proportion of cells of a given kind is likely to vary from experiment to experiment and will thus make close comparison of data very difficult. It may be noted that Pavlidis (1969) has considered a population of interacting oscillators and shown that the whole system can oscillate with a much longer period than if the cells were not communicating.

A further possibility, consistent with the large number of cellular control systems, is that the irregularity is the result of the superimposition of, or interaction between, several distinct periodicities (which could be of essentially constant characteristics). In this discussion it will be assumed that the different rhythms arise in distinct intracellular systems and that double periodicities are not present. Such factors, cell heterogeneity, chaos and aliasing could all contribute towards results presented here. However, there is definite evidence for the existence of multiple oscillations. We therefore feel that the main cause of the irregularity is the

multiplicity of intracellular rhythms which is born out of LDH studies, although other factors almost certainly contribute towards the observed waveform.

Self-modulation

In common with the morphological, enzyme and protein oscillations, the rhythms modulating the period and amplitude of the phosphorylation processes have periods similar to those of the primary oscillations themselves. The reactions in a cell are highly integrated through common substrates and cofactors, thus a signal from one part of the system can cause a response in another which feedbacks to the first system and modifies its behaviour. Gilbert (1978c, 1980) has shown how reactions of this kind can control the frequency of an oscillator and suggested that they may be a cause of cellular and physiological ageing (parametric dampening). One possible mechanism of this kind was discussed by Visser et al (1992) and Gilbert and Visser (1993) who showed that insulin stimulated morphological dynamics and the latter raised the possibility that these in turn could decrease the thickness of the surrounding diffusion layer and hence increase the uptake of components that can affect the oscillation responsible for the shape changes.

Chaos

We have previously decided (Ferreira et al, 1994a,b) that chaos is unlikely to be a major factor, one argument being that in view of the extreme sensitivity of chaotic oscillators to disturbances,

agreement between split cultures should never be good yet often it is so (Figs. 65, cf Tsilimigras, 1970) and even in different experiments (Fig. 19). One argument being that we can observe discrete bands in the periodograms, whereas chaotic behaviour gives rise to a wide range of peaks in the power spectrum and in the periodograms (Olsen and Degn, 1985; also Gilbert and Visser, unpublished data). Then again, one could expect to see marked fluctuations of the present kind only if the chaotic behaviour was essentially the same in individual cells otherwise they would cancel out. I am not aware that chaotic cells in a population can synchronise and still be chaotic (Klevecz, 1992, Lloyd and Lloyd, 1993). However, we have asked if characteristics of the kind shown here and elsewehere (Ferreira et al, 1994 a,b) can be explained in such terms. The detection of particular patterns of temporal organisation, the existence of distinct phase relationships between different enzyme oscillations, (Gilbert, 1968, 1969, 1974a, 1984a) also argue against the involvement of chaotic behaviour as a universal feature.

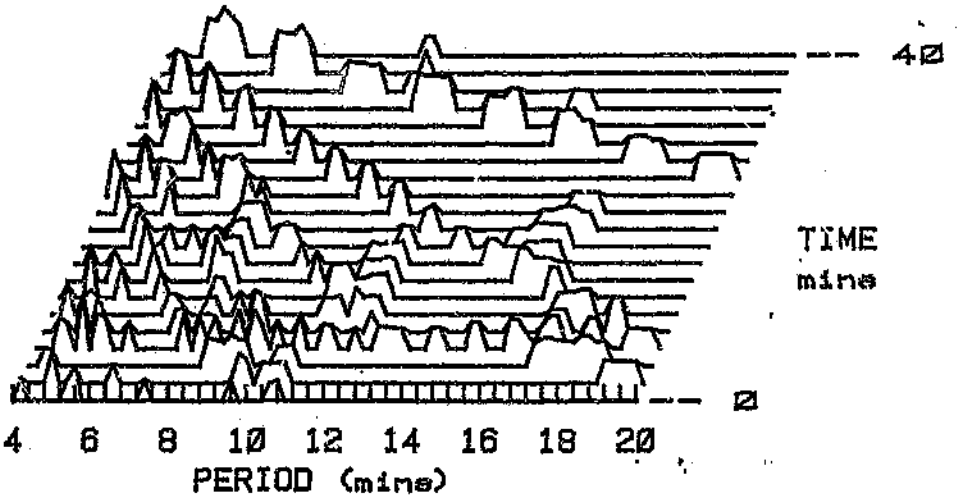
However, we do have data suggesting the transient occurrence of chaos in several oscillating systems for short duration bands containing a wide range of frequencies (see Fig. 68). Similar behaviour has also been noted in the morphological and redox oscillations studies (Gilbert personal communication). He believes that this represents the occurrence of transient chaos. Under such conditions the concentrations of nutrients will vary with time and one can expect the rates of at least some internal reactions to change in

Fig.68 TRANSIENT CHAOS ?

There is some controversy regarding the nature of the irregularity of cellular oscillations (see discussion). Unlike Brodsky we do not believe it to be due to chaotic behaviour but occasionaly as at the initial stages of this diagram a broad range of periods is present in the data which is short lived. This is characteristic of chaotic behaviour and we believe that the simultaneous presence of a wide range of periods may be due to transient chaos as the rates of reactions governing the oscillations passes through a chaotic region of values.

LACTATE DEHYDROGENASE
COMPARISON OF RATE RATIOS
INVERSE PERIODOGRAM

B/P RATIO



Py. 3/Py. 7

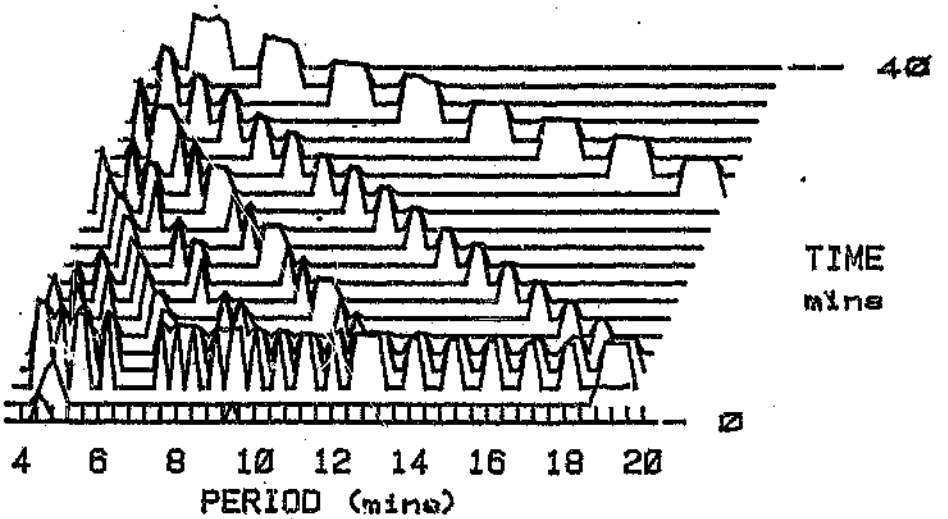


Fig. 68

response. The state of the system will change with time and may pass through a region of state-space which supports chaotic behaviour. As the region is generally small compared with the conditions supporting regular oscillatory behaviour,(see Decroly and Goldbeter, 1982) the chaotic behaviour would last only for a short time.

THE MEAN VALUES OF OSCILLATIONS

As can be seen from some diagrams (Figs. 9a, 35b, 37a,37b 38), there is often a sudden change in the mean values of oscillations but more generally, the means of the various rhythms also vary in pseudo-periodic fashion (e.g., Fig.37a, 38) as found in other, unpublished observations on cellular rhythms (Gilbert personal communication). However, this aspect of the present work is not considered further in this thesis, but the rhythms concerned would seem to be distinct from those involved in the modulation of the periods and amplitudes and thus seem to complicate the picture even more.

THE PHASINGS OF OSCILLATIONS

Consideration of this aspect is limited mainly to comparison of the timing of the oscillation potentials for proteins X and Y (Fig.7a,b) and the LDH isozymes in HL-60 cells (Fig. 40) but phase changes or variations can be seen in other situations because of the complexity of the rhythms. However, it is not known if they were temporary or permanent (Gilbert, 1974b, 1984a, Gilbert and MacKinnon 1992).

THE NATURE OF THE OSCILLATORS

The presence of very high frequency fluctuations would seem to indicate that we are observing the effects of metabolic oscillations (Lax 1972, Duffy 1971, Duffy and Sanderson 1971). The periods are in the range of glycolytic substrate and co-enzyme oscillations seen in many studies (e.g. in MEL cells, Visser et al, 1992). Both the kinetic rhythm (compare Duffy 1971; Duffy and Sanderson 1971) and the gel periodicities seem to be partly due to fluctuating levels of regulators arising in the glycolytic pathway itself, but may not be limited to them. The activity stain measures the reverse reaction,

lactate ---> pyruvate

to the kinetic method, but it seems reasonable to presume that the temporal pattern of changes in the former should be reflected in the latter. However, if this is so, the difference between the electrophoretic and kinetic assays must be due to the involvement of regulators which are removed during electrophoresis. The variations in the amount of active isozyme could be due to co-migration of another regulator which oscillates in level but this seems unlikely. An alternative is that the regulator is tightly bound to the lone isozyme. The abortive ternary complexes with NAD/NADH (e.g., Dixon and Webb 1988) are possible candidates but it is not known if they would dissociate under these conditions. We do not know if there are any other naturally occurring regulators which meet this requirement. It

would seem obvious that such a regulator would also have to associate and dissociate quite freely in the intact cell otherwise rapid periodic changes in the stain intensity are unlikely to be seen.

Similarly, the association and dissociation (see Markert and Massaro, 1968) of the LDH subunits seems too slow to be a likely mechanism unless both the reactions are catalysed. Although reversible binding of LDH isozymes to cytoskeleton components seemed a good possibility but in view of the fact that the amount of active isozyme still oscillates in the cell and particle free system shows that other reactions can only contribute to a minor degree.

