# REVERSIBLE PHOSPHORYLATION OF PROTEINS IN PROLIFERATING AND DIFFERENTIATING CELLS: CYCLIC VARIATIONS AND THE EFFECT OF GROWTH REGULATORS

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# ABSTRACT

Living cells are highly auto-dynamic entities which means that the underlying biochemistry is equally dynamic, a reality which is ignored by most researchers. Theoretical studies indicate that such a state must be due to the existence of oscillatory variations in the levels and activities of key components in the cell. In this study, the dynamic behaviour of four major, interrelated areas of cell biochemistry (phosphorylation, dephosphorylation, the terminal reaction of glycolysis and the amount of soluble protein) were examined and all systems found to oscillate in murine erythroleukaemic cells (MEL) and, where examined, also in the human HL-60 leukaemic cell line.

Certain processes have been shown to be oscillatory for the first time (the phosphorylation potential, the lactate dehydrogenase active isozyme level and aspects of the regulation thereof). While others have been shown to occur at a higher frequency than previously reported (phosphotyrosine phosphatase activity, the activity and apparent isozyme pattern of lactate dehydrogenase, the amount of extractable protein). All rhythms are shown (for the first time) to be complex and to involve several contributing periodicities, some modulating the period and amplitude of the observed oscillation. The frequencies are very high (periods of 1-20 minutes and probably less) and the amplitudes are equally high (variations in magnitude of as much as a hundred fold).

Phosphorylation processes, currently of particular

interest with regard to the nature and control of cell proliferation are thus found to be more highly dynamic than previously believed, a fact which throws some doubt on the current ideas on cell proliferation. The actual lactate dehydrogenase (LDH) active isozyme pattern is shown not to be constant (as generally believed) but to vary at high frequency (possibly due to phosphorylation of the the enzyme) while the kinetics and specificity of the lone isozyme in murine erythroleukaemic cells appear to be varying at equally high frequency due to the action of regulators (perhaps arising elsewhere within the glycolytic pathway). Similar results were obtained with HL-60 leukaemic cells with at least two of the isozymes varying in level, to some extent independently. The hormone, insulin, and the inducer of cell differentiation, HMBA (hexamethylenebisacetamide), have been found to affect the dynamics of the four systems although, because of the complexity of the rhythms the actual effects on the dynamics are not easily defined. Insulin has a marked effect on the mean level of the activity of the LDH isozyyme.

The fact that all oscillations ~~is~~ are seen despite no attempt being made to synchronise the cell population suggests the existence of communication between cells but how this can occur when the rhythms are of such high frequency is intriguing.

All the results add further support for the long standing view of my supervisor, that the properties and behaviour of cells reflect the internal dynamics and that differentiation, cancer and intracellular signalling occur through changes in the pattern of temporal organisation of cellular oscillations.

# DECLARATION

Apart from the help of my supervisor, Dr. D.A. Gilbert, regarding the writing or modification of computer programmes to plot the experimental data in various forms, the drawing of some graphs on his personal computer and plotter (for which the analysis programs were written) and the interpretation of the results so obtained, I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the Faculty of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any other degree or examination in any other University, nor has it been prepared under the aegis or with the assistance of any other body or organisation or person outside the University of the Witwatersrand, Johannesburg.

GRACINDA MARIA NUNES FERREIRA

22 day of JUNE, 1994.

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Completing this thesis has taken a big slice out of my life. It has been engrossing, exciting and interesting. My parents, Mr. and Mrs. Ferreira and my brother, Carlos, have given me a great deal of emotional and practical support to be able to do this work and I want to thank them for their unfailing encouragement, love and confidence in my ability to carry through, even when I was most frustated and despairing about it.

Grateful thanks are acknowledged to:

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Colin, for reminding me to set short term objectives, " CHEWABLE CHUNKS"; "BITABLE BITS" and for his patience and understanding while this thesis was completed.

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# PREFACE

I undertook this field of work because I believed that experience in this advanced field of cell biochemistry would supplement and reinforce my pharmaceutical qualifications which lacked depth in this subject.

I am therefore most greatful to my supervisors, Professor K.D. Hammond and Dr. D.A. Gilbert for providing me with the opportunity to undertake the projects despite my limited knowledge in the subjects. I trust that their faith in my abilities is justified by this thesis.

I also wish to thank Professor Hammond for her continuous support, help and encouragement throughout the course of the work.

Especial thanks are due to Dr. D.A. Gilbert for much help in giving me the necessary background in the subjects which included the loan of books, lecture notes, articles on different aspects, access to the draft manuscript of his monograph, the use of his personal computer and plotter, for computer programs for plotting and analysing the data, for actual plotting of some diagrams while modifying programmes and for the aid regarding the interpretation of results. In view of the large amount of information obtained and its complexity, I also appreciate the advice of Dr. Gilbert regarding the organisation of the thesis.

The project was based on much earlier theoretical and experimental studies of Dr. Gilbert indicating an oscillatory basis for cell dynamics and his view of the rates of rhythmic processes in differentiation, cancer and intracellular signalling.

While the initial intention was to study just phosphorylation dynamics, novel and interesting results led into other areas (that might yet prove to be involved in phosphorylation processes) which emphasise, even more, how highly dynamic and complex cellular reactions really are. Those who ignore this fact (and that includes most cell biochemists), may thus be producing results of doubtful value.

Two articles on the subject matter of this thesis are in the press (as given below) although additional interesting results in the areas of phosphorylation and extractable protein oscillations have been obtained since submission of the articles. Presentations of the work have been given at various conferences (as listed below) and a number of other articles are already in preparation .

OSCILLATIONS IN PHOSPHOTYROSINE PROCESSES IN MURINE ERYTHROLEUKAEMIC CELLS : INSULIN STIMULATION OF THE DYNAMICS by G.M.N. Ferreira, H.D. Hammond and D.A. Gilbert at 33rd Annual Congress of the Federation of South Africa Societies of Pathology, Sun City, Bophuthatswana, Africa, 1993.

HIGH FREQUENCY LACTATE DEHYDROGENASE ACTIVITY AND DIFFERENTIAL ISOZYME DYNAMICS IN LEUKAEMIC CELLS by G.M.N. Ferreira, K.D. Hammond and D.A. Gilbert at South African Biochemical Society 12th Congress, Stellenbosch, 1994.

**PUBLICATIONS GENERATED BY THIS WORK**

**ARTICLES IN PRESS**

INSULIN STIMULATION OF HIGH FREQUENCY PHOSPHORYLATION DYNAMICS IN MURINE ERYTHROLEUKAEMIC CELLS by, G.M.N. Ferreira, K.D. Hammond and D.A. Gilbert (1994). Biosystems, (in press).

OSCILLATORY VARIATIONS IN THE AMOUNT OF PROTEIN EXTRACTABLE FROM MURINE ERYTHROLEUKAEMIA CELLS: STIMULATION BY INSULIN. by G.M.N. Ferreura, K.D. Hammond, D.A. Gilbert 1994 Biosystems, (in press).

POSTER PRESENTATIONS AT MEETINGS

A CONTINUOUS ASSAY FOR PHOSPHOTYROSINE PHOSPHATASE: OSCILLATIONS IN THE ACTIVITY IN MURINE ERYTHROLEUKAEMIC CELLS. by G.M.N. Ferreira, K.D. Hammond and D.A. Gilbert at 31st Annual Congress of the Federation of South African Societies of Pathology, Warmbaths, South Africa 1991.

PHOSPHORYLATION DYNAMICS IN RELATION TO INDUCED DIFFERENTIATION, MITOGENESIS AND THE REVERSAL OF MALIGNANCY by G.M.N. Ferreira, K.D. Hammond and D.A. Gilbert at Federation of S.A. Societies of Pathology 32nd Annual Congress. Wild Coast Sun, South Africa, 1992.

**The following articles are in various stages of preparation:**

1) DISTINCT, VERY HIGH FREQUENCY OSCILLATIONS IN THE ACTIVITY AND AMOUNT OF LACTATE DEHYDROGENASE IN MURINE ERYTHROLEUKAEMIC CELLS.

2) HIGH FREQUENCY OSCILLATORY REGULATOR MODULATION OF THE KINETICS AND SPECIFICITY OF LACTATE DEHYDROGENASE IN MURINE ERYTHROLEUKAEMIC CELLS.

3) INDEPENDENT HIGH FREQUENCY PERIODIC REGULATION OF INDIVIDUAL ISOZYMES OF LACTATE DEHYDROGENASE IN HL-60 CELLS.

4) INDEPENDENT OSCILLATORY REGULATION OF THE AMOUNT AND ACTIVITY OF LACTATE DEHYDROGENASE IN CELL-FREE EXTRACTS OF MURINE ERYTHROLEUKAEMIC CELLS

5) TRANSIENT CHAOS IN THE DYNAMICS OF MURINE ERYTHROLEUKAEMIC CELLS ?

# LIST OF ABBREVIATIONS

| | |
|---|---|
| Fetal calf serum | FCS |
| Phosphotyrosine | PT |
| Phosphotyrosine phosphatase | P-T-Pase |
| Murine erythroleukaemic cells | MEL |
| Human leukaemic cells | HL-60 |
| N'N- hexamethylenebisacetamide | HMBA |
| Hydroxymethyl-methylamine | Tris |
| Dulcecco's modified Eagles minimum essential medium | MEM |
| Trichloro acetic acid | TCA |
| Lactate dehydrogenase | LDH |
| Foetal calf serum | FCS |
| Dimethyl-sulphoxide | DMSO |
| Epidermal growth factor | EGF |
| Platelet derived growth factor | PDGF |
| Phosphofructose kinase | PFK |
| Fructose diphosphate | FDP |
| Fructose monophosphate | FMP |
| Adenine diphosphate | ADP |
| Adenine monophosphate | AMP |
| Lactate Dehydrogenase | LDH |
| Nicotinamide adenine dinucleotide | NAD |
| " " (reduced form) | NADH |
| Maturation promoting factor | MPF |

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# INTRODUCTION

# CHAPTER I

# INTRODUCTION

## GENERAL COMMENTS

The studies reported here cover a range of distinct but related aspects of cell biochemistry. Therefore this introduction is equally wide ranging in scope if not in depth. The major aspects are given in Table I which also attempts to indicate some of the links between successive topics but others should become apparent as the thesis is developed. The subject of phosphorylation and dephosphorylation of cellular constituents is considered first, partly for historical reasons (such experiments being the first undertaken) and also because of the ever growing recognition of its importance for every aspect of cell behaviour, properties and function. The general aim was to prove the autodynamic aspects of the subject as relatively little is known about them. However, the studies diverged into areas where the connections may not be obvious, namely the regulation of lactate dehydrogenase isozymes which could occur through phosphorylation and dephosphorylation of the enzymic forms. Circumstances prevented this view from being substantiated but it seems even more likely to be a factor. What should become evident is the fact that the various systems are even more highly autodynamic and complex than even we expected them to be.

# Table I OUTLINE OF INTRODUCTION

| SUBJECT | OUTLINE | LINK |
|---|---|---|
| PHOSPHORYLATION AND DEPHOSPHORYLATION | General involvement in metabolic processes<br>Phosphorylation of serine, threonine and tyrosine | ---------- Reversal of phosphorylation |
| PHOSPHO-TYROSINE PHOSPHATASE | Particular enzyme catalysing dephosphorylation | ---------- Known actions of insulin on phosphorylation processes |
| INSULIN ACTION | Important hormone | ---------- Insulin as a mitogen |
| CELL REPLICATION | Phosphorylation processes and cell cycle | ---------- Dependence on cell replication |
| DIFFERENTIATION | Nature of phenomenon | ---------- Blocked differentiation |
| CANCER | Continued proliferation<br>Leukaemias | ---------- An example of blocked differentiation |
| MEL CELLS | Characteristics | ---------- Reversed malignancy |
| INDUCED DIFFERENTIATION | Differentiation reinitiated | |
| HMBA | Inducing agent | ---------- Dependence on control processes |
| CONTROL PROCESSES | Cellular controls<br>Glycolysis | ---------- Living cells are highly dynamic |
| AUTODYNAMIC BEHAVIOUR OF CONTROL SYSTEMS | Oscillations<br>General properties<br>Action of disturbances | ---------- Oscillator concept of cancer and differentiation and cell replication |
| MEL REDOX AND MORPHOLOGICAL DYNAMICS | Observed oscillations in MEL cells | ---------- Glycolysis- the source of ATP<br>Phosphorylation of LDH |
| LACTATE DEHYDROGENASE | Role in glycolysis<br>Observed oscillations<br>1 isozyme in MEL cells | ---------- Another cell blocked in differentiation<br>Contains 4 LDH isozymes |
| HL-60 CELLS | Human leaukaemic cells | ---------- This occurs in all cells and influences data interpretation |
| EXTRACTABLE PROTEIN OSCILLATION | | |

## PHOSPHORYLATION AND DEPHOSPHORYLATION

As any text book shows, early biochemical studies soon recognised the metabolic importance of substrate and coenzyme phosphorylation and transfer of the phosphate group from one such molecule to another. Only in the last decade or so has it been appreciated that similar reactions occur with and among many proteins and that this class of reactions is of great significance to the behaviour, properties and functions of all cells. The protein phosphorylation reaction was the first example of control through covalent modification and has been found to occur in almost every type of cellular process including synthesis of cell constituents, metabolic pathways and even gene expression (Krebs, Beavo, 1979).

In particular, the activation and deactivaton of a wide range of enzymes by kinases and phosphatases (including covalent modification of those enzymes themselves) form a complex set of regulatory reactions without which cells could not survive (Flochart and Corbin 1982; Li 1982; Krebs 1985). They include signal processes at the level of receptors in the plasma membrane through to genetic and associated reactions and many intermediate processes. To give a general overview of this field of research would require more space than this thesis provides. Therefore in this introduction attention will be focussed on one aspect of the subject which is particularly relevant to the topics of my own research, namely the role of these processes in cell differentiation and proliferation (or more correctly, in mitosis). It should be noted, however, that similar

reactions are involved in other major aspects of cell metabolism, such as transcription initially, however, something should be said about the nature of the reactions involved in protein phosphorylation. The subject will be related to virtually all aspects of the thesis although that fact may not be obvious. One aim of the work presented here was to see if the phosphorylation potential of cell extracts varied in periodic manner and if it is so, to determine the true period and obtain some indication of the amplitude, as these factors are important with regard to current ideas on mitosis .

**PROTEIN PHOSPHORYLATION**

As indicated, reversible protein phosphorylation is an important feature of many biological processes including signal transduction, metabolism and growth control (Bourret et al 1989). The reactions involved in the enzyme-catalyzed phosphorylation and dephosphorylation of proteins may be depicted as:

$$\text{Protein} + n\ \text{NTP} \xrightarrow{\text{kinases}} \text{Protein-P}_n + n\ \text{NDP}$$

$$\text{Protein-P}_n + n\ \text{H2O} \xrightarrow{\text{phosphatases}} \text{Protein} + n\ \text{P}_i$$

where NTP is one or more of the nucleotide triphosphates and $P_i$ is inorganic phosphate (see, for example, Flochart and Corbin 1982, Li 1982, Krebs 1985). However, this is a much simplifed picture in that it does not take into account the nature of the protein

residue affected, the multiplicity of such sites on many proteins, the order in which they become phosphorylated or the importance of the timing at which they all occur and the factors determining those times. More often than not attention is focused merely on the extent of the covalent modification.

Most studies have been concerned with phosphorylation and the more frequent sites for this regulatory action are the hydroxyl groups of seryl and threonyl residues of the target proteins, tyrosyl residues are also affected but to a lesser extent, making up only 0.01%-0.05% of the phosphoamino acid content of a normal cell (Hunter and Sefton 1980; Sefton et al 1980; Ushiro and Cohen, 1981). While the mammalian phosphoseryl and phosphothreonine kinases are ~~extremely~~ not only in the control of glycogen metabolism, but also in replication, the phosphorylation of the tyrosyl residues has taken on a significance far out of proportion to the frequency at which it occurs. This stemmed from the fact that there is a 10 to 60 thousand fold increase in the extent of tyrosyl phosphorylation on infection by viruses causing transformation. More recent studies have revealed additional reasons for stressing the importance of the latter reactions. The view of the relative significance of the different target sites changes almost daily as new information becomes available but presumably all are of some significance depending on the topic of interest. Indeed, some proteins (e.g., those involved in mitosis - see later) appear to undergo multiple phosphorylation at all three residues and the sequence of reactions may be important in determining their overall function (see

Roach, 1991, for a review of multiple phosphorylation reactions). What is clear is that all kinds of phosphorylation and dephosphorylation reactions are of the utmost importance to cell proliferation, transformation and differentiation, three inter-related problems.

By way of example, a phosphoprotein with protein kinase activity was found to be required for transformation by the Rous sarcoma virus. Increased protein tyrosine phosphorylation has also been observed in blood platelets following treatment with thrombin and collagen which activate platelet aggregation. The role of the protein tyrosine kinases in non-proliferating, terminally differentiated cells is not known, they may induce cell aggregation in response to the agonists. Some of these aspects will be amplified as the theme is developed.

It has been found that a change in the extent of the phosphorylation of protein tyrosine residues occurs during differentiation of the virally transformed MEL cells (see later). It has been suggested (Hammond et al 1985) that dephosphorylation processes could be involved in the reversal of malignant characteristics which is produced by inducers of differentiation. However, recognising the importance of time dependent processes and rhythmic behaviour in the regulation of cellular metabolism, they proposed not a simple shift in the extents of phosphorylation but a change in the dynamic "equilibrium" (Hammond et al 1989). It was argued that to be of significance, the kinase and phosphatase enzyme systems must constitute controlled processes and as such

the systems could have the ability to behave in an autodynamic manner, transformation and the reversed malignancy could then result from changes in the pattern of dynamic behaviour (Gilbert, 1968,1984a). Such concepts are discussed more fully in the subsequent sections of this introduction.

**PHOSPHORYLATION AT TYROSINE RESIDUES**

Phosphorylation at tyrosine in proteins is important if only in that specific tyrosine kinases have been found to reside in membrane receptors for various growth factors (e.g., insulin) and in oncogene products; it was suggested that the reaction may correlate with cell proliferation (Sefton et al 1980; Kolata 1983. Ramachandra and Ullrich 1987). To this end, tyrosine phosphorylation by specific phosphotyrosyl kinases has been associated with the regulation of proliferation and also differentiation and transformation. Moreover, the intrinsic tyrosine kinase activities of the receptors for various growth factors [epidermal growth factor - (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor-1 and insulin are changed by the binding of the agent and the receptors even phosphorylate themselves and other proteins at tyrosine residues (Piga et al 1984). Thus, in at least some instances, the stimulation of the tyrosine kinase activity probably plays a role in the signalling process.

Tumour promoters induce tyrosine-specific protein phosphorylation and it has been found that high levels of tyrosine protein kinase activity exist in murine and

human T lymphoma cell lines while lower levels were detected in normal resting lymphocytes. It has been observed that many oncogene products have tyrosine kinase activities and also an extensive sequence homology with several known growth factor receptors (Ferrari and Baserga, 1987; Bradshaw and Prentis, 1987)

**PHOSPHOPROTEIN PHOSPHATASES**

For protein phosphorylation to be of importance as a regulatory mechanism, the reverse step must take place, the levels of both phosphorylated and dephosphorylated forms of enzymes or proteins are important if a regulatory function exists.

At present, relatively little is known concerning the possible role of phosphoprotein phosphatase-mediated dephosphorylation in regulating growth, differentiation and neoplastic transformation. Phosphoprotein phosphatases apparently exist in some tumour and normal tissues and cell lines; sometimes these enzymes also appear to be related to alkaline phosphatase activity while others are acid phosphatases, (Swarup, Cohen, Garbers,1981, Leis and Kaplan, 1982). Alkaline phosphatases exist in several isozymic forms. Dephosphorylation by acid and alkaline phosphatases has been studied in several tumour lines and changes have been observed which appear to be related to the rate of cell proliferation, [Seale, Sach, 1974; Cox and Park, 1982; Kikichi, Takagi, Parmley, Ghanta, Hiramoto 1982; Takhara, Herz, Singer, Hirano, Koss 1982]. One hypothesis suggests that protein tyrosine phosphorylation feeds into or regulates the

much more abundant serine (threonine) phosphorylation that occurs in cells but recent cell cycle studies indicate more complex patterns of behaviour.

Phosphotyrosyl protein phosphatase activities have been found in eukaryotic cell lines and are widely distributed in various tissues, in association with cell membranes and with the cytosol, supporting the argument that they do play an important role in cellular regulation processes. Multiple pH specificities have been observed, displaying both acid and alkaline phosphatase activities and they appear to exist in at least 3 distinct forms, each with a different $M_r$ value. It is not clear whether these different forms represent distinct and different isozymes, the association of one enzyme or catalytic subunit with different regulatory proteins or the proteolysis of a single enzyme form to yield several different enzymes with a range of $M_r$ values.

It has been reported that some phosphoseryl protein phosphatases can also exhibit P-T-Pase activity but studies on substrate specificities have proved that phosphotyrosine phosphatases are totally distinct enzymes and, indeed, the activities can be separated from each other using various chromatographic techniques.

It should be noted that the suggestion has been made that the specificities of kinases and phosphatases are too low for the reactions to be biologically significant if acting alone but that they interact with other proteins (termed targetting subunits) which convey a

much higher degree of specificity (Hubbard, and Cohen, 1993, Kennelly and Krebs 1991). This would make the system comparable with the ubiquitin reactions governing protein degradation (Jantsch, 1992). If correct, this mechanism further complicates the issues well beyond those considered here although not invalidating them.

Our argument is that IF phosphorylation at tyrosine residues is so important with regard to the regulation of replication, cancer, etc then it follows that P-T-Pase must also be a key enzyme for these various phenomena.

As indicated, the dominance of either phosphorylation or dephosphorylation will depend on the balance between the activities of the kinases and phosphatases. The regulation can occur by simply altering the ratios of the rates of these processes or by changing the relative times or frequency at which they act. If the latter, then what determines the time at which a reaction occurs or its repetition interval? Is regulation of these covalent reactions achieved by changes in their dynamics rather than through simple changes in rates or levels of components? If the phosphorylation potential oscillates then one can expect the phosphatase activities to oscillate also.

Phosphorylation at tyrosine in proteins seems to be important if only in that specific tyrosine kinase activity has been found to reside in membrane receptors for various growth factors and in oncogene products, it has been suggested that the reaction may correlate with

cell proliferation (Sefton et al 1980; Kolata 1983; Ramachandra and Ullrich 1987). Phosphorylation and dephosphorylation of proteins appears to be a factor in the control of MEL cell differentiation. Studies done on MEL cells have shown that they contain protein kinases and that protein phosphorylation patterns change during induced differentiation (Gazitt and Friend 1981; Earp et al 1983; Schwartz and Rubin 1983).

**PHOSPHOAMINO ACID PHOSPHATASES**

Initially there were no suitable substrates for phosphoprotein phosphatase activities so, Hammond et al 1989 used the phosphoamino acids and reported the presence of phosphoamino acid phosphatases in MEL cells with similar but not identical cyclic changes in the activities of all three of them in both control cells and those treated with DMSO or HMBA (see later for abbreviations). They obtained results suggesting that there might be a change in dephosphorylation status associated with differentiation (Hammond et al 1985). If both phosphorylation and dephosphorylation processes are autodynamic, the balance could be modified by altering the dynamics (for example, the co-ordination between the periodicities). Such a possibility did not seem impossible for, as will be discussed shortly, differences in phasing of isozyme oscillations have been reported for several cells.

**PHOSPHOTYROSINE PHOSPHATASE**

Limitations to the studies of Hammond et al (1985) were (a) the discontinuous nature of the assays

originally used, and (b) the fact that the chemica determinations of the liberated phosphate were time consuming; one of the thesis tasks was the development of a continuous spectrophotometric assay for the hydrolysis of phosphotyrosine and a comparison of the two assay methods (see Appendix IV).

In their studies, Wolbrandt et al,(1985) used sampling times which were very long but they considered the possibility that the frequencies were much higher than they appeared at that time. Proving this to be so was one of the tasks forming part of this thesis. Although the situation is even more complex than this picture indicates, it serves as a basis for much of the following introduction.

## Role of $Ca^{2+}$ ions

Apart from the "targetting subunits" mentioned above, many of the kinases and phosphatases are themselves regulated by the $Ca^{2+}$-dependent protein calmodulin and hence by the cytosolic calcium concentration. Changes in the calcium levels influence the ability of cells to grow and divide, the ion thus being essential for both metabolism and as a trigger of cell division (Campbell, 1983). To illustrate this point we may note that in sea urchin eggs, it has been shown (using the $Ca^{2+}$ indicator Aequorin) that a substantial rise in intracellular free calcium occurs within 60 seconds of fertilization after which begin the first cell divisions of embryogenesis. The steady state (average) calcium concentration in the cytosol of mammalian cells is of the order of $10^{-8}$M to $10^{-7}$M but

upon stimulation the intracellular calcium concentration reaches μM levels, which is sufficient to stimulate phosphatase activity. In some situations, cAMP can also stimulate cell division through an interaction with the intracellular $Ca^{2+}$. It is clear that a complex signal transduction pathway exists, the initial stimulus being a gradual change in the calcium levels, this results in the activation of various enzymes by phosphorylation and dephosphorylation by the kinases and phosphatases respectively, which respond to a stimulus. In the experiments referred to, the rise in free calcium ion level can spread like a wave across the cell raising the point that spatial effects, even at the intra-cellular level can be important at least in some instances (Gilbert, 1985).

**INSULIN ACTION**

Insulin is a hormone of general interest but despite much study its mode of action at the cellular level is still inadequately understood, particularly as it can exhibit specific and non-specific effects (Gilbert and Visser, 1993).

Other studies from these laboratories have confirmed that it stimulates morphological dynamics (Visser et al, 1992) and, in particular, it enhances fluctuations of the plasma membrane in a frequency dependent manner. The more dynamic surface is believed to stir the microenvironment more effectively thereby decreasing the effective thickness of the surrounding diffusion layer. This in turn would increase the rates of uptake of nutrients and excretion products and so produce a

non-specific stimulation of metabolic processes (Gilbert and Visser,1993).

The hormone has been shown to act as a mitogen in many studies (King and Kahn, 1985) although Gilbert (1978b) suggested a mechanism for the action and interaction of regulators which shows that such effects can depend on the cell species concerned and the presence of other regulators, as seems to be the case.

A number of other studies have shown that insulin enhances phosphorylation processes through its action on the membrane receptor which activates its kinase properties. Its mitogenic action is thought to operate through this reaction which then triggers a series of other phosphorylations and dephosphorylations. While insulin, stimulates cell replication in general, the agent HMBA reduces the proliferative ability of MEL cells (see later). One reason for studying the action of insulin in the present work was to see if its action was opposite to that of HMBA on MEL cells.

One might note that a daily rhythmic nuclear protein kinase activity has been reported for rat liver (Langner and Rensing, 1979).

## CELL REPLICATION

With regard to their proliferative ability, cells can be classified as being:

1) in the active state of growing and dividing,
2) in a quiescent state but from which they can be

stimulated to undergo division by certain stimuli,

or,

3) apparently unable to replicate any more.

Most cells are capable of proliferating under the right conditions (which differ for different cells) and thus fall into either of the first two catagories. At this point it should be stressed that replication (which implies the true duplication of all components) may or may not be the same as proliferation (which involves the formation of daughter cells whether or not they have the same characteristics as the parent). Thus differentiation involves the latter as the population of cells are becoming "different". Here the words will be used interchangably without making a distinction between the two, although it is obviously important whether or not the daughter cells are identical to each other and to the original cell. While Gilbert (1988) has considered this aspect from the theoretical point of view, most other concepts of replication processes seem to ignore it.

The actual process of division (cytokinesis) is of short duration and can be used as a reference point in time. The overall set of reactions taking place between one division and the next is termed the cell cycle an aspect that will be considered in the next section. At the moment discussion will focus on the regulation of proliferation.

As indicated, quiescent cells can be stimulated to

replicate by the addition of agents which, because they normally also induce mitosis, are termed mitogens. In some cases, the same effect can be achieved by removing inhibitors of the process. Gilbert (1978b), MacKinnon and Gilbert (1993) have pointed out that agents can exhibit both kinds of action depending on the circumstances and the former has proposed a mechanism accounting for such properties in terms of the initiation or suppression of dynamic behaviour in an intracellular control system (see later). Such a view is now largely substantiated by observations on periodic mitosis in cell free systems (e.g. Kirschner 1992, Norbury and Nurse 1992). Like other rhythmic processes, the rates and kinetics of the system determining replication (see later) must fall within certain boundary conditions thereby requiring threshold levels for any regulators. This results in switch-like characteristics thus giving reason to discuss the initiation of replication in terms of triggering effects (Gilbert, 1978b). The latter has shown how the magnitude of the threshold is related to the maximum density to which cells grow in culture and he has also explained the effects of the malignant transformation in such terms (Gilbert, 1977, 1978a).

## MITOGENS AND THE REGULATION OF PROLIFERATION

Although an enormous amount of work has been done on the action of mitogens relatively little is known about how they actually achieve their effects. MacKinnon and Gilbert (1993) have suggested that this is because of a lack of a clear concept of the processes underlying proliferation, namely what results are important with

regard to determining the change from quiescence to active replication. However, a number of important factors have been discovered. Thus Stoker (1973) showed that the diffusion layer thickness is a factor which limits the replication of quiescent cells in a confluent monolayer of 3T3 cells, while Koren 1976 has correlated proliferation with the rate of uptake of nutrients. Folkman and Moscona (1978) have thought about the possibility that replication can be induced by an increase in the rate of uptake of nutrients when the shape of a cell changes. These aspects are important from the present point of view because insulin is mitogenic for many cells, Gilbert and Visser (1992) have shown that the hormone stimulates the dynamic behaviour of the surface membrane which may result in a decrease in the thickness of the surrounding diffusion layer. They also point out that variations in surface area might accompany increased membrane dynamics.

Cells sometimes have to react quickly to certain signals, if the response (eg. proliferation) needs a generalised activation of metabolism, it could be advantageous to have a non-specific mechanism by which this may be achieved rapidly (Gilbert and Visser, 1993). The latter point out that if the process involved also activates locomotion, then several distinct actions could be initiated simultaneously by a change in the dynamics. The signal can be a chemotatic one (as with insulin) thereby causing the cell to move towards the origin of a signal in an attempt to reach more beneficial conditions (Durham and Ridgway 1976, Matsumoto, Ueda and Kobatake 1988).

A number of mitogens appear to stimulate the uptake of metal ions (Glaser, Whitely and Rothenberg 1980). Epidermal growth factor rapidly produces morphological changes (Chinkers, Mckanna and Cohen 1979; Brunk, Schellens and Westmark 1986), whereas chemotatic agents are associated with oscillatory behaviour (Wyman et al 1987) and have been reported to induce damped oscillations in the intensity of light scattered by cells (Matthias, Wyman and Deranleau 1987), which occur at a frequency comparable with those observed by Visser et al (1993) as discussed later.

The actions of mitogens can thus be very complex and involve processes which are not obviously connected with proliferation. It seems reasonable to expect insulin to have complex effects on cellular dynamics.

## THE CELL CYCLE

Considering cells in the process of replication, it may be noted that the cell cycle is generally divided into several stages:

1) the $G_1$ (first "gap") phase in which general protein and RNA synthesis are stimulated,
2) S phase, defined by the actual onset of DNA and chromosomal protein synthesis,
3) $G_2$ (or second "gap") phase wherein the RNA's and proteins required for mitosis and division are formed,
4) M and D phases when mitosis and cell division take place.

In the above, $G_2$ is followed by mitosis, (M) (heralded by the disappearance of the nuclear membrane) which can be further subdivided. The cytoskeletal microtubules are arranged in a spindle formation on which the condensed chromatids are arranged, thereby forming the metaphase plate. The anaphase stage is seen by the separation of the daughter chromosomes in response to a chemical signal, the Maturation Promoting Factor (MPF), which is responsible for the dissolution of the nuclear membrane prior to mitosis and also meiosis in Xenopus oocytes. Upon the separation, two membranes form around the daughter nuclei (telophase). This is then followed by the division of the cytoplasm to create two new daughter cells. Each of the reactions mentioned can influence others not actually involved in replication processes (see later comments).

The last two of these are the shortest in duration while the $G_1$ phase is the most variable and appears to include a quiescent state from which the cells are spontaneously triggered into active replication by random processes (Gilbert, 1978b, 1978c, 1982a, 1982b, for example).

In the classical view, most quiescent cells are arrested in the $G_1$ or $G_2$ (but not S) phases (as determined by the DNA content). These have been reclassified as $G_o$ and $R_2$ phases because many quiescent cells undergo chemical and morphological changes not normally found in cycling cells during $G_1$ or $G_2$ stages. $Ca^{2+}$ levels play a major role in the decision to leave $G_o$ or $R_2$ and enter the cell division cycle.

Quiescent cells can also be stimulated to proliferate by viruses, certain chemicals and some other hormone and thus "rejoin" the cell cycle.

At this time one is reminded that cells do not always replicate continuously but appear to wait between cycles for random lengths of time. It is not evident how the cyclin concept accounts for such behaviour but a detailed explanation has been presented in terms of another concept to be discussed shortly.

Ideally, the cell division cycle should be studied by observing all those processes which occur when a single cell grows and eventually divides (Edwards 1981) but it is generally necessary to use populations. The cell cycle is characterized by a recurrent sequence of discrete events (Edwards 1981). DNA replicates and growth leads to an overall approximate doubling in the amount of each cellular constituent prior to segregation at cell division, one can therefore say that the time domain of the cell cycle is thus a higher order of the temporal hierarchy and nesting within it are the ultra fast, the metabolic and the epigenetic domains (Lloyd, Edwards and Poole 1982). The time taken for completion of the cell cycle varies for both prokaryotes and eukaryotes and is not fixed for either group, eg. Bekena Natriegens, 10 minutes (Eayon 1962); water mould Achlya bisexualis, 57 minutes (Griffin et al 1974); amphibian embryos (eg. Xenopus), 15 minutes (Graham and Morgan 1966); mammalian embryos, 10-20 hours in mice (Graham 1961). In embryos the initial division times are generally short and they become progressively longer. One has to keep in mind that cell cycle times relies on

nutritional status and on temperature and can therefore be controlled by the external environment. Klevecz (1976) has suggested that cells in culture exhibit cell cycle times which are multiples of some base period.

It is also necessary to note that although cell growth and division are normally coupled they can be dissociated, as with division and mitosis (Gilbert, 1981).

**PHOSPHORYLATION AND REPLICATION**

With many of the earlier results, the evidence for involvement of phosphorylation in cell replication was indirect and by association simply because little was known about cell cycle processes. As the malignant transformation results in abnormal proliferation characteristics, any correlation with cancer has been taken to mean that the phosphorylation reactions under study must be related to replication. More recent results have been more direct.

We have mentioned that viral transformation often stimulates both phosphorylation and cell replication. The gene products of many oncogenes have been shown to be associated with protein kinase activities. A remarkable feature of the majority of these activities is that tyrosine is phosphorylated. Phosphotyrosine is a rare amino acid derivative in contrast to phosphoserine and phosphothreonine (Sefton, Hunter, Beemon, Eckhart, 1980). Studies with Rous sarcoma virus transformed cells have indicated that transformation may be mediated by a phosphoprotein which displays the activity of a protein

kinase and specifically phosphorylates tyrosine in protein substrates. It has also been shown that isozymes of enolase, phosphoglycerate mutase and LDH are phosphorylated at tyrosine in cells transformed by those viruses whose transforming proteins are, or include, tyrosine protein kinase [Pincus, Beckman and George (1984)]. However, the significance of these latter effects is unclear.

One view of cell proliferation relies on the coupling of primary mitogenic signals to the initiation of DNA and general protein synthesis, phosphorylation appears to play a crucial role in this signal transduction pathway. Many proteins, including histones, lamins and cytoskeletal components, undergo marked changes in phosphorylation state during the M phase. These phosphorylation events are now believed to mediate mitotic processes such as chromosome condensation, nuclear envelope breakdown and spindle assembly and elongation (Cyert and Thorner, 1989). Several gene products centrally involved in the regulation of mitosis have been identified by genetic analysis of the cell cycle mutants in a number of species, many have been identified as protein kinases or related structures. In Aspergillus, a positive regulator of mitosis (the nimA product) is a protein kinase. In the bimG11 temperature sensitive cell cycle mutant of this organism, the mutation in the gene sequence coding for the bimG11 product blocks the cells from exiting mitosis. The bimG11 product shows an extraordinary sequence homology with the mammalian type-1 phosphoprotein phosphatase. In the fission yeast, three mitotic regulators ($cdc2^+$, $wee1^+$ and $nim^+$), have homology to protein kinases. The

$cdc^{2+}$ product is required for the transition from $G_2$ to the M phase and its counterpart in bakers yeast has also been found to have kinase activity. The $cdc^{2+}$ product has a homologue in higher eukaryotes which has proved to be an integral part of the maturation promoting factor, (MPF). MPF as also been found to induce the transition from $G_2$ to the M phase. Another mutation found in the fission yeast, dis, which results in the loss of chromosomes and impaired entry into the new cell cycle also occurs in the gene sequence coding for a product with protein phosphatase activity. In work done on mouse FM3A cells, it was found that DNA synthesis could be controlled by phosphorylation and dephosphorylation of histone H1 (Takada et al 1989). It was found that histone H1 stops the activity of the enzyme while phosphorylation of the histone reduces this inhibitory effect. Dephosphorylation of the phosphorylated histone reinitiated inhibition of the DNA primase. Further, the change from interphase to mitosis is associated with the hyper-phosphorylation of histones (Doonan and Morris, 1989). Therefore the dominant feature of the cell cycle is that of interconversion of components between phosphorylated and dephosphorylated forms, in which the distinct forms themselves can also have protein kinase activity.

It has been proven that protein phosphorylation at the tyrosine residue is associated with the products of several retroviral transforming genes, and epidermal growth factor, platelet-derived growth factor, insulin, polypeptide mitogens and tumour promoters were also found to increase phosphorylation at the tyrosine residue; it is also known that the tyrosine

phosphorylation is associated with stages of B-cell differentiation in human lymphoid leukaemias [Kuratsune et al (1985)]. The biological significance of protein phosphorylation is discussed by 1985 [Karlund, 1985] with regard to a number of systems, one of which is the system in which $Ca^{2+}$ ions and diacylglycerol serve as a second messengers of hormonal action; these hormonal signals generate cellular responses as a result of changes in the phosphorylation state of the key proteins and second messengers are involved which are formed as a result of stimulation of the phosphatidylinositol cycle.

## PHOSPHOTYROSINE PHOSPHATASES AND REPLICATION

As indicated earlier, phosphorylation processes could not be involved in any kind of control reactions if dephosphorylation did not also occur. The first report on the existence of P-T-Pase came in 1980 when studies were being carried out on tyrosine kinase activity in A431 human epidermoid carcinoma cells (Lau et al 1989). It was observed that cell membrane proteins labelled with $^{32}P$ at tyrosine residues, slowly released $^{32}P$. This tells us that enzymic dephosphorylation may have been occuring. This possibility was confirmed during work on cells infected with Rous or Fujinami sarcoma viruses which can undergo a temperature sensitive transformation. At allowed temperatures a quick accumulation of phosphorylated tyrosine residues was seen in the transformed cells, while at non-permissive temperatures the levels of tyrosine (P) declined to basal, untransformed levels. Since protein

denaturation was not present, this shows that a protein dephosphorylation activity was indeed present and involved in cell replication. Phospho-tyrosyl protein phosphatase (P-T-Pase) activities have been found in eukaryotic cell lines and are widely distributed in various tissues, in association with cell membranes and with cytosol, supporting the theory that they may play an important role in cellular regulation processes. Phosphorylation and dephosphorylation of proteins, mediated by kinases and phosphatases, respectively, has been recognised as a widespread mechanism by which essential metabolic pathways and physiological processes are regulated (Flockhart and Corbin 1982, Li 1982, Krebs 1985). Phosphorylation at tyrosine in protein seems to be important in that specific tyrosine kinases have been found to reside in membrane receptors for various growth factors and in oncogene products, it has been suggested that the reaction may correlate with cell proliferation (Sefton et al 1986, Kolata 1983, Ramachandra and Ullrich 1987).

As mentioned before dephosphorylation may be important with respect to the reversal of the malignant transfomation (and consequential inhibition of replication) a study was made of certain dephosphorylation enzymes in relation to the induction of differentiation (Wolbrandt, Hammond and Gilbert, 1985). These aspects of cells are intimately related to replication as discussed later.

## OSCILLATORY NATURE OF THE CELL CYCLE

The classical views in the cell cycle given above

are no more than descriptive and do not explain the driving force which initiates and then suppresses the reactions outlined. Recent experimental studies have supported the earlier suggestions by Gilbert (1968, 1974a), Sel'kov (1970) and later by others (see the review by Edmunds, 1988) that an oscillatory process is involved. However, the recent studies implicate a cyclic series of reactions in which the compound(s) called cyclin is phosphorylated and dephosphorylated. It may be noted, however, that thiols and disulphides can regulate phosphofructose kinase (PFK).

Relatively recently it has been shown that mitosis can occur repeatedly in cell free extracts (Kirschner 1992, Norbury and Nurse 1992). On the basis of studies in such systems several theoretical models of the processes have been proposed in order to account for the oscillatory variations in the levels of, in particular, the cyclins and which involve phosphorylation and dephosphorylation reactions.

Gilbert has shown (1974a, 1977, 1978a, 1978b, 1978d, 1982a, 1982b) that all major facets of proliferation can be explained, even in detail, in terms of limit cycle oscillatory behaviour. Developed before the existence of cyclins was known, this concept depends on the inter-conversion of coenzyme (like) components, the thiol-disulphide system (Sel'kov, 1970) was used to illustrate the arguments but Gilbert (1974a, see also MacKinnon and Gilbert, 1993) pointed out that the phosphorylation-dephosphorylation system could behave similarly. Inter alia, the concept provides explanations as to why cells attain a maximum density in culture

without exhausting the nutrients, how this limit is influenced by transformation and hence how cells become malignant (Gilbert, 1977, MacKinnon and Gilbert, 1993). In explaining the random variations in the cell generation time mentioned above, Gilbert (1978a,1978b,1980) and MacKinnon and Gilbert (1993) have also provided a mechanism whereby replication can be regulated in continuous fashion, hence accounting for the variability in the growth rates of tumours. Gilbert and MacKinnon (1993) feel that such an oscillation drives the cyclin system, a point which will be raised again in the discussion.

## HIGH FREQUENCY OSCILLATIONS AND CELL REPLICATION

Conventional ideas on the regulation of replication rely on simple discrete changes in the levels of constituents (although it is unclear how replication is thereby regulated).

On this basis that the cell cycle is a rhythmic process, Gilbert (1980) and Gilbert and MacKinnon (1992) have shown how metabolic oscillations with periods much shorter than the cell cycle, can alter the replication frequency. All that is required is that the components involved can modify the reaction rates of the cell cycle control system. As Gilbert implicates a coenzyme control process for the latter, there seems no reason why this should not be so, a wide range of intracellular constituents will influence a coenzyme system and vice versa, which may be necessary if duplication of all components is to occur (Gilbert, 1980, 1988, MacKinnon and Gilbert 1993). In support of such a view, a number

of periodicities have been detected in cells (see later) and it has been shown that the complex mitogen actually alters the pattern of temporal organisation of cells (Gilbert, 1984a, Gilbert and MacKinnon, 1992). It seems impossible for them to exist and to be modified without replication being affected, irrespective of the nature of the latter (Gilbert, 1984a). Similar considerations could apply in respect of the cyclin system but the ways in which the control system (Tyson, 1991) can be modulated by other rhythms may be more limited if the cyclins do not have coenzyme like actions.

**DIFFERENTIATION**

Multicellular organisms, such as man, consist of multiple kinds of cell having distinct properties, characteristics and functions, yet they all arise from a single cell, the fertilised ovum or zygote. The process whereby these various cells are formed is termed differentiation, it is poorly understood other than that it is due to changes in cellular composition. Presumably a number of factors contribute to the forces causing earlier cells to alter their properties through changes in the levels and activities of constituents and numbers of organelles (e.g.,mitochondria). It is widely believed that differentiation involves gene switching but Gilbert (1973b) has suggested that it also depends on metabolic switching. In the associated phenomenon of development, spatial organisation of the cells takes place and depends on the migration of groups of cells from one site in the embryo to another, the overall processes are organised in time and take place despite the apparent existence in individual cells of control

processes considered to be responsible for maintaining consistency of their properties. Gilbert (1968, 1973a, 1974b, 1984a) has also suggested that the phenomenon of differentiation not only involves changes in the organisation of cellular processes in time but actually depends on them in that particular disturbances of the dynamic state are responsible for differentiation along different paths. It is argued that to maintain a particular composition all the reactions must be co-ordinated in time, if such co-ordination is altered then the compositions will change and the process can be irreversible if those changes further modify the dynamic state. Among other features, such a view solves the apparent conflict about cellular regulatory systems by incorporating their ability to behave in an autodynamic manner. These aspects will be considered again when discussing the nature of the autodynamic state.

Differentiation is a prolonged process (according to the complexity of the organism) and may take years to complete, Gilbert (1968,1984a) suggests that ageing is merely the final stages of the approach to a new steady state. The phenomenon is essentially a continuing process but in some instances it appears to take place in distinct stages, cells waiting in an intermediate, partially differentiated state until receiving a signal to proceed to the next, and often terminal form. However, in so far as the population of cells is concerned, this is probably an incorrect view (Gilbert, personal communication) for a small percentage of the cells will be differentiating at all times. There is evidence that this is because of a random triggering reaction.

The process is thus a matter of the relative "rates" at which cells are formed and pass on to the fully differentiated state, with the signals influencing the proportion of cells affected, as and when the organism requires it to be so. The phenomena seems to be similar to replication in culture where individual cells are not cycling non-stop but are also randomly triggered into the proliferative state, as described earlier, the mechanism may be similar. The phenomenon is thus always essentially continuous but can be conceptually considered to be discontinuous.

Some undifferentiated stem cells are pluripotent, that is, they are capable of differentiating along distinct lines (thereby producing different cells) according to the nature or relative magnitudes of different signals. These signals are generally produced by the more or terminaly differentiated cells, in that way the organisms can maintain an effectively constant level of the latter cells. In particular, these considerations apply to the formation of various blood cells, such as erythrocytes.

In all cases, differentiation also depends markedly on changes in the numbers of particular cells, in most circumstances many more differentiated cells are needed than there are stem cells and some of the latter must always remain for future requirements. Cell replication is thus an essential part of the phenomenon. With the blood tissue, an increase in the rate of terminaly differentiation is therefore preceded or accompanied by an increased proliferation of the stem cells although, in the case of the erythrocyte system

especially, the induction of further differentiation then leads to a loss of the proliferative ability.

## CANCER

Although cancer is frequently discussed as if it is one disease, it is more correctly considered as a multitude of diseases with certain common properties. One major reason for this view is that it can be caused by diverse agents, while another is that there is almost inevitably a high degree of heterogeneity among the cells within any tumour while distinct tumours can be produced by certain reagents according to the dosage and temporal pattern of treatment with those agents (Gilbert, personal communication). Moreover, no common universal lesion has been observed despite much research. Although many agents have long been identified as being carcinogenic, their modes of action remain obscure, one needs to know how the effects they produce are expressed as cancer (Gilbert, 1978a, 1981).

The dominant view in the study of carcinogenesis has always been, and still is, genetic. This is presumably because the malignant characteristics can be passed from each cancer cell to its progeny and many researchers can only view heredity in genetic terms. Mutation is the main example whereby a defect can be inherited but there is no one site of action of all mutagenic agents and no single mutation responsible for malignant properties, the process is all-or-none in character and cannot in itself account for heterogeneity in a cancer cell population (Gilbert, 1984b).

Similar arguments hold with respect to other genetic defects such as gene switching, that is unless the process is switched on and off repeatedly. In which case the characteristics can depend on the frequency and phasings of the switching process (Gilbert, 1966, 1968, 1973b, 1984a). But switching can also occur at the metabolic level (Gilbert 1973b, 1984a) and different cellular characteristics can be obtained according to which switches have been affected. If switching (genetic or metabolic) is a repetitive process then one can expect cellular characteristics to depend on the frequencies and co-ordination of all such processes, i.e. by the patterns of temporal organisation (Gilbert, 1968, 1984a). At the metabolic level, the magnitude of any switching process can also be affected by changing the kinetics involved thereby giving a greater range of properties for a given set of active genes (Gilbert, 1968, 1984a). In these latter situations heterogeneity can arise through the switching of different sets of processes, changes in the frequency, amplitude of timings of switch actions and through the influence of random fluctuations (Gilbert, 1966, 1968, 1973b, 1985). It may also be noted that random metabolic fluctuations can also account for the continuous nature of differentiation.

Cancer is characterised by two particular aspects of cell behaviour, neither of which is, in itself, abnormal, these are the abilities of the cells concerned to proliferate, and, in many cases, to infiltrate tissues as well. Obviously, many normal cells are able to replicate and embryonic cells, in particular, are capable of doing so faster than most, if not all,

cancer cells (Gilbert, 1984b). Then again, white blood cells must invade tissues in order to combat infections. The first factor is the most important as infiltrating cells are unlikely to be a problem if they cannot also proliferate. Gilbert (Gilbert, 1984) has stressed that the defect is more correctly stated by saying that cancer cells replicate when they should not do so and has proposed a quantitative rather than qualitative abnormality. He suggested (Gilbert, 1974, 1977, 1978a, 1978b, MacKinnon and Gilbert, 1993) that cell replication has an oscillatory basis (now confirmed, see later) and that cell cycle heterogeneity is due to random fluctuations of metabolic rates. On that basis he showed that cells need not replicate continuously but may do so just once at random intervals. He also proposed (Gilbert, 1977, 1978a, 1980; MacKinnon and Gilbert 1993) that cancer involves any change in metabolism which alters the rates of the cell cycle control reactions so as to shift the state nearer to, or within, the boundary supporting rhythmic behaviour, will alter the frequency at which the cell divides under a particular set of conditions. The corresponding normal cell will either not divide or will do so less frequently whereas, under other conditions (as when in culture, or, in response to injury), both can replicate continuously at about the same frequency or rate. According to this concept, cell replication is a property of the whole cell and not merely of a particular set of reactions.

To some extent at least, metabolic changes affecting the replicative state must also alter cellular

function and behaviour (Gilbert, 1984a, 1988; MacKinnon and Gilbert 1993) although some people seem to consider cell proliferation reactions as being completely independent of all else. In some cases, among the other characteristics which could be modified cell motility and hence the metastasising ability. Under some conditions normal cells could thus be stimulated to replicate even more frequently than the malignant cells without influencing their normal behaviour. Yet, after the stimulus is removed, the normal cells would become totally quiescent whereas the cancer cell could continue to proliferate.

**MEL CELLS**

As indicated above, sometimes the process of differentiation can be blocked effectively thereby producing cells with characteristics intermediate between those of the stem cells and the fully differentiated progeny. This is the case with the murine erythroleukaemic (MEL) and the human HL60 cells studied here. The former are produced by the action of the Friend virus. Both retain the ability to proliferate, both in vivo and in culture. They spontaneously differentiate further only at a very low rate (frequency - see above comments). In vivo the result is that they are leukaemic cells, the stem cells are being continually stimulated to divide and differentiate but are successful only in the first of these. The failure to form the normal fully differentiated cell increases the stimulus to form more and more cells, the host dies through, inter alia, a lack of the erythrocytes (in the case of MEL cells) and a surfeit of the partially

differentiated (leukaemic) cells (which continues to replicate).

Having a blood cell origin these cells grow easily as stationary suspension cultures and can be adapted to stirred suspension conditions although this was not done in the present studies. Of great importance is the fact that MEL cells can be induced to undergo further differentiation by a wide variety of agents. This system thus serves as a model for the reversal of the malignant transformation. Further differentiation is not completely normal and can take somewhat different paths with different agents but the induction process usually results in (a) the formation of haemoglobin (which has been inhibited by the Friend virus), and (b) loss of proliferative ability.

Both these process could be due to changes in the phosphorylation status of cells hence the reason for some of the studies reported here. In view of the theoretical concepts of differentiation and cancer discussed in this section, and the fact that previous studies had revealed oscilatory changes in P-T-Pase activity in MEL cells, attention has been focused on the fluctuations in the activity of P-T-Pase and on protein phosphorylation patterns and their response to insulin an inducer of differentiation. It was thought that these two agents might act in opposition since one is known to stimulate phosphorylation processes while the other produces effects which can be attributed to a depressive action on phosphorylation. Because of the complexities of the response to the individual agents

alone attention was eventually limited to the separate actions.

## MEL CELL DIFFERENTIATION

The regulation of differentiation remains one of the central unresolved issues in modern biology. In vitro inducers of MEL cell differentiation are chemicals of widely different structures and properties (in that way they compare with mitogenic and oncogenic agents) and they include planar-polar compounds of which HMBA is the prime example. These compounds are particularly active inducers, HMBA has low molecular weight. The process whereby cells enter the terminal stage of differentiation is known as commitment. Cell division occurs in the absence of inducer but after a period of exposure to an inducer (the lag period) the cells become irreversibly committed. The time of onset and the rate of commitment vary both with the type and concentration of inducer. There is evidence that the inducer mediated commitment of MEL cells is a multistep process. Differentiation in the MEL cell line is characterized by a change in morphology which is accompanied by an increase in iron uptake and heme synthesis, accumulation of haemoglobin, appearance of erythrocyte-specific membrane antigens, a decrease in size and a limitation in proliferative ability (Reaben, Rifkind and Marks 1980). Induced differentiation has many similarities to erythropoeitin-regulated differentiation, including chromatin condensation and other morphological changes, terminal cell division and accumulation of globin mRNA. Induced MEL cell differentiation is similar in many ways to normal erythropoeisis but MEL cells are transformed

and there are a number of aspects of MEL cell differentiation and proliferation that are not normal. MEL cells have the capacity to proliferate with erythropoeitin. They rarely proceed in vitro to the non-nucleated stage of differentiation characteristics of normal erythropoeisis and they may exhibit patterns of erythrocyte expression that are different from those in normal erythropoeisis [Marks, and Rifkind, 1978].
One of the most significant alterations in induced MEL cell is a decrease in oncogenicity of the leukaemic cells [Friend, Scher, Holland and Sato (1971)].

**HL-60 CELLS**

There is thus a belief that some malignancies are due to a block in differentiation which if set free would result in a more differentiated and therefore benign or nearly normal condition. HL-60 has been a useful model human system in the search for substances that are active as inducers of differentiation.

The human HL-60 cell line derived from a patient with acute promyelocytic leukaemia serves as a useful model of differentiation of human leukaemia cells (Collins, Gallo, and Gallagher 1977). Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture (Collins, Gallo, Gallagher 1977). The majority of HL-60 cells are promyelocytic, but 5-10% of them show morphological characteristics of more mature myeloid cells (Collins, Ting and Gallo 1980). Induction of morphological and functional differentiation of human promyelocytic leukaemia cells (HL-60) by compounds which induce differentiation of MEL cells

(Gallager 1979). Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukaemia (Gallager 1979) were studied.

However, in the present experiments HL-60 cells were used because they are similar to MEL cells and because it was believed they contained more than one isozyme of LDH. The aim was to compare the isozymes in the HL-60 cells in order to find out

(a) if the pattern of behaviour was like that in MEL cells,

and if so,

(b) whether the variations for the individual isozyme are identical.

**INDUCED DIFFERENTIATION**

There are a number of examples of tumour cells which appear to arise through a blockage in the expression of their differentiation "program" but which can be induced to differentiate further in response to a variety of agents [Marks, Sheffery, Rifkind (1987); Friend, Scher, Holland, and Sato, (1971); Tanaka, Levy, Terada, Breslow, Rifkind and Marks (1975); Reuben, Wife, Breslow, Rifkind and Marks (1976); Lotem and Sachs (1975) Sach (1978); Metcalf (1985); Hubermann and Callaham (1979); Lotem and Sachs (1979) Reuben, Wife, Breslow, Rifkind and Marks (1976)].

**HMBA**

The initiation of MEL cells to undergo erythroid differentiation (with suppression of oncogenicity) by the use of polar/apolar compounds has proved a useful model for the study inducer-mediated differentiation of transformed cells [Marks, Sheffery, Rifkind (1987); Friend, Scher, Holland and Sato, (1971); Tanaka, Levy, Terada, Breslow, Rifkind and Marks (1975); Reuben, Wife, Breslow, Rifkind and Marks (1976)]. Of these agents HMBA is the most frequently used, and until recently, was the most potent known inducer of MEL cell differentiation and was thus selected for the present experiments (particularly as it had been used for other studies in these laboratories (Hammond et al 1989). Despite much research its mechanism of action is still not known but it seems that HMBA-induced MEL cell terminal erythroid differentiation is a multistep process even though only one stimulus is required.

As indicated, the action of HMBA on MEL cells reduces their proliferative ability, an *in vitro* effects which mimics terminal differentiation *in vivo* - erythrocytes do not replicate. Insulin on the other hand is mitogenic and might thus antagonise the action of HMBA on metabolism and replication. The affects on the phosphorylation potential of these two agents, individually, and in combination , was initially undertaken but, as the present results show, each agent alone produces complex effects on cellular dynamics. Interpretation of the combined action thus proved even more difficult. Therefore, this latter line of study was

not pursued after the initial experiments though, as some results consistent with this view were obtained, it might prove worthwhile examining the biological effects of the combination.

## THE DYNAMIC CELL

A characteristic of all living entities is that they are autodynamic. As is evident from any time lapse observations, living cells are no exception, fluctuations occur in various features. The surface moves rapidly and there are variations in the gross shape particularly when cells are dividing. Internal organelles, such as mitochondria move and nuclei can rotate (Paddock and Albrecht-Buehler, 1988). Many cells can migrate, a dynamic process in its own right but one which also involves more clearly defined changes in shape (this aspect is of great importance to the metastatic features of cancer -see later). All the major outstanding biological phenomena are dynamic processes. These observations imply that the underlying metabolic reactions are also highly dynamic. But since a dynamic state is required for entities to be alive, it seems highly likely that there are less obvious features of cell behaviour which are also dynamic (Gilbert, 1968, 1984a). It is clearly necessary to understand how the dynamic states arise, how they are regulated and how the cellular properties and characteristics depend on the dynamics.

Dynamic behaviour can best be explained by the existence of oscillatory variations in the levels of cellular constituents (Gilbert, 1968, 1974b, 1980), as

they represent the only kind of characteristics which can be self generated and can persist indefinitely under suitable conditions. Such behaviour is also a reflection of the fact that living cells are far from equilibrium (and must be so) as there is a constant flux of components into and out of each one. A system in equilibium must be unchanging.

It is known that a number, possibly many, metabolic and genetic control systems exist in cells, negative feedback regulatory reactions were initially considered necessary for the maintenance of cell properties through their potential ability to maintain a constant level of constituents. Such a view seems paradoxical in view of the highly dynamic nature of living cells, but Gilbert (1968) pointed out that they can also give rise to rhythmic behaviour and suggested that under most circumstances they can operate in their oscillatory mode. In that way they can keep cell characteristics not absolutely constant but yet keep them within certain limits. He therefore also suggested that permanent changes (as occur during differentiation and cancer, for example) involve changes in the dynamic state, that is, in the pattern of oscillatory behaviour. Temporary alterations in cell properties, behaviour and function can be perceived as being dependent on similar affects on control system dynamics but without moving outside the stability boundaries so that the original dynamic state is regained (Gilbert, 1968, 1974b, 1984a). If more than one control system oscillates then the overall pattern of temporal organisation must play a part in determining cellular function and behaviour as well as differentiation and cancer (Gilbert, 1968, 1984a,

Gilbert and MacKinnon 1993).

As indicated (Edmunds 1988), oscillatory variations occur in the activity and apparent isozyme patterns of a number of glycolytic enzymes and distinct phasing relationships have been reported in untransformed and polyoma transformed BHK cells (Gilbert 1968, 1974b, 1984a, Gilbert and Tsilimigras 1981, Gilbert and MacKinnon 1992). Tsilimigras and Gilbert 1977, Gilbert and MacKinnon 1981 have shown that the extractable protein oscillation cannot entirely account for enzyme periodicities though it probably contributes toward them (see later). The present experiments were carried out with these various aspects in mind and the results add further credit to the concepts. The means whereby oscillations can arise is discussed shortly.

## BEHAVIOUR OF BIOCHEMICAL SYSTEMS

Biochemical systems have properties not exhibited by isolated enzymes in the test tube and not covered by most biochemical text books or in university lectures. The different characteristics arise because of the interdependence and interaction with other reactions. In the cell, one of the most important aspects is that the reactions are an open system, at least, under normal conditions. This means that there is a flux of material through the cell it takes up nutrients from its environment and excretes products into the surroundings. This flux ensures that the cell remains far from equilibrium, when it stops the cell dies. This state permits somewhat distinct thermodynamic properties. Whereas non-living systems tend to disorder, living

entities are associated with the creation of order in space and in time. However, this can only be achieved at the expense of energy and for that reason they are often referred to as dissipative systems as energy is dissipated as the cell creates organelles, other cells etc. Another aspect is that living systems also exhibit structure in time, for example, the levels of consituents are not constant but vary with time and not in a random manner, as will be discussed later.

Other aspects of biochemical systems relate to response of the reactions to a signal, where the latter may mean any change in the level of some other cellular constituents, to variations in the concentration of an external agents, such as a hormone, or to an alteration in the conditions, for example the temperature, thus the terms signal, disturbance and perturbation can be used interchangably although the first will generally refer to a specifc disturbance. These aspects are not generally described and are those which Gilbert (1973a, 1973b) has termed the amplitude, frequency and phase discriminatory properties. Most people understand some features of the first of these which relates to the dependencies of the response on the magnitude of a signal. Thus it is understood that the response of a cell can be different if the amount of agent is increaed but it is not necessarily understood in what way the response may be distinct. One characteristic of importance is that different systems will generally exhibit different response times, some can react quickly to, say a change in the environment while others will do so very slowly (due to the distinct kinetics of the reactions concerned). This could mean that a metabolic

change could be produced without, for example, the genetic systems being affected. However, such "time-separation" as it has been termed, is not absolute. If the disturbance is large enough in magnitude significant effects can also occur in the slowly responding system. But, among other things, the response also depends on the duration of the disturbance (if it persists long enough then eventually both systems will be affected), if the perturbation is repeated and if so at what frequency. If several changes occur, say in the environment, the overall can depend on the relative timings of the changes. If biochemical systems can exhibit such characteristics then it seems certain that these various factors will be important in one set of circumstances or another. Attention is usually focussed only on the nature of reactions produced not the kinetics involved although the latter can determine if those reactions occur and when.

**HYSTERESIS**

Although much attention has been paid to sigmoid co-operative kinetics with regard to enzyme kinetics, it is not widely recognised that multienzyme systems, in particular, can exhibit S-shape rate curves as shown in Fig. 1. Such dependencies are important in that they permit the relevant system to exhibit multiple steady states and which state exists depends on the history of the system. Suitable transient disturbances can cause switching between the states. These effects are explained in the caption to Fig.1 (Gilbert 1974b, Katchalsky and Spangler 1968, Sel'kov 1966a).

**Fig.1 HYSTERESIS IN BIOCHEMICAL SYSTEMS**

It is well known that regulation of enzymes can give rise to sigmoid kinetics rather then those obtained by simple Michaelis-Mintell equations. It is less well known that more complex rate equations can give rise to even more highly non-linear rate relationships, even S-shape shown here. In this diagram we illustrate the existence of hysteresic behaviour and indicate how such a system can generate oscillations. For substrate (S) concentrations with values between those corresponding to points $S_d$ and $S_b$, three rate values are possible, i.e. three alternate steady states exist and the system exhibits switch characteristics. Thus if S increases from very low values, the rate increases according to section d-a on the curve. As S increases further the rate will jump from "a" to "b". A reduction in the level of S thereafter, back to original values will cause the rate to vary according to the path b-c-d. The system thus exhibits hysteresis. In fact that the foward path is different from the back path. If the rate of the previous reaction is V as shown, the steady state will be given by the point O, but this is unstable. Thus a random disturbance increases S, so that it approaches $S_b$, the rate J becomes greater then V therefore S increases further still and will continue to do so until point "a" is reached, when the state switches to point "b", now J is greater than V therefore S will increase again up to the point given by $S_b$ when the switch a-b will take place again and the process carries on repeating itself. The systems thus undergoes automatic repetitive switching i.e. it oscillates in the level of S and in the rate J as shown on the right.

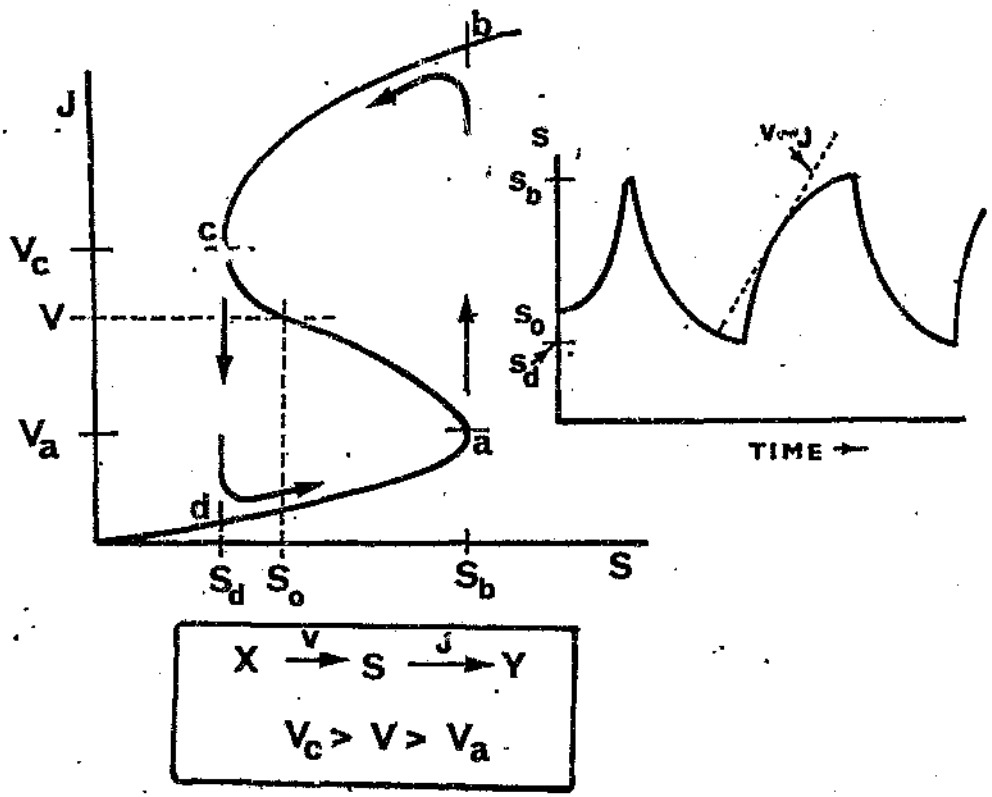



Fig. 1

In general, switch systems can be tripped in more than one way (e.g. by a change in the level of a substrate, or, in the kinetics of the relevant reactions). Multiple signals acting at different points can be synergistic or antagonistic with regard to triggering depending on their nature, sense, amplitude, frequency and duration. In addition, their relative timing(s) can also govern if a change in state occurs. Gilbert (1973b) has considered these aspects and the possible involvement of hysteretic metabolic switching (in a system exhibiting substrate inhibition kinetics) in relation to certain aspects of differentiation and cancer.

## CONTROL SYSTEMS

In the past there was much discussion on the role of negative feedback control systems to maintain constancy; superficially this is inconsistent with the view that life reflects dynamic behaviour. Moreover, such controls would have to be over-ruled during differentiation and cell replication. Alternatively they could play a more intimate part in determining the existence of life and all of its facets. The latter is a more appealing idea and is possible if the pertinent control systems act in the periodic rather than static mode of operation (Gilbert, 1968, 1984a; compare Savageau 1976; Tyson,1991). Control is then still maintained but more relaxed so that there is more scope for changes to take place. In fact they would probably contribute toward them, especially if the oscillating components have widespread action throughout the cell (e.g. coenzymes),

such agents can thus start, terminate and co-ordinate various metabolic processes (the rhythm can acting as a timing clue). On the other hand, theoretical studies (Gilbert, 1973b) indicate that metabolic and genetic biochemical switch processes (which exhibit thresholds) can be triggered by simple super-critical changes in the level of some component although there is also an inverse dependency on the duration of the change. Normally it is assumed that this is the only factor concerned but as switch systems also exhibit amplitude, frequency and phase discriminatory properties (Gilbert, 1973b) more subtle characteristics may be even more important.