COMPARISON WITH MORPHOLOGICAL OSCILLATIONS

Although the observations reported here are on different systems, the various oscillations appear to show the same characteristics as the morphological and redox changes in MEL cells described by Visser et al (1992) in that they too exhibit periodic modulation characteristics with periods similar to those of the primary rhythms. The waveform similarities to NADH results would seem to support the view that the present oscillations may be at least partly due to the glycolytic substrate oscilllations.

If the stimulating action of insulin on the morphological dynamics does account for its non-specific effect on metabolism (Gilbert and Visser, 1993), then some of its effects on the present rhythms may occur

through that mechanism, perhaps by influencing metal ion dynamics. Most of the surface fluctuations are of much higher frequency than some of the present changes but morphological oscillations with periods of the order of 30 minutes were detected (Visser et al 1990), which covers the rhythms frequencies observed here.

COMPARISON WITH OTHER OSCILLATIONS

Oscillations in the activities of other glycolytic enzymes, in their apparent isozyme patterns and in the amount of extractable protein are also rhythmically modulated in similar, complicated fashion (Gilbert 1974b; Tsilimigras and Gilbert 1977; and unplublished data). All these parameters may be determined by the same unkown processes although it has been emphasized that the enzyme variations are not simply a reflection of the variation in the amount of extractable protein (Gilbert and Tsilimigras 1981). The present results support this view in respect of LDH (including the isozyme variations) and also for the phosphotyrosine phosphatase activity. Thus "specific activities" also oscillate and no particular phase relationships can be detected between the protein rhythm and any other. However, this is not surprising if (as appears to be the case) each observed oscillation is due to the summation or interaction of several rhythms and these differ in frequencies.

It can also be that the glycolytic enzyme oscillations are at least partly due to phosphorylation

(Cooper et al 1987), an aspect that should be given further attention, particularly with regard to the LDH studies.

PHOSPHORYLATION POTENTIAL

Figures (8-13) show variations obtained for the oscillations in the phosphorylation potential for both protein X and protein Y and the stimulatory effect of insulin (Fig.41-43) as described and discussed in Ferreira et al (1994a). The remaining diagrams in that group (Figs. 12-13) are concerned with attempts to determine if the phosphorylation potential rhythm is due to changes in the levels of the unphosphorylated proteins involved. The latter results cannot be considered conclusive but allow some preliminary conclusions to be drawn as indicated shortly.

In so far as we are aware, oscillations in this parameter have not been reported previously; the shortest period detected is 20 minutes but the fitted curves suggest values slightly less then this (not shown). However, one is likely to find shorter periods if one uses shorter sampling intervals.

The fluctuations seen could be the result of changes in the activities of both kinases and phosphatases, in the levels of the protein being phosphorylated or in the level of ATP. The P-T-Pase oscillation shows that there may well be a contribution from that or other phosphatases. ATP levels are known to oscillate in cells (the glycolytic substrate studies, Brodsky et al, 1992) Gilbert, unpublished results) so

that too might be involved. So all factors may play contribute toward the phosphorylation results and each may be responsible for different rhythms in the raw data.

With regard to the Coomassie Blue protein staining results we note that oscillations are known to occur (i) in protein synthesis (recently shown NOT to be dependent on an ATP rhythm, Brodsky et al, 1992), and (ii) in the rate of incorporation of amino-acids into protein (e.g., Brodsky et al, 1992; Tsilimigras, 1982). The Coomassie Blue stain results (Figs. 12a,b,13a,b) suggest that rhythmic changes in the protein levels contribute toward the phosphorylation potential results. However, two factors, namely (a) the differences between the waveforms of the Coomassie [illegible] periodicities and the corresponding phosphoryl[illegible]on potential changes, and (b) the distinction between the two periodograms (Fig. 13a), would seem to prove that the other factors play a part in the phosphorylation processes.

Although not of direct, or obvious relevance to this thesis, we note that the uptake of ^{32}P by the "soluble" oscillating yeast glycolytic system occurs in pulses (Betz, 1968). With rising NADH levels the phosphate is incorporated into FDP whereas with falling NADH level the inorganic group is incorporated into ATP (Betz, 1968) (see introduction). The ATP and FDP levels have been reported to be partially out of phase (Boiteaux and Hess, 1973). It would seem important to find out if this

occurs in MEL cells and if the timing relationship is always kept or if it can be modified and can be related to the results given here.

The effect of ATP on cell free extract LDH oscillations suggests that it may be worth while checking to see if the phosphorylation potential of extracts (and the P-T-Pase activity) also vary with time in that system.

Comparison of X and Y data

Summarising the results (see Figs. 8-13 and Ferreira et al, 1944a):

(a) oscillatory variations occur in both cases,

(b) there is a rapid and delayed effect of insulin (see later) which makes the behaviour of treated cells differ markedly from the control,

(c) the waveforms are essentially the same for the two corresponding proteins under some conditions, and initially are so in the insulin treated cells, (Fig.13b)

but

(d) that under other circumstances, the curves are distinct, as in the later stages of Fig.13b.

All the data so far obtained make it evident that the in vitro phosphorylation potential for the two different protein bands both vary in a periodic manner even in cells not deliberately synchronised in any way. The pattern of oscillations is essentially the same for both proteins but not the details; the characteristics are

not constant with time but are rhythmically modulated (Fig.9a,b) with regard to their amplitudes, frequencies and mean levels , these factors presumably contributing to the often noted burst-like nature of the rhythms. Oscillations with frequencies similar to those of the rhythms modulating the period and amplitude of the primary oscillations can be detected in periodograms, but there is evidence in the data for existence of lower frequency oscillations. Longer duration experiments are required to confirm this.

Despite the similarity of the X and Y oscillations, the lack of any distinct phase relationship between them (Fig.7a) indicate tnat differences exist although their nature is not evident; a simple delay affecting one or other of them would produce a cyclic phase curve. This view is supported by the fact that the ratio of the staining intensities for the two proteins is not constant throughout an experiment, but also varies in pseudo-periodic fashion with variations of up to fifty fold (Fig. 10). Moreover, distinctions can be seen in periodograms for X and Y obtained from the same experiment. These various differences between these two fluctuations again support our contention that several rhythms contribute toward the observations. On the other hand, the Coomassie Blue rhythms for protein X and protein Y can be identical in waveform, yet in the insulin treated cells the two curves gradually become distinct (Fig. 13b): this suggests that the processes involved are not the same but, they can become synchronised and appear as one rhythm.

At this stage we do not know the identity of X and Y

nor can we decide the nature of the underlying reactions though we note that Rozengurt, Rodriguez-Pena and Smith (1983) reported the stimulatory effect of growth factors on the in vivo phosphorylation (possibly auto-phosphorylation) of an 81kD protein. We also note that a p65-phosphatase has been implicated in the regulation of mitosis (Meikrantz and Schlegel 1992). If protein Y is this component than the present results must throw even more doubt on current ideas on the cell cycle. It may also be noted that a number of the heat shock proteins (HSPs) have molecular masses in the 60-65kD region (Lucassen and Van Eden, 1994).

These results would seem to suggest that both common and specific effects contribute toward the observed rhythms. At this stage several possible explanations exist for the complexity even if, as here, one assumes that both X and Y bands consist of single proteins. The variations can be due to changes in any or all of the following: (a) the activities of the relevant kinase(s), (b) the levels of the proteins (see Ferreira et al, 1994a), (c) the extents and multiplicity of the phosphorylation (how many phosphate groups are bound and how many different kinases are involved), or, (d) in the activities of some phosphatase(s), (e) possibly, oscillations in the ATP level, although ATP was added for the determination (it could be worthwhile repeating studies using only hot ATP without the cold compound). As kinases are generally regarded as being very specific (but see the Introduction), it seems probable that those catalysing the reaction for protein X and protein Y are different; several enzymes could be involved in each

case as may be phosphatases. If both activities vary with time in a periodic manner, the complexities observed can be understood, especially if the frequencies are distinct. However, more than two oscillations seem to be involved for both protein X and protein Y. This could account for the lack of a particular relationship in the phase plots. However, it begs the question as to how two (and possibly more) kinase activities can be independently regulated in periodic manner.

PHOSPHOTYROSINE PHOSPHATASE

As mentioned earlier, oscillations in the dephosphorylating processes can be expected to behave similarly to the phosphorylating reactions if this system is of significance from the control point of view. Periodic variations in the activities of phosphoamino acid phosphatases have been reported from this laboratory(Hammond et al, 1989a,b), but the sampling times used were long and hence the studies could not detect rapid changes. Now there is evidence that the P-T-Pase enzyme rhythm, at least, has a period in the region required to match the phosphorylation potential changes.

The results of Figs. 14,15 show that the activity of the enzyme oscillates at high frequency and high amplitude and that it too seems to be periodically modulated with respect to frequency and amplitude. Of particular interest is the obvious biperiodicity (Fig. 14b,c, 15, 47) seen several times. Occasionally one

can see what appears to be multiperiodicity (Fig. 14a, 9a,9b, 56), although the studies concerned were not long enough in duration to be sure that this is the case.

With regard to the possibility that some or all of the rhythmic processes discussed involve regulation by the glycolytic substrate oscillations it is important to note that bi and tri periodicity has been observed in both theoretical and experimental studies of glycolytic oscillations (Decroly and Goldbeter, 1982; Goldbeter and Decroly, 1983; Markus 1984).

POSSIBLE RELEVANCE TO REPLICATION.

The results obtained clearly show that phosphorylation processes in cells are far more dynamic than even cell cycle studies have shown (e.g Norbury and Nurse 1992, Kirschner 1992). Marked variations occur in the phosphorylating potential which can and do change as much as 100- fold within the sampling time of ten minutes. Thus the amplitudes are high and so the changes seem significant, especially in view of the effects of insulin (see later) and the fact that the concentrations will be higher in the intact cells. The fluctuations are clearly oscillatory though generally very irregular, (a characteristic of all periodicities seen in this study). This fact supports the view that cell metabolism, function and behaviour cannot be sufficiently understood without due consideration of the relevant dynamics at the time in question and under the conditions then known. If sampling intervals are too long, fluctuations are missed and if the protocol used

involves averaging of data from several experiments (Gilbert 1974b, 1984a) then interpretations need to be revised.