When discussing the subject of switching it is necessary to consider how such systems can achieve immunity from noise while maintaining adequate sensitivity. If the amplitude threshold is high and the response times are long then noise is far less likely to cause random triggering but a large signal will be required. On the other hand, the system could be far more sensitive to deliberate triggering by a change in frequency of a signal or in the timing between two signals. Bearing in mind the fact that disturbances rarely if ever affect only one system in the cell, these other dynamic aspects could be most important with regard to tripping a switch. Specificity of action of a perturbation (signal) can thus depend on the relative response times of systems even though they are each capable of reacting to a signal, only a system with a fast response time can react to a rapid change of short duration. It seems more realistic that multiple signals exist, particularly in vivo.

Bearing these various aspects in mind, Gilbert (1968, 1984a) suggested that:

(a) fertilisation is a process initiating rhythmic behaviour,
(b) that, under defined conditions, each cell species exhibits a characteristic pattern of periodocities, reflecting its unique pattern of metabolism,
(c) that changes in the pattern of temporal organisation underlie both differentiation and cancer,
(d) that ageing (at both cellular and physiological levels) is the result of parametric dampening of rhythmic behaviour (as a result of changes in the rates of relevant reactions)

and

(e) that some hormones act by altering the dynamic state of the cell.

That oscillations exist in cells is not now in doubt and it has been shown (i) that various agents, including viruses, can produce rapid changes in the pattern of temporal organisation, (ii) that different patterns exist in distinct cells, (iii) the patterns can be produced by changes in conditions or the action of agents, and (iv) that the phasing between two effective isozyme pattern rhythms in virally transformed cells differs from that seen in untransformed cells. The results presented here add further support for these concepts.

For protein phosphorylation (or, for that matter, any similar process) to be of importance as a regulatory mechanism, the reverse step must take place;

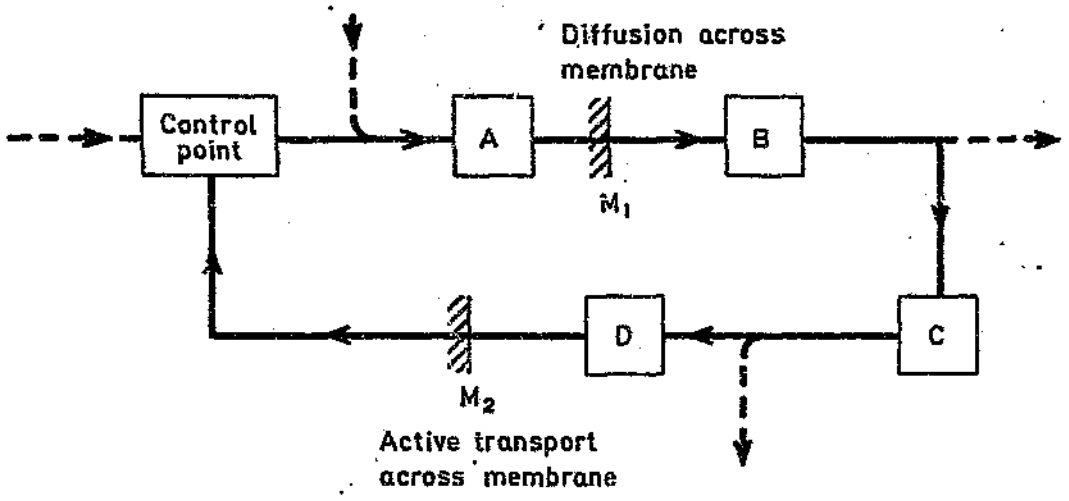
In such a simple system no control exists, and the behaviour will be determined solely by the rates of the two reactions irrespective of whether one considers specific reactions or the overall phosphorylation status of the cell. Unless some time delays exist there will be a simple equilibrium will be set up between the two.

In general, true control can only occur where a cyclic system exists so that the regulatory process can sense the magnitude of the effect it produces. In biochemical terms this implies that an enzyme reaction governing the formation of a component is regulated by that component (see Fig. 2). Such reactions comprise a feedback loop exists, if the effect is one of inhibition then it is termed negative feedback whereas if the action is stimulatory it is called a positive feedback loop component. Negative feedback processes thus tend to maintain constancy in the amount of the compound concerned and were invoked as a means whereby the stability of a differentiated state might be maintained. However, as Gilbert (1968) pointed out, such behaviour would inhibit any change in cell properties or function and therefore differentiation. Moreover, cells would have difficulty in being dynamic. The paradox can be

**Fig.2 A FEEDBACK CONTROL LOOP**

This is a diagrammatic representation of a feedback loop showing how a component can control its own level by regulating an enzyme involved in its synthesis. Various processes, such as membrane transport, can cause time delays as can a large number of intermediate reactions. These can destabilize the control reactions and cause oscillatory behaviour especially if the controlled enzyme is very sensitive to the level of the component as when cooperative kinetics are involved. The enzyme may be activated or inhibited by the component, these are respectively known as positive and negative feedback.

# BIOCHEMICAL FEEDBACK CONTROL SYSTEM




A, B, C, D, $M_1$, $M_2$ represent various steps in Feedback Pathway.

e.g. Control point = gene, A, B = RNA, C = Enzyme, D = substrate or product.

Dotted arrows represent interactions with other systems.

Fig. 2

resolved if the control systems operate in their autodynamic, i.e., oscillatory mode (Gilbert, 1968).

The simple negative feedback control given earlier can generate oscillatory variations in the levels of related constituents if there is a time delay in the response of the controlled enzyme to the signal (as may occur if the number of intermediate reactions is relatively large, and/or if the enzyme is very sensitive to the regulator. Ultimately the behaviour is dependent on the overall rates and kinetics of the set of reactions and the rate at which the initial substrate is supplied by previous reactions. If the enzyme (or set of enzymes) being controlled exhibits highly non-linear, S-shaped kinetics the system can exhibit hysteresis, with alternate states being possible for certain sets of conditions. Switching thus occurs in metabolic processes.

**AUTODYNAMIC BEHAVIOUR OF CONTROL SYSTEMS**

When considering the generation or existence of any cellular rhythms, several factors should be taken into account and these include:

(a) the existence of co-operative kinetics leading to amplification, that is, proportionally greater changes in rate with a given change in regulator concentration;
(b) time delays due to the involvement of a number of sequential reactions or diffusion processes;
(c) the existence of hysteretic kinetics (see earlier section) which, for suitable rates of the previous

enzyme, can undergo automatic repeated switching between such states, the system oscillates.

(d) the coupling of metabolic processes through common substrates, coenzymes and even temperature effects (Gilbert, personal communication);
(e) resonance, the ability of systems to amplify minor fluctuations and even select a rhythm of particular frequency out of a mixture;

and, where several distinct periodicities exist,

(f) interaction between different rhythms in a given cell [see (d)], between the same and different oscillations in adjoining cells;
(g) entrainment, the ability of one rhythm to change the frequency of another until the two become the same,
(h) frequency and phase locking wherein particular relationships between oscillations are enforced by the interactions [see (g)]
(i) the existence of frequency doubling and double periodicities, wherein a given system exhibits alternate stable periods and complex waveforms (this may lead to chaos, i.e, totally irregular oscillations);
(j) frequency demultiplication, the ability of a system to generate an oscillation with a period which is a multiple of its natural period.

The above have all been used either to explain the origin of particular oscillations, some facet of their behaviour, or some significant aspect of cell biology. Automatic repetitive switching between alternative steady states (resulting from hysteresis in the kinetic

characteristics) is one fundamental generating mechanism, it is described in Fig. 1 and mentioned later in relation to glycolytic oscillations.

Time delays (as a result of the involvement of several intermediate reaction steps or diffusion) and high amplification in a negative feedback control system can make the system unstable so that it too oscillates. This is true of mechanical, electronic and other systems not merely biochemical processes. This raises an important aspect of the subject. It is not generally appreciated that oscillations in totally different systems and processes, biological and non-biological, can exibit similar characteristics, such as those outlined above, this is because they can all be described by similar sets of differential equations (Gilbert, personal communication).

Hysteretic characteristics can exist for other types of regulatory systems, for example, reactions involved in the interconversion of two forms of a cofactor, such as thiols-disulphides, (Sel'kov 1970; Gilbert, 1974a) or NAD and NADH, phosphorylation and dephosphorylation (Gilbert, 1974a). Such kinetics can be attributed to the interdependence of the rates of conversion on the levels of the components themselves, that is, the existence of more complex control loops. This latter type of control reaction have been invoked by Sel'kov (1970) and by Gilbert (1974a, 1977, 1978a, 1978b, 1978d, 1980, 1982a, 1982b) to account for the cell cycle and various aspects of cell replication. The system (see Fig. 1) gives rise to a particular kind of oscillations termed a limit cycle, the characteristics of which are stable providing

the rates of the reactions remain fixed.

## HIGH FREQUENCY OSCILLATIONS

Rapp (1979) and Berridge and Rapp (1979) have given a comprehensive list of high frequency (periods from about 1 min upward) cellular oscillations (including calcium ion rhythms) and discussed some of them. A more recent review is that by Edmunds (1988) and Berridge and Irvine (1984) has considered the phosphoinositol.

The oscillations concerned here have apparent periods of the order of minutes but it is important to point out that recent fast reaction studies have shown the existence of sub-second oscillations in the levels of phosphorylated inositols period (Raha et al 1993) . It does not seem impossible for other rhythms to have similar high frequencies.

## DISTURBANCE OF DYNAMIC SYSTEMS

The regulation of metabolism in response to an external stimuli is usually considered to occur through simple changes in the levels of constituents. Living cells are not static; function and behaviour of cells are determined by the levels and activities of constituents, cellular behaviour must show the way these factors actually vary with time, the metabolic state is expected to be affected by agents, this includes initiation/ suppression of particular rhythms or changes in mean levels, frequencies, amplitudes and phasings. These can occur through permanent or temporary changes in the rates of the control reactions or transient changes in the levels of the components involved, either

through the direct addition of the components forming the oscillating components of the control process, or, through other reactions which can achieve that effect.

The latter type of action is especially important in that it can cause rephasing of an oscillation. This rases another important issue, namely that the effect produced by an agent on a dynamic system depends critically on the state of the system at the time it acts (Winfree 1980). This kind of response can lead to the synchronisation of a population of oscillators or their entrainment (see below). The effect is illustrated in Fig.3 - taken from Gilbert (1980).

In several cell species, oscillations occur in the activities and effective isozyme patterns of a number of enzymes associated with glycolysis (Gilbert,1968, 1974b, Tsilimigras and Gilbert 1977, Gilbert and Tsilimigras 1981, Gilbert and Mackinnon 1992); in the protein content of cells (Brodsky 1992, Brodsky et al 1992) and in the amount of protein extractable from them (Tsilimigras and Gilbert 1977, Gilbert and Tsilimigras 1981). Enzyme rhythms were found to be influenced by the addition of serum and other agents. The pattern of temporal organisation can differ in different cells, in that the phasing between isozyme rhythms in untransformed BHK fibroblasts was distinct from that in polyoma virus transformed cells [Gilbert 1968, 1973, 1984a, Gilbert and MacKinnon 1992]. ~~One~~ They also found rhythmic variations in (a) the activities of phosphoamino acid phosphatase [Hammond et al 1989], and (b) in cell morphology and redox state [Visser et al 1990]. Insulin enhanced the morphological periodicities

**Fig.3 THE IMPORTANCE OF THE TIMING OF ACTION OF A DISTURBANCE ON AN OSCILLATING SYSTEM**

A particular disturbance can produce a different response according to the time of its action in relation to the synchronised population of oscillators which are initially not synchronised and that effect is illustrated here. Shown are a number of identical oscillations which are out of phase with one another and the effect of a particular disturbance at time T. At the top is vn the peak times of 40 such oscillations before and after the disturbance and it can be seen that they are highly synchronised afterwards (Gilbert 1980).

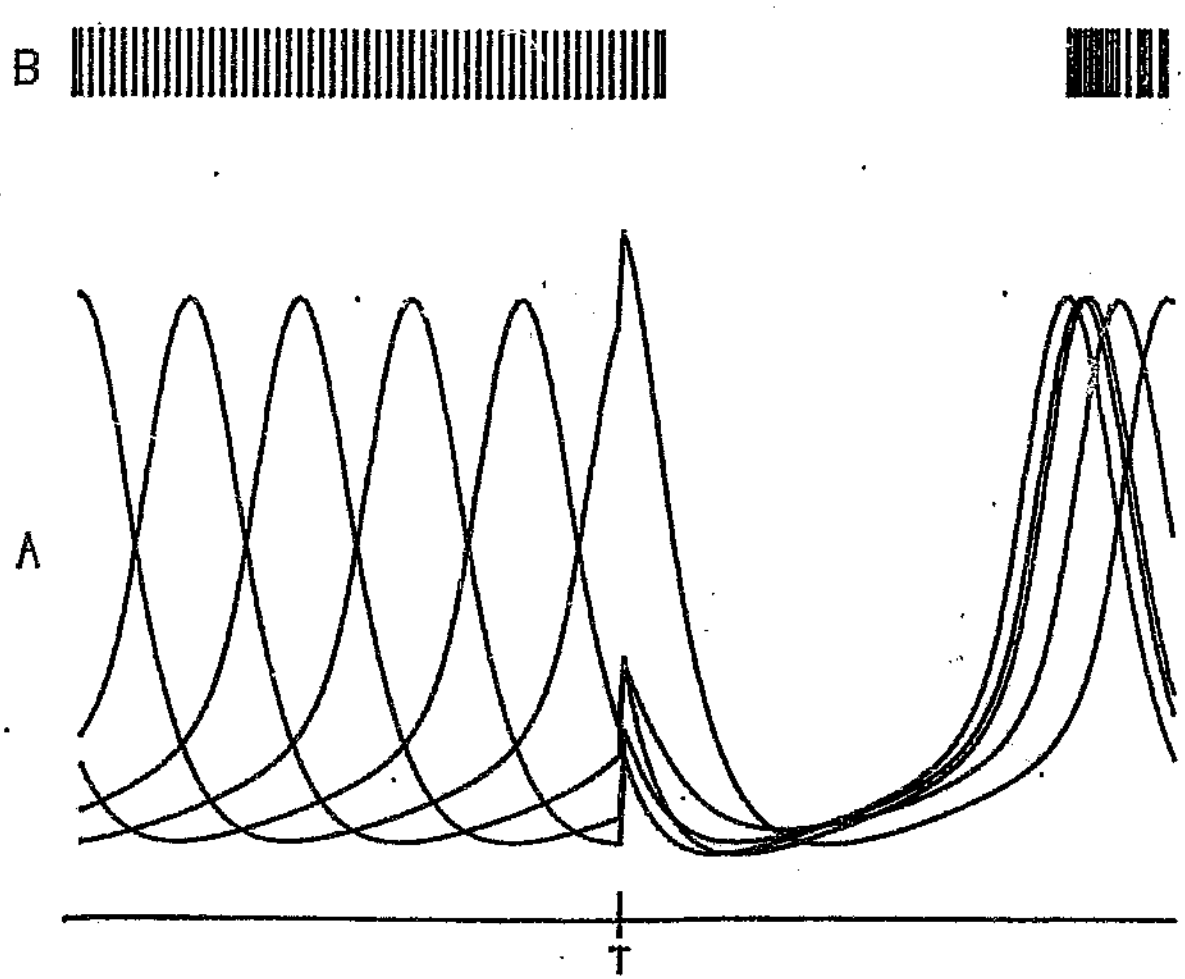

B
A
T
TIME
ALPHA= 6
BETA = 1.8
GAMMA= 3
DELTA= 1.5
Vo = .058
DISPLACEMENTS
SH -0.2
SS 0.5

Fig. 3

and it was speculated that the effect may explain the non-specific action of the hormone on metabolism (Gilbert and Visser 1993].

**SYNCHRONISATION AND ENTRAINMENT**

Cellular processes are highly integrated through the existence of common metabolites and co-enzymes (Gilbert, 1968 1984a). It is such interactions which allow the various reactions to become co-ordinated thereby permitting the cell to function more efficiently. Without them cells may not be able to survive, some processes could proceed faster than others and thereby deprive others of the components they require in order to preserve the viability of the cell. For example, if cellular membrane components were not produced at a rate similar in that of other constituents the cell might burst.

The interaction between reactions thus forms control reactions which continually adjust in order to maintain the co-ordination, and it is clear that the co-ordination must exist on a time basis. The regulatory reactions cannot impose rigid control (total homeostasis) otherwise nothing could change with time (Gilbert, 1968) and these points alone suggest that the different control systems act in their oscillatory mode (Gilbert, 1968). It seems reasonable therefore, to expect the dynamics to be affected not only by agents of external origin but also through the action of the common constituents thereby bringing about the co-ordination in time.

It is a well known fact (Pavlidis 1967) that interacting oscillators can synchronise with each other and this can occur whether they are both (all) internal or not. Rhythms of different periods can assume the same frequency, although whether or not this occurs depends on their initial periods and the number, nature and magnitudes of their interactions. Normally their frequencies need to be similar but cellular systems have the ability to adapt which mechanical systems (for example) normally can not. In principle at least, this means that even though two rhythms may initially have distinct frequencies, the various interactions can lead to one or both changing their kinetics so that they become similar enough for them to eventually take up the same time course. Where this adaption is not possible, two interacting periodicities can still synchronise, particularly if the signals are repeated periodically, as they can generally, do if they both exist within the same cell. The oscillations can "lock" into particular phase relationships.

An external signal may be one imposed by the observer in which case all cells in the population will normally be similarly (but not necessarily absolutely simultaneously or identically) affected. On the other hand each cell may be signalling to each and every other cell in a continuos manner as occurs in aggregating amoeba of Dictyostelium). The process whereby one rhythmic process becomes synchronised by another is termed entrainment. In this case the driving oscillation (zeitgeber) is unaffected by the other.

The time taken for intracellular synchronisation to

occur depends on a number of factors, as indicated above but clearly it may take some considerable time. And, presumably the time taken will be far longer if more than two rhythms are trying to come into a similar state. Gilbert (1974b, 1984a) has pointed out that when this steady state has not been achieved the temporal variations in the levels and activities of components will be changing with time in a non-periodic manner and that therefore the biological characteristics of the cell concerned will also change with time. This aspect will be considered further when the topics of differentiation and cancer are discussed but it may be noted that if such a common state cannot be achieved then it is unlikely that the cellular processes will become co-ordinated to the extent required for survival and the cells can be expected to die (Gilbert 1984a).

With regard to the last aspect, it may be noted that Astumian et al (1987) and Chen (1987) have provided theoretical evidence that control systems may be more efficient when operating in their oscillatory mode. One can speculate that the cell as a whole is most efficient when it is in a steady state and one set of phase relationships may be more efficient than another (Gilbert, personal communication).

## HIGH FREQUENCY OSCILLATIONS and CELL REPLICATION

On this basis that the cell cycle is a rhythmic process, Gilbert (1980, 1982a) has also shown that higher frequency metabolic oscillations can alter the replication frequency providing that the components involved can modulate the rates of the cell cycle system

reactions. As he implicates a coenzyme control process for the latter, there seems no reason why this should not be so as a wide range of intracellular consituents will influence such a system and a number of periodicities have been detected in cells (see later). Similar considerations apply in respect of the cyclin system but the ways in which the control system can be modulated by other rhythms may be more limited. In agreement with his theoretical studies, Gilbert (1984a), Gilbert and MacKinnon (1992) have presented evidence that the complex mitogen, foetal calf serum, can alter a number of higher frequency enzyme rhythms.

## CHAOS

Brodsky (1975) and Brodsky et al (1992) have commented on the irregularities in the protein rhythms and they suggest that the systems are behaving chaotically (see for example, Olsen and Degn 1985). However, we have found no evidence for continuous chaos in the case of any of the rhythms studied here (Ferreira et al, 1994 a,b) but we have obtained results which may indicate that it does occur for short periods of time.

The binding of insulin to its receptor elicits a diverse array of effects on cell metabolism and growth. At present four major hypotheses are being considered for its mode of action, individually or in combination, these may carry signals from the insulin receptor to a

variety of final targets. The hypotheses examined are:

1) that insulin acts through stimulation of the tyrosine protein kinase activity of the insulin receptor
2) that it activates serine kinase(s)
3) that it produces a second messenger derived from a glycolipid, and
4) it modulates the activities of G- proteins (Graham 1988).

## GLYCOLYSIS

Glycolysis is the nearly universal metabolic system which utilizes glucose in order to provide various metabolites required for other processes and, in particular, energy in the form of ATP, partly through glycolysis itself and through the oxidation of the product, pyruvate via mitochondrial reactions. Under aerobic conditions the latter pathway is dominant and under partial anaerobic conditions much of the pyruvate is converted to lactate. The reactions involve phosphorylation of different substrates and oxidation of NADH and in certain reactions carbon-carbon bond cleavage.

Many glycolytic enzymes (if not all) exists in isozymic forms which exhibit different kinetics and/or different substrate specificities. These allow one to estimate very quickly the apparent isozyme pattern in cell extracts by expressing these in terms of rate ratios wherein the activities of the enzyme are measured

using different substrate concentrations or different substrates (see the section on LDH).

## GLYCOLYSIS AND CANCER

There is still poor understanding of the molecular mechanisms underlying carcinogenesis, that process by which normal tissue gives rise to cancer cells either spontaneously or by experimental induction. One of the original theories on the nature of the processes responsible was that of Otto Warburg, who proposed that cells became malignant because of a high rate of anaerobic glycolysis. Despite much experimental support the idea was abandoned largely because of exceptions and because the mechanism could not be uncovered (see Greg, 1972. Moreover, it has never been shown how the high rate of anaerobic glycolysis could account for the malignant characteristics of cancer cells, although this was and is a failure of all other concepts. Thus, many genetic mutations have been associated with cancer but exactly how they alter the replication characteristics is not evident. In proposing a general mechanism for the nature of cell replication and the effect thereon of transformation, MacKinnon and Gilbert (1992) suggested that earlier failures were mainly due to the lack of an adequate understanding of the nature of the cell cycle. Gilbert's concept of replication and that of Sel'Kov (1972) involves redox reactions, and his experimental support comes from studies on glycolytic enzymes and thus a link between anaerobic glycolysis and proliferation can be distinguished, the suggested mechanism provides detailed explanations for a number of

facets of replication and the effect thereon of transformation. Some other relevant comments are made in the following sections.

## LACTATE DEHYDROGENASE

### Its metabolic role

This enzyme is generally considered to be the last in the glycolytic pathway in eukaryotic cells, its nominal role being that of converting pyruvate to lactate although this view does not explain why the cell should make use of this step when many intermediates and much energy could still be obtained from the these components. For this reason its primary role is considered to be that of re-oxidation of the coenzyme (NADH) under, in particular, anaerobic conditions, thereby enabling glycolysis to continue to supply at least a limited amount of the ATP needed for viability and for functioning.

Lactate dehydrogenase (LDH) is found in both prokaryotes and eukaryotes (although they are not comparable) and it catalyses the interconversion of pyruvate and lactate. It is a tetrameric enzyme and there are two kinds of sub-unit, the so-called M type (occurring predominantly in muscle) and H type (found predominantly in heart). These subunits give rise to a family of five isozymes, LDH- $H_4$ (or LDH1), $H_3M$ (LDH2), $H_2M_2$ (LDH3), $HM_3$ (LDH4) and $M_4$ (LDH5) forms. Studies on the enzyme in most mammalian tissues have revealed five active forms [Yasin and Bergel (1965); Rider and Taylor (1980). The isozymes can be separated

by chromatography and electrophoresis and have been shown to possess distinct physical, chemical and immunological properties (Nisselbaum and Bodansky 1963; Rider and Taylor, 1980). Most tissues contain unequal quantities of the different, inactive subunits which appear to combine randomly to form a binomial distribution of the 5 possible enzymatically active tetramers of MW 140 000 (Eventoff and Rossman 1976). There is a strong species specificity in the LDH isozyme pattern, in mouse, the most abundant embryonic isozyme is LDH 5, whereas in other species (eg. chicken and man ) the embryonic isozyme is LDH 1.

The M and H subunits are the products of different genes (Shaw and Barto 1963) and there is a distinct change in the relative distribution of the enzyme during foetal and postnatal maturation (Singh and Kamungo 1968). Changes in the activity of LDH and in the isozyme patterns were observed in rat mammary tissues during pregnancy and lactation (Lee, Oliver, Coe and Oyasu 1979). In malignant tissues isozyme patterns are changed (Goldman, Kaplan and Hall 1964), generally being re-orientation in the direction of the M-form, with the $M_4$ or $M_3H$ isozymes becoming predominant. Thus Yasin and Bergel (1965) and Leese (1965) showed such abnormalaties in human stomach carcinoma and Rosado, Morris and Weinhouse (1969) noted similar changes in a number of rat tumours.

At the normal assay temperatures ($30^0$C) the $H_4$ enzyme exhibits substrate (pyruvate) inhibition, which is associated with the H chain such that the extent of

inhibition is directly proportional to the percentage of H subunit in the enzyme (Dawson, Goodfriend, Kaplan 1964). It was thought that this inhibition may favour a constant flow of pyruvate into the pathways of oxidative phosphorylation, the LDH pattern of tissues having high aerobic metabolism shows a higher proportion of the H subunit than tissues where the metabolism may be anaerobic (Villar-Palasi and Larner 1970). However, it has been reported that the substrate inhibition characteristics exhibited by the $H_4$ LDH is absent if the assay is carried out at $37^0$C as opposed to the $30^0$ C usually used for assays. This suggests that there is little difference between the extreme isozymic forms of the enzyme in vivo. In addition, Gilbert (1974) has pointed out that substrate inhibition characteristics can give rise to unstable steady states which can result in total and possibly irreversible inhibition of the enzyme.

In some cells (at least) LDH is distributed between particulate-bound and free forms, with the kinetic properties of the enzyme being altered by binding (Nitisewajo and Hultin 1976; Clarke and Masters 1976 and Berlet and Lehnert 1978). Ross and Hultin 1980 (unpublished data) noted that $H_4$ isozyme does not bind to the particular fraction. Ehman and Hultin 1973, reported that chicken breast LDH ($M_4$) is not inhibited by nicotinamide adenine dinucleotide (NAD) when it is bound to particulate structures and suggested that this binding may prevent the dissociation of the enzyme into monomeric forms.

Isozymes are generally considered to be good

markers of cell type. They are of developmental interest not only because their final tissue patterns may give clues regarding metabolic regulation but also because the transient isozyme pattern changes, which are discernable at particular times in neonatal and embryonic life, may be essential for our understanding of the dependence of development on metabolism. The isozyme patterns of several enzymes have been studied in plants as well as invertebrates. In the former observations have been made on enolase, aldolase, pyruvate kinase as well as LDH.

The LDH subunits found in yeast differ from those found in vertebrates. The muscle type (M-LDH) is found in enzyme that is active at high pyruvate concentrations and the heart type (H-LDH) is the enzyme that is inhibited by high pyruvate concentration.

One of the more promising areas of research has been to look at enzymes present in normal and cancerous cells in order to find differences between the two. An enzyme which has been thought to occur in a single form, may exist in two or even more isozymic forms. Every cell type - including each kind of cancer cell, appears to have its own pattern of isozymes, the observed changes between isozymic forms of a given enzyme being the result of structural variations in the molecules. A specfic example of an enzyme is LDH.

As indicated above, there is still poor understanding of the molecular mechanisms underlying malignancy. A study of isozymes in relation to cancer initially suggested that the patterns are more like

that of the corresponding embryo. Although this appears to account (in some unclear way) for the abnormal replicative characteristics of the cancer cells, like the Warburg theory, exceptions also exist for this idea and it does not take into account the species differences in patterns for particular tissues. Nevertheless, when compared with the tissue of origin, tumours often exhibit different LDH isozyme patterns (Yasin and Bergel, 1965; Leese, 1965), glucose-6-phosphate dehydrogenase and alkaline phosphatase (Rider and Taylor 1980).

Although it is known that changes in the LDH isozyme pattern occur in relation to differentiation and cancer (Rider and Taylor 1980), it is widely believed that the pattern is constant in differentiated cells and established cell lines, this despite evidence suggesting the contrary (Gilbert, 1966, 1968). It is also believed that the pattern in such cells is determined by the total proportions of the M and H subunits This thesis throws doubt on that simple view.

Changes in the concentration of oxygen cause rapid changes in the metabolic patterns of most cells and it could thus influence differentiation according to the relative respiratory properties of cells in the embryo. At low concentrations of oxygen, carbohydrate is converted to lactate by the glycolytic enzymes present in cells via LDH yet Gilbert (1974) observed a rapid fall in the LDH activity of HaK cells on exposure to nitrogen. Low concentrations of oxygen also cause a slower response in the biosynthesis of some glycolytic

enzymes, including LDH, which are increased in concentration (Labeytir, Slonimski and Naslin 1959, Adebonojo, Bensch and King, 1961). However, the oxygen tension is not the sole regulator of LDH synthesis; chelating agents increase the rate of the enzyme synthesis despite exposure to high concentration of oxygen.

As mentioned earlier, like, for example, hexokinase, the different isozymes of LDH exhibit different substrate specificities. In particular, the $H_4$ isozyme will oxidise alpha-ketobutyrate at nearly the same rate as pyruvate whereas the relative rates are about 0.2 for the $M_4$ isozyme (human LDH - e.g., Lax, 1972). As discussed later, this fact has been used to study oscillations in the glycolytic apparent isozyme patterns.

## GLYCOLYTIC SUBSTRATE OSCILLATIONS

In intact organisms and in a variety of studies in vitro, it has been repeatedly observed that glycolysis is stimulated by an increase in pH. One of the glycolytic enzymes likely to be responsible for this effect is phosphofructokinase (PFK) as its activity is very dependent on the pH and it is a major regulated enzyme. PFK is affected by a number of other metabolites, including citrate, creatine phosphate, ATP, ADP and AMP. It is inhibited at low pH but activated by phosphate and by ammonium and potassium ions (Abrahams and Younathan 1971, Kemp 1971). In particular, PFK is activated by its products, FDP, ADP and AMP. As these

are formed they stimulate the enzyme to produce more with the result that sudden bursts of activity occur. If the rates of the earlier reactions cannot produce the substrate, FMP, fast enough, the FDP level will drop because it is used by the subsequent reactions. Therefore the activating effect of FDP on the PFK will also decrease. So, as a result of the low rate of formation of FMP, the PFK activity decreases and the original state is restored and the cycle repeats itself; the PFK activity becomes less than that of the earlier reactions, FMP and hence FDP increase and the process re-occurs.

The feedback activation of PFK by its products thus cause automatic and repetitive switching in the activity of the enzyme between very low and very high values. This takes place in a short space of time and occurs because of the existence of hysteretic kinetics. These regulatory reactions thus cause oscillatory behaviour of PFK and hence rhythmic variations in the concentrations of all glycolytic intermediates and cofactors.

PFK oscillations have been observed in both intact cells and in cell extracts; yeast has been particularly well studied. The first glycolytic oscillations were observed in yeast cells and extracts by Betz and Chance (1965), by Schoener and Elsaesser (1965), and by Hess and Boiteux (1971). The glycolytic oscillations have also been observed in heart and other muscle extracts, all being dependent, in particular, on adenine nucleotide regulation of PFK. In skeletal muscle extracts the oscillatory behaviour produces similar changes in purine nucleotide levels. Obviously, these effects can only occur if the cells or extracts are supplied with glucose or some other precursor of fructose monophosphate, the substrate of PKF, and phosphate in the form of ATP. Glucose is phosphorylated by hexokinase utilising ATP. The level of the latter therefore decreases while the concentration of FMP and AMP increase. These changes activate PFK and thus stimulate the formation of its products which further activate the enzyme. Oscillations in the concentration of total adenine nucleotide result from oscillatory operation of the purine nucleotide cycle in response to the changes in the ATP/ADP ratio associated with the glycolytic oscillations.

Although glycolytic oscillations occur in both the skeletal muscle and yeast system and are generated by transient bursts of PFK activity, there are some differences between the two systems. In muscle, PFK is activated by several factors due to autocatalytic activation by FDP once it has reached a critical point due to accumulation of AMP and FMP and loss of ATP, the concentration of FDP oscillates in muscle. In yeast

cell extracts, however, the concentration of FDP remains high throughout the oscillation period therefore FDP can not be involved in the changes in PFK activity in yeast cell extracts; the sudden burst of PFK activity results from autocatalytic activation by AMP rather than by FDP.

There are two important differences between the muscle and yeast systems:

(i) in muscle, the terminal step of glycolysis (which reoxidizes NADH) is the lactate dehydrogenase reaction, which is (apparently) very active. In yeast, pyruvate must first be decarboxylated to acetaldehyde (which is the substrate for alcohol dehydrogenase) and the decarboxylation is a relatively slow reaction (Betz and Chance, 1965)

(ii) FDP activates yeast pyruvate kinase by decreasing the $K_{app}$ for phosphoenolpyruvate [Haeckel, Hess, Lauterborn and Wuster (1968). Hunsley and Suelter (1969)].

The concentration of FDP in the oscillating yeast system remains too high to affect the activity of PFK but in whole cells, the changes in FDP could have a strong affect on the activity of pyruvate kinase.

Glycolytic oscillations in heart extracts (Frenkel 1968a) have some similarities to yeast and skeletal muscle systems. The behaviour of FDP in heart is oscillating similarly to that of muscle.

There are differences in the way of generating oscillations in yeast and skeletal muscle systems. Oscillations of glycolysis in yeast, cells as well as in cell-free extracts of yeast, have been observed in a number of laboratories (Hess and Boiteux,1971). They can be induced over a large flux range, the oscillation is not only of interest for its eventual physiological significance and as a model of how nature produces oscillatory states but also as a condition for the study of glycolytic dynamics and control (Hess, 1971).