The present ideas on the cell cycle (e.g. Norbury and Nurse 1992, see also the section on cell replication in the introduction), are based on a sequence of phosphorylation and dephosphorylation reactions in which cyclin (in particular) is covalently modified (at the tyrosine residue) in a rhythmic manner with a period of one per cell cycle (which may be as long as 24 hours). The present results throw some doubt on that concept in view of the rapidity and high amplitude of the P-T-Pase and phosphorylation potential results. The cell cycle data seem compatible only if the magnitude of the cyclin variation is much higher than those determined here (which may be even higher in the intact cells); comparison is difficult but the sampling times used in the cyclin studies (mainly carried out on non-mammalian cells) seems to be considerably longer than here. Their data may thus be subject to aliasing (compare MacKinnon and Gilbert , 1993).

Although the phosphoserine and phosphothreonine phosphatases have not been examined in this study it seems quite probable that they also oscillate at a much higher frequency than it seemed originally. If this is the case then the doubt regarding the cell cycle aspects is increased even further. In some cyclin studies, if not all, the cell extracts are supplemented with ATP whereas there is evidence (see Brodsky et al, 1992, for example) that this cofactor oscillates, also at high frequency (see MacKinnon and Gilbert , 1993). This adds

more confusion to the situation as does the involvement of inhibitors of the enzymes involved in mitosis (Meikrantz and Schlegel 1992).

LACTATE DEHYDROGENASE

Cellular systems (Figs.17-40,48-53 and 58)

The kinetic assay results are similar in characteristics to both published (see the introduction) and unpublished data (Gilbert, personal communication). The electrophoretic activity values, not previously described, also vary in an oscillatory manner; all rhythms are irregular and complex and appear to involve modulation with respect to all parameters, i.e. mean, period, amplitude. The oscillations thus seem universal.

Proposals (based on kinetic measurements) that the isozyme pattern oscillates (Gilbert, 1968, 1971) are now justified but it is also shown that the processes are more complex than then believed. Thus superimposed on the actual isozyme oscillation are rhythmic variations in the levels of regulators which can apparently alter the specificity of the isozyme as well as the kinetics (see Fig.22a,b, 27, 31, 32). These can be seen under our conditions but may not be if steady state exists when all periodicities may be synchronised. The original studies (Gilbert, 1968, 1971) suggested a period of around 3-4 hours, but it is not clear if this means that a slow rhythm also exists or if that value is incorrect and arises through aliasing, that is, too long a sampling time.

Klevecz (1969) has used an immunological method to conclude that periodic synthesis of LDH occurs while the rate of degradation remains constant. However, the rhythm he followed appeared to have a period of some hours and would not seem to relate to the present results, that is, unless his data was subject to aliasing (Gilbert, 1974b). The very large confidence limits in his data are consistent with the view that the period might be considerably shorter than he realised. A similar process which could be much faster than de novo synthesis is the association and dissociation of sub-units, particularly if the process is catalysed in some way.

Reversible covalent modification could be involved in the oscillation in the amount of active LDH; in vivo phosphorylation has been reported (see introduction). We have presented evidence for high frequency oscillations in the phosphorylation potential of two proteins in these cells and for rhythmic changes in the activities of phosphoamino acid phosphatases which may be relevant. However, the extract activity changes seem to be due to regulation by dissociable components (see Duffy and Sanderson, 1971) which we find can affect both the kinetics of the enzyme and its specificity (see earlier comments).

Duffy and Sanderson (1971) suggested that the regulators may be peptides so it is necessary to note that oscillations also exist in the protein content of cells, and in the rate of protein synthesis (eg Edwards and Lloyd, 1930; Brodsky et al 1992). The level of extractable protein also varies in a cyclic manner

(Gilbert and Tsilimigras 1977, Tsilimigras thesis 1982; Ferreira et al 1994b, see later comments) but the oscillatory character of either the isozyme level changes or extract activity change is not lost by "correction" for the rhythm in extractable protein (as concluded from other results, Gilbert and Tsilimigras 1981). In any case it could not explain the difference between the kinetic and activity stain intensity oscillations.

Finally, in studies on other (HL-60) cells we find that individual isozymes of the enzymes are independently (Fig.34,35a,b 38,39,40) regulated. The significance of all these results with regard to the control of glycolysis (and possibly other systems) is not evident, but they confirm and emphasize the fact (see above) that living cells are very dynamic and more complex than generally known.

To conclude, electrophoretic and kinetic determinations of the activity of LDH in MEL cells and HL-60, sampled at 1-5 minute intervals, reveal distinct oscillations in the activity and apparent and actual levels of the isozymes. All oscillations have periods in the range of 2-10 minutes, or less, and appear to be rhythmically modulated with respect to period, amplitude and mean. Yet differences exist in the periodicities which suggest the involvement of distinct processes.

CELL FREE SYSTEMS

Although much of the early work on glycolytic oscillations was carried out on cell free systems (see

the Introduction for a summary), we were surprised to observe them under our conditions and, in so far as we know, no one has reported them as occuring in cultured mammalian cell extracts. Unfortunately the finding came too late to allow any detailed studies but it was found (for the first time) that the oscillation in the amount of isozyme, as studied by electrophoresis, also occurs under these circumstances. This seems to rule out cyclic synthesis and degradation as a mechanism and more strongly supports the idea that reversible covalent modification, perhaps phosphorylation, is responsible especially in view of the effect of ATP. The process seems too rapid for association and dissociation of subunits.

These results also seem to rule out rhythmic synthesis and degradation of peptides as a mechanism as suggested by Duffy and Sanderson (1972). Covalent modification might activate or inhibit peptides present in the extracts.

The use of cell free system should be very valuable for future studies on the LDH isozyme dynamics and one might simultaneously examine the phosphatase in order to see if phosphorylation is involved in the LDH changes. However, they also show the danger of not checking extracts for constancy in behaviour.

How many cell extracts have the glycolytic system still functioning in this way and thus may have given or will give rise to incorrect conclusions?

Many studies on cell extracts probably depend on the levels of glycolytic substrates but it seems to be widely accepted that once prepared the composition of the extracts remain constant. This may mean that the results obtained in such studies are open to doubt.

EXTRACTABLE PROTEIN OSCILLATION

Detailed aspects of this topic are given in Ferreira et al (1994b) and therefore only some issues are discussed here.

The present results make one believe that this is a universal phenomenon (at least with cultured cells) and therefore of fundamental importance. These arguments are supported by the facts that;

(a) we get essentially the same results despite the use of different procedures in the various studies;
(b) in the stationary suspension culture studies, samples were randomly assigned to the different groups and selected in the same way;

and,

(c) we can get good agreement between parallel culture series even though they are not perfect (Fig.19, 65).

"Specific activities" (the ratio of an activity to the corresponding protein concentration) vary with time showing that these various oscillations are not due to

the extractable protein oscillations. Then again, if the latter were the only factor, all the oscillators, (like the LDH activity and isozyme rhythms) should be the same.

It seems probable that changes in the protein content would contribute towards the extractable protein oscillation, in which case there should be good correlation between the results of Brodsky and those discussed here. Another factor could be rhythmic polymerisation and depolymerisation of cytoskeleton components known to be oscillatory in some circumstances and to have a period of several minutes in vitro (Mandelkow et al, 1988) with consequential release of enzymes which bind to polymers (Clark and Masters 1975 and compare Ross and Hultin 1980). Despite that issue, the latter mechanism does not seem sufficient to account for (a) different patterns of temporal organisation in cells (mentioned above), nor (b) the apparent variations in the protein composition in the cell extracts, as judged by (i) the use of different methods for estimating protein concentration (Tsilimigras and Gilbert 1977; Gilbert and Tsilimigras 1981), and (ii) the different variations in the levels of protein X and protein Y.

Another factor could be the existence of substrate rhythms; the variation in the level of a substrate could alter the extent of aggregation of the corresponding enzymes or their binding to insoluble cellular components.

The present results reinforce the points made

earlier (Tsilimigras and Gilbert 1977; Gilbert and Tsilimigras 1981; Hammond et al 1989), that in view of the high amplitude of this rhythm, one should not simply "correct" all enzyme activities for the protein concentration in the extracts. This can lead to totally incorrect conclusions being drawn. Moreover, valuable information may be lost if one ignores the possible oscillatory variations in the amount of extractable protein in the composition of the extract. Much doubt must exist about the significance of many studies where protein values have been used in this way.

In view of the erythroid nature of the MEL cell it is pertinent to point out that the rate of haemoglobin synthesis in rat reticulocytes has been shown to oscillate with a period about 1 min. (Tepper et al, 1969).

EFFECTS OF INSULIN

Perhaps the most dramatic result seen is the stimulating effect of insulin on the mean level of the lone LDH active isozyme in MEL cells (Fig. 17), which does not seem to be reflected in the corresponding extract kinetic activity values, presumably due to the action of the regulators present on the latter. In so far as we are aware, such results have not been reported previously. However, the hormone also markedly stimulates phosphorylation processe studied here nearly as dramatically and also affects the extractable protein oscillation, (these two effects

being considered in articles in the press). Here I have concentrated on some more recently appreciated aspects and consider the other oscillatory processes not so far published.

Insulin appears to modify the dynamics of the other systems to a greater or lesser extent but the results are difficult to interpret and define in view of the complexity. The response may be an increase in amplitudes, the initiation of new rhythms, the suppression of others or changes in the frequencies of particular oscillations, as well as action on amplitudes, means and the modulation characteristics. It also seems to stabilise drifts in frequencies (e.g. Figs.52a,b,55). But this study does show that more in-depth studies are both needed and warranted, especially as the hormone has been found to have similar affects on MEL cell morphology (Visser et al 1993). Gilbert and Visser (1993) have suggested that enhancement of cell surface movements can account for the non-stimulation of transport processes and this can give complex effects on metabolic processes being observed.

Effects of insulin on phosphorylation.