Despite an apparently widespread belief that glycolytic oscillations are observed only under anaerobiosis (but see, the next section), glycolysis is a dynamic process which is ruled by its intrinsic control mechanisms mediated by metabolic intermediates serving as controlling ligands. However, it is important to remember that there are other regulators like mono- and divalent ions, especially potassium and magnesium ions. Moreover, the glycolytic state of a cell is dependent on the substrates present in the environment (eg. carbohydrates, phosphate, ammonium ions), and furthermore, the enzyme pattern is set by environmental conditions via induction and repression mechanisms.

Summarising the differences in the way oscillations are generated in yeast and skeletal muscle:

(1) oscillatory behaviour of the glycolytic pathway and the purine nucleotide cycle in skeletal muscle extracts involves periodic bursts of PFK activity which are caused by AMP dependent, autocatalytic

activation of the enzyme by FDP; this is totally different from what occurs in yeast cells and extracts where glycolytic oscillations are generated by autocatalytic activation of PFK by AMP. Whether steady state or oscillatory behaviour occurs depends on the PFK activity.

(2) The autocatalytic activation of PFK by FDP only begins once a certain critical concentration of that component accumulates.

(3) Glycolytic oscillations in yeast extracts and cell suspensions involve control of PFK by AMP and FMP; the concentration of FDP remains very high.

(4) Glycolytic oscillations in heart extracts are considered to be similar to those of the yeast system.

One may comment that many people who are aware of oscillations in glycolysis seem to believe that they only exist under special laboratory conditions. This thesis again dispels such a view.

## MEL CELL PHOSPHORYLATION, MORPHOLOGICAL AND REDOX DYNAMICS

Some related studies on MEL cells have been carried out in these laboratories. They fall into two groups: the first consisted of discontinuous, destructive observations on the activities of the phosphoamino acid phosphatases in extracts while the second involved essentially continuous, non-destructive monitoring of

MEL cells (and amoeba, Visser 1994 ) which yielded information on changes in shape and redox state.

In the former it was found that oscillations occur ~~In the former it was found that oscillations occur~~ in the activities of phosphoserine, phosphothreonine and P-T-Pases, the apparent periods being essentially the same and of the order of 60 minutes (Hammond, et al 1989). The possibility that the frequencies may be even higher was considered in view of the fact that the sampling times used were long and comparable to the apparent periods. From unpublished data (Janura and Gilbert, personal communication) it appeared that the phosphotyrosine phosphatase rhythm was inhibited at 4$^{o}$C and affected by insulin.

In the other studies (Visser et al, 1992), spectrophotometric and fluorometric methods were used to follow periodic changes in shape and oxidative state of MEL cells. The morphological variations were deduced from variations in the intensity of scattered light while the redox rhythms were detected through changes in the absorption or fluorescence of NADH and FAD. By making use of the Hewlett Packard diode array spectrophometer and hence difference spectrophotometry, it was possible to minimise effects due to fluctuations in the numbers of cells in the light beam and to gain higher specificity for the components of interest. It also made it possible to determine three parameters simultaneously. In all cases, resort was made to smoothing and time series analyses in order to confirm the oscillatory nature of the variations observed. Evidence was obtained that a range of oscillations exists in the shape and in the redox state of the cells

(the periods being of the order of 10 sec. to 30 min.), and that they tend to be modulated in pseudo periodic (i.e., pulsed) manner. (The latter findings have a bearing on some of the results presented here.) Moreover, the patterns of behaviour could be modified by agents.

In the latter studies (Gilbert and Visser, 1993) it was found that insulin (and some other chemotactic agents -(Visser 1994) rapidly enhance (in particular) the morphological oscillations in a frequency dependent manner, some rhythms becoming lower in amplitude, some being unaffected but the majority being stimulated (see also Visser et al, 1993; Gilbert and MacKinnon, 1993). Some of the effects could be explained by the hormone changing the frequencies of particular oscillations. It was suggested (Gilbert and Visser, 1993) that the more marked changes in cell surface movements and shape could decrease the net thickness of the surrounding diffusion layer and thereby cause a non-specific stimulation of metabolism.

## GLYCOLYTIC ENZYME OSCILLATIONS

### Activity oscillations

The PFK activity variations mentioned above have been largely deduced from changes in the levels of its substrate and product. In so far as we are aware there have been no direct measurements on the activities of the glycolytic enzymes themselves other than for lactate dehydrogenase (LDH) (see next section). Here we are particularly concerned with LDH activity

determinations where samples of the enzyme in cell extracts are assayed by adding to buffered substrates and the activity determined in some way, for example, by following the rate of oxidation or reduction of a coenzyme, NADH in the case of LDH. The enzyme samples so used may contain substrates, coenzymes, activators and inhibitors of the enzyme of interest in addition to other enzymes which could modify the enzyme in some way, for example, by phosphorylation. These additional components will be diluted when compared with the cellular levels and hence, in general, any affects on the enzyme which are detected will probably be less than occurs in the cell. This is the situation in the present studies. For an adequate assessment of the overall effects on the enzyme, the rates of the reaction need to be determined by, for example, simultaneous studies on the levels of substrate and products of the enzyme.

Edmunds (1988) has recently reviewed the subject of cellular oscillations so here we will focus on the rhythms of direct interest. Gilbert, Tsilimigras and Gilbert (1968, 1969, 1974b, 1984a) and Gilbert and Tsilimigras 1981, and Tsilimigras and Gilbert 1977) have reported oscillatory variations in the activities and apparent isozyme patterns of a number of glycolytic enzymes and the related glucose-6-phosphate dehydrogenase. Kinetically determined oscillations in the activity of lactate dehydrogenase in intact cells have been observed in several other studies (e.g., Lax, 1972; Duffy, 1971; Duffy and Sanderson, 1971; Klevecz, 1976). The periods have been variously reported as being

from around a minute to several hours, this probably being due to equally varied sampling interval and timing of the sampling relative to the true oscillation (keep in mind the subject of aliasing Gilbert 1974; see Methods section). Kinetic determination on its own gives no indication as to whether the amount of enzyme varies or whether there is some other reason for the observed fluctuation.

Of particular interest were the timing relationships found by Gilbert (1968, 1974b, 1984a; Gilbert and Tsilimigras, 1981) between the oscillations for different enzymes; these are discussed further in the next section.

A factor affecting these processes 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 the polymers (see for example, Clark and Masters, 1975 and compare Ross and Hultin, 1980 and comments on LDH above). The latter mechanism does not seem sufficient to account for different patterns of temporal organisation in cells, as mentioned above, nor the apparent variations in composition of proteins in the cell extracts as judged by the use of different methods for estimating protein concentration (Tsilimigras and Gilbert, 1977; Gilbert and Tsilimigras, 1981). At least two independent processes would seem to be necessary, a view which is supported by some periodogram analyses (Gilbert and Tsilimigras, 1981; Ferreira et al, 1994a; see also Visser et al, 1990 and

Gilbert and Visser, 1993 for power spectrum analyses of morphological and redox data).

Reports on the phosphorylation of LDH (Williams et al 1985 and Cattaneo et al 1985) and on the DNA helix destabilisation action of $M_4$ LDH (Termonia et al 1982), coupled with our observations on oscillations of the enzyme and in the phosphorylation potential (to say nothing about the limited information on mechanisms), led us consider the possibility that covalent modification might at least contribute toward the LDH oscillations. Although the results of the present experiments do not supply evidence on this possibility, they do provide additional reason why further studies should be undertaken on the involvement of phosphorylation in the LDH oscillations. As will be seen, they also led to the observation on the cell free systems.

## Isozyme oscillations

Because of the simplicity of the kinetic method for estimating isozyme patterns changes, Gilbert (1968, 1974) used it to study oscillations in the apparent isozyme patterns of LDH in mammalian cell extracts. This approach was also extended for the study of the isozyme patterns of other glycolytic enzymes, see above. Adequate electrophoresis and scanning equipment were not then available, but these have now been used and comparison of the results of the two methods reveals some interesting aspects of the regulation of LDH activity, specificity, kinetics, real

and effective isozyme patterns; in so far as we are aware these have not been reported previously.

Most importantly, distinct timing relationships and switching have been described for the glycolytic isozyme oscillations (Gilbert, 1968, 1974, 1984). In particular, the phase relationship between aldolase and LDH "isozyme" periodicities were found to be different in untransformed and polyoma transformed BHK cells (Gilbert 1968, 1971, 1974, 1984) supporting the views of the latter that changes in the pattern of temporal organisation can be involved in both differentiation and oncogenesis (see earlier comments). Moreover, various agents and changes in conditions were found to affect the patterns of behaviour. In the present studies, carried out on LDH in MEL and HL-60 cells, several distinct issues arise, all related, yet it is best to look at them separately in the different sections of the thesis.

## THE EXTRACTABLE PROTEIN OSCILLATION

When determining the amount of a cellular component it is common practice to express the result relative to the concentration of protein in the extract (as with enzyme specific activities for example). This is done in the belief that the procedure will correct for variations resulting from minor differences in the number of cells from sample to sample or in the extraction efficiency. Elsewhere (Tsilimigras and Gilbert 1977, Gilbert and Tsilimigras 1982, 1982; Ferreira, Hammond and Gilbert. 1994) it has been shown that high amplitude, high frequency oscillations occur

in the protein content of extracts of several cell lines. As indicated in those articles, this throws grave doubt on the validity of the use of such corrective methods.

On the other hand, Brodsky (1975) and Brodsky et al (1992) have studied rhythmic changes in the protein content of intact mammalian cells while Edwards and Lloyd (1980) have examined protein levels in amoeba. The nature of the processes involved are not understood but metabolic oscillations may also contribute towards the extractable protein rhythm by affecting the aggregation of their relevant enzymes or their binding to polymers (see earlier comments). It is not clear if such reactions can contribute towards the results of Brodsky et al (1992) or those of Edwards and Lloyd (1980).

As the extractable protein oscillation has been well studied, it was not intended to consider the phenomenon further but determinations of the protein concentration of extracts was carried out routinely as the rhythm provided a an internal reference parameter for the other studies. However, examination of the information gained provided interesting results, which are therefore included in the thesis. Here we present evidence that the oscillation also occurs in extracts of Friend-virus transformed murine erythroleukaemic cell line and show that it has characteristics similar to the other periodicities. An article on the subject is in the press at the time of writing (Ferreira, Hammond, Gilbert. 1994). It seems likely that variations in protein content account for, or contribute toward, the extractable protein oscillation. Brodsky (1975) and

Tsilimigras (1982) have reported oscillations in the rate of incorporation of amino acids into protein in intact cells and tissues and in cell extracts.

Elsewhere (Gilbert and Tsilimigras, 1981) it has been shown that the extractable protein oscillation cannot entirely account for enzyme periodicities though it probably contributes toward them. The view is re-examined in the present studies.

# METHODS

# CHAPTER II

# METHODS

## GENERAL COMMENTS

An overview of the experiments carried out is given in Table II. It shows the main topics, the cells concerned, the conditions used, the agents studied and the techniques applied. The rest of the thesis will roughly follow the arrangement given there. In this section only an outline is given of the methods and procedures, the full experimental details can be found in the appropriate appendix.

## CELL CULTURE MAINTENANCE

MEL cells do not normally attach to surfaces but exist as individual cells and they were therefore maintained as stationary monolayer suspensions in 250 ml or 50 ml plastic flasks according to requirements. They were fed every 2-3 days by replacing one third of the volume with fresh medium. When more cells were needed, the culture size was increased every two-three days by adding 1/3 volume of fresh medium.

# TABLE II EXPERIMENTAL OVERVIEW

| SUBJECT / ASPECT | PHOSPHORYLATION POTENTIAL | ENZYME ACTIVITY | | GENERAL | CELL FREE SYSTEM |
|---|---|---|---|---|---|
| | | p-TYROSINE PHOSPHATASE | LDH | EXTRACTABLE PROTEIN | |
| Parameter | Protein-X<br>Protein-Y<br>Chaos | Activity | Activity<br>Ant isoz.<br>Regn. isoz.<br>Independent regn. isoz.<br>Chaos | Total ant.<br>Chaos | Activity<br>Ant. isoz. |
| Cell | MEL | MEL | MEL<br>HL-60 | MEL<br>HL-60 | MEL |
| Culture | Stationary monolayer | Stationary monolayer | Stirred suspen. | Stationary monolayer<br>Stirred suspen. | -- |
| Agent | Insulin<br>HMBA | Insulin<br>HMBA | Insulin<br>HMBA | Insulin<br>HMBA | ATP |
| Methods | Autoradiog<br>Electroph. | Quant.<br>Kinetic<br>Electroph. | Kinnetic<br>Electroph. | Quant.<br>Electroph. | Kinetic<br>Electroph. |
| Plots | Time<br>Mean<br>Period<br>Amplitude<br>Periodog.<br>Phase<br>Ratio | Time<br>Mean<br>Mean<br>Amplitude<br>Ratio | Time<br>Mean<br>Period<br>Amplitude<br>Periodog.<br>Ratio | Time<br>Mean<br>Period<br>Amplitude<br>Periodog.<br>Ratio | Time<br>Mean<br>Period<br>Amplitude<br>Periodog.<br>Ratio |

Abbreviations:
p-TYROSINE -- PHOSPHOTYROSINE
Trans -- Transient
Regn. -- Regulation
Periodog. -- Periodogram
Autoradiog. -- Autoradiography
Quant. -- Quantitative
Ant. -- amount
Isoz.. -- Isozyme
suspen. -- suspension
Electroph. -- Electrophoresis

## EXPERIMENTAL PROCEDURES

### (i) Phosphorylation potential

These studies were carried out in MEL cells in stationary monolayer. Aliquots of a freshly fed stirred suspension were placed in a series of flasks, sufficient in number for the experiment undertaken. These were randomly assigned to control, insulin treated, HMBA treated, or, insulin with HMBA treated groups. The appropriate agent, sterile filtered was added to each of the relevant flasks and an equal volume of buffer to the controls. Immediately after, one flask from each group were selected at random for immediate determination of phosphorylation potential or P-T-Pase assays and additional flasks taken at 10 minute intervals thereafter. Cells from each flask were centrifuged at 2000 rpm for 10 minutes to form a pellet which was resuspended and washed in saline, centrifuged further with these steps being repeated twice more. The pellet was resuspended in extraction buffer, homogenised and centrifuged at high speed. Samples were kept at $-70^{o}C$ until required.

### (ii) Phosphate incorporation and electrophoretic separation

Supernatants were incubated with $\gamma^{32}$P-ATP in the presence of magnesium acetate for 2 minutes at $30^{o}C$. A solution contaning TRIS-HCl, SDS, glycerol, mercapto-ethanol and bromophenol blue was added and the samples were boiled for 2 minutes. Equal volumes of each extract was then electrophoresed on SDS-PAGE gels (in some

experiments equal amounts of protein were loaded into each lane, see text) the procedure being that of Laemmli (1970) using a mini-system. A current was applied for 2 hours, gels were fixed, washed twice in glycerol, dried under vacuum and autoradiographed. Autoradiographs were scanned using a laser densitometer using the area under the curve as a measure of the extent of incorporation of $^{32}P$ (see appendix III). In some cases some of the flasks were left for 24-72 hours to determine longer term effects.

The amounts of protein on the SDS gel corresponding to the phosphoproteins of interest (protein X and protein Y) were determined by staining the gel with Coomassie Blue before carrying out the autoradiograph (see Appendix III).

It is assumed that the variation in the autoradiograph intensity is proportional to the amount of phosphoprotein and not to varying degrees of phosphorylation of the proteins. It is not known if the dye stain intensity is affected by differences in the extent of phosphorylation.

Every effort was made to make the collection of cells and subsequent treatments as uniform as possible.

Disadvantages of this approach are (i) it is slow, and (b) that one is using individual cultures for each data point and they could be behaving differently. However, Tsilimigras (1982) found that there was good correlation between cultures (with regard to LDH activity and isozyme data), at least in the short term.

We believe that our 3D data, showing the persistence of particular rhythms supports this view otherwise one would not expect to obtain such a result.

LDH STUDIES

For these suspension studies, freshly fed cells (MEL or HL-60 cells) were placed in a closed (but fitted with a cotton wool plug), magnetically stirred culture vessel placed in a water bath at 37°C. Sampling was either started immediately or, in a few cases, the next morning ( 24 hours later) according to whether the cells were to be in a disturbed state or to be given a chance to reach a steady state. Sampling was via a Cornwall syringe fitted with a narrow bore silicone rubber tube the tip of ~~which~~ which was submersed in the culture. Cells were thus rapidly collected on Millipore 0,45 µm cellulose filters under suction and twice washed quickly with saline. The cells and membrane were then immediately immersed in liquid nitrogen in which they were stored until required. They were then taken out singly and thawed and refrozen twice in liquid nitrogen before adding the extraction buffer. The bottle was shaken on a Vibromix after which they were frozen and then thawed again to form the crude extracts. The latter were then centrifuged at low speed to remove debris and frozen in liquid nitrogen until examined. After thawing they were immediately kept on ice while enzyme activity and isozyme pattern determinations were undertaken. (see Appendix V).

## EXTRACTABLE PROTEIN

Protein determinations were routinely carried out on all extracts, the method used being determined by the topic of interest. The protein concentrations were measured by the standard Lowry method (see appendix VI).

## ENZYME ACTIVITY

### (i) Phosphotyrosine phosphatase

Before undertaking the P-T-Pase experiments, a comparison was made between a continuous assay and the discontinous method used previously. The former involved a spectrophotometric monitorings of the hydrolysis of phosphotyrosine to tyrosine. The discontinous assay is a non enzymatic method based on the determination of liberated phosphate. As described in Appendix IV, excellent agreement was obtained and therefore the continuous method was used for all further studies. Cell extracts were prepared as for the phosphorylation experiments. A blank was performed on the phosphotyrosine to find out the endogenous rate of phosphate cleavage so that the reaction rates could be corrected if needed but the rate was found to be negligible. Assays were performed using the cell extract to start the reaction, the activity being determined from the rate of change in absorbance at 280 nm (see appendix IV).

### (ii) LDH assays

LDH activity was measured by a spectrophotometric

method following the rate of oxidation of NADH at 340 nm. The standard procedure used 0.7mM pyruvate (P or Py.7) as substrate, however, for the apparent isozyme (kinetic) studies the rate of oxidation of NADH was measured using in, addition, 0.35 mM pyruvate (Py.3) and 3.3mM alpha-ketobutyrate (B). The apparent isozyme pattern was expressed by the ratios of the pairs of rates, Py.3/Py.7 and B/P. (see appendix V). The former value was used in case the B/P ratio was affected by the presence of a specific ketobutyrate dehhydrogenase but no evidence for the latter was found.

(iii) LDH electrophoresis

The actual isozyme patterns were measured using the Beckman Paragon agar gel electrophoresis system followed by formazan blue activity staining according to their kit instructions. The intensities of the isozyme bands were determined by means of a laser scanning densitometer (using the area method), the values obtained being taken as a measure of the amount of active isozyme.

CELL FREE SYSTEM

For the cell free studies, the extraction method assays and isozyme determination were carried out as for the LDH studies [see Appendix V ]. For the cell and particle free experiments the extract was first centrifuged at a speed sufficient to remove mitochondria.

**ASSAY REPRODUCIBILITY**

In order to gain confidence in results, the usual approach in such studies is either to (a) take multiple samples in any experiment at each time point and to take the average for each, or, (b) to repeat experiments and again take the means for corresponding time points. Such methods expect (i) that the pattern of behaviour of the cells is reproducible from experiment to experiment and (ii) that any changes occurring in the extract are slow: both circumstances may not be true and can only be determined by experimentation. In the present studies it was not possible to take multiple samples for each time value particularly in view of the rapidity of the changes and for such high frequency fluctuations slight time differences can make marked differences in the data values; averaging would completely blur the picture but such circumstances may account for many experiments where very large standard deviations are seen. Gilbert (1974b, 1984a) has considered problems of this kind including the point that one cannot normally know the phase existing at the time one starts sampling, only after the measurements have been completed.

Where one has multiple small cultures of the cells it is difficult (without special equipment) to take more than two samples at the same time but rather than assessing the confidence in each point, one can estimate the reproducibility over the whole experiment by arbitrarily assigning the cultures to one or other of two series and plotting the two values against each other and determining an overall correlation coefficient. Tsilimigras (1982) used this

method to show that very high coefficients could be obtained thereby supporting the view that what happens in one culture happens in the others throughout the course of the experiment. Unfortunately in these studies where monolayer cultures were used, it was necessary to use large volumes which meant delays in processing a second sample and therefore such an approach was not used.

However, in order to give an indication of the confidence in both the assay procedures (where assays were repeated) and the practical ability of the experimenter, we give in Fig.4, examples of where we have graphs where the first assay value is plotted against the second. In Fig.4 (left diagram) the results is a composite for two P-T-Pase experiments whereas right diagram the results are for one LDH experiment. Also given is the line of unit slope which should be obtained for perfect agreement.

Other aspects of the reproducibility problem are considered in the Discussion.

**DATA ANALYSIS**

Only if the periodicity being observed is pure and constant with time can one obtain a definitive picture of the dynamics. The difficulties of analysis and interpretation increase with increasing complexity. In an effort to glean as much information as possible from data (which is often very irregular (see discussion) we use various other methods to study the time series (Gilbert and Tsilimigras, 1981, Visser et al, 1990),

**Fig.4 ASSAY REPRODUCIBILITY**

Particularly when the rhythm being studied is of high frequency, as here, it is dificcult to take multiple samples and average the values and so obtain some indication of the confidence limits.

What one can do is to carry out the assays in duplicate and plot the two values against each other and this gives an overall idea of the reproducibility of the assay method and the ability of the person carrying them out. This is done here for the LDH pyruvate assay (right diagram) and the P-T-Pase assay (left diagram). In the latter we have combined the results from three experiments. It can be seen that the biggest errors are seen where the activities are high because it was more difficult to obtain an accurate measure of the slope and, where different chart rates are used one multiplies small errors by a factor for adjusting values to the same scale.

# ASSAY REPRODUCIBILITY

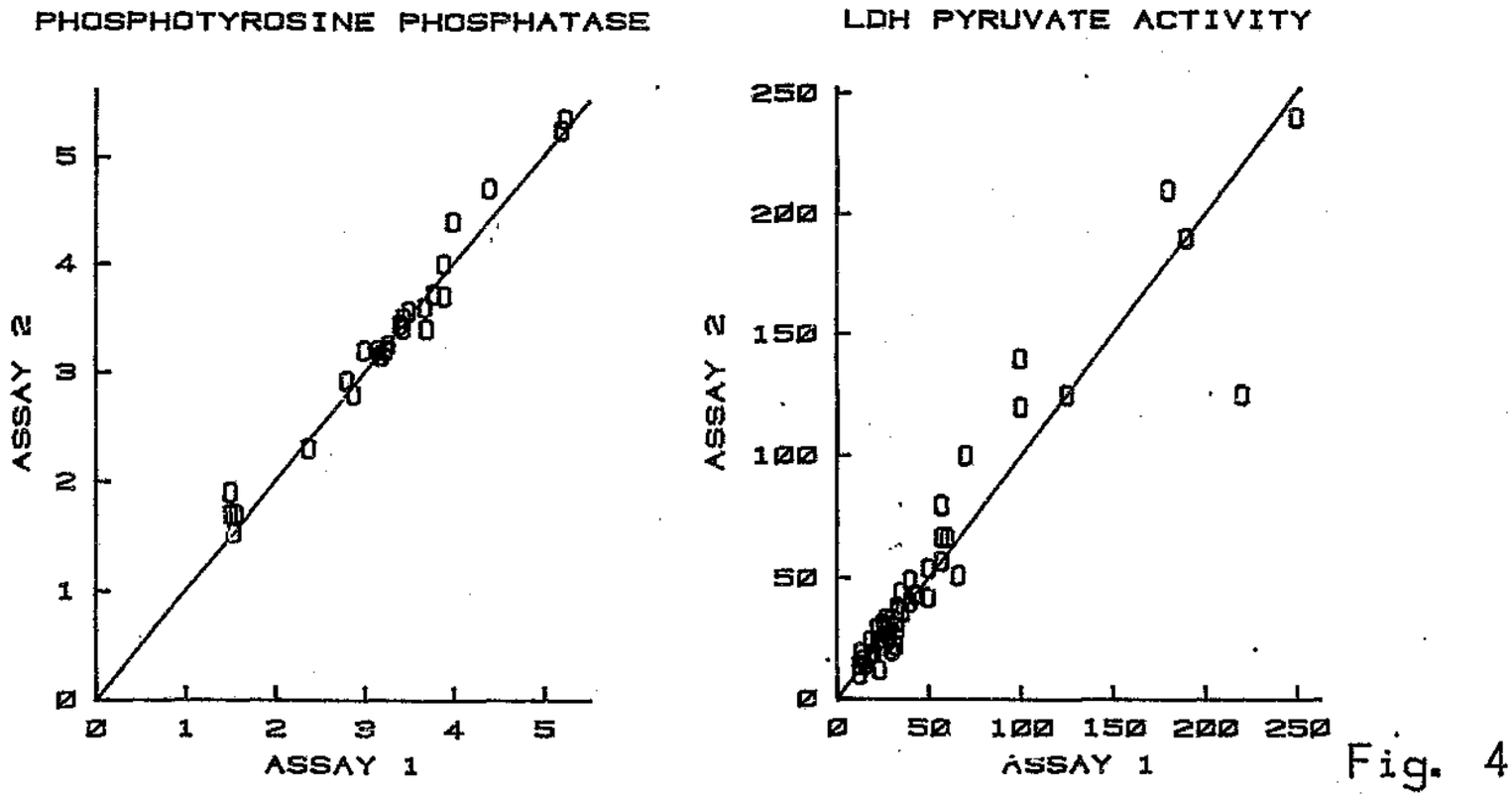



Fig. 4

using Per-amp plots (cf Tsilimigras and Gilbert, 1977) and by applying variations of the periodograms due to

(a) Lamprecht and Weber (1970), and, (b) Enright (1965). These methods were applied either to the overall data set or, to a small section (window) of the time series which is moved sequentially along the whole set of values and thus gives an indication of the variation in the pattern with time (e.g., Visser et al, 1990). Because the period range covered depends on the sampling interval and the number of points used for the window, this latter approach covers a smaller period range than when all the values are examined (see Visser et al, 1990).

In studies such as those reported here, one obtains a series of values for the parameter of interest which are obtained at (preferably) regular time intervals. If plotted against time they yield fluctuations about some mean level, which itself may vary with time. The primary aims of the thesis was to determine the magnitude of any fluctuations, to determine if they have a rhythmic basis, to see if more than one oscillation is involved in each case, to estimate the frequencies of any oscillations that are present and to see whether the periodicities are affected by the selected agents.

CURVE FITTING.

The usual procedure is to plot the values as a function of time and to show the pattern of behaviour by joining the points by straight lines, it being argued that it is wrong to draw smooth curves as that involves

interpolation and one does not know the true intermediate values. However, Hammond et al (1989) have commented that the same arguments apply to the use of straight lines and, as biological changes are unlikely to be linear and involve sudden disjointed alterations in slope that they imply, it is better to draw an objective smooth curve through the data values. Hence the first step in the present analyses has been to do this using a fourth degree polynomial curve which passes through the data values. Similarly, other time curves obtained from the original data plots have been fitted in the same way.

This procedure may not be a truly accurate representation of the true time course but it does give an objective picture which in essence, conforms to that which would be drawn by eye. It also allows a reasonable estimate of the the positions of the peaks and troughs in the data which permits the apparent period, amplitude and mean values to be calculated (next section).

## GRAPHICAL ESTIMATION OF THE INSTANTANEOUS PERIOD AND AMPLITUDE (Per-amp plots)

A rough estimate of the period can be obtained from the number of peaks seen in a given time and the amplitude can often be estimated by eye. However, these parameters have been calculated by computer according to the methods of Tsilimigras and Gilbert (1977). The period is determined from the time between successive

peaks and troughs of the fitted curves. The values so determined are plotted against some suitable point in time such as the second peak (or trough), or latterly, against the time of the intermediate trough (or peak). A curve is then fitted through the set of values as just described.

The amplitude is determined from the vertical distance between a peak (or trough) to the line joining adjacent troughs (or peaks). The value so obtained is plotted against the time of the relevant peak or trough and a curve again fitted through the data points.

The mean values are taken as the midpoints of the vertical lines used to calculate the amplitudes, the apparent time course again being obtained by fitting the polynomial curve. However, in order to simplify the diagrams these plots are not generally given though they usually show signs of periodic variation.

All the above curves were automatically calculated by the computer programme made available by Dr D.A. Gilbert.

**PERIODOGRAMS**

**(i) The Enright periodogram.**

In addition to determining the period by graphical means, attempts have been made to calculate this parameter by the use of the Enright (1965) and Inverse [Gilbert and Joosting, (1993)] periodograms. The former determines the variance at a selected period (relative

to the total variance) in the chosen set of data values. If the fluctuations are completely due to an oscillation of period P then a plot of the relative variance against period will yield a peak at the point given by P of unit magnitude, the width being governed by the period interval used for the calculations. If more than one oscillation is responsible for the fluctuations then, ideally, several peaks will be obtained, each of lower, fractional magnitude. In principle, random contributions should be eliminated because they should not be repeated at regular time interval. With short lengths of data (as used here) this effect is not completely achieved but the residue can be improved by plotting only variance values greater than some arbitrary threshold.

The common practice is to apply a periodogram to the whole set of values and then to argue about the significance of each peak obtained on statistical grounds (e.g, Fry, Humphrey and Isles, 1981). We prefer to use the window method and to judge significance from the persistence of a given oscillation during the course of the experiment, or, from its repeated presence in different experiments. Accordingly, for each set of data we use additional forms of the periodograms to study the time dependency; we thus also follow (a) the variance change with time for individual oscillations, and, (b) the sum of the corresponding variances for individual oscillations whose periods fall within arbitrary ranges (Visser et al, 1990). As indicated in the latter article, we take this second approach in an effort to accomodate slight changes in frequency during the experiment. By examining several adjacent ranges one can often detect the occurrence of burst activity wherein a

number of oscillations appear and disappear simultaneously over a short interval of time.

By choosing a data set which is smaller than the total number of values collected, then by moving this "window" along the total set one can obtain some idea of (a) whether a particular rhythm is present throughout the duration of the experiment, and, (b) whether its period remains constant, varies with time and is affected by an agent. This can be achieved by means of a three dimensional plot of variance vs period and time. However, this can only be done at the expense of covering a smaller period range since the limits are determined by values given by twice the sampling interval and half the total duration of the experiment (the so called Nyquist limits); practically,however, the upper limit is less than this as one requires several cycles of the oscillation to calculate the variance with any accuracy (Visser et al, 1992).

We also vary the window width and calculation interval (the amount by which the period is incremented during the calculations). It will be appreciated that if the latter is large then oscillations can be missed or appear minor if the step between period intervals is large. As described elsewhere (Visser et al, 1990) one can effectively eliminate minor components by plotting values only if they exceed some arbitrary set threshold. Similarly, this can be done by increasing the window size since the contribution of the minor component to the total variance will be smaller if other rhythms are thereby included; this approach is limited by the number of data values obtained in our experiments. Wide windows

and short data sets also limit the time scale obtained by this method since the window can be shifted by fewer points. On short data sets we increment the window movement by a fraction of the sampling interval in order to emphasise the pattern but this does not interpolate.

It should be noted that where (as here) one uses a window for the determination, it is not possible to accurately define the point of addition of any agent in a 3D plot because the set of values in the window will include a range of values. In the present diagrams the time of addition of the agent is taken to be that time which corresponds to the midpoint of the window. In periodograms the time scale will not appear to correspond with that of time plots of the data.

Several options are possible in the available programs; one is the setting of a threshold as discussed above, other are the window size (in terms of the number of data values used) and the (step) interval between calculations on the time scale, that is, by how much the window is moved along the data set for each calculation of period spectrum. Yet another is the period calculation interval; as pointed out by Enright (1965) this can be smaller than that corresponding to the sampling interval. Although a fractional step interval merely repeats the previous period spectrum its use can help emphasise certain features in the 3D plots. The threshold option can also be used to eliminate minor oscillatory components or, if required, to remove the major components so that attention can be focussed on the minor ones.

In addition to the options, several distinct Enright periodogram plots are available. One gets, in effect, a plan view of 3D graph (Fig. 5), indicating only at what period an oscillation is detected during the course of the experiment; the magnitude of the variance is lost. This allows one to concentrate on the frequency aspects. Another form is the period range diagram (Fig. 6a,b) in which the variances are summed over arbitrarily set period limits; this can be useful when the period of an oscillation is drifting or varying slightly during the course of the experiment. It can also give a clearer indication of burst characteristics where in oscillations with various periods are simultaneously affected, e.g., by an agent. Some of these aspects have been considered by Visser et al (1992) and Ferreira et al, (1994a,b).

Neither the Enright nor the Inverse periodogram (see next section) are really suitable for use with short lengths of data but in the absence of any other adequate method they have been used in an effort to gain the maximum information out of the data available. Larger windows should give better results in terms of, for example, resolution and sharper peaks but will lead to elimination of smaller components and rapid variations in period whereas these aspects are important to studies of the present kind, particularly where the initial affects of agents are of concern. Large windows thus have the effect of smoothing the data in which fine detail becomes lost. When one uses a fractional period calculation interval the method can be compared with the

**Fig.5 PERIODOGRAM PLAN PLOT**

If one is simply concerned with the periods of oscillations that are present and not the magnitude of the peaks in the periodogram, one can use plots of this kind where a line is drawn if an oscillation of the particular period occurs (and greater than a set threshold); the magnitude of the variance is lost. This kind of diagram is useful for seeing if an agent affects the frequency. Here the addition of insulin seems either to change the rhythms with initial period of 20 mins or stabilise the drift at around 25 mins. A similar conclusion may be drawn with the rhythms having final period of about 40 mins.

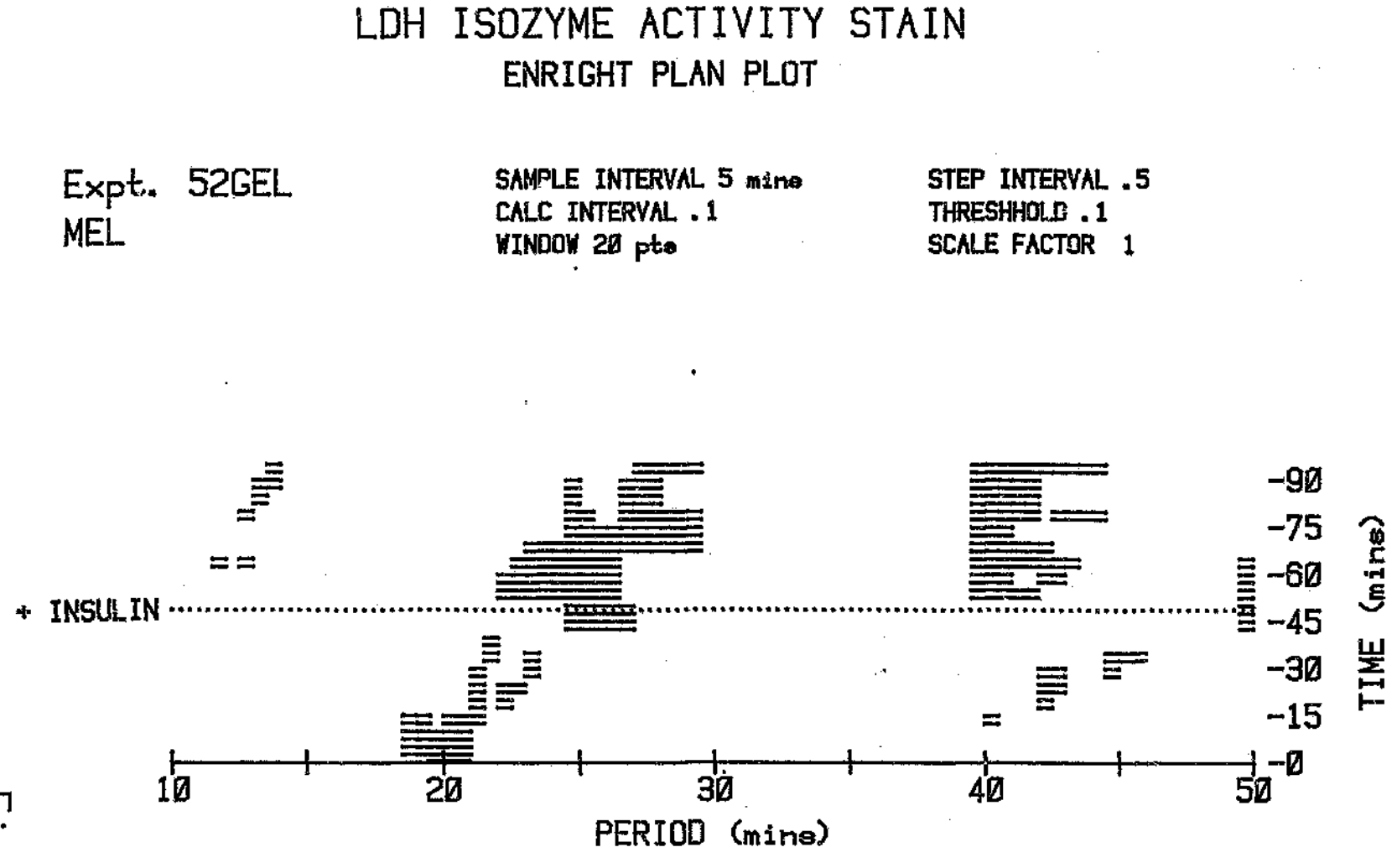

LDH ISOZYME ACTIVITY STAIN
ENRIGHT PLAN PLOT
Expt. 52GEL
MEL
SAMPLE INTERVAL 5 mins
CALC INTERVAL .1
WINDOW 20 pts
STEP INTERVAL .5
THRESHHOLD .1
SCALE FACTOR 1
+ INSULIN
-90
-75
-60
-45
-30
-15
-0
TIME (mins)
10
20
30
40
50
PERIOD (mins)

Fig. 9

**Fig.6a,b KNRIGHT PERIODOGRAM: PERIOD RANGE SUMMATION**

This figure is an example of a variation on the 3D periodogram with the difference that the variances at any particular time are summed over a selected range of periods so that one has, in effect, a mean variance for that period range at that time. This is repeated for each time value to give the plots of the kind shown above (see Visser et al 1993, Ferreira et al 1994a). The aim is to overcome minor frequency heterogeneity in the population of cells and also slight period fluctuations. However, no other such diagrams will be presented here.

PROTEIN X

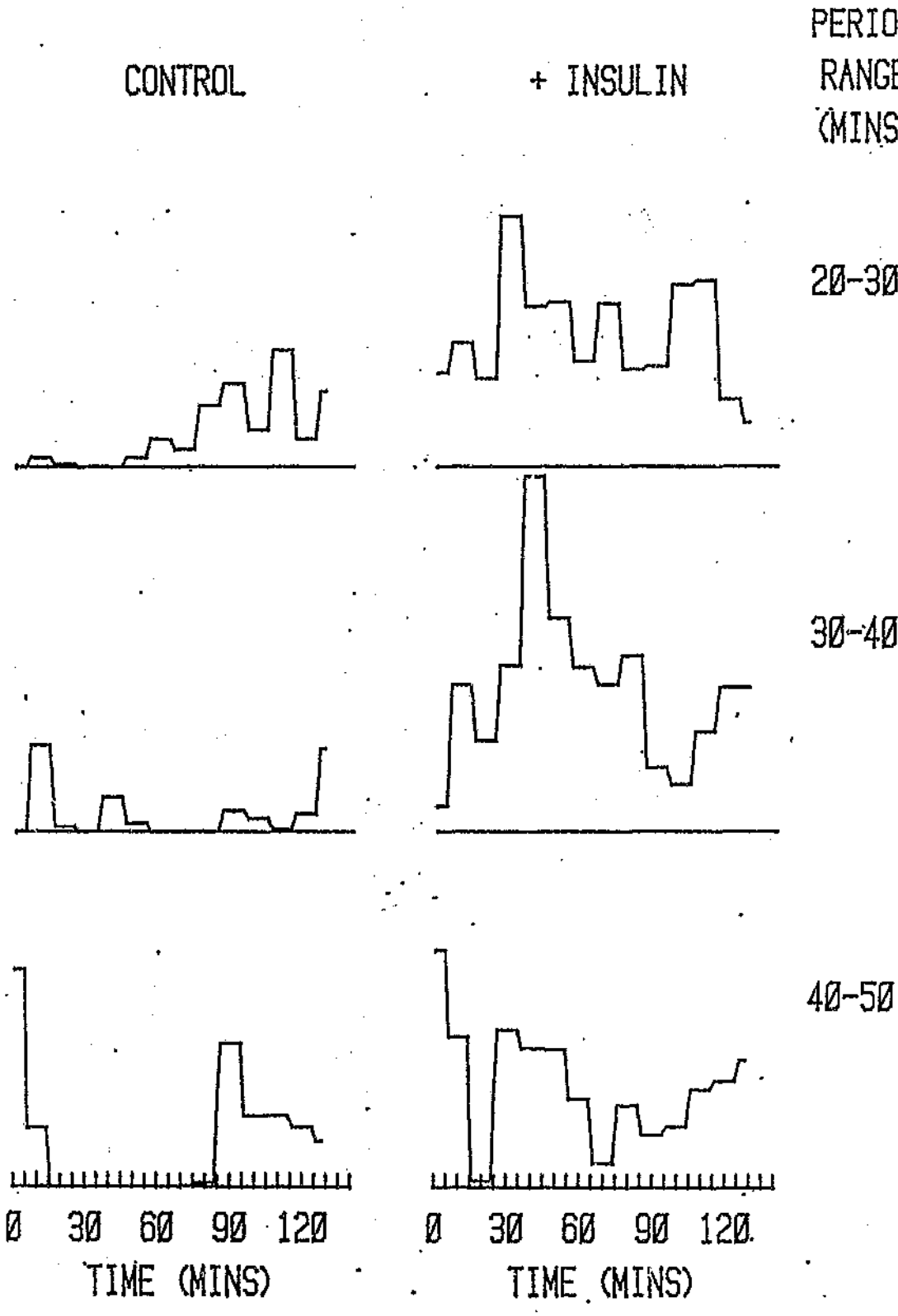



Fig. 6a

PROTEIN Y

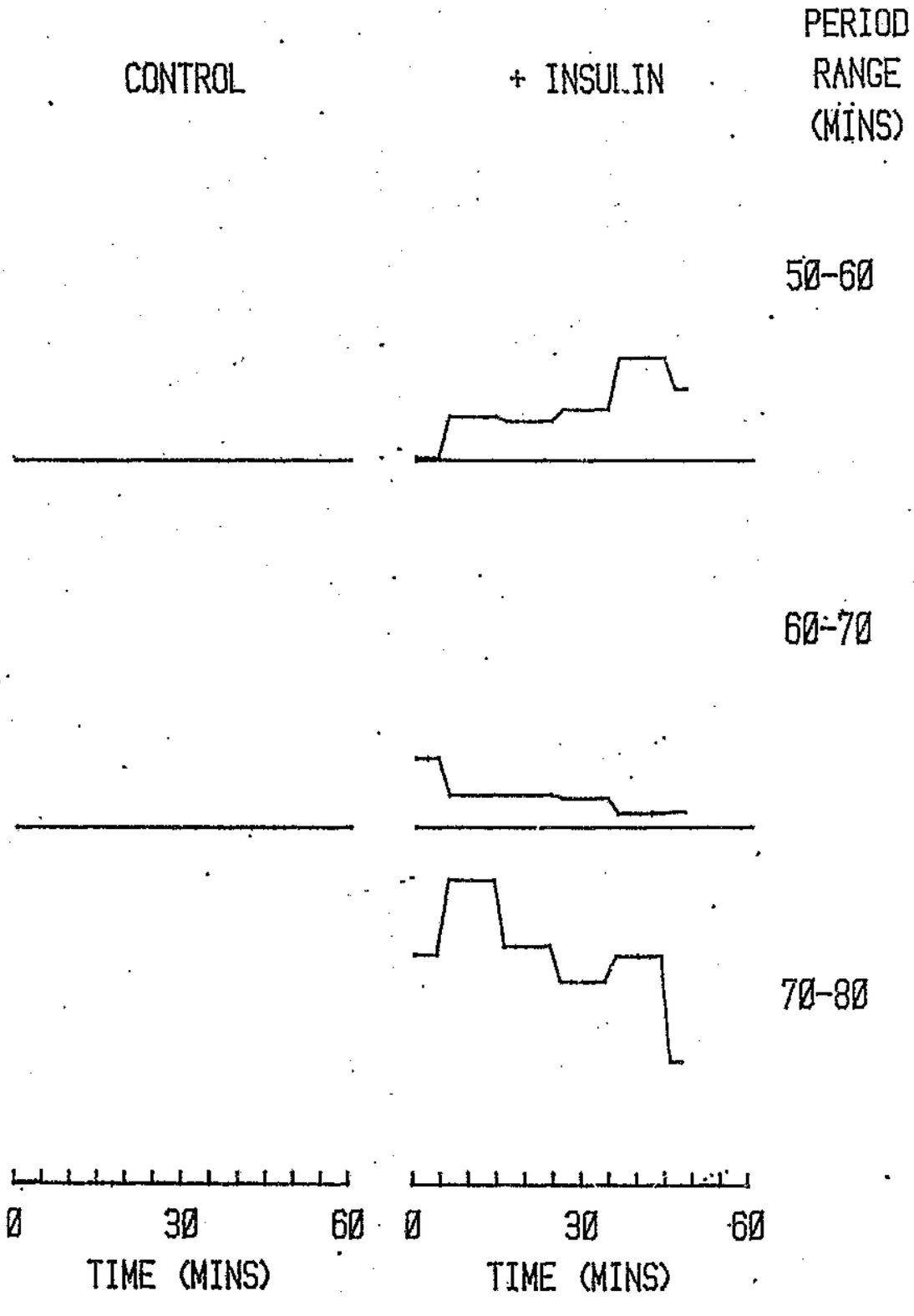



Fig. 6b

graphical method given above wherein one uses reference points other than the peaks and troughs but averages the values over two or more cycles of the oscillation depending on the actual window used in relation to the period observed.

If one covers a very wide period spectrum one can include the harmonic values, if the oscillation stays sufficiently constant over the time interval corresponding to the period of the harmonic. In some instances what appears to be harmonics are observed but it is not always possible to prove that they are not distinct rhythms. Some times the primary oscillation changes in period and this makes any harmonic more easily seen, because the harmonic will also change in period at twice the rate (Fig. 52a,52b,68).

**(ii) The Inverse periodogram.**

In essence this is a modification (due to Gilbert and Joosting, 1994) of norm analysis (Lamprecht and Weber, 1970) which linearises the base line and inverts the plot thereby permitting more easily interpretable 3D plots to be obtained. As such it is based on the auto-correlation function in which the set of data values are compared with itself for different time lags. The 3D plots again allows one to get some idea of whether a particular oscillation is present in the data throughout the duration of the experiment or whether there are frequency drifts etc, as for the Enright periodogram. The same basic options and forms are

available. The method is also subject to similar limitations.

## PHASE PLOTS

The phase plane plot is another method for studying time relationships in glycolysis (see introduction), has also been used for a similar purpose with regard to enzyme and apparent isozyme rhythms. The possible significance of the phasing of processes will be considered later. (Gilbert, 1969, 1974b, Gilbert and Tsilimigras, 1981). In this instance, the corresponding values for two rhythms are plotted against each other. In this case, the oscillations concerned are those for the two proteins X and Y, as determined in the same cell extracts. In the steady state situation, where the behaviour is essentially constant with time, the resultant graphs can clarify the timing relationship between the two periodicities. Thus a line of positive slope would indicate an in-phase relationship whereas a line of negative slope shows that the rhythms were out-of-phase. On the other hand, a circular pattern shows that an intermediate timing relationship existed (Gilbert, 1969, 1974b). The method becomes less useful as the complexity of the rhythms increase.

The phase plots are used to illustrate the timing-relationship between oscillations in different parameters, the value of one being plotted against the corresponding value of the other (both determined at the same time point). In a steady state, one would get plots of positive or negative slope where the two

oscillations are in phase or out of phase, respectively, or a cyclic curve for other stable time relationship. These conclusions really only apply in the simplest situations but the method can be used for various ways, for example, to illustrate change in the behaviour as a result of the action of an agent (Gilbert 1971, 1974, 1984; Tsimiligras, thesis 1982). These phase plots have been used to identify phosphofructokinase as the main oscillator in glycolysis by showing the time-relationship between changes in the different substrates. In Fig 7 and 40 are examples of our data but in view of the complexity of the oscillations studied here, the method has not been generally used, these phase plots confirm other interpretations given, for example, that periods often vary and that several oscillations are involved.

In general, no approach is perfect and one needs to examine the data in several ways in order to determine the self-consistent aspects. In this section we give examples of these different methods and compare and contrast results from different experiments.

**Fig.7a,b PHASE PLANE PLOTS**

Where two oscillations exist in the same cell (as for protein X and protein Y (Ferreira et al 1994a) rhythms and the variations in the two LDH isozymes in the HL-60 cells), it is necessary to determine the timing relationship between them (if constant). One can study this aspect by plotting one value against the other in a phase plane plot, as here. If two rhythms are involved and the system is in a steady state one should obtain simple plots providing the frequencies are the same. In that case a linear plot will be obtained and this will be of positive or negative slope for the in-phase and out-of-phase situations. If an intermediate phase relationship existé one can get a cyclic curve. As can be seen here, no particular phase relationship can be detected for the two sets of values shown which implies the lack of a steady state, different frequencies, the involvement of multiple rhythms or all three possibilities.

PHASE PLOTS
PHOSPHORYLATION POTENTIAL

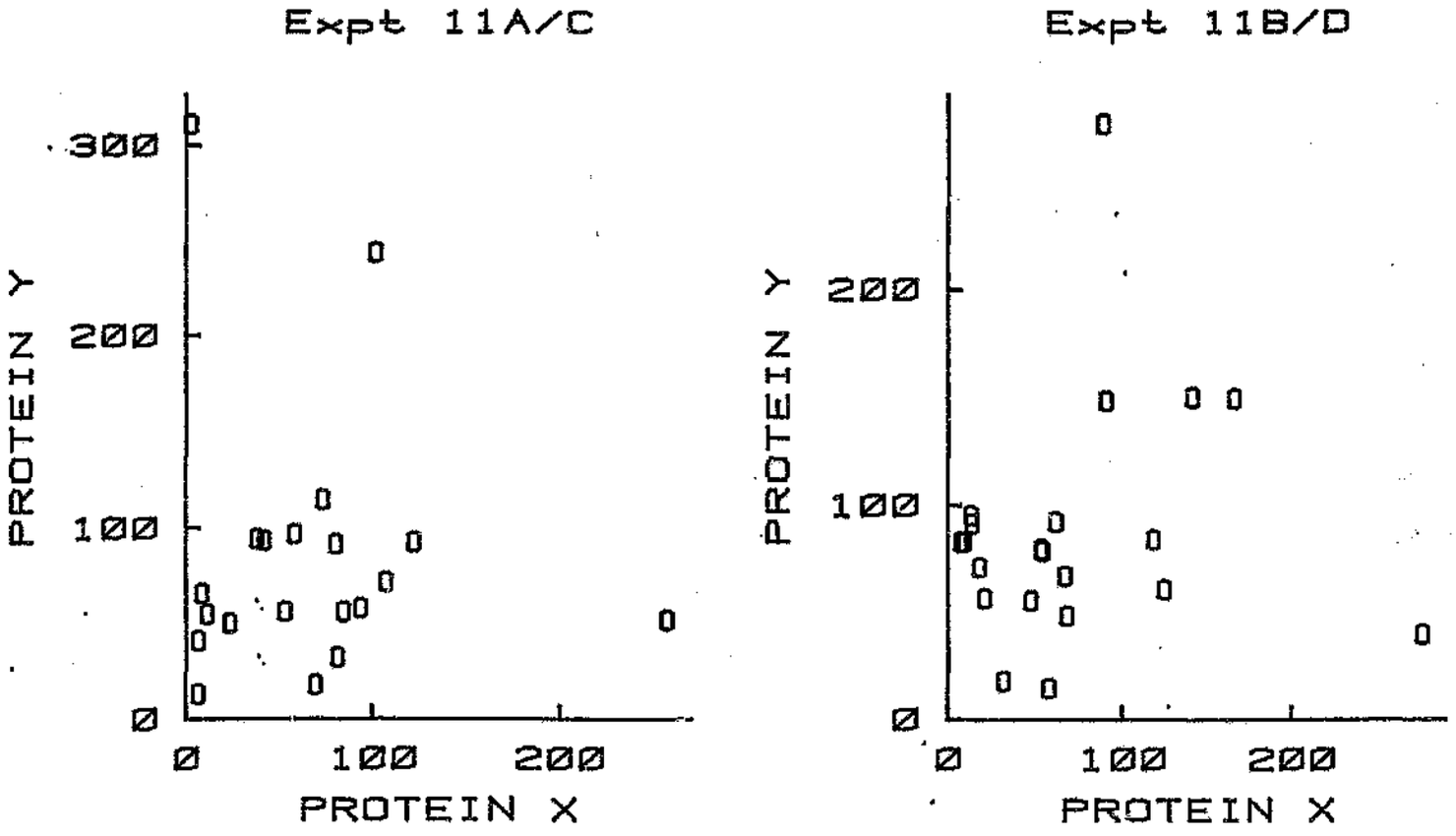



Fig. 7a

PHASE PLOTS
PHOSPHORYLATION POTENTIAL

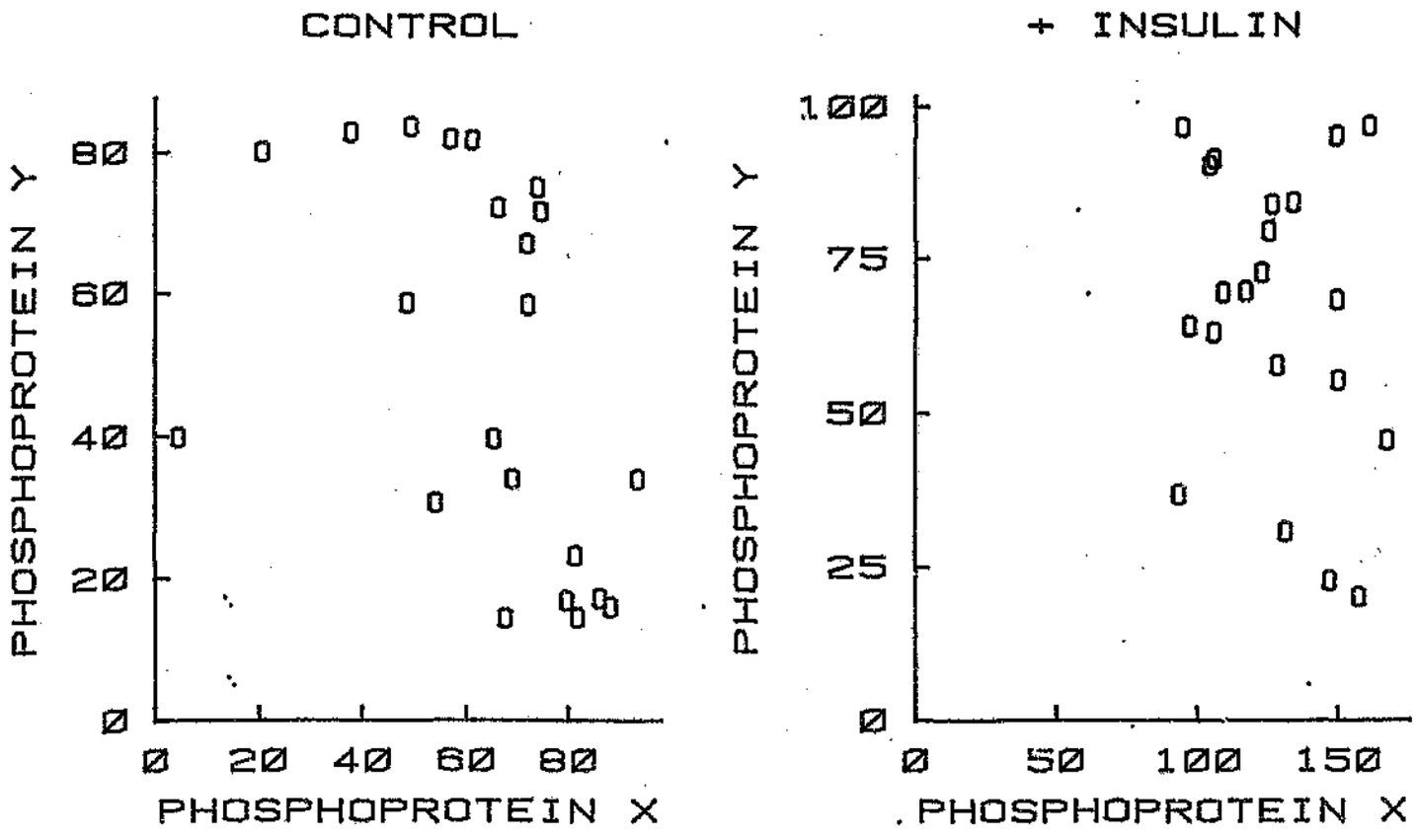



Fig. 7b

# RESULTS

# CHAPTER III

# RESULTS

## GENERAL COMMENTS

In this section diagrams are presented (a selection from some 300) which give:

(i) fitted curves to raw data plotted as a function of time showing the rhythmic nature of the data,

(ii) Per-amp plots indicating the apparent instantaneous period and amplitude the fitted curves from which I deduce that the oscillations are being modulated with respect to period and amplitude,

(iii) overlays where different time curves are superimposed thus allowing one to decide differences in their amplitudes, phasing, period and mean value,

(iv) the occasional phase plot which make me believe that there is a lack of stable temporal patterns of behaviour,

(v) Enright and Inverse periodograms which indicate (a) that several rhythms are generally involved (b) that similar oscillations

are distinct, or (c) that the periods of rhythms are changing with time either spontaneously or as the result of the action of an agent.

Such diagrams are used to illustrate and analyse oscillations in :

(1) Phosphorylation potential,
(2) P-T-Pase activity,
(3) LDH activity, real and apparent isozyme patterns in MEL cells and then in HL-60 cells,
(4) LDH activity and isozyme pattern in cell free systems,

and

(5) in extractable protein levels,

in addition, the plots are used to study the response of those parameters towards insulin or HMBA.

In monolayer serial culture experiments these agents were added at time zero to cultures randomly assigned to control or treated groups and the cultures were than randomly selected at intervals for analysis. This procedure convinces us that the rhythms occur in all cultures. In the suspension culture studies the agent was added (half way through the experiment) to the whole culture. The standard concentrations used in all experiments were:

Insulin: 1 $\mu g/\mu l$ final concentration

HMBA: 5mM final concentration

Some results and discussion for the protein phosphorylation and extractable protein studies are in the press. Attention here is focused on more recent results.

**PHOSPHORYLATION POTENTIAL** (Fig.8-13)

These results show that oscillations occur in the ability of extracts to incorporate $^{32}P$ from labelled ATP into two proteins of $M_r$ 81kD (protein X) and 63kD (protein Y) and that several rhythms contributed toward the observed oscillations. Although the patterns of behaviour for protein X and for protein Y can be the same, at times they are very distinct. Evidence is presented which seems to indicate that the protein X and Y oscillations are partly due to variations in the levels of the corresponding proteins which may vary in unison under some circumstances. The stimulating effect of insulin reported in Ferreira et al 1994b has been further confirmed, (Figs. 8-13, 41-43) and the hormone may synchronise the changes in the 81kD and 63kD protein levels for a short time.

The complex effects of HMBA at different times are shown in Fig.56.

**PHOSPHOTYROSINE PHOSPHATASE OSCILLATIONS** (Figs. 14-16)

Oscillations in the activity of this enzyme (and also in the activities of phosphoserine phosphatase

and phosphothreonine phosphatases) were first reported to occur (in MEL cells) some time ago but, using a much shorter sampling time and a new kinetic assay, it is now shown that the frequency is far higher then appeared to be the case. Of particular interest is the birhythmicity and trirhythmicity frequently seen in this oscillation.

The immediate and long term effects of insulin (Fig.16, 44-46) and HMBA (Fig.57) and also the combined action of insulin and HMBA (Fig.47) are also described.

**LACTATE DEHYDROGENASE** (Figs 17-40)

It is shown firstly that oscillations in the total LDH (pyruvate) activity occur at high amplitude in MEL cells (as with many others) and that the frequency is much higher than described in most studies. It is then shown electrophoretically (for the first time) that oscillations also occur in the actual intensity of the lone active isozyme in MEL cells and these too are of high frequency and high amplitude. Evidence is then presented that the apparent isozyme pattern, as determined by the kinetic method (B/P ratio), also oscillates in MEL cells and does so at a much higher frequency than previously reported for any cells. As only one isozyme is detectable the ratio should be constant and so this rhythm is considered to be the result of variations in the amount of active isozyme and/or to the action of regulators present in the extracts which change

the specificity, or, even to the existence of a ketobutyrate dehydrogenase which must also oscillate in level. In an effort to check this point a second kinetic method (the Py.3/Py.7 ratio) has also been used to determine the apparent isozyme pattern but instead of staying constant as it should do for a single isozyme, one can see that it too oscillates at high amplitude and high frequency. From this data it is considered that the regulators change both the kinetics and the specificity of that isozyme.

Similar changes are detected in extracts of HL-60 cells which exhibit four isozymes of LDH: data is presented which indicates that the two main isozymes are also modulated (as for MEL cells), but to some extent the regulators act on the individual isozymes independently. Curiously, the amplitude of the variations in the apparent isozyme pattern (B/P ratio) is smaller than for the MEL cells where only one isozyme is found.

It is thus shown that, contrary to general belief, the actual and effective isozyme patterns of cells are not constant but change rapidly as a result of a number of different factors. Of particular importance is the fact that, inter alia, insulin rapidly and markedly increases the mean level of the active LDH isozyme in MEL cells.

**CELL FREE SYSTEM**

In addition to observing variations in LDH activity, apparent and actual isozyme patterns in intact cells, similar studies were undertaken in cell and particle free extracts of MEL cells, these are described in Fig.60-63.

**EXTRACTABLE PROTEIN RHYTHM** (Figs.64-67)

The oscillation in the total extractable protein in MEL cells, and the stimulating effect of insulin, have been discussed in Ferreira et al (1994b). Here, for two experiments, we merely show that it occurs at a higher frequency than we previously reported for MEL cells (or any other) and that the modulation characteristics are as for all other oscillations. For the first time it is found also in HL-60 cells. Inverse periodograms are also given for the rhythm in MEL cells where in which show both the enhancing and stabilising effects of insulin. For the first time it is shown that oscillations occur for two particular proteins, those corresponding to protein X and protein Y in the phosphorylation studies (Fig.12a,b).
The results showing the action of insulin are given in Fig.54-55 and that of HMBA are given in Fig.59.

The studies presented can only be considered exploratory and were carried in order to confirm the highly dynamic nature of the cell and to form a basis for future studies. The complexity prevented me from giving definite answers to some questions but it seems possible to prove the existence

of certain rhythms and to show that the oscillations are complex and that the agents have some action on them even if the actual response is not simply described.

All experiments, with the exception of the cell free studies, were carried out at least three times.

# PHOSPHORYLATION POTENTIAL

**Fig.8 AUTORADIOGRAPHS OF SDS-PAGE GELS SHOWING PROTEIN X AND PROTEIN Y IN MEL CELLS FOR CONTROL AND INSULIN TREATED**
(cells in stationary monolayer)

Extracts of cells taken at 10 minute intervals were prepared and each was incubated with [$\gamma$-$P^{32}$] ATP (0.5 X $10^3$ cpm/pmol) in the presence of magnesium acetate for 2 minutes at 30°C. A solution containing TRIS/HCl, SDS, glycerol, mercaptoethanol and bromophenol was added. Samples were boiled and then electrophoresed together with rainbow markers. (see appendix IX). Autoradiographs were carried out. (details see appendix IX). These photographs show representative results with the 81kD (protein X) and 63kD (protein Y) bands studied here. For the following graphs the intensities of the bands were measured by scanning densitometer; the values obtained were multiplied by a factor (usually 0.001) in order to make the labelling of the Y-axis simpler. The area under the curve was taken as the amount of phosphorylated component.

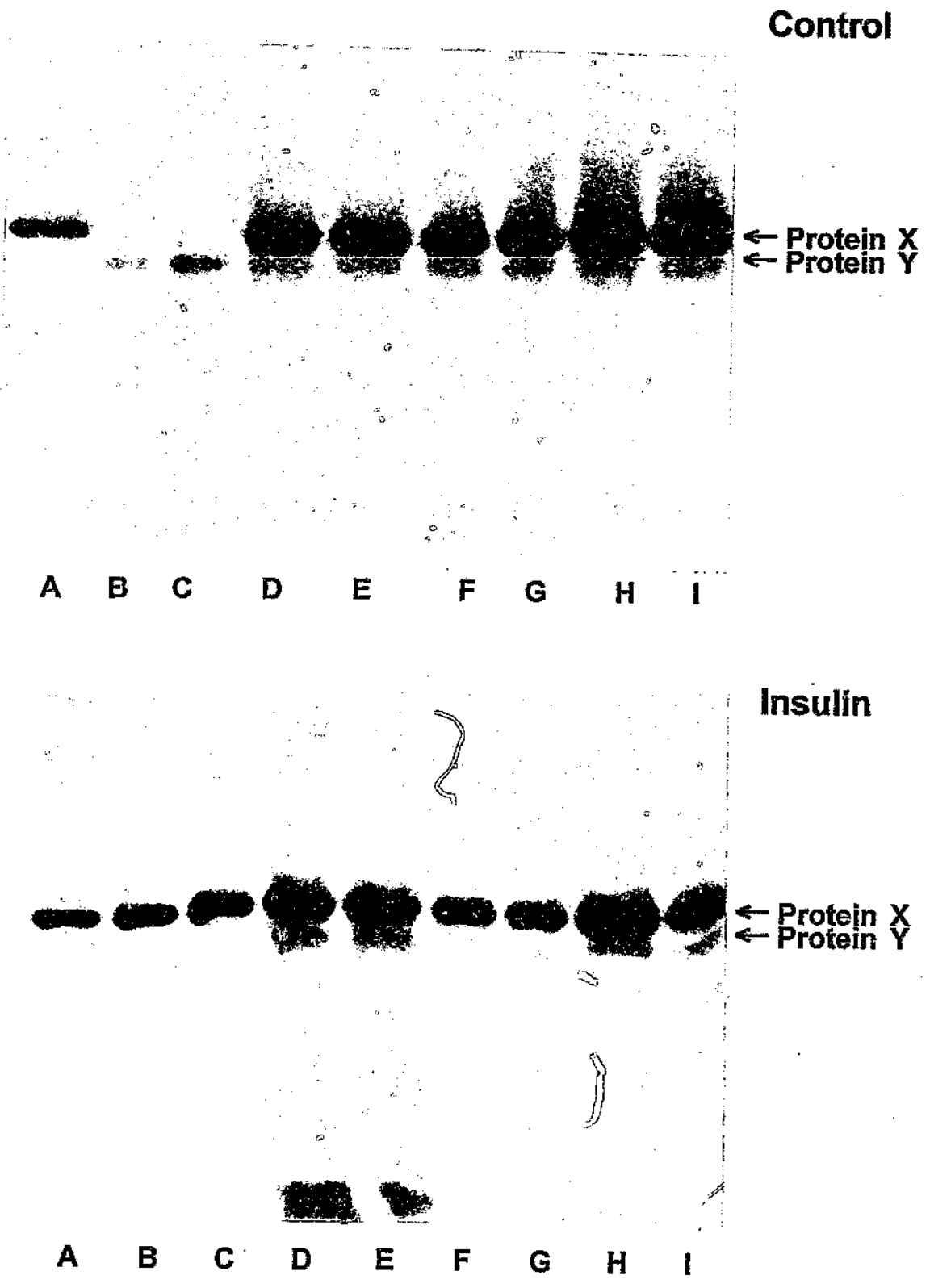

Control
Protein X
Protein Y
A B C D E F G H I
Insulin
Protein X
Protein Y
A B C D E F G H I

Fig. 8

**Fig.9a,b TEMPORAL CHANGES IN THE PHOSPHORYLATION POTENTIAL FOR PROTEINS X AND Y IN MEL CELL EXTRACTS: IMMEDIATE ACTION OF INSULIN**
(MEL cells in stationary monolayers)

These diagrams show examples of variations in the intensities of autoradiographs for the extent of incorporation of $P^{32}$ from labelled ATP into the two proteins of molecular masses 81kD (protein X) and 63kD (protein Y) in the absence or presence of insulin. The X and Y intensity values obtained were measured on the same gel. All phosphorylation potentials were determined using MEL cell extracts. The phosphorylation potential results are discussed further in an article in the press (Ferreira et al 1994). Some more recent results are presented here.