The results presented suggest (i) that the hormone may have both rapid and delayed effects on the protein phosphorylation dynamics, and (ii) that oscillations in the levels of more than one protein contribute towards the overall dynamic behaviour. Furthermore, it would seem that several distinct rhythms modulate the two particular phosphoprotein rhythms examined, but that

insulin can affect them in a differential manner. This would seem to imply that, under some conditions, the different periodicities can become co-ordinated so that the net effect on the two proteins is essentially the same, but that the coupling between the various rhythms can change thereby allowing desynchronisation. On the other hand, the results shown in Fig.12a and b, suggest that the hormone has a differential action on the oscillations in the protein levels corresponding to proteins X and Y.

Insulin is mitogenic so it is important to mention that some of the effects seen on these high frequency rhythms may be due to the initiation of the cell cycle oscillation (see Introduction). This applies especially to the long term studies where an increase in cell number may have occurred.

Effect of insulin on P-T-Pase.

Insulin appears to have a rapid effect on the activity and specific activity of the enzyme, though there is a difference between the two parameters (Figs. 44-47). Thus the hormone seems to increase the mean, to change the waveshape and to increase the period of the activity rhythm but decrease the mean of the specific activity periodicity. The action of the hormone on the activity is maintained at 36 hours (Fig. 45) and the amplitude is low at that time, but some sort of recovery is seen at 48 hours. (Fig. 45). Unfortunately the cell numbers were not

determined at the later times so it is not clear if the mitogenic action is influencing these results.

Fig. 47 shows that the amplitude of the P-T-Pase oscillation is decreased by the combined action of insulin and HMBA and an effect is still seen 48 hours later but is not as marked.

Effect of insulin on the extractable protein rhythm.

This aspect is covered in Ferreira et al (1994b) where it is shown that the hormone also stimulates this periodicity. It is not discussed again here but two periodograms are included (Figs. 54,55).

EFFECTS OF HMBA

Action of HMBA on protein X phosphorylation.

As can be seen from Fig. 56, the inducer rapidly influences the mean level and waveshape of this rhythm and the effect is still seen at 36 hours. The apparent difference between the means of the control and treated data at 72 hours could be due to fewer cells in the latter, due to the action of HMBA on cell replication although the mean of the control has not significantly changed in that time. An alternative explanation could be that, since HMBA causes a loss of viability, dead cells in the culture may have disintegrated thereby lowering the number of cells contributing to the determination: however, there is no evidence for that in any of the following diagrams. The lower mean could thus

be real.

The effect of HMBA on phosphotryosine phosphatase.

The picture here (Fig. 57) is very similar to that just discussed for the action of the inducer on the phosphorylation potential. The only difference seems to be that the amplitude of this rhythm is higher than that of the control at 72 hours.

The action of HMBA on LDH.

This aspect has not been studied to any extent but from Fig. 58 it would seem that the inducer does have some influence on the waveshape of the isozyme oscillation at 72 hours.

Effect of HMBA on the extractable protein oscillation.

From Fig. 59 it would seem that HMBA alters the oscillation in that the curves for control and treated cells differ but no particular effect can be distinguished.

SUMMARY

SUMMARY OF FINDINGS

The studies reported here show that previously reported oscillations (namely in the activities of P-T-Pase and LDH and the rhythm in the level of extractable protein) are of much higher frequencies than hitherto believed. They have also yielded several novel findings. It has been found that the phosphorylation of two proteins in cell extracts vary at high frequency, partly due, it seems, to changes in the levels of the proteins themselves but not entirely due to that effect. Particularly in view of the P-T-Pase results it seems probable that variations in the enzymes catalysing the dephosphorylation of these proteins may also contribute toward the observed phosphorylation rhythms. It was also shown that differences exist between the oscillations in protein X and Y so that there are specific factors affecting the individual components.

The phosphotyrosine phosphatase oscillation is of high frequency and of high amplitude and therefore sufficient to throw some doubt on current ideas about the involvement of cyclins in the cell cycle, and other metabolic reactions for that matter. The results support the idea that regulators of phosphorylation processes act by modifying the dynamic balance between the forward and reverse reactions (Hammond et al 1989).

It has been found that regulators of LDH exist which can apparently affect the activity, the kinetics and specificity of the enzyme. In addition it

has been shown that rhythmic variations occur in the amounts of active isozymes and that different isozymes appear to be independently affected. These periodicities are also observed in cell and particle free extracts and may influence other studies in which it is assumed that once prepared the composition of the extract is constant.

The results confirm the periodic modulation of some rhythms and show that this still occurs when a shorter sampling time intervals are used. In addition the data indicates that other rhythms are similarly affected.

Both insulin and the differentiation inducer, HMBA, affect the oscillations although in some instances the effects are difficult to define but the hormone clearly enhances phosphorylation dynamics and the extracable protein rhythm and causes a marked increase in the mean of the LDH isozyme oscillation.

The results add further support for the various concepts of the oscillatory basis of cellular processes.

A summary of the major individual findings is given in Table III

TABLE III

MAIN FINDINGS

The effects of insulin and HMBA on the various oscillations are too complex to include in this table, it is just indicated where their action has been examined.

I PHOSPHORYLATION

1. Evidence for high frequency (periods <10 min) oscillations in the phosphorylation potential of two protein.
2. That the two oscillations are independent,
3. Evidence that several rhythms involved with period, amplitude and mean are modulated.
4. Oscillations enhanced by insulin in a distinct manner
5. HMBA results (see text)
6. Shown that these rythms are not explained by oscillation in the level of extractable protein
7. Coomassie Blue stain suggest X variations partly due to rhythmic change in the level of the protein

II. PHOSPHOTYROSINE PHOSPHATASE

1. Confirmed existence of oscillation in the activity
2. I Have shown that the frequency of oscillation much higher than originally believed (period <= 5 min)
3. Evidence that several rhythms involved with modulation of period, amplitude and mean.
4. I have shown that this rhythm is not explained by the extractable protein oscillation
5. Insulin results (see text)
6. HMBA results (see text)

III. LACTATE DEHYDROGENASE

(A) EXTRACT ACTIVITY (MEL)

1. Confirmed oscillation in MEL cells
2. Shown that frequency very high (period < 2 min) as in human erythrocytes and HeLa cells,
3. Evidence that several rhythms involved with modulation of the period, amplitude and mean
4. Shown that changes partly due to variations in the amount of active isoenzyme
5. Shown that both isozyme and activity oscillations distinct from extractable protein oscillation in agreement with data from other cells
6. Insulin results (see text)
7. HMBA results (see text)

(B) ACTIVITY (HL-60)

1. Shown that the pattern of behaviour as for MEL

(C) ISOZYME (MEL)

1. Shown that B/P ratio oscillates as in other cells
2. Shown that frequency much higher than previously observed
3. Shown that periodicity shows same modulation characteristic as other rhythms
4. Shown that the B/P oscillation not due to variations in the number and activity of isozymes and that regulators may alter specificty as well as activity
5. Ratio of rates at two pyruvate concentration also oscillates despite only one isozyme and is not identical to B/P ratio rhythm indicates mutliple regulations

6. The rhythm is not determined by extractable protein oscillation

7. Insulin markedly increases the mean level of the isozyme activity stain (see text for other effects)

8. HMBA results (see text)

(D) **ISOZYME** (HL-60)

1. Shown that the amounts of two active isozymes both oscillate

2. Phase plots show little correlation between the two isozyme rhythms

3. Shown that the ratio of the two active isozymes oscillate also indicating independent regulation of the isozymes

4. That periodicities show the same modulation characteristic as other rhythms

5. The rhythms are distinct from extractable protein oscillation

IV. **EXTRACTABLE PROTEIN OSCILLATTION**

1. Shown that this also occurs in MEL and HL-60 cells

2. Shown that frequency higher than previously observed (period also ca 1-2 min))

3. The periodicity shows same modulation characteristic as other rhythms studied

4. Coomassie Blue stain shows that this rhythm not due to variations in level of the X protein

5. Insulin results (see text)

6. HMBA results (see text)

V. CELL FREE SYSTEM (MEL)

1. Evidence that LDH activity oscillates in cell free and cell and particle free systems

2. The period (<2 min) is within the range observed for glycolytic oscillations seen in cell free extracts of yeast, bovine heart muscle and skeletal muscle

3. The periodicity shows same modulation characteristic as other rhythms studied

4. Shown that ATP increases mean and appears to affect periods

5. Shown that the amount of active isozyme also oscillates at high frequency even in this system (due to phosphorylation?)

GENERAL ASPECTS

1. Behaviour of all systems very similar suggesting same basic underlying oscillatory processes, but differences indicate involvement of additional factors

2. Although the rhythms modulating the period and amplitude are generally very distinct, in some instances where cells undisturbed for some time, they are almost identical suggesting that the different modulating rhythms can become synchronised

AN INTRIGUING ISSUE

Of the more general issues arising from this and the other related studies mentioned above, one is of particular interest; the fact that oscillatory behaviour can be detected in all our experiments despite the fact that the cultures are not deliberately treated in order to produce synchrony. This seems to imply either that disturbances (such as feeding the cells) can act as a timing clue, or, that communication exists between the cells which is able to cause (partial) synchronisation of oscillations in different cells. However, desynchronisation can be expected to occur rapidly: the fact that this does not happen would seem to imply that the response to feeding, for example, is more prolonged then might have been expected. On the other hand, if the partial synchronisation is due to chemical communication between cells then any manipulation of the cultures is likely to disturb the pre-existing pattern of communication and thus alter the metabolism, a possibility which is not widely appreciated. Certainly movement of cultures will generally perturb the oxygen and other diffusion processes. Hence cells are not likely to be in a steady state when examined (Gilbert and Visser, 1993).

However, it seems clear that there are a number of distinct rhythms in cells and it seems impossible for all of them to become easily synchronised at the same time because of all the interactions; are cells hardly ever in a true steady state? The only way this might be feasible is if there really is a master oscillator in each cell (glycolysis ?) and only one component of that

system which acts as the communication molecule. Against this idea is the very high frequency of the rhythms which seems to imply a compound which can diffuse throughout the culture very rapidly even when the latter is stationary.

CONCLUSIONS

CHAPTER V

CONCLUSIONS

The analysis of time-dependent data, i.e. periodic processes, is not easy, especially when each rhythm is complex; when observations have been made discontinously; when they have been irregularly spaced; when they cover a limited period of time (number of cycles) and where the frequencies and/or amplitudes vary. Some of the experimental, analytical and interpretative problems of such studies have been looked at elsewhere (Gilbert 1974b, 1980), but others also exist. Often the requirements are conflicting; for example, longer experiments require more cells, more time, more effort and the repeated sampling can influence the results. No analytical method is adequate; in view of the very large and rapid changes in mean levels often seen one might get better results by detrending the data but that can introduce other frequencies and so was not done. In general, no methods are entirely suitable but despite the limitations the periodograms have been invaluable.

Time dependent processes and rhythmic behaviour are clearly vital components in the regulation of cellular metabolism and it does not seem unreasonable to believe that cell transformation, differentiation and proliferation depend on them (as discussed earlier). The

studies of those who ignore this point (and that includes most researchers) are of doubtful value.

The living cell is even more highly dynamic than I believed it to be when starting this thesis, and therefore it is harder to interpret results than I expected it to be. Better analytical methods are required but, even if available they would be of little use if the experimental methods are inadequate. In view of the high frequency of all the oscillations it may be very difficult to obtain suitable data without specialised equipment.

APPENDICES

APPENDIX I

LIST OF CHEMICALS AND SUPPLIER

Acetic acid	BDH
Acrylamide	BDH
Ammonium sulphate	Biorad
ATP (adenosine-5'-triphosphate)	Boehringer
[γ-^{32}P] ATP	Amersham
Bisacrylamide(N'N-methylenediacrylamide)	Merck
Coomassie Brilliant blue	Sigma
Bromo-phenol blue	BDH
Cellulose acetate membrane	Whatman
Cellulose membrane filters (type H.A. 0.45μm)	Millipore
Copper sulphate ($CuSO_4$ $5H_2O$)	Ass. Chem.
Eagles Minimum Essential Medium (with Hank's salts)	Highveld Biologicals
EDTA (ethylene diamine tetra-acetic acid)	Boehringer
EGTA (ethylene glycol tetra-acetic acid)	Boehringer
Fetal calf serum	Highveld
Folin - Ciocalteus phenol reagent	Merck
Glycine	BDH
HEPES	Boehringer
HMBA (N'N hexamethylenebisacetamide)	Sigma
Hydrochloric acid	BDH
Insulin (from Bovine pancreas)	Boehringer
α-Ketobutyrate	Sigma
Lactate dehydrogenase (from beef muscle)	Boehringer
Magnesium acetate	SAAR Chem.
MEM (Dulbeco's modified Eagles minimum essential medium with Hank's salts)	Highveld
2- Mercaptoethanol	Biochemical
Methanol	Ass.Chem.
Millipore water	Whatman
NADH (reduced nicotinamide adenine dinucleotide)	Boehringer
Na_2CO_3	SMM Chem.
NaF (sodium fluoride)	BDH
Nunclon flasks (250ml and 50ml)	Nunc
Parogen LD electrophoresis kit	Beckman
Penicillin	Sigma
DL-Phosphotyrosine	Sigma
PMSF (phenylmethyl-sulfonyl-fluoride)	Sigma
Potassium sodium tartrate	SAAR Chem.

Pyruvate (sodium salt)	Boehringer
Rainbow markers	Amersham
RPMI 1640	Highveld
Sodium dodecyl sulphate	BDH
Sodium acetate trihydrate	BDH
Sodium carbonate	SMM Chem.
Sodium chloride	Ass. Chem.
Sodium hydroxide	SAAR Chem.
Sodium potassium tartrate	SAAR Chem.
Streptomycin	Sigma
TEMED	Stratagene
Trichloroacetic acid	BDH
Tris (hydroxymethyl)-aminomethane	Merck
Triton-X 100	Merck

Abbreviations (local firms only)

Ass. Chem.	Ass. Chem. Enterprise
Biorad.	Biorad Laboratories
Highveld	Highveld Biologicals RSA
SAAR Chem.	SAAR Chemicals
SMM Chem.	SMM Chemicals

APPENDIX II

CELL CULTURES, MEDIA and TECHNIQUES

ORIGIN of MEL cells

These had been maintained in these laboratories for several years being orinally obtained from an unknown commercial source.

MAINTENANCE of MEL cells

These cells were grown in stationary suspension cultures in 250 ml Nunclon culture flasks and were kept in an incubator at 37°C. The cells were fed every second day by diluting 1:3 with fresh, warm medium. Excess cells were discarded if necessary. For routine maintenance the serum concentration used was 5% but this was increased to 10% when cells were required for an experiment which was undertaken at this concentration.

MEL cells were maintained as stationary monolayer suspension (unattached to the flasks) cultures in Eagles Minimal Essential Medium with Hank's salts, and antibiotics (penicillin and streptomycin). Fetal calf serum was added to a concentration of 5% between experiments and this was increased to 10% when larger numbers of cells

were required for study and this level was used during each experiment.

EXPERIMENTAL cultures

For the actual experiments, cells were prepared in one or other of two ways:

i) for the stationary culture studies, a reservoir of cells was kept in the incubator at 37°C. Aliquots of cells were quickly removed and transferred to individual culture flasks for carrying out an experimemt.

or

ii) cells from stationary culture were transferred to a culture flask fitted with a Teflon-coated magnetic flea mounted on a vertical shaft (Bellco Ltd) and stirred using a low speed, regulated magnetic stirrer (Bellco Ltd) placed under the stirred water bath and kept at 37°C, overnight. Aliquots were taken at regular intervals over a convenient period of time using a Cornwall syringe.

EAGLE'S MINIMUM ESSENTIAL MEDIUM (MEM)

(with Hank's salts)

This solution was used for MEL cells and contained:

Dulbecco's modified Eagle's minimum essential medium	10.72g/l
Penicillin	0.270g/l
Streptomycin	0.450g/l
$NaHCO_3$	0.350g/l

MEM has a high concentration of glucose and pyruvate and contains L-glutamine and the indicator phenol red. The ingredients were dissolved in distilled deionised water, pH adjusted to 7.4 using 3N NaOH, filtered under sterile conditions using 0.22µm millipore (Sterivex G5) filter, left in incubator for 12 hours to test for presence of bacterial, viral and mycoplasma contamination (by visual inspection) following which it was kept at 4°C until required. It was then kept at 37°C before use When required, fetal calf serum of 5 - 10% was filtered into the medium, this was then used to feed cells every second day.

ORIGIN of HL-60 CELLS

HL-60 were obtained from Dr. Avri Davidoff of department of haemφtology, University of the Witwatersrand and were grown in a stationary suspension culture. Frozen cells were thawed out at 37°C in water bath. Quickly wash HL-60 in RPMI 1640 without fetal calf

serum, centrifuged at 1200g for 10 minutes (Beckman model TJ-6 centrifuge), decant supernatant, resuspended pellet in RPMI 1640 with 20% fetal calf serum.

MAINTENANCE of HL-60 cell cultures

Cells were grown in stationary suspension cultures with RPMI medium using 50ml Nunclon culture flasks and were kept in an incubator at 37°C, CO_2 at 5°C. HL-60 were fed twice a week. To remove any debris, HL-60 cells were spun at 900rpm (Beckman model TJ-6 centrifuge) for 5 minutes, the pellet was resuspended in RPMI 1640 with 10% FCS. Cell viability and counts were routinely undertaken.

EXPERIMENTAL cultures

HL-60 cells were examined in stirred suspensions as for the MEL cells.

RPMI MEDIUM

This was used for HL-60 cultures and contained:

RPMI 1640 (with glutamine)	10.4g/l
HEPES	2.38g/l
Bicarbonate	1.85g/l
Penicillin	0.063g/l
Streptomycin	0.106g/l
~~Merc~~	~~1.92g/l~~
water	to 1000ml

The above ingredients were dissolved in distilled deionised water and then filtered using Whatman cellulose filter under sterile conditions. It was stored at 4^{o}C until required. The medium was warmed to 37^{o}C before feeding the cells. For the growth and maintenance of the HL-60 cells, fetal calf serum was filtered using a millipore filter into RPMI to a concentration of 10% and cells were fed every third day by diluting 1:3.

SERUM

Fetal calf serum (FCS) was purchased from Highveld Biologicals and had been screened for mycoplasma and virus contamination. FCS was used for the routine production of all tissues culture cells and for the experiments reported in this thesis. All serum was kept frozen at -20^{o}C and was

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SERUM

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heated inactivated at 56°C for 30 minutes before use and filtered using Millipore filters under sterile conditions.

VITAL STAINING

Cell viability was assessed by staining with trypan blue and counting the percentage of viable and non-viable cells using a haemocytometer. The viability was always above 90% in experimental cultures.

CELL COUNT

Samples of the cell suspension were taken and counted at regular intervals during maintenance to evaluate the growth and also immediately before an experiment to determine the cell number and viability in order to enable comparison of results.

Cell number was determined by using an improved Neubaur haemocytometer. The method enables the estimation of cell viability by the Trypan blue exclusion method. Here a small sample (20µl) of the suspension was taken and mixed with an equal volume of the Trypan blue dye. This mixture was allowed to stand for 1 minute and then loaded onto the haemocytometer and observed at 400X magnification. Living cells have the ability to exclude the dye, therefore the viability of the population can be determined by the ratio of clear to the total number of cells.

APPENDIX III

PHOSPHORYLATION STUDIES

GENERAL OUTLINE OF EXPERIMENTS

The experimental cells were kept in monolayer as a series of individual cultures randomly assigned to control or treated groups (in some cases two control series for comparison purposes). An agent (insulin or HMBA or both) was added to the treated group and an equal amount of solvent added to the controls (details of all aspects are given below). At regular intervals one flask from each group was selected at random, cells were collected and homogenised, centrifuged and treated with [γ-^{32}P] ATP. After stopping the reaction samples were electrophoresed on SDS-PAGE and autoradiographed. The intensity of the bands corresponding to 63kD and 81kD were scanned using a scanner. In some experiments the SDS-PAGE gels were first stained with Coomassie Blue and scanned before autoradiography in an effort to estimate the actual amount of protein corresponding to protein X and protein Y.