From the lower diagrams it can be seen that each oscillation appears to be rhythmically modulated with respect to period and amplitude. Elsewhere (Ferreira et al, 1994a) it is shown that insulin enhances these dynamics but from the present plots it seems that the oscillations are more regular after addition of the hormone. Multiperiodicity is exhibited in the insulin treated cultures. The curves for insulin treated cells are given here just to show that the hormone does not affect the general pattern of behaviour: the action of the hormone are considered in more detail in a later section.

In various diagrams given here, the intensity values has been multiplied by suitable factors (usually 0.001) in order to make the labelling of the y-axis simpler.

In all the per-amp plots of this kind the amplitude changes are in terms of the corresponding raw data curve (i.e. the relevant upper diagram).

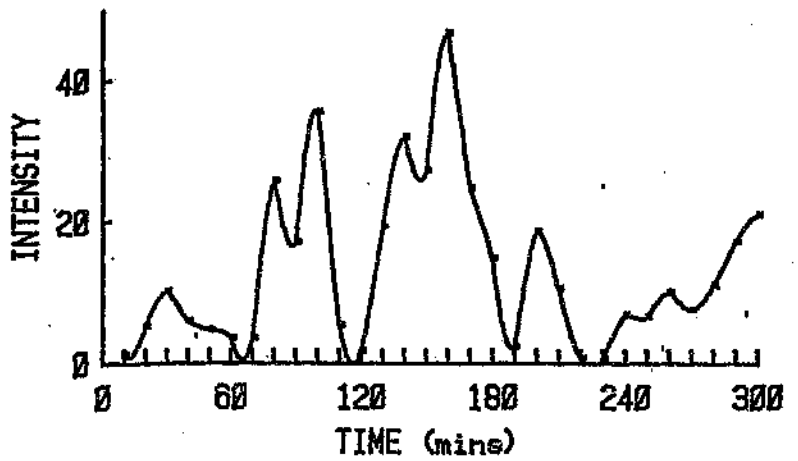
Cell number = $6.80 \times 10^6$ cells/ml

# PHOSPHORYLATION POTENTIAL

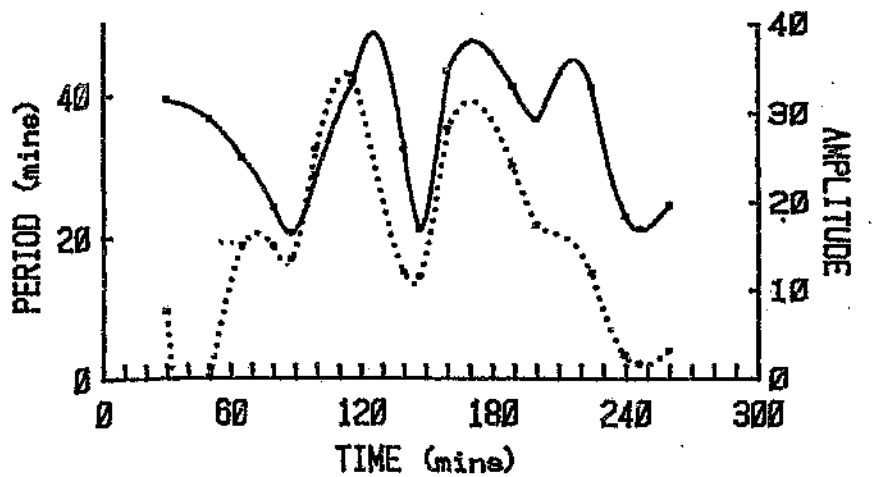
AUTORADIOGRAPH SCANS

Fig. 9a

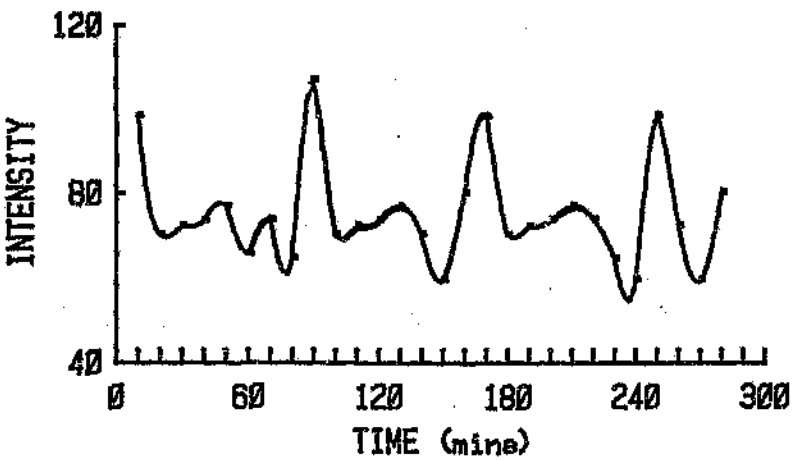
PROTEIN X: CONTROL



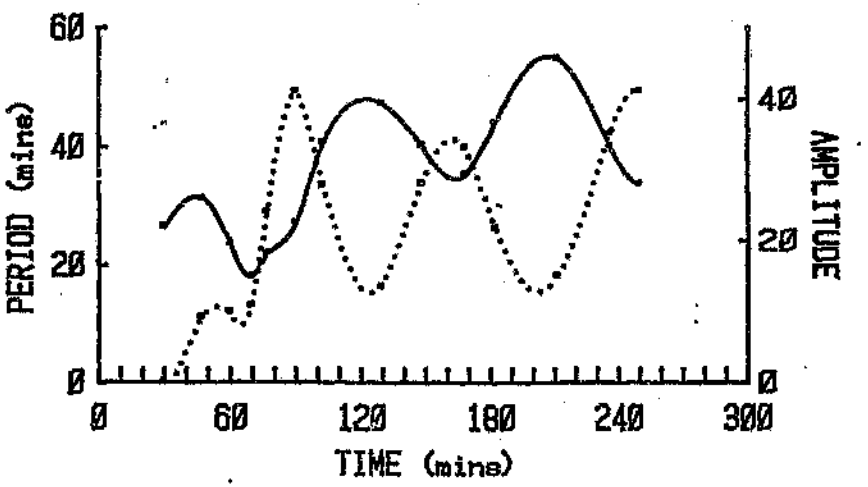

PERIOD ——— AMPLITUDE .....




PROTEIN X: + INSULIN




PERIOD ——— AMPLITUDE .....



Y1-C

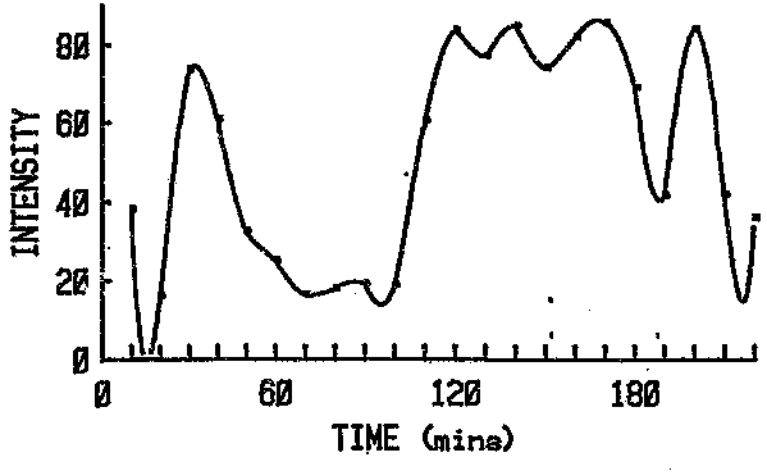
Y1-I

# PHOSPHORYLATION POTENTIAL

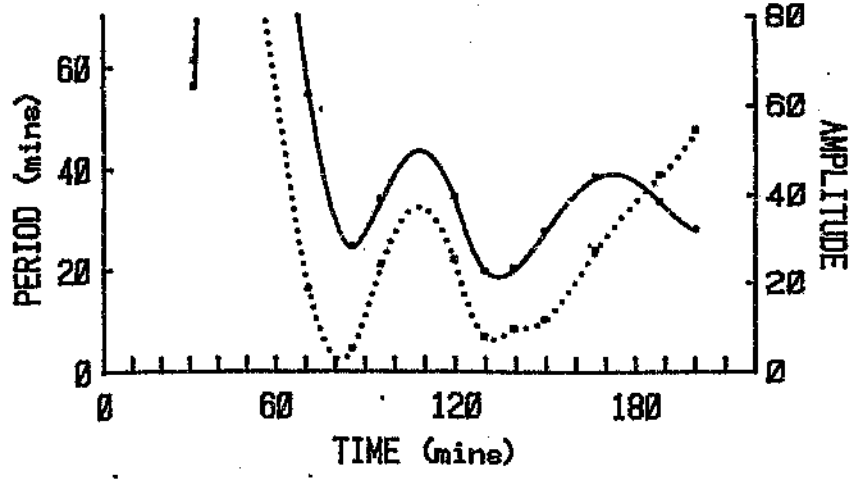
## AUTORADIOGRAPH SCANS

Fig. 9b

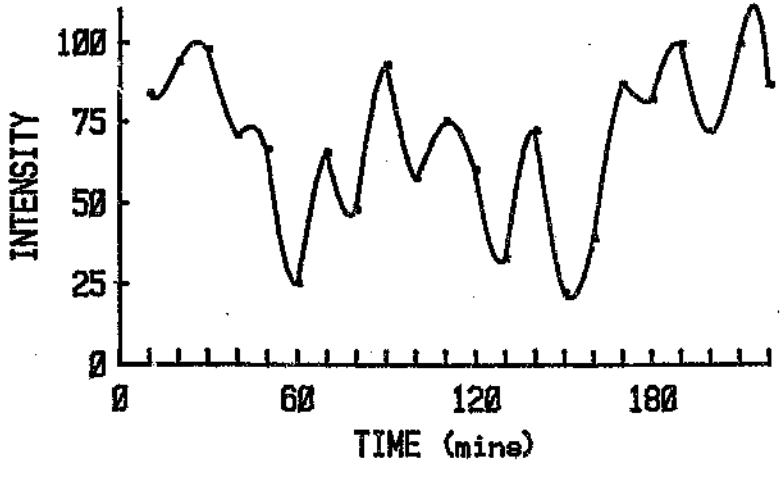
PROTEIN Y1 CONTROL




PERIOD ——— AMPLITUDE .....




PROTEIN Y1 + INSULIN




PERIOD ——— AMPLITUDE .....

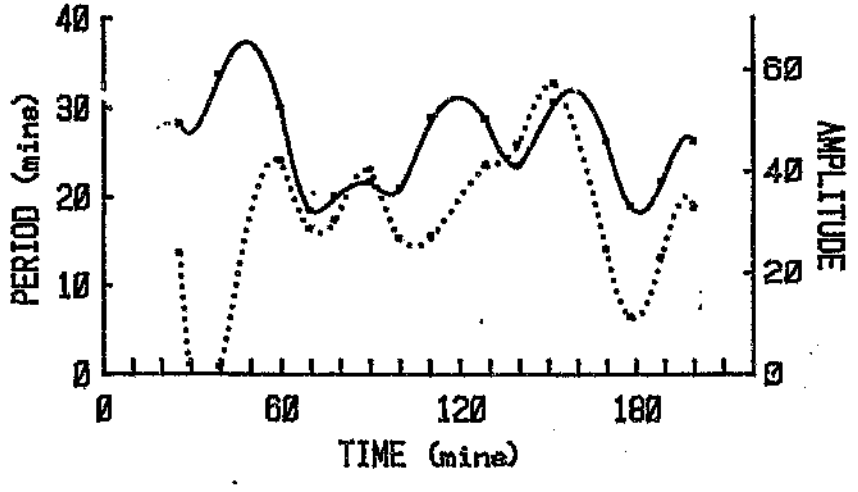


**Fig.10 TEMPORAL VARIATIONS IN THE RATIO OF THE PROTEIN X / PROTEIN Y PHOSPHORYLATION POTENTIALS**
(MEL cells in stationary monolayer)

This diagram shows oscillations in the ratio of the autoradiograph stain intensities for protein X to protein Y for two different experiments in the absence or presence of insulin. The corresponding X and Y values were measured on the same autoradiograph. The oscillatory nature shows that the oscillations of X and Y are independent of one another but the fact that the amplitude is sometimes smaller than at other times is probably due to a changing phase relationship because the two individual oscillations are of different frequencies.

Cell number = 6.80 x $10^6$ cells/ml

# PHOSPHORYLATION POTENTIAL

## PROTEIN X / PROTEIN Y RATIO

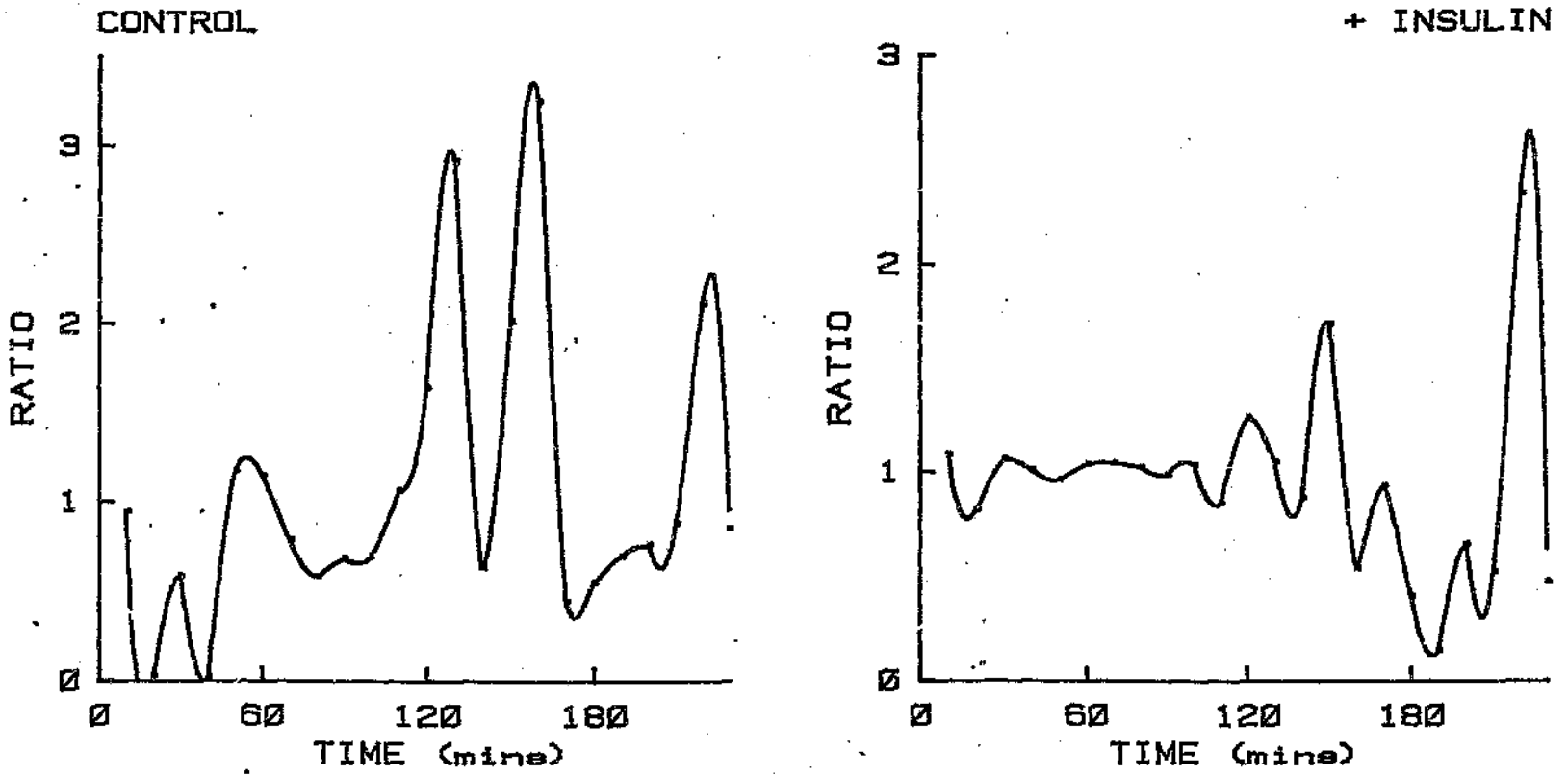



Fig. 10

**Fig.11a,b ENRIGHT PERIODOGRAMS FOR VARIATIONS IN THE PHOSPHORYLATION POTENTIAL OF PROTEIN X AND PROTEIN Y**
(MEL cells in stationary monolayer)

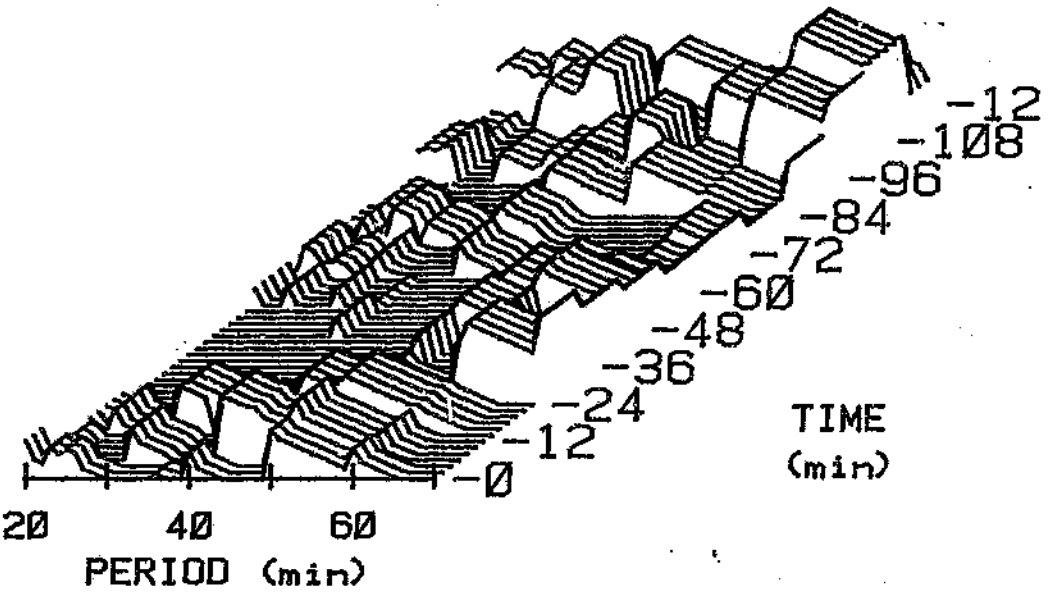
This shows changes in the variance for different oscillation periods as a function of time during the course of one experiment in untreated and insulin treated cells. The complex pattern of behaviour can be seen, several oscillations apparently contributing to the observed time curves (such as those shown in Fig.9). These diagrams are presented in Ferreira et al 1994b.

Cell number = $6.80 \times 10^6$ cells/ml

Window: 15 pts
Period calculation interval: 0.1
Threshold: 0.1
Step interval: 0.2

# PHOSPHORYLATION POTENTIAL
# PROTEIN X

CONTROL




INSULIN

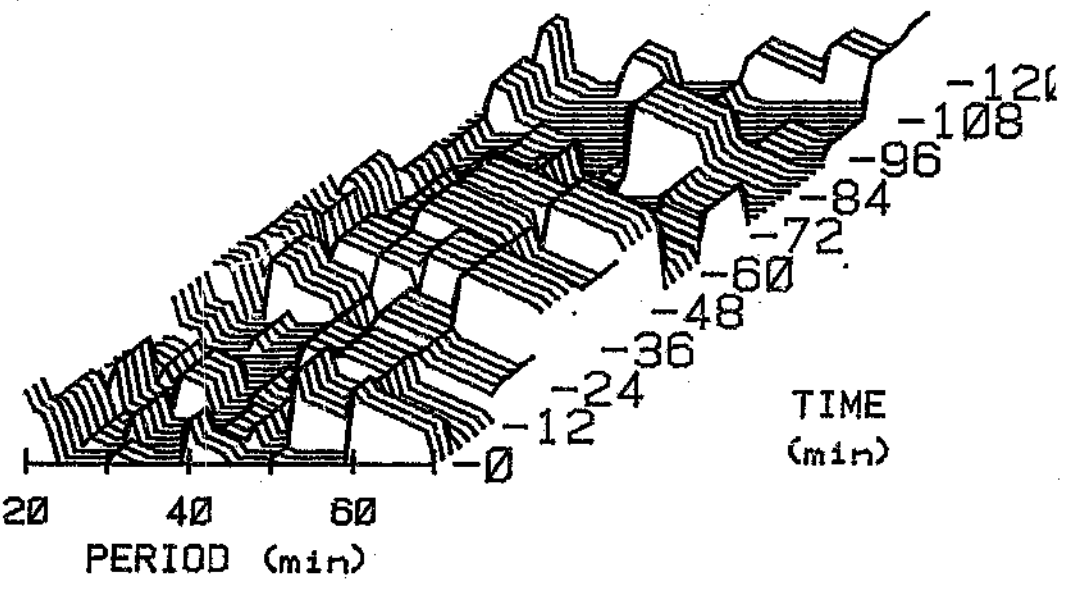
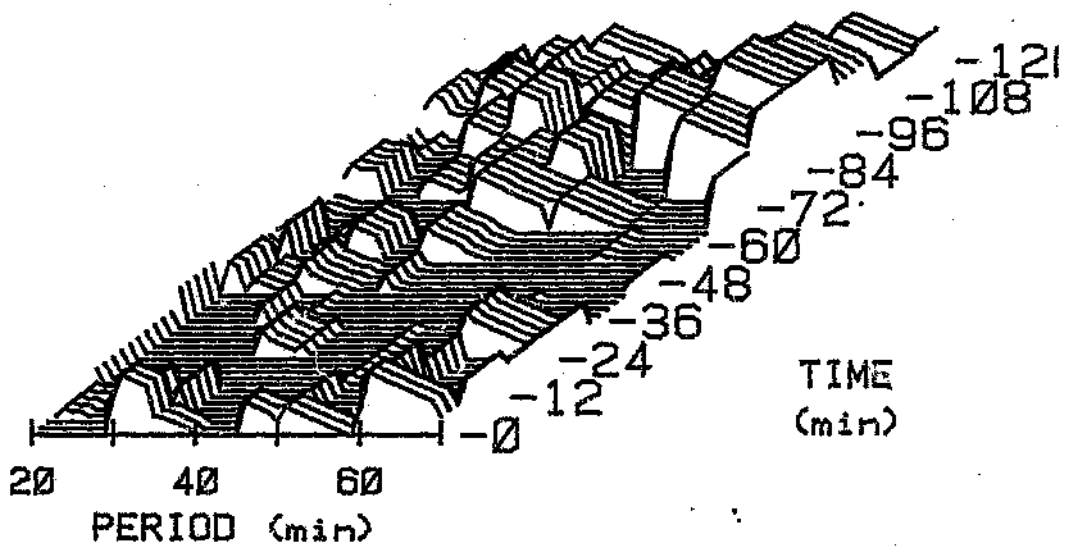



Fig. 11a

# PHOSPHORYLATION POTENTIAL
# PROTEIN Y

CONTROL




INSULIN

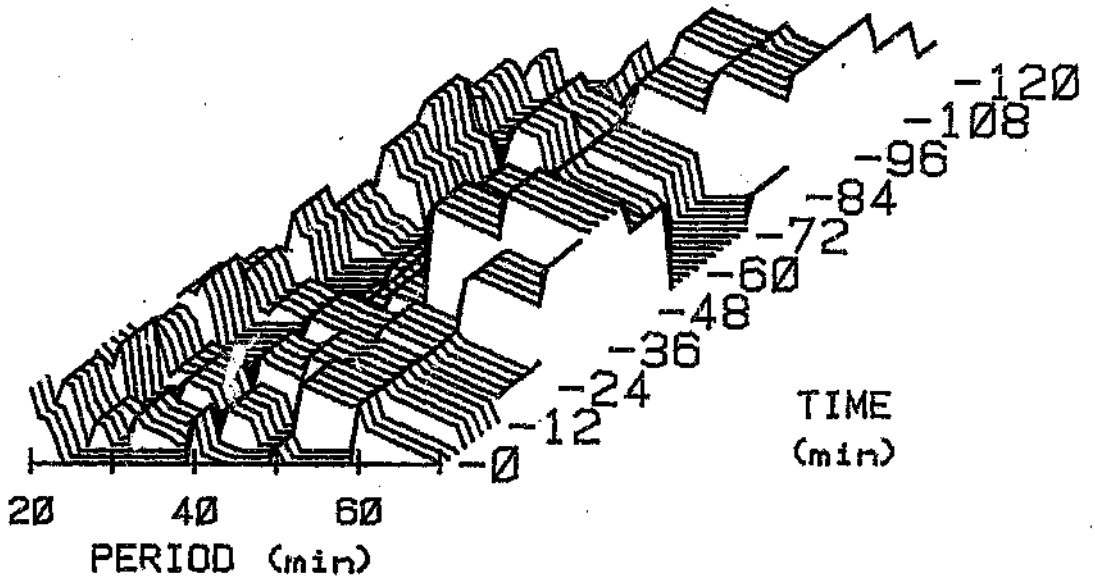


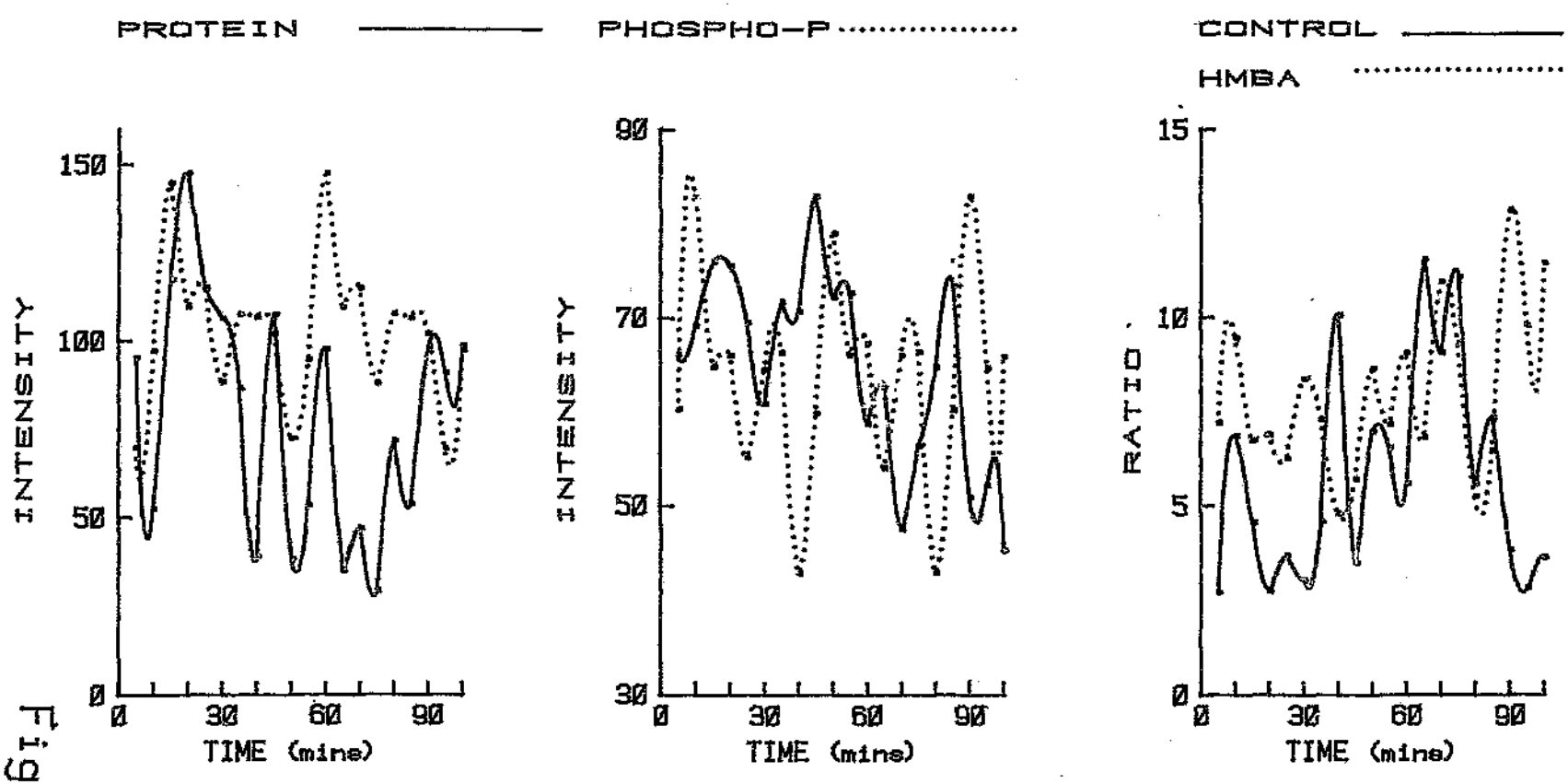

Fig. 11b

**Fig.12a VARIATIONS IN THE AMOUNT OF THE PROTEIN 1 CORRESPONDING TO PHOSPHOPROTEIN X AS MEASURED BY COOMASSIE BLUE STAINING**
MEL cells in stationary monolayer)

In these experiments the SDS-PAGE gels were stained with Coomassie Blue to determine variations in the level of the protein corresponding to the 81kD band. After scanning to determine the intensities the gels were then autoradiographed. The phosphorylation intensity for protein X and the corresponding Coomassie Blue stain intensity variations are shown in graphs 1 and 2 for control and HMBA treated cells in parallel studies. It can be seen that both oscillate but the patterns of behaviour are not the same for the amount of protein and phosphorylation potential. This view is confirmed by plotting, in graph 3, the ratios of the values for the two cultures and it is evident that this parameter also varies in rhythmic fashion. From these and other results (see also next diagram, Fig. 12b) we conclude that the phosphorylation potential oscillation is partly due to changes in the level of the protein being phosphorylated but other factors, perhaps variations in the activities of the kinase or phosphatases also play a part.

The phosphorylation potential values are multiplied by factors of 0.002724 and 0.001271 for graphs 1 and 2, respectively, in order to produce the same maximum values as the protein level oscillations. The values for both ratio curves have been multiplied by a factor of 0.01. Cell number = $6.80 \times 10^6$ cell/ml.

# PROTEIN X PHOSPHORYLATION POTENTIAL
# VARIATIONS IN THE AMOUNT OF 81kD PROTEIN
## COOMASSIE BLUE STAIN INTENSITY




CONTROL

Graph 1

HMBA

Graph 2

Graph 3

Fig. 12a

Fig.12b **VARIATIONS IN THE AMOUNT OF THE PROTEIN CORRESPONDING TO PHOSPHOPROTEIN X and Y AS MEASURED BY COOMASSIE BLUE STAINING IN INSULIN TREATED CELLS**
(MEL cells in stationary monolayer)

Details are as for Fig.12a, with the exception that the data refers to both the 81kD (protein X) and 63kD (protein Y) band. In the control cultures the waveforms for the two proteins were identical as in the initial stages shown here. However, the two curves eventually diverge as can be seen in this diagram. Insulin thus appears to have caused a gradually increasing mean value and produced a more regular oscillation (see Fig.13b,c).