EXTRACTION FOR PHOSPHORYLATION studies
(stationary monolayer cultures)

In each experiment, control and treated cells were derived from the same stock suspension but kept in individual flasks for the duration of the experiment. Flasks were arbitrarily assigned to control or treated groups and samples were selected at random from these groups. Cell suspensions (50ml) were centrifuged at 300g (2000rpm) for 15 minutes (using a Beckman model TJ-6 centrifuge). The pellet was washed with 5ml homogenizing buffer (50mM TRIS/HCl pH 7.5) and centrifuged a further 15 minutes, the washing was repeated twice more. The pellet was re-suspended in 1.5ml 50mM Tris/HCl pH 7.5. This was left on ice for 15 minutes, homogenised using a Polytron homogenizer, 3 bursts of 10 seconds each. After centrifugation at 100 000g (40 000rpm) at $4^{o}C$ for an hour (using Beckman L8-M ultracentrifuge), the cytosolic fraction was used. Extracts were stored at $-70^{o}C$ until required.

HOMOGENIZING BUFFER for PHOSPHORYLATION studies

This solution contained:

Tris/HCl buffer	50 mM pH 7.5
EDTA	2 mM
EGTA	2 mM
NaF	2 mM
Triton-X 100	0.1%
2-mercaptoethanol	0.5%
PMSF	1 mM

The chemicals were dissolved in distilled deionised water and the pH adjusted to 7.5 with 1N HCl. The buffer was kept at $4^{o}C$ until required.

ATP solution for PHOSPHORYLATION studies

TRIS/HCl pH 7.5 (20mM)	0.121g/50ml
Magnesium acetate	0.536g/50ml (50mM)
ATP	0.004g/50ml buffer containing magnesium sulphate (50mM)
[γ-^{32}P] ATP	2 x 10 cpm/pmol

(final specific activity in reaction mixtures = 0.5×10^{3} cpm/pmol)

Tris was dissolved in distilled deionised water and the pH adjusted to 7.5. Magnesium acetate and ATP

were added to this buffer. Every 10µl ATP solution, contained: 2.5µl [γ-^{32}P] ATP + 7.5µl cold ATP

Preparation of INSULIN (from Bovine pancreas, sterile lyophilised)

Insulin was freshly prepared just prior to carrying out experiment

Stock solution: 1 mg/1ml water
Final solution: 0.01 mg/ml

A trace of 1N HCl was added to help solubilise the hormone. It was used at a 1 in 100 dilution, being filtered through a 0.45µm membrane under sterile conditions directly into the cell suspension.

Preparation of HMBA solution
(N N' hexamethylene-bisacetamide)

Stock solution: 0.5 M
Final concentration: 5 mM

This stock solution contained:

HMBA 0.1 g/ml
MEM

The HMBA was dissolved in MEM (containing foetal calf serum and antibiotics) over a water bath (30°C) for a few seconds to aid the dissolution of the HMBA. This was then filtered under sterile conditions using a 0.45μm membrane filter into the MEL cell suspension when needed. For 5mM final concentration, 0.1ml HMBA was added for each 10ml of MEL cell suspension.

Because the biological effects of HMBA become marked only after 48-72 hours, sampling was undertaken for short durations at four stages: immediately after the addition of the agent and 36, 48, 72 hours later.

TREATMENT of cells with INSULIN and HMBA

MEL cells were grown in stationary monolayer, fed 48 hours before experiment was carried out. Two agents were investigated, namely insulin and HMBA. There are two ways in which insulin was used during the experiments.

a) monolayer cultures, two sets of flasks derived from the same stock solution, to one insulin was added, to the other equal volume of MEM (under sterile conditions),

or,

b) for the stirred suspension studies, insulin was added half way through the experiment (under sterile conditions).

RUNNING GEL

STOCK ACRYLAMIDE

This solution contained:

Acrylamide	30g
Bisacrylamide	0.8g
water	to 100ml

Acrylamide and bisacrylamide were dissolved in distilled deionised water and stored in amber glass bottles at 4^{o}C until required.

SDS/TRIS buffer

The solution contained:

Tris pH 8.8 (1.5M)	18.17g
SDS	0.40g
water	to 100ml

TRIS was dissolved in distilled deionised water, pH adjusted to 8.8 using 1N HCl, SDS was then added and the solution stored in amber glass bottles at 4°C until required.

STACKING GEL

STOCK ACRYLAMIDE

The solution contained:

Acrylamide	8.0g
Bisacrylamide	0.256g
water	to 50ml

Acrylamide and bisacrylamide were dissolved in distilled deionised water, stored in amber glass bottles at 4°C until required.

SDS/TRIS buffer

The solution contained:

Tris pH 6.8 (0.5M)	3.03g
SDS	0.20g
water	to 50ml

TRIS was dissolved in distilled deionised

water, the pH adjusted to 6.8 using 1N HCl, SDS added, solution storred in amber glass bottles at 4°C until required.

AMMONIUM SULPHATE

The solution contained :

Ammonium sulphate	0.1g
water	to 1ml

Ammonium sulphate was dissolved in distilled deionised water, made to volume, freshly made on the day of carrying out SDS-PAGE electrophoresis.

RUNNING BUFFER for SDS-PAGE

The solution contained :

Tris/HCl pH 8.3 (0.025M)	3.03g
Glycine (0.192M)	14.40g
water	to 1000ml

Tris was dissolved in distilled deionised water, pH adjusted to 8.3 using 1N HCl, glycine was added, solution made up to volume. This solution was kept at 4°C until required.

DESTAINER for SDS-PAGE

The solution contained:

Methanol	45ml
Acetic acid	10ml
water	to 100ml

Methanol, acetic acid and distilled deionised water were mixed and stored at room temperature until required.

FIXER for autoradiographs

The solution contained:
methanol:acetic acid:water (4:1:5)

Methanol, acetic acid and distilled deionised water were mixed, solution was stored in amber glass bottle at room temperature until required.

GLYCEROL solution

The solution contained:

glycerol	10ml
water	to 100ml

Glycerol was dissolved in distilled deionized water, freshly made on the day that it was required.

PHOSPHATE INCORPORATION and ELECTROPHORETIC SEPARATION

Supernatants (100μl) were incubated with 10μl of ATP (containing [γ-^{32}P] ATP 0.5 x 10^3 cpm/pmol) in the presence of magnesium acetate (50mM) for 2 minutes at 30^{o}C. A solution (100μl) containing TRIS -HCl (0.0625M), pH 6.8, SDS (2%), glycerol (10%), mercaptoethanol (0.5%) and bromophenol blue (0.1%) was added. The samples were boiled for 2 minutes and then eletrophoresed

on SDS-PAGE. Equal amounts of protein (20μg) were loaded into each lane. The procedure was that of Laemmli, using a mini-system with 4% stacking and 7.5% resolving gels. A current 30mA/gel was applied for 2 hours. Gels were fixed overnight in a mixture of methanol:acetic acid:water, washed twice in 10% glycerol for 30 minutes, dried under vacuum and autoradiographed for 4 days. Autoradiographs were scanned using a laser densitometer.

For the Coomassie Blue protein stain studies, the gels were first dyed and scanned before carrying out autoradiography.

Procedure for SDS-PAGE ELECTROPHORESIS and AUTORADIOGRAPHY

The samples were loaded using a micropipette unto a minisystem (7 x 8 cm gel sandwiches, thickness 0.75mm) with 4% stacking and 7.5% resolving gels. A current of 30mA/gel was applied for 2 hours. Gels were fixed overnight in a mixture of methanol : acetic acid: water, washed twice in 10% glycerol for 30 minutes, dried under vacuum and autoradiographed for 4 days; the films were then developed and fixed using the necessary solutions. Autoradiographs were scanned using a laser densitometer (soft laser scanning densitometer model SL-2D/1D UV/VIS). Molecular weight positions were determined with rainbow markers added to one lane.

COOMASSIE BLUE stain for SDS-PAGE gels

The solution contained :	
Acetic acid	10ml
Methanol	30ml
Coomassie Brilliant Blue	0.1g
water	to 100ml

Coomassie brilliant blue was dissolved in distilled deionised water, acetic acid and methanol were added, made up to volume and stored at room temperature until required.

APPENDIX IV

PHOSPHOTYROSINE PHOSPHATASE STUDIES

GENERAL OUTLINE OF EXPERIMENT

These were carried on MEL cells in stationary monolayer. Experimental procedures were as for the phosphorylation studies (see appendix III) up to the point at which cell extracts were prepared (see the following sections for details). Phosphotyrosine phosphatase activities were initially measured using a discontinuous method based on the determination of free phosphate released during the reaction. Because of the limitation of this method, a continuous assay was developed and applied in subsequent studies. Only the latter results are presented in the thesis, but details of both methods and a comparison are given below in order to show that the continuous method is valid. Where required agents were added to the monolayer cultures as in the phosphorylation studies.

EXTRACTION FOR PHOSPHOTYROSINE PHOSPHATASE studies

MEL cell suspension (50ml) was taken from monolayer stationary flasks kept at $37^{o}C$, filtered by suction unto glass fiber filters. The cells were then washed with 2.5ml 0.9% saline

solution. The membranes containing the cells were put in scintillation vials. The cells were washed from the filters using a 0.1M sodium acetate buffer pH 6.0 in a 1.5ml volume. The filters are removed, the suspension is freeze-thawed twice in acetone at -70^{o}C, spun at 10000rpm (Beckman model TJ-6 centrifuge) for 10 minutes to remove any debri. The supernatant is then used for phosphotyrosine phosphatase assays. 0.5ml solution is kept for protein determination.

SODIUM ACETATE(0.1M) pH 6

This solution contained:

Sodium acetate	0.1M
HCl	

Sodium acetate was dissolved in distilled deionised water and the pH adjusted to 6.0 with 1N HCl. The solution was kept at 4^{o}C until required.

DISCONTINUOUS ASSAY

In the initial studies, a discontinuous assay was used for this enzyme. However, a continuous kinetic method was developed, compared with the original method and found to be much quicker and cheaper. A subsequent literature search eventually uncovered two other articles proposing this method and therefore only an outline is given of these earlier experiments. The method adopted for the later studies is then described.

DISCONTINUOUS ASSAY method

This is based on the determination of the free phosphate liberated by the phosphatase

phosphotyrosine ----------> tyrosine + P_i

and has thus been used to study the activity of enzymes acting on phosphoserine and phosphothreonine as well as that hydrolysing phosphotyrosine (Hammond et al. 1985). The assay was performed in the extraction buffer. 0.3ml of the buffer was added to 0.1ml 50mM phosphotyrosine solution in extraction buffer. A final concentration of 10mM phosphotyrosine was thus obtained. 0.1ml of the enzyme preparation was used. The reaction was allowed to proceed for 20 minutes and was then stopped using 0.5ml ice-cold 10% trichloro acetic acid (TCA). The mixture was centrifuged for 10 minutes at 2500rpm (using Beckamn model TJ-6 centrifuge) to pellet the protein precipitate. 0.9ml of the supernatant was used for the phosphate determination procedure. A blank was performed to quantify the endogenous phosphate cleavage by adding the TCA before the enzyme preparation and incubating as before.

PHOSPHATE determination

The method used for determining the free phosphate as a result of the hydrolysis of phosphotyrosine is that described by Lindbert and Ernster in 1956. To find the concentration of

phosphate released during the assay, a standard curve is required. For this, a stock solution of disodium hydrogen orthophosphate was used to prepare a series of standards: 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2ml aliquots of the standard were pipetted into duplicate tubes and made up to 3ml with distilled deionised water. Reaction mixtures (0.9ml) were pipetted into duplicate tubes and also made up to 3ml with distilled deionized water. 0.5ml 5M H_2SO_4, 5.0ml isobutanol-benzene (1:1) and 0.5ml 10% ammonium molybdate were added to the tubes and the tubes were mixed well using a vibrating mixer (Whirlmixer). The phases were allowed to separate and 3ml aliquots of the organic layer were transferred to clean tubes. These were made up to 5.0ml with 3.2% H_2SO_4 in absolute alcohol.

0.5ml of a 10% solution of stannous chloride in concentrated HCl (diluted 200 times with 0.5M H_2SO_4 immediatelly prior to use) was added, tubes mixed immediately. Absorbance was measured at 640nm and the phosphate concentration in the experimental samples were read from the standard curve obtained.

DEVELOPMENT of a CONTINUOUS ASSAY method

The phosphate determination method was found to be tedious, expensive and time consuming. A continuous assay was therefore developed. The spectrum of phosphotyrosine between 230nm and 320nm was scanned at 15 minutes intervals, following the addition of commercial acid

phosphatase, using the same solution, without the enzyme, as a reference. The difference spectrum so obtained showed peaks at 230nm and 280nm as a result of phosphate cleavage. The wavelength of 280nm was used for the continuous assay. Articles in the literature also suggested this wavelength (Lau et al 1989, Padros et al, 1984). A 10mM phosphotyrosine was used to find out if there is a marked endogenous rate of phosphate cleavage but it was found unnecessary to correct reaction rates.

Assays were carried out using commercially available acid phosphatase to observe the time course of the reaction, it being followed for 20 minutes to determine over what period the rate of reaction is linear. Assays were performed in a stirred cuvette using a 2ml 10mM phosphotyrosine and 0.1ml cell extract. The reaction was followed for 1 minute at 280nm and the rate of phosphate cleavage was calculated and found to be proportional to the amount of extract used over the narrow range studied.

COMPARISON OF CONTINUOUS AND DISCONTINUOUS ASSAY METHODS

The rates of reaction obtained using both methods were found to be proportional confirming that the spectral changes observed are due to phosphate cleavage. 2ml 10mM phosphotyrosine in 0.1M acetate buffer pH 6.0 was placed in a stirred cuvette in a jacketed cuvette holder maintained at 37^{o}C. 0.1ml of the phosphatase was added and the

absorbance was measured every 3 minutes. A parallel, scaled reaction was performed using a stirred, jacketed vessel at 37°C from which 0.5ml aliquots were removed at 3 minute intervals for the phosphate determination method. 0.5ml of ice-cold TCA was used to stop the latter reaction as indicated above. Results of phosphate assay were plotted against the rate of the continuous spectrophotometric assay carried out in the cuvette. Linear regression analysis was performed to determine the agreement between the two sets of values.

PHOSPHOTYROSINE solution

This solution contained:

DL- Phosphotyrosine	0.262g/l
Sodium acetate buffer, pH 6.0	0.1M

Sodium acetate was dissolved in distilled deionised water pH adjusted to 6.0 using 1N HCl. DL- phosphotyrosine was added to the sodium acetate solution. (The substrate solution was made immediately prior to use).

PHOSPHOTYROSINE PHOSPHATASE: standard assay

The method adopted for the studies reported here involved the determination of the rate of change in absorption at 280nm using 2.5ml of 10mM phosphotyrosine in 0.1M sodium acetate buffer pH 6.0 (using the same solution as a blank), to which 0.5ml of cell extract

was added. The substrate solution was kept on ice until required and it was then warmed to 30°C in a water bath. The cuvette was kept at this temperature during the course of the assay. The reaction was followed for a period of 5-10 minutes sufficient to allow accurate measurement of the slope. Determinations were carried out using a Perkin-Elmer 330 spectrophotometer with a normal chart sensitivity of 0 to 0.1 absorbance units, the sensitivity being varied according to the activity of the extract. Corrections were made for the actual sensitivity used when calculating the rate. The activities are expressed as μmol/min/ml using an absorbance difference of 1.2 for the 10mM substrate.

APPENDIX V

LACTATE DEHYDROGENASE STUDIES

GENERAL OUTLINE OF EXPERIMENT

Experimental cells were stirred suspensions for both MEL and HL-60 cells studies. A pool of cells were put into a stirred suspension culture flask and left overnight at 37^{o} C; the following morning (ca 15-16 hours) sampling was commenced at 1, 2 or 5 minute intervals using a Cornwall syringe. Cells were collected and washed quickly on cellulose acetate membrane under suction, cells and membranes were immediately frozen in liquid nitrogen and extracted for the LDH studies. When required, insulin was added to the culture halfway through the experiment. The procedure was the same for MEL and HL-60 cells, except for the different medium.

EXTRACTION FOR LACTATE DEHYDROGENASE studies
(stirred suspension cultures)

Aliquots (2mls) of the cell suspension were taken using a Cornwall syringe connected to the culture by a short, narrow bore silicone rubber tube and the cells collected under suction on cellulose acetate filter membranes (Millipore type H.A. 0.45μm previously soaked in 0.9% saline). The cells were

washed twice on the membrane with 2.5ml 0.9% saline. The membranes with cells were placed in small glass bottles (Wheaton) and immersed immediately into liquid nitrogen. At a convenient time, they were thawed and then frozen-thawed three more times, followed by the addition of 1ml 0.05M Tris/HCl pH 7.4. For further extraction of cytoplasmic components, each sample was shaken for 30 seconds on a vibrating mixer (Whirlimixer) and then centrifuged at 2000g (Beckman model TJ-6 centrifuge) for 20 minutes at 5°C to remove cell debris. The supernantant fractions were stored in liquid nitrogen for enzyme activity and isozyme pattern determinations, or, at -20°C for protein measurements.

CELL FREE SYSTEMS

200ml of MEL cell suspension were centrifuged at 2000rpm for 10 minutes (Beckman model TJ-6 centrifuge). The pellet was washed with 2.5ml 0.9% saline then centrifuged at 2000rpm for 10 minutes, this was repeated twice more. The flask was left to stand on ice for 15 minutes, the pellet was resuspended in 1ml homogenizing buffer, homogenized using a Polytron homogenizer, three bursts each of 10 seconds. The homogenate was then centrifuged at 40 000rpm (100 000g) at 4°C for 1 hour (Beckman L8-M ultracentrifuge).

KINETIC DETERMINATION OF ACTIVITY

LDH ASSAY: standard method

The activity of lactate dehydrogenase is measured directly by the rate of decrease in the level of NADH at 340 nm according to the following reaction:

pyruvate + NADH ------> lactate + NAD

For the standard assay the initial pyruvate concentration is 0.7 mM and NADH 0.1mM. The temperature used is 30^{o}C, the reaction being carried out in 0.05M TRIS/HCl buffer, pH 7.4.

Stock **PYRUVATE** solution (70mM)

This solution contained:

Sodium pyruvate	77.2mg
Tris/HCl buffer, pH 7.4 (0.05M)	10ml

TRIS was dissolved in distilled deionised water and the pH adjusted to 7.4 using 1N HCl. Sodium pyruvate was added to the buffer; kept at 4^{o}C until required.

Final PYRUVATE solution (0.7mM)

This solution contained :

Stock pyruvate solution	1ml
buffered NADH	100ml

The stock pyruvate solution was added to buffered NADH. Solution kept at 4°C until required (solution is stable for five days).

NADH solution (0.116mM)

Dissolved NADH in 0.05M Tris/HCl pH 7.4 to give an absorbance of 0.700 units using Beckman spectrophotometer.

KINETIC ESTIMATION OF THE (APPARENT) ISOZYME PATTERN

The normal kinetic method for estimating the isozyme pattern depends on the different forms exhibiting distinct specificities or different kinetics. Both methods have been used here although the standard method is based on the fact that LDH isozymes can reduce both pyruvate and α-ketobutyrate (α-oxobutyrate) abbreviated to (P) and (B) respectively:

Pyruvate ----------> Lactate (P)
α-ketobutyrate ----------> α-hydroxybutyrate (B)

Carrying out assays with the standard pyruvate concentration (0.7 mM) and others with

3.3mM α-ketobutyrate as the substrate, B/P rate ratios have been obtained of approx. 0.8 for human H_4 type LDH and approximately. 0.2 for human M_4 type LDH (Lax, 1972). The ratio is taken as a measure of the "average" isozyme pattern in cell extracts (Gilbert, 1968, 1974) and is also used here for that purpose.

In one study, however, an additional measure of the isozyme pattern was obtained by determining the ratio of the activities toward two different pyruvate concentrations, namely the standard value (P, or, here Py.7) and half that concentration (0.35 mM, or, Py.3). Here too, the ratio adopted (Py.3/Py.7) should be constant but different for each isozyme. The assay solutions used were as follows.