Cell number = $6.80 \times 10^6$ cells/ml

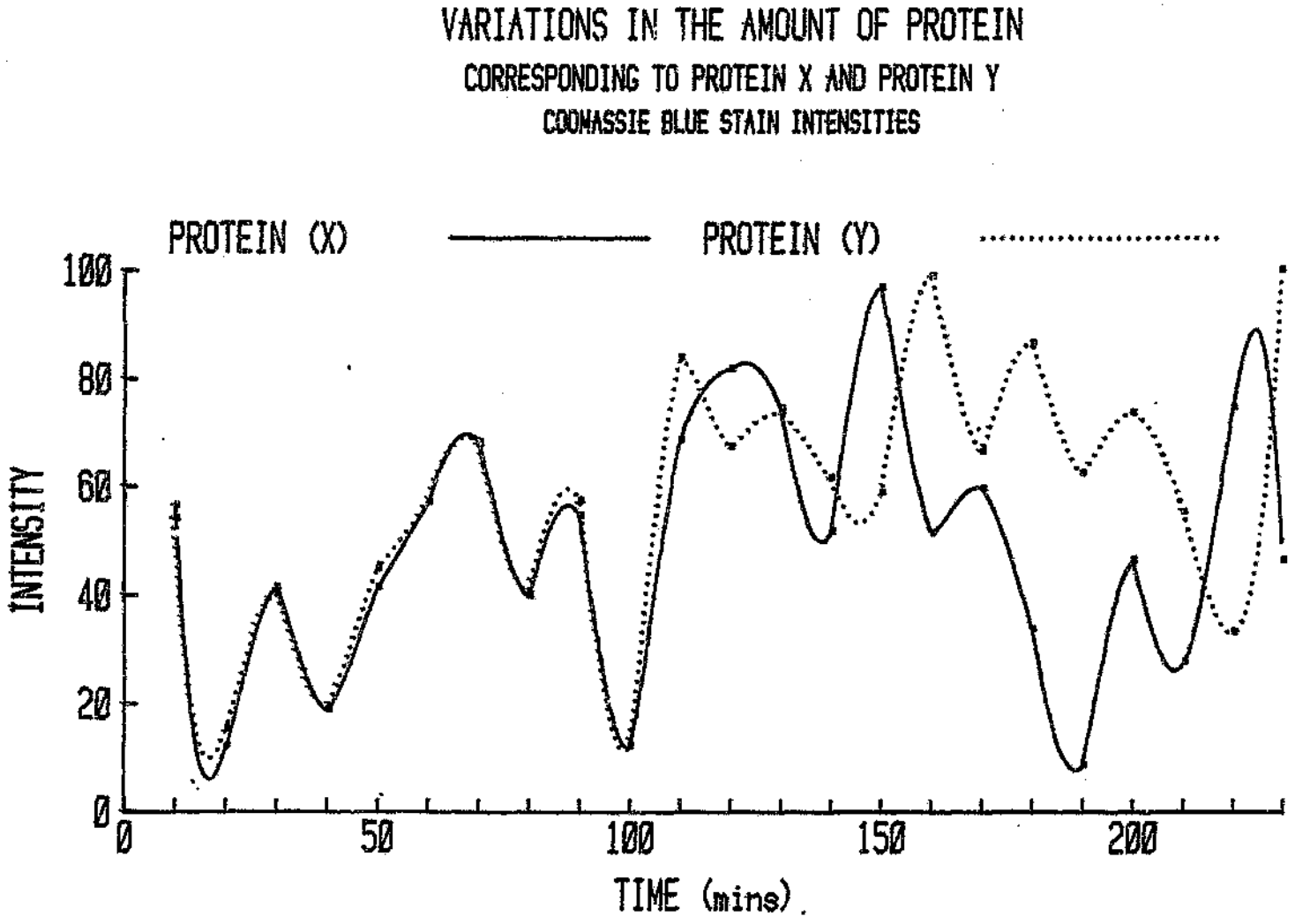

VARIATIONS IN THE AMOUNT OF PROTEIN
CORRESPONDING TO PROTEIN X AND PROTEIN Y
COOMASSIE BLUE STAIN INTENSITIES
PROTEIN (X)
PROTEIN (Y)
INTENSITY
100
80
60
40
20
0
0
50
100
150
200
TIME (mins)

Fig. 12b

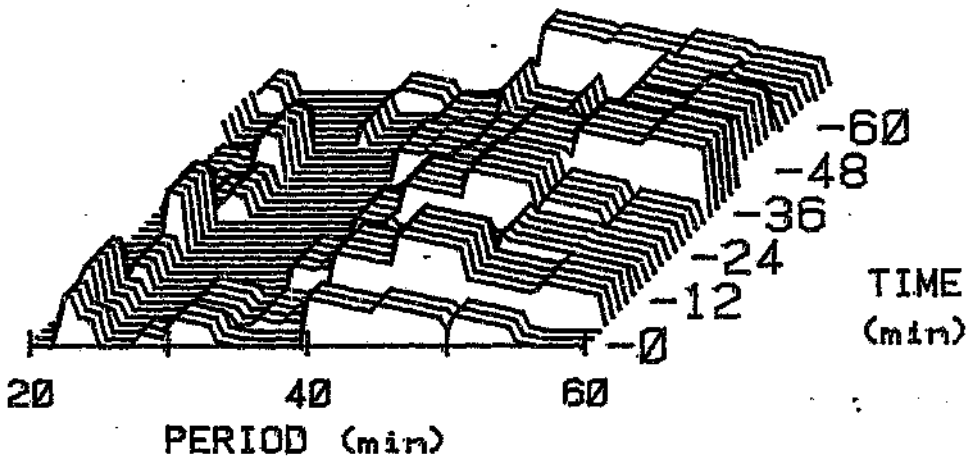
**Fig.13a ENRIGHT PERIODOGRAMS FOR OSCILLATIONS IN PROTEIN X AND THE CORRESPONDING COOMASSIE BLUE STAINED BAND**
(MEL cells in stationary monolayer)

To confirm the belief that the Coomassie Blue stain (81kD) oscillation differs from the corresponding autoradiograph (protein X) rhythm, presented here are these Enright periodograms for the data shown in Fig.12. Despite similarities it can be seen that the phosphorylation potential variations contain additional components supporting the view that factors other than fluctuations in the level of the protein are also involved.

Cell number = $6.80 \times 10^6$ cells/ml

# PHOSPHORYLATION POTENTIAL
# ENRIGHT PERIODOGRAMS

AUTORADIOGRAPH




COOMASSIE BLUE

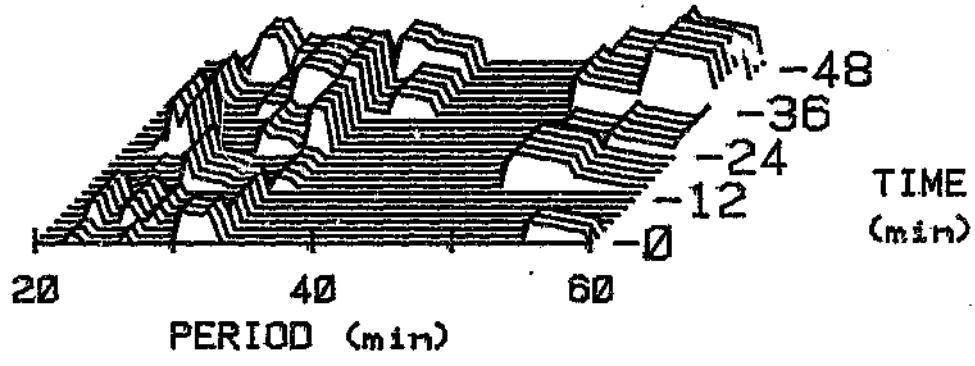


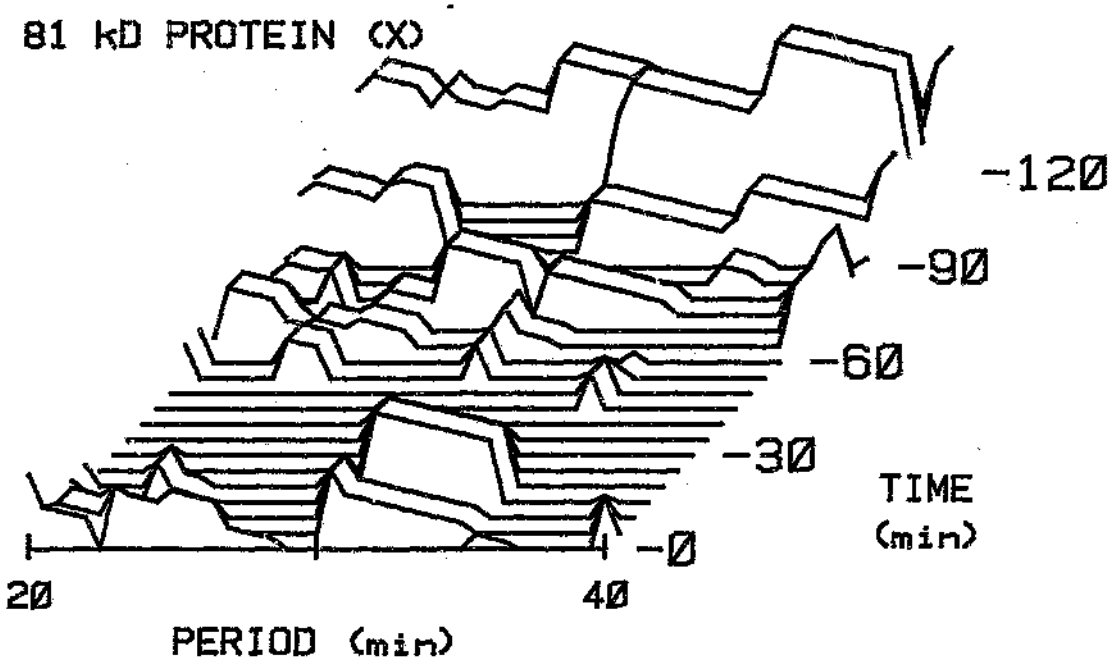

Fig. 13a

**Fig.13b ENRIGHT PERIODOGRAM COMPARISON OF THE OSCILLATIONS IN THE LEVELS OF THE PROTEINS CORRESPONDING TO PROTEINS X AND Y IN CONTROL CULTURES**
(MEL cells in stationary monolayer)

As indicated in the caption to Fig.12b, in control cultures the waveforms for the oscillations in the levels of the 81kD and 63kD proteins corresponding to phospho-protein X and Y (as determined by Coomassie Blue staining) were identical. As can be seen from this diagram the periodograms are also very similar as might be expected.

Cell number = $6.80 \times 10^6$ cells/ml

# COOMASSIE BLUE STAINED PROTEINS
# ENRIGHT PERIODOGRAMS: CONTROL



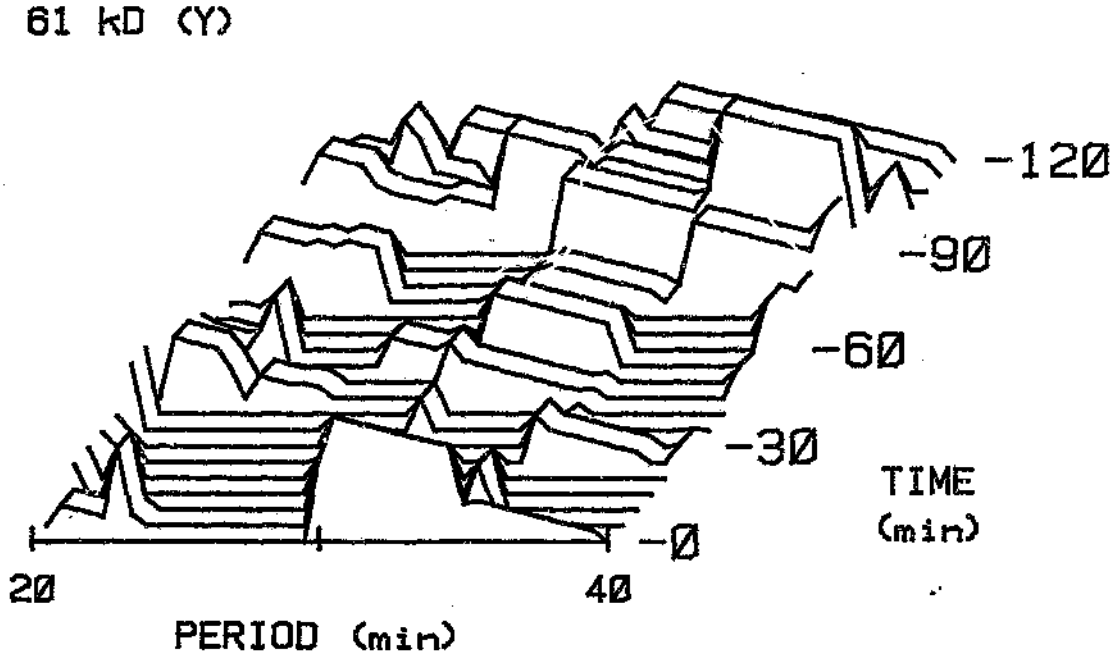
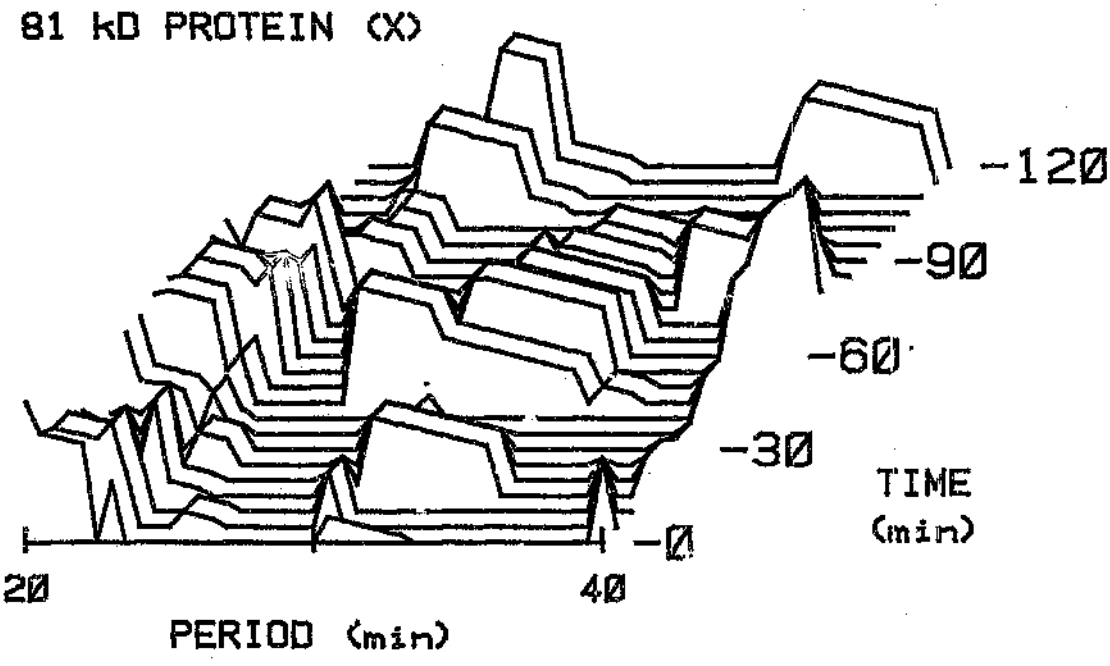




Fig. 13b

**Fig.13c ENRIGHT PERIODOGRAM COMPARISON OF THE OSCILLATIONS IN THE LEVELS OF THE PROTEINS CORRESPONDING TO PROTEINS X AND Y IN INSULIN TREATED CULTURES**
(MEL cells in stationary monolayer)

This diagram gives the Enright periodograms for the oscillations in the levels of the two proteins corresponding to proteins X and Y for which temporal variations are given in Fig.12b. As can be seen, the two diagrams differ in the later stages of the experiment particularly with regard to the rhythm with period about 30 minutes. Therefore, both common and different factors are acting on the two proteins. It would appear that insulin has a delayed and differential effect on this periodicity although it is not clear if the rhythm is stopped or if its period is changed out of the range covered.

# COOMASSIE BLUE STAINED PROTEINS
# ENRIGHT PERIODOGRAMS: INSULIN



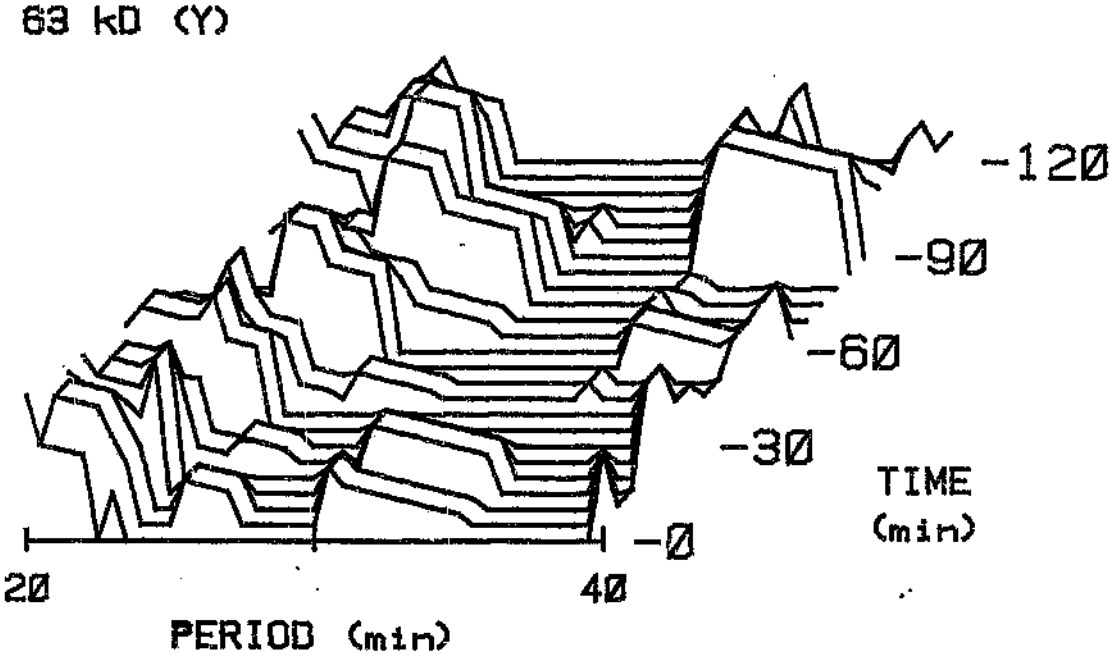




Fig. 13c

# PHOSPHOTYROSINE PHOSPHATASE

**Fig.14a,b,c ACTIVITY AND SPECIFIC ACTIVITY OF PHOSPHOTYROSINE PHOSPHATASE IN MEL CELLS IN STATIONARY SUSPENSION**
(Control cultures: 0 hours.)

These plots show the high frequency (when compared with published data) oscillations in both the activity and specific activity of phosphotyrosine phosphatase in MEL cells in three different experiments. Also given in these PERAMP plots are the variations in the instantaneous period and amplitude for each, as described in the methods section. The specific activities were determined by taking the ratio of the activity of a sample to the concentration of protein in the corresponding extract but as pointed out in the text, (see also later diagrams in this results section) the protein levels also oscillate and hence this "correction" procedure is invalid. Irrespective of this point it is evident that the procedure does not abolish the oscillatory behaviour though it seems to affect the characteristics to some extent as might be expected as the protein level also oscillates.

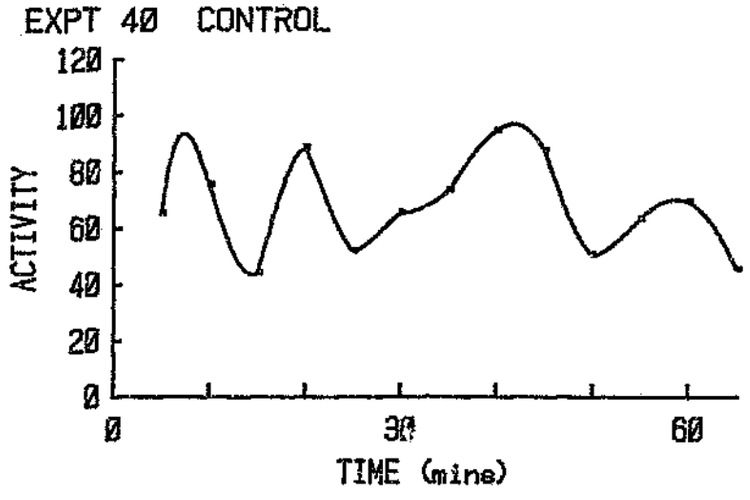
The results show clear evidence for bi- and tri-rhythmicity and to confirm the generality of this behaviour two more plots are given in Fig. 15 a,b. The effects of insulin or HMBA on the behaviour in parallel cultures are given later in sections dealing with the actions of the agents. It would be useful to carry out such determinations and the phosphorylation potentials in the same extracts to compare the frequencies and phasings under different circumstances. Cell number 5.9 - 6.1 x $10^6$ cells/ml.

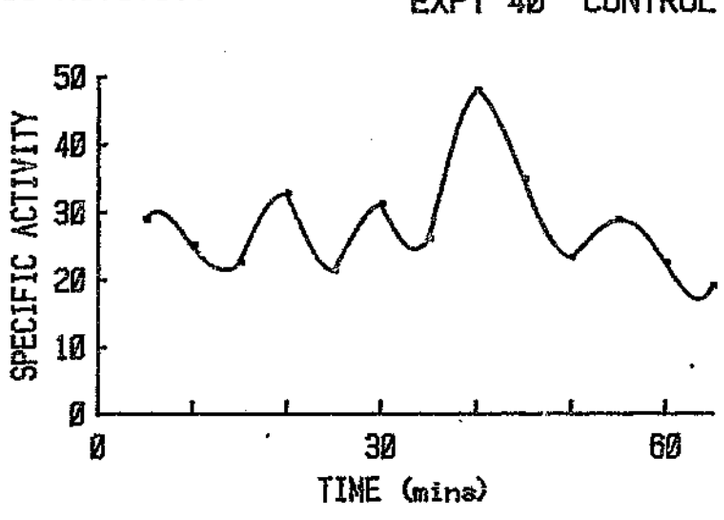
40TPC

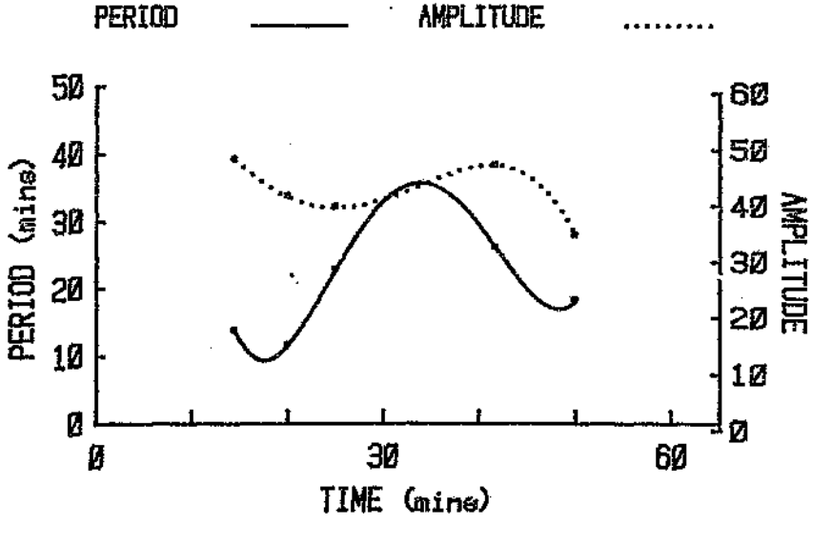
40CT/P

# PHOSPHOTYROSINE PHOSPHATASE

## ACTIVITY vs SPECIFIC ACTIVITY



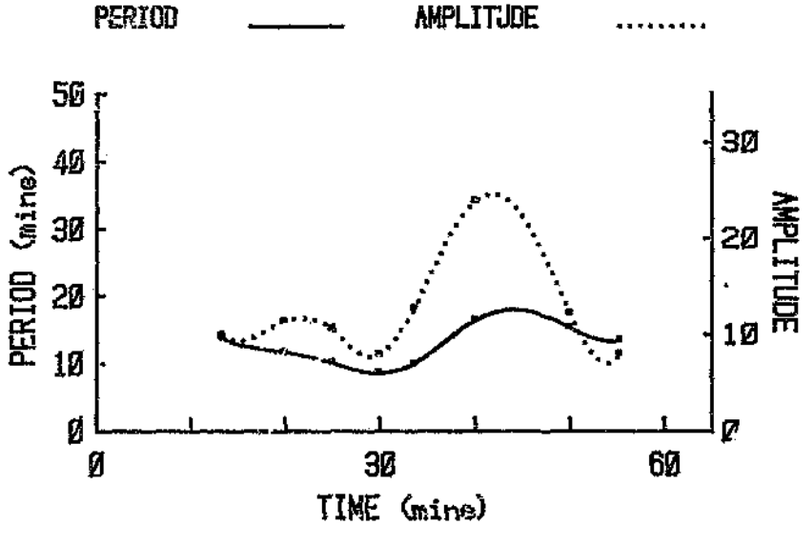




Fig. 14a

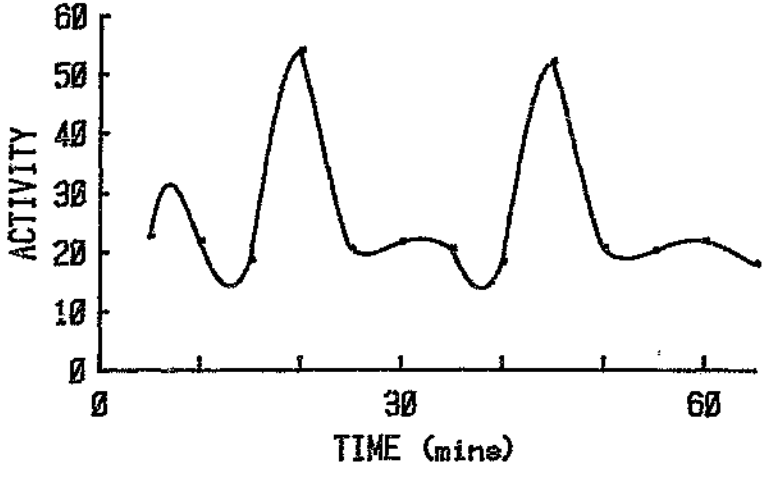
41TPC

41CT/P

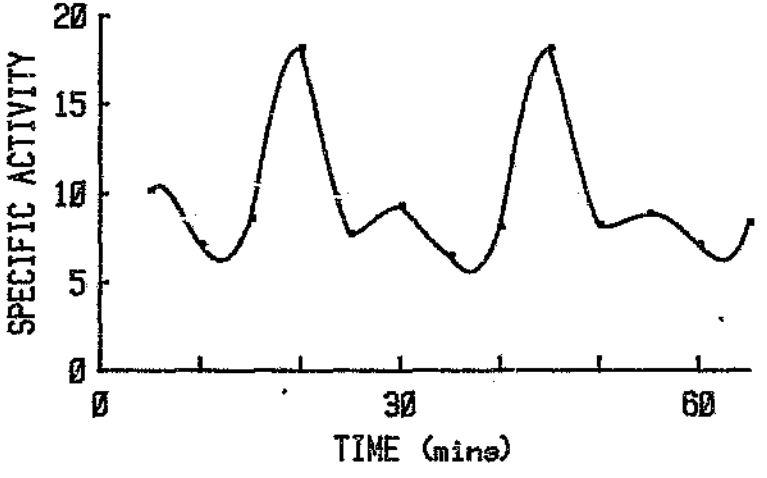
# PHOSPHOTYROSINE PHOSPHATASE

## ACTIVITY vs SPECIFIC ACTIVITY

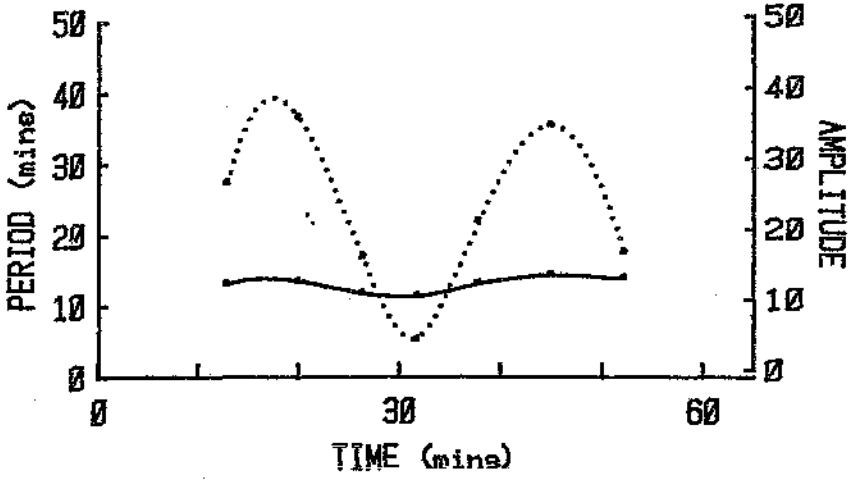
EXPT 41 CONTROL




EXPT 41 CONTROL




PERIOD ——— AMPLITUDE .....




PERIOD ——— AMPLITUDE .....

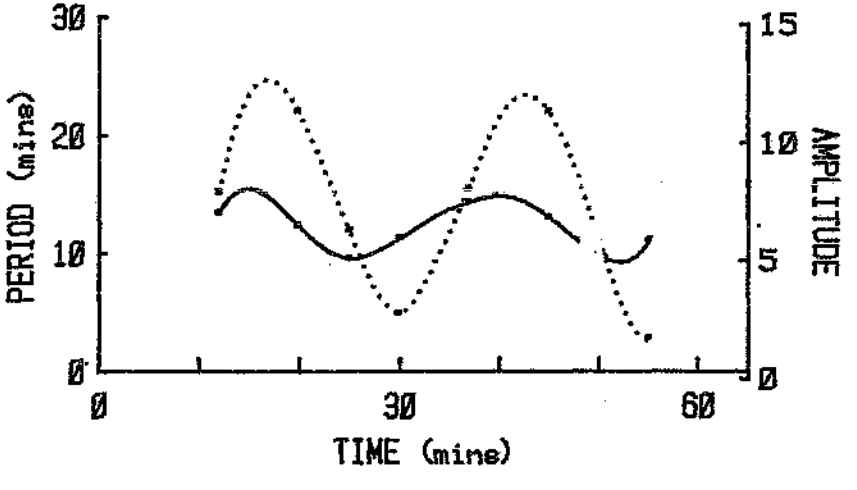


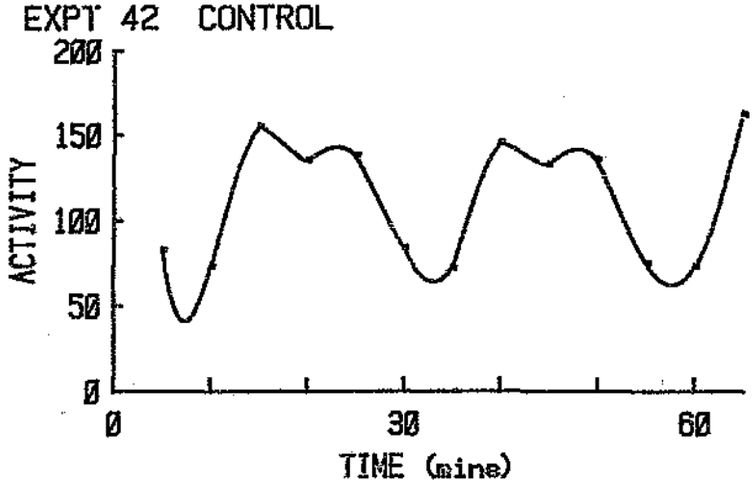

Fig. 14b

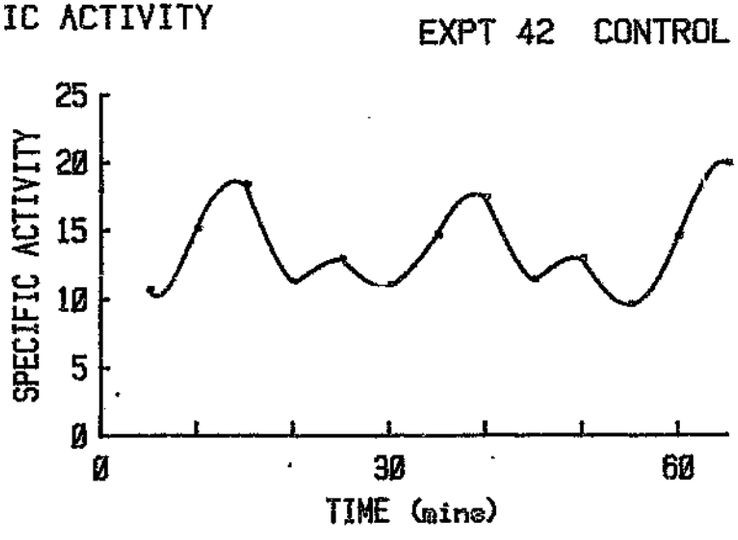
42TPC

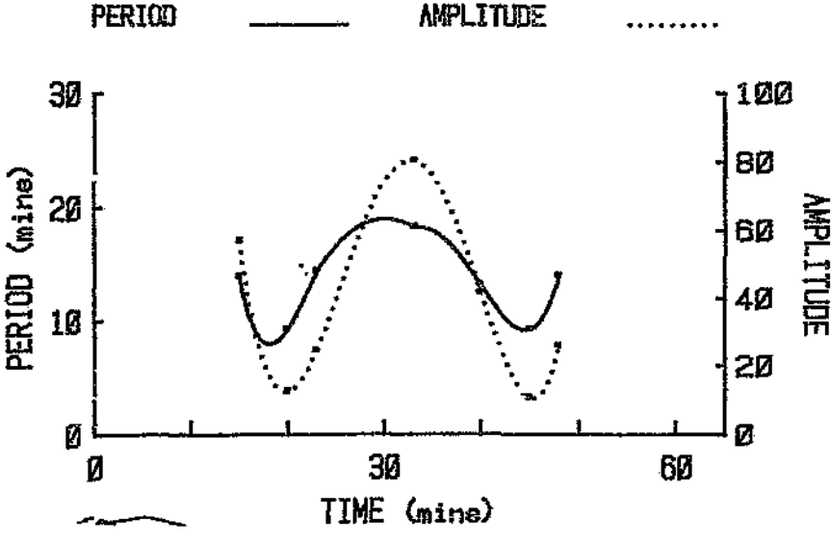
42CT/P

# PHOSPHOTYROSINE PHOSPHATASE

## ACTIVITY ve SPECIFIC ACTIVITY



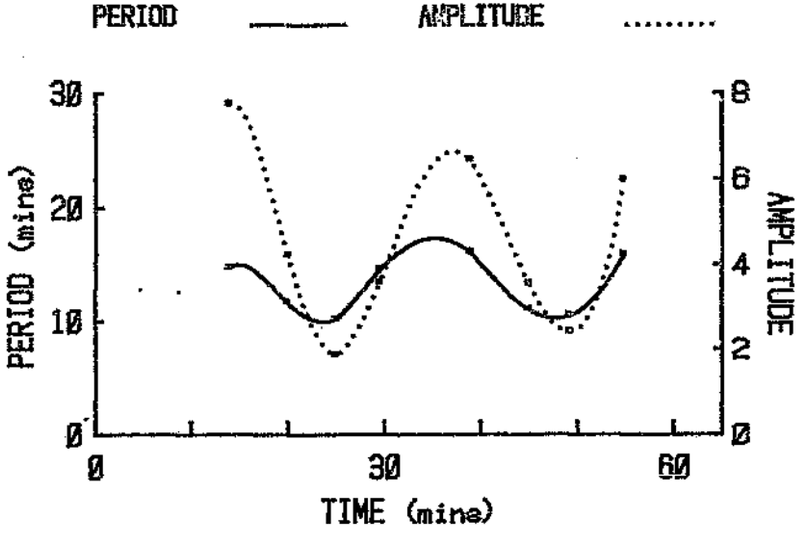




Fig. 14c

**Fig.15a,b. OSCILLATIONS IN THE ACTIVITY AND SPECIFIC ACTIVITY OF PHOSPHOTYROSINE PHOSPHATASE IN MEL CELLS IN STATIONARY SUSPENSION**
(Control cultures: 0 hours)

Two further examples of the phosphotyrosine activity and specific activity oscillations (together with the corresponding changes in period and amplitude) are presented in these diagrams. The details are as given in the caption to Fig. 14.