Stock α-KETOBUTYRATE solution (0.1M)

This solution contained:

2-oxobutyric acid	125mg
Tris/HCl pH 7.4 (50mM)	10ml

TRIS was dissolved in distilled deionised water, pH adjusted to 7.4 using 1N HCl. 2-oxobutyric acid was added, this was stored at $4^{o}C$ until required. (this solution is stable for 5 days).

Final α-KETOBUTYRATE solution (3.3mM)

This solution contained:

2-oxobutyric acid stock solution	1ml
buffered NADH	30ml

Stock solution of 2-oxobutyric acid was added to buffered NADH, kept at 4°C until required (this solution is stable for five days).

Second PYRUVATE concentration (0.35mM)

For the determination of the apparent isozyme pattern using two different concentrations of pyruvate as substrate, for the second pyruvate concentration the standerd pyruvate solution was diluted 1 to 2 with the 0.05M THIS/HCl buffer, pH 7.4 thus giving a concentration of 0.35mM pyruvate (referred to as Py.3).

Storage of substrate solutions

All solutions were stored at 4°C until required but not longer than five days.

Assay procedure

Aliquots (1ml) of each substrate solution were placed in separate test tubes and kept on ice until actually used when the individual tube was warmed to 28°C in a circulating water bath. Cytoplasmic extract (100µl) was added to each tube, the contents well mixed and the transferred to a cuvette maintained at the same temperature. The decreasse in absorbance was measured continuously at 340nm. (Perkin Elmer 330 spectrophotometer). The chart slope of the trace is directly proportional to the enzyme activity in the

sample. Each assay was carried out twice (see Fig.4) for each substrate and an average taken to give the rate of the reaction, expressed as the rate of change in absorbance at 340 nm/min.

ELECTROPHORETIC DETERMINATION OF (ACTUAL) ISOZYME PATTERN
(Using The Beckman Paragon kit)

The Paragon Lactate Dehydrogenase (LD) Isozyme Electrophoresis kit is intended for the electrophoretic separation of the human isozymes of lactate dehydrogenase but was found suitable for the present work. The differences in the subunit structure of each isozyme produce a different surface charge, and this distinction forms the basis by which the isozymes are separated by electrophoresis.

The principle of electrophoresis is based upon the fact that when placed in an electrical field, proteins will migrate towards one or other of the electrodes at rates depending on their surface charge under the conditions used, in particular the pH and the nature of the buffer ions. By allowing sufficient time, individual proteins may thus be separated.

The Paragon LD isozyme kit provides for the electrophoretic separation of LDH isozymes in a buffered agarose gel. After electrophoresis, the isozyme in the gel are detected by the following specific colourimetric chemical sequential reactions:

lactate + NAD ----------> pyruvate + NADH

NADH + NBT ----------> NAD + NB-formazan dye

The nitro blue formazan dye is formed at the site of each isozyme band. The isozyme pattern may be interpreted or quantitated by scanning with a densitometer, as here.

Procedure for electrophoresis

Samples (5µl) of each extract were electrophoresed on the agarose and stained for enzyme activity using this system as described more fully below. Gel stain intensity was determined using a laser densitometer (soft laser scanning densitometer model SL-2D/1D UV/VIS), taking the area under the curves as a measure of the amount of isozyme.

Contents of the Paragon kit:

LD gels
LD buffer
LD substrate
Templates
Template blotters
Gel blotters
Drying blotters

Reactive ingredients:

LD gel : 1.0% agarose, 1.4% AMPD bicine - barbital - aspartate buffer, 0.1% sodium azide, and non-reactive ingredients necessary for optimum performance.

LD buffer: 14g: 2-amino-2methyl-1,3-propanediol, 38mmol/l reconstituted; DL-aspartic acid, 23mmol/l reconstituted; N,N-bis (2-hydroxyethyl) Glycine, 25mmol/l reconstituted; 5,5-diethylbarbituric acid, sodium salt,15mmol/l reconstituted.

LD substrate:

reconstituted: lithium L-Lactate, 208mmol/l; Nicotinamide Adenine Dinucleotide (NAD), 5.6mmol/l; p-Nitro Blue Tetrazolium Choride (NBT), 2.4mmol/l; Phenazine Methosulphate (PMS), 0.33 mmol/l and non-reactive ingredients necessary for optimum performance.

Reagent preparation , storage and stability

LD gels : Just prior to use, gels are carefully removed from the foil package. Gels should be stored at room temperature, 18°C to 26°C, until expiration date.

LD buffer: Contents of the buffer bottle were dissolved in 1000ml distilled deionised water. Unopened buffer should be stored at 2°C to 8°C, until expiration date. Reconstituted buffer is stored in a closed container at room temperasture, 18°C to 26°C, and is stable for 60 days or until expiration date, if sooner.

LD substrate:

Reconstitution is accomplished by the addition of 2ml of LD buffer. Restopper the vial and mix by gentle inverson. Continue mixing intermittently for 20 minutes before using. Just prior to use,

mix thoroughly to obtain complete solution. Reconstituted substrate is stable for 24 hours when stored in the dark at 2°C to 8°C. Avoid freezing.

Acetic acid: solution, 5% — To 2850ml of distilled, deionized water, add 150ml of glacial acetic acid. Mixed thoroughly. The solution is stored in a closed container at room temperature (18°C to 26°C).

Electrophoretic procedure

1) all samples and controls were prepared;

2) each compartment of the Paragon electrophoresis apparatus was filled with 45ml of LD buffer;

3) both containers of Paragon wet processor were filled with 300ml each of 5% acetic acid solution;

4) LD gel was removed from the foil package and place on a paper towel. Blot gently with gel blotter. Discard blotter;

5) template application was as follows:
   a) The template was folded lengthwise;
   b) The template was aligned with "A" position dots located on edges of gel;

c) the template was applied to the gel such that template slots contact gel surface first;
d) the template was sealed by gently rubbing a finger across it.

6) a fixed volume (normally 5 µl) of each sample was applied across each template slot. Five minutes was allowed for diffusion after the last sample has been applied;

7) the template was gently blotted with the template blotter. Both blotter and template were discarded;

8) the gel bridge assembly was placed on to the gel aligning the positive(+) and negative(-) sides of the gel with the corresponding positions marked on the gel bridge assembly. The assembly was placed into the Paragon electrophoresis cell and the latter covered;

9) the Paragon electrophoresis cell was placed into the power supply, the voltage set to 100 volts and the power applied for 20 minutes;

10) upon completion of the electrophoresis, the gel was removed from the Paragon electrophoresis cell and placed on top of a gel blotter. Both gel and gel blotter were placed into a Paragon incubation box;

11) another gel blotter was saturated with the contents of one bottle of

reconstituted LD substrate, and the blotter placed onto the gel surface, care being taken to avoid bubble entrapment between blotter and the gel;

12) the incubation box was sealed and placed in a preweated Paragon incubator and left at 45°C for 30 minutes (care being taken to reproduce these conditions for all gels)

13) after incubation, the gel blotter was removed from the gel surface and the gel placed into the gel frame provided;
14) the gel was then immersed in 5% acetic acid solution # 1 for 1 minute;
15) the gel was removed from the frame;

16) a small paper towel was placed on the base of the press dryer assembly and the gel on the paper with the agar face upwards. A gel blotter was then placed over the gel and moistened with acetic acid solution this being followed by two drying blotters. The dryer weight was placed over the assembly and the gel press dried for 3 minutes. A second gel may be pressed dry at the same time;

17) the gel was removed from the press dryer and placed into the gel frame. (The blotters were discarded.)

18) the gel was then immersed in 5% acetic acid solution # 2 for 1 minute;

19) after removing the gel from acetic acid solution #2 and removing the excess solution by wiping the backsurface, it was placed in the Paragon dryer until completely dry;

20) Finally the gel bands were scanned with the laser densitometer at 600nm in order to determine the stain intensity by the area method.

CELL FREE SYSTEM

Aliquots of 100ul were transferred from the extract supernatant every 2 minutes, to a small bottle and rapidly frozen and stored in liquid nitrogen, Each sample was individually thawed immediately before measuring the activity of the extracts kinetically as described above. Aliquots (5µl) of the extracts were also electrophoresed and stained for enzyme activity using the Beckman Paragon system and the stain intensities determined with the laser densitometer. Care was taken to ensure reproducibility at all stages.

APPENDIX VI

EXTRACTABLE PROTEIN

EXTRACTION FOR EXTRACTABLE PROTEIN studies

No special extraction procedure was used for these studies, the protein concentrations being measured in all extracts that became available irrespective of the method used to obtain them.

PROTEIN DETERMINATION

The Lowry (1951) method was used for all determinations according to the following procedures:

Lowry-Folin reagent

Reagents:

Albumin	1%(w/v)
NA_2CO_3 in 0.1M NaOH	2%
$CUSO_4.5\ H_2O$	1%
Sodium potassium tartrate	2%
Folin reagent	1 in 1 dilution

(commercially obtained Folin reagent contains sodium tungstate, sodium molybdate, H_3PO_4, concentrated HCl, lithium sulphate and a small quantity of bromine).

Procedure

An albumin standard was prepared at a suitable concentration and duplicates placed in a series of tubes as follows:

tube number	diluted standard (mls)	H_2O(mls)
1	1.0	0
2	0.75	0.25
3	0.50	0.50
4	0.30	0.70
5	0.10	0.90
6	0.00	1.0

5.0 mls of a mixture containing 50ml NA_2CO_3 in 0.1M NaOH, 0.5ml 1% $CuSO_4.5H_2O$, 0.5ml 2% sodium tartrate (this solution is made immediately beforehand because it is only stable for a few hours). The tube contents were mixed well and left for 15 minutes at room temperature. The Folin reagent was diluted with an equal volume of distilled deionised water and 0.5ml of diluted reagent was added to each tube and immediately mixed well. The reaction mixtures were kept in the dark for 30 minutes and read against a blank at 750nm within half-hour. Protein values were estimated from plots obtained for the absorptions of the standards as a function of concentration.

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