The activities in all phosphotyrosine phosphatase diagrams are expressed as μmoles/min/ml and the specific activities as μmoles/min/μg protein.

Cell number for a) $6.80 \times 10^6$ cells/ml (exp.1)
b) $5.50 \times 10^6$ cells/ml (exp.2)

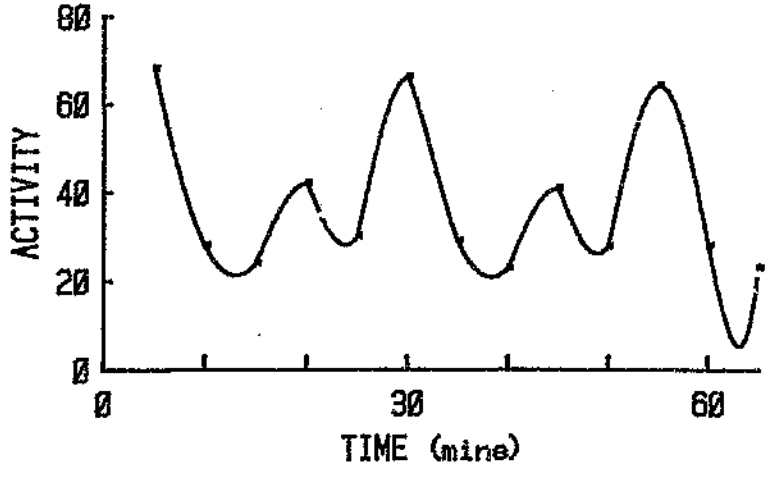
43TPC

43CT/P

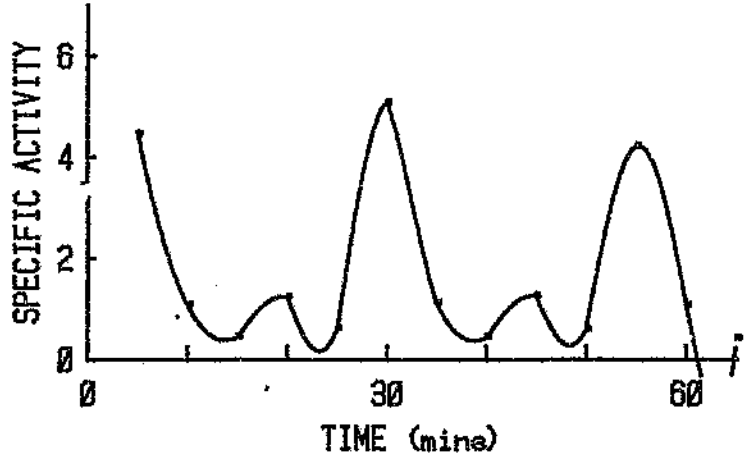
# PHOSPHOTYROSINE PHOSPHATASE

## ACTIVITY ve SPECIFIC ACTIVITY

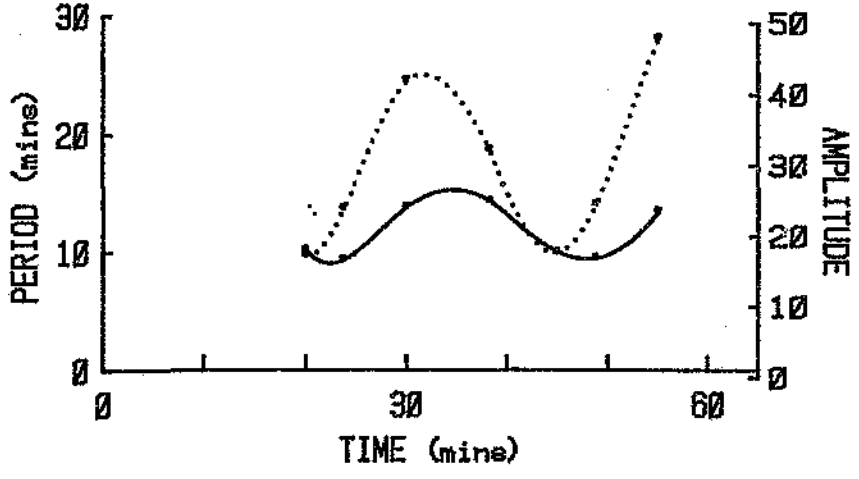
EXPT 43 CONTROL




EXPT 43 CONTROL




PERIOD ——— AMPLITUDE .....




PERIOD ——— AMPLITUDE .....

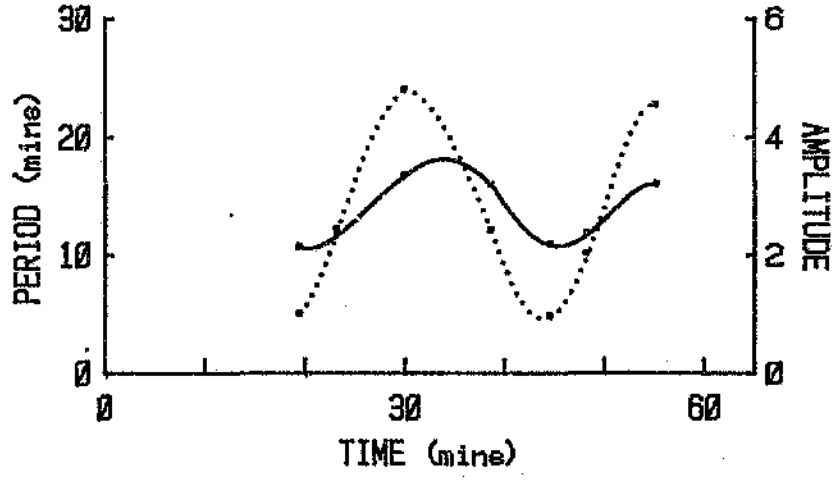


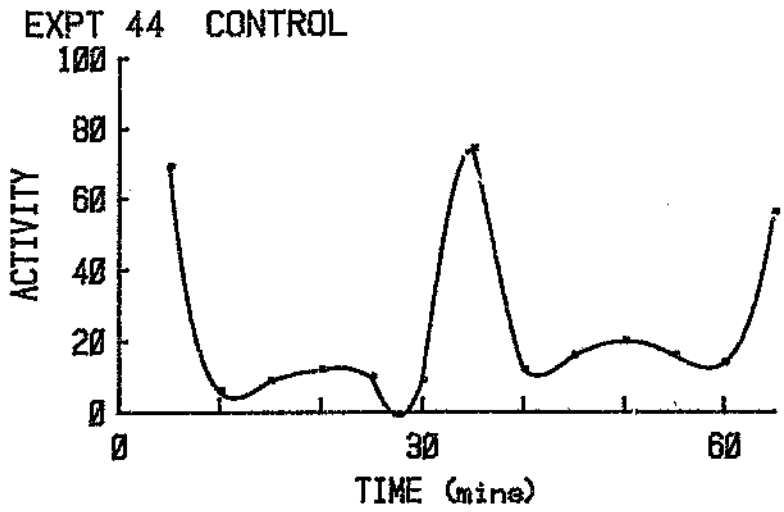

Fig. 15a

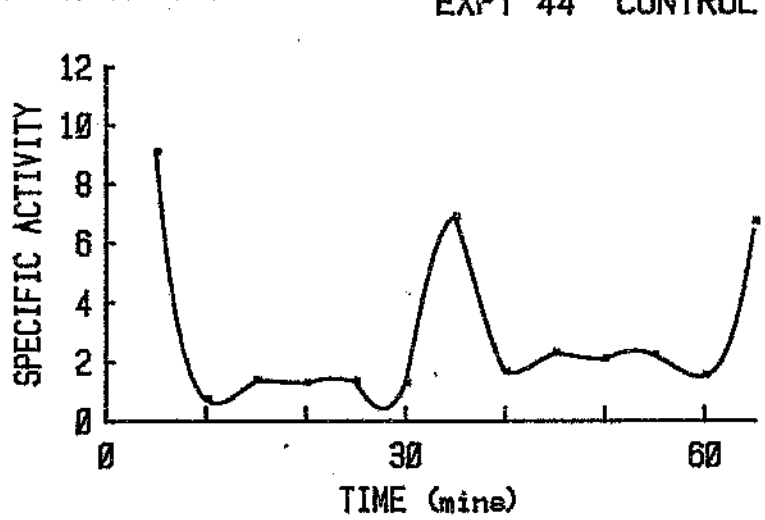
44TPC

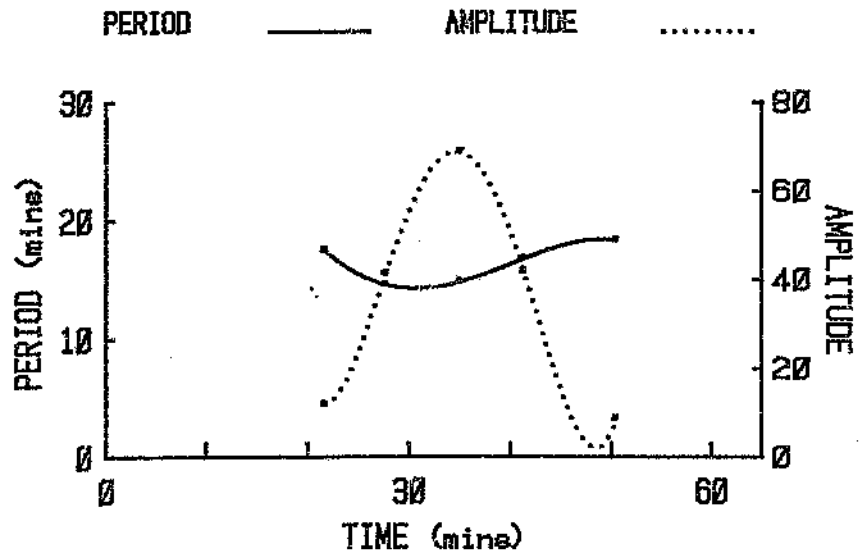
44CT/P

# PHOSPHOTYROSINE PHOSPHATASE

## ACTIVITY vs SPECIFIC ACTIVITY



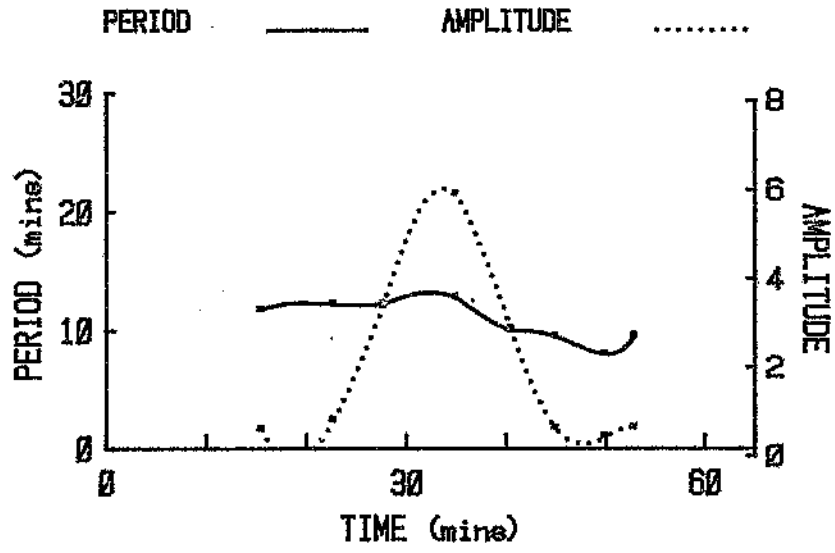




Fig. 15b

**Fig.16 ENRIGHT PERIODOGRAMS FOR PHOSPHOTYROSINE PHOSPHATASE SPECIFIC ACTIVITIES FOR TWO EXPERIMENTS IN THE ABSENCE AND PRESENCE OF INSULIN**
**(MEL CELLS IN MONOLAYER CULTURE)**

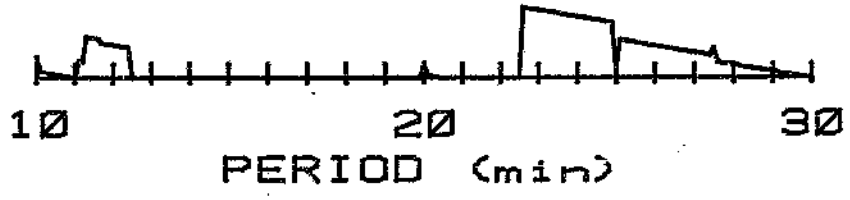
This diagram shows the overall Enright periodograms (the number of data values is too small for 3D time plots) for the two experiments shown in Fig.14a and 14c. It can be seen that a rhythm of about 12 minutes appears in both controls, the broad bands in the longer period regions (22-30 minutes) are probably harmonics. In the insulin treated samples, the twelve minute rhythm is no longer evident but the bands in the 20-30 minute range probably reflect an increase in the period produced by the hormone (compare with the period-amplitude plots of fig.14a,b,c).

Cell number for a) $5.90 \times 10^6$ cells/ml (exp.40)
b) $6.10 \times 10^6$ cells/ml (exp.42)

Window: 12pts
Period calculation interval: 0.2
Threshold: 0

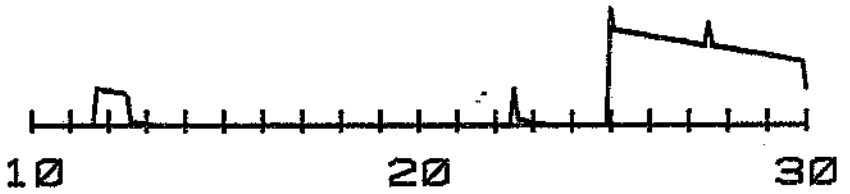
# PHOSPHOTYROSINE PHOSPHATASE SPECIFIC ACTIVITIES

CONTROL
Expt 40




+ INSULIN
Expt 40

CONTROL
Expt 42




+ INSULIN
Expt 42

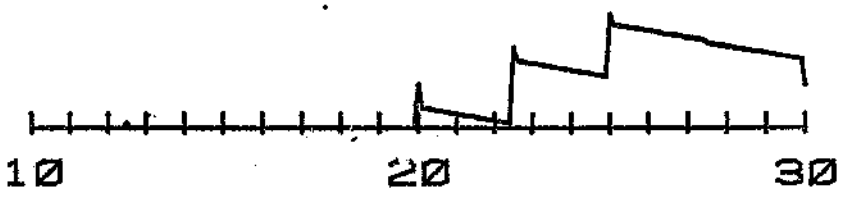



Fig. 16

# LACTATE DEHYDROGENASE

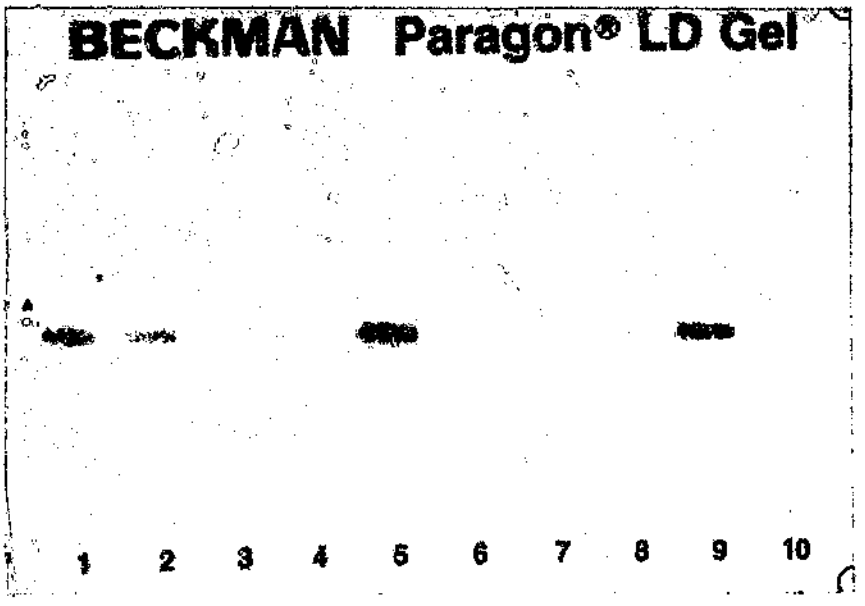
**Fig.17a,b HIGH FREQUENCY VARIATIONS IN THE ISOZYME BAND INTENSITY OBTAINED BY ACTIVITY STAINING AFTER ELECTROPHORESIS**
(MEL cells in stirred suspension)

Shown here, for two different experiments, are photographs of agarose electrophoresis gels (Beckman Paragon LDH kit ,as described in appendix VII), after staining for LDH activity. In both diagrams the top figure is for control cultures and the lower figure is for cells treated with insulin at time zero (control and treated studies carried out in parallel on cells derived from the same initial culture). The sampling interval was 1 minute for each of the ten lanes (1-10) but similar results have been obtained with sampling intervals of 2,5 and 10 minutes, showing that the variations are persistant for 3 hours or more without any evidence of dampening. These results should be compared with those obtained with HL-60 cells (Figs.34). Only one isozyme is evident, despite prolonging the duration of electrophoresis and the staining time (results not shown).

Contrary to general belief, it can be seen that the amount of active isozyme fluctuates with time. Plots of the intensity variations are given in the following diagram.

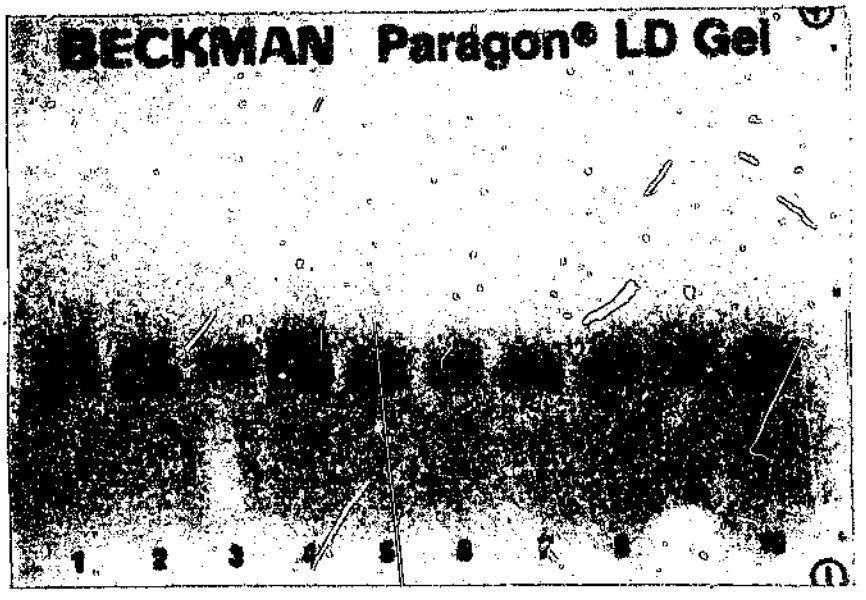
As can been seen in both diagrams, insulin caused a rapid and very marked increase in the mean activity; the metabolic importance of this novel effect is unclear. The oscillation still continues as is more evident in figure 17b.

Cell Number = 2.23 X $10^6$ cells/ml




Control

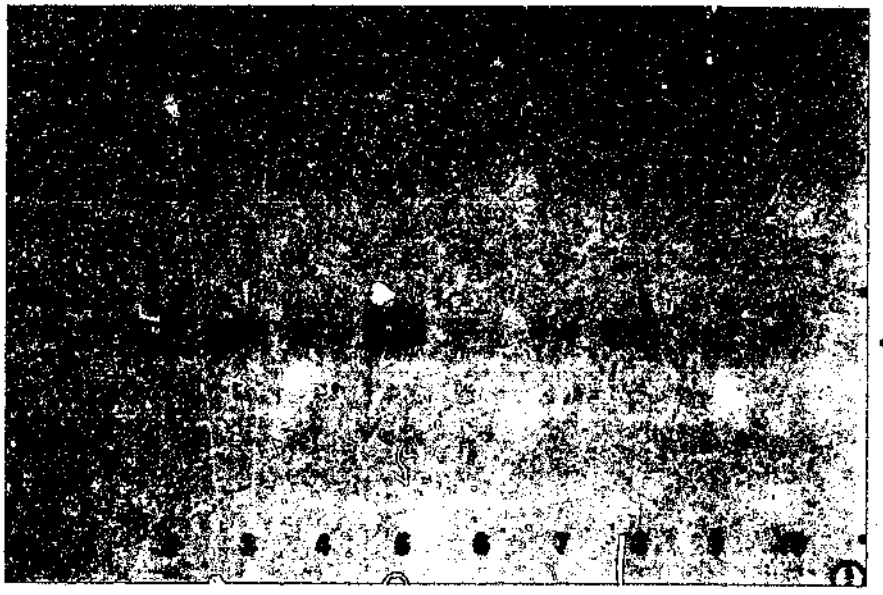
← LDH isozyme band




Insulin

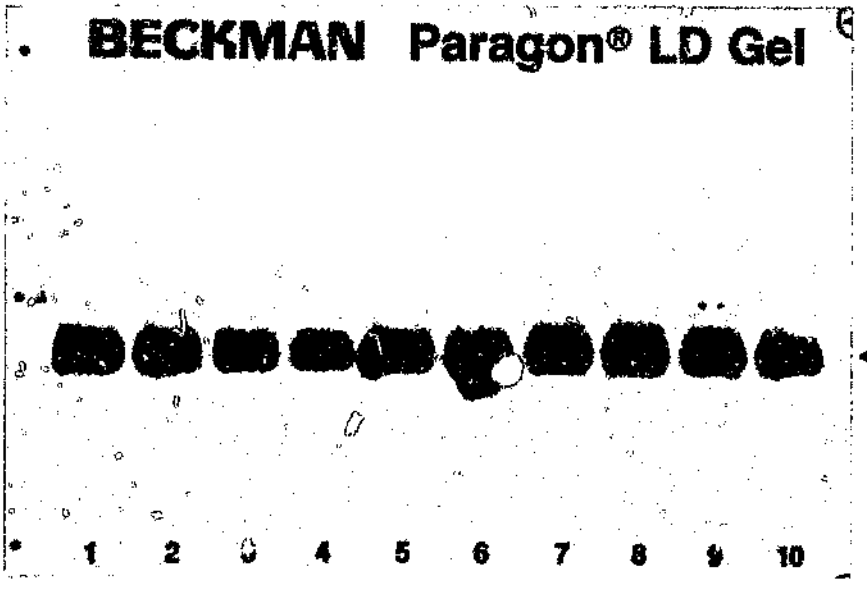
← LDH isozyme band

Fig. 17a



Control

← LDH isozyme band




Insulin

← LDH isozyme band

Fig. 17b

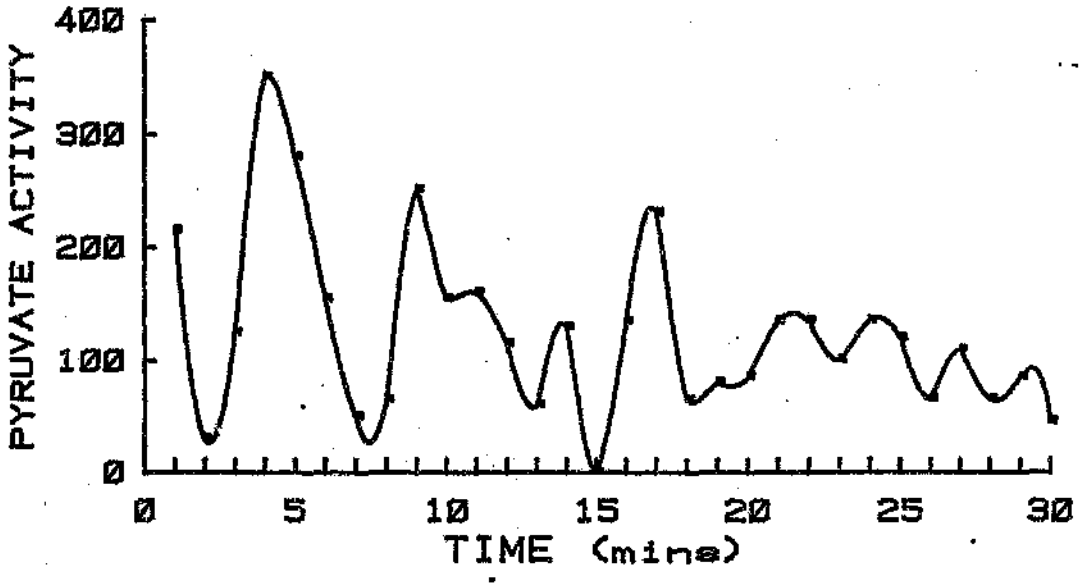
**Fig.18 OSCILLATIONS IN THE EXTRACT LDH PYRUVATE ACTIVITY IN MEL CELLS**
(cells in stationary monolayer)

This diagram shows that the activity of LDH oscillates as it does in many other cell lines but the rhythm has not been reported for MEL cells. However, the period (of the order of 1-2 minute or less) is less than given for most cells because of the shorter sampling time used here. The results also show that the period and amplitude are periodically modulated as for variations seen in other cells (Gilbert, unpublished). Oscillatory variations occur in the protein level as discussed in other sections. Later it will be shown that oscillations are still observed even if one determines the specific activity for the extracts.

In all the LDH diagrams the activity is expressed as the rate of change in absorbance at 340nm per minute.

Cell number = $1.23 \times 10^6$ cells/ml

# LDH PYRUVATE ACTIVITY



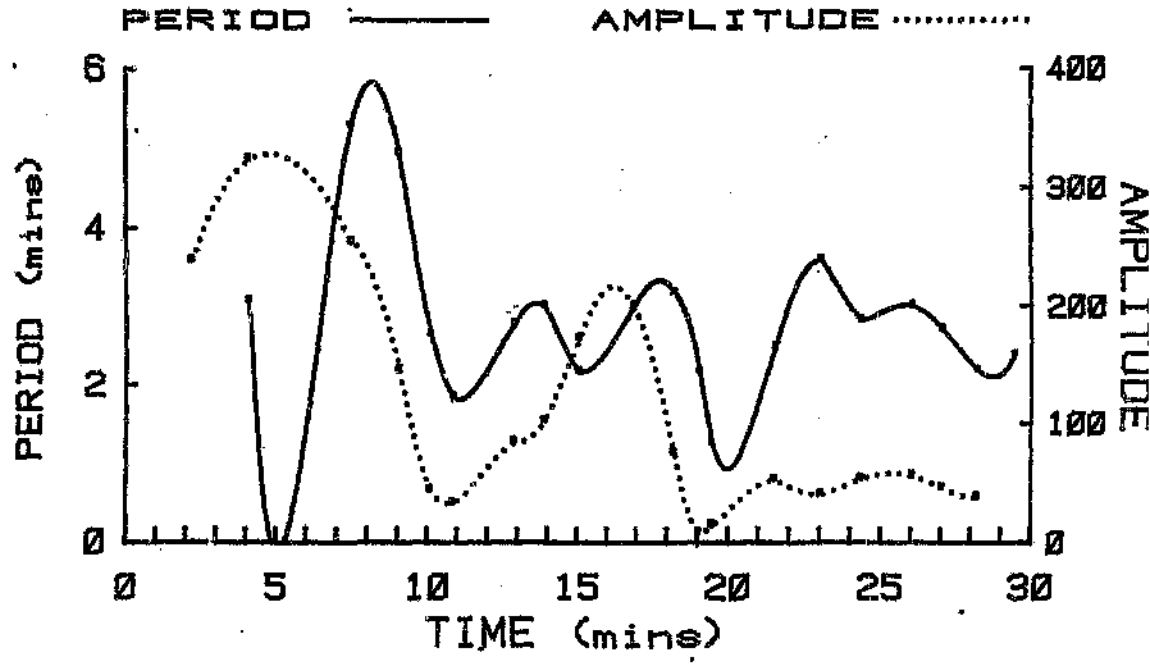




Fig. 18

**Fig.19 HIGH FREQUENCY VARIATIONS IN THE ISOZYME BAND INTENSITY OBTAINED BY ACTIVITY STAINING AFTER ELECTROPHORESIS**
(MEL cells in stirred suspension)

Shown here are plots of the intensity variations for the activity stain corresponding to gel photographs of the kind given in Fig. 17 for two different experiments. The very good agreement is evident and it is clear that the period is of order of 2-3 minutes (or less - see text). It can also be seen that the variations are quite marked in magnitude.

Cell number for a) $7.25 \times 10^6$ cells/ml (exp.53)
b) $6.99 \times 10^6$ cells/ml (exp.54)

LDH ELECTROPHORESIS
ACTIVITY STAIN

53GEL
54GEL

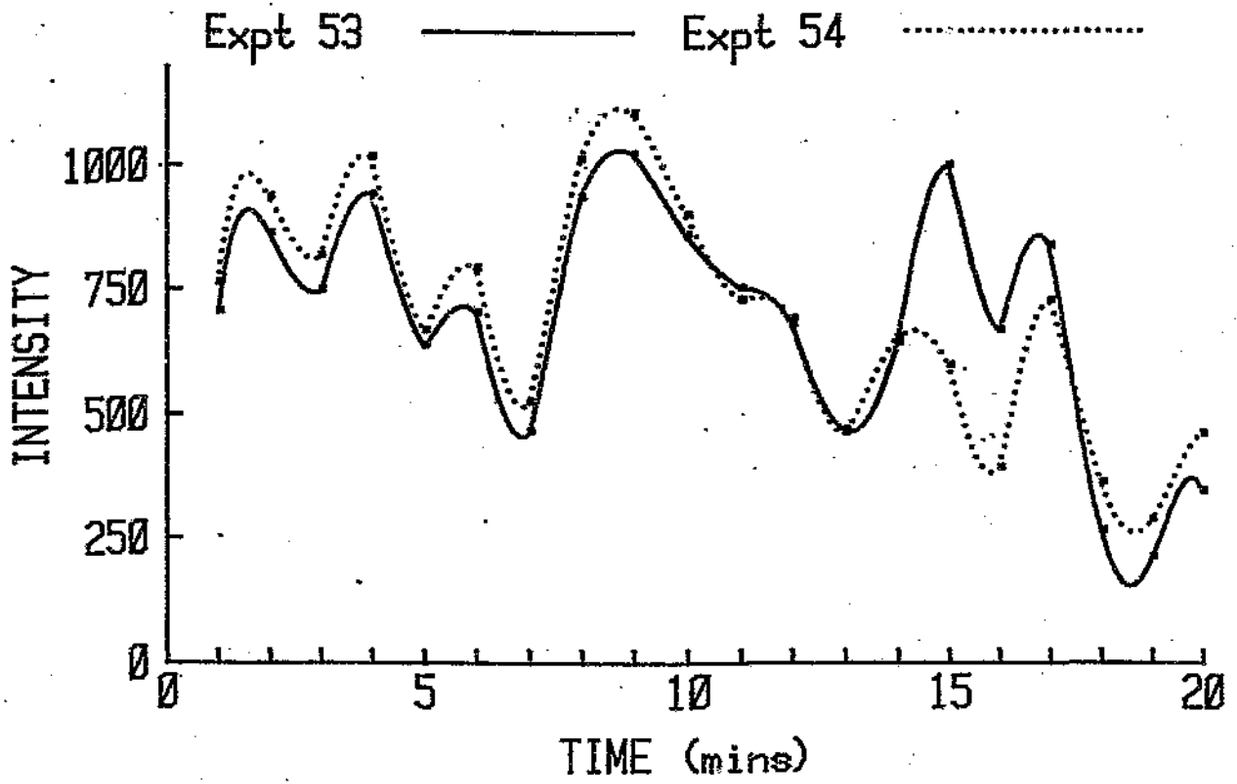


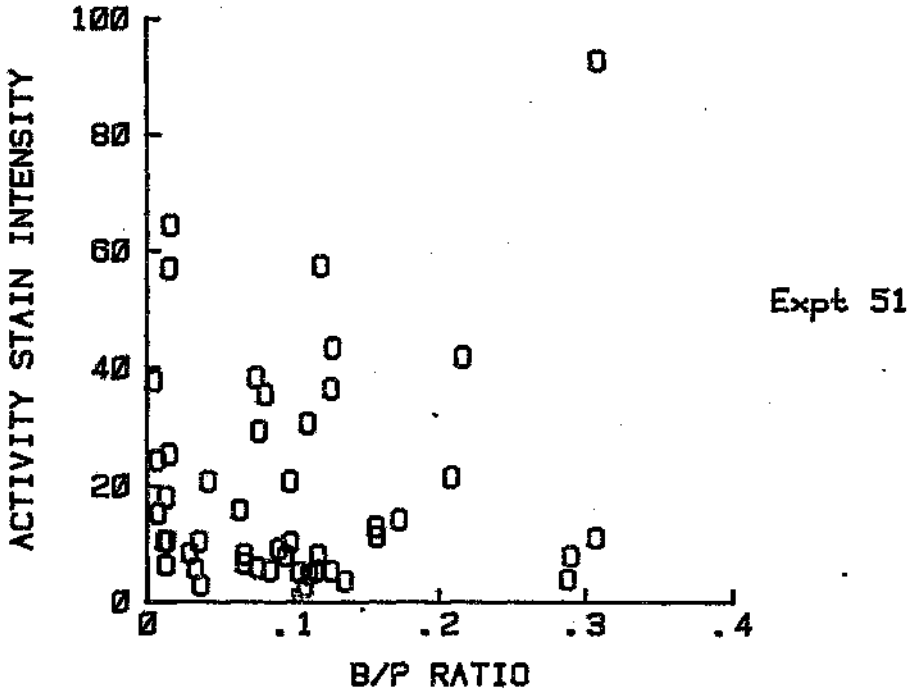

Fig. 19.

12.6.93 CYN 4

**Fig.20 PHASE PLANE PLOTS FOR THE TWO RATE RATIOS: APPARENT LDH ISOZYMES**

Given here are the phase plane plots for data from two experiments showing the lack of a stable time relationship between the two rate ratios, (B/P and Py.3/Py.7), the values of which should give a measure of the LDH isozyme pattern. These diagrams add further weight to the arguments (see Fig.32) that the two parameters are actually measuring different processes so that the ratios are not equivalent. In turn, this agrees with the view that regulators are affecting both the kinetics and the specificity of the enzyme.

# LACTATE DEHYDROGENASE ISOZYME PHASE PLOTS: APPARENT vs ACTUAL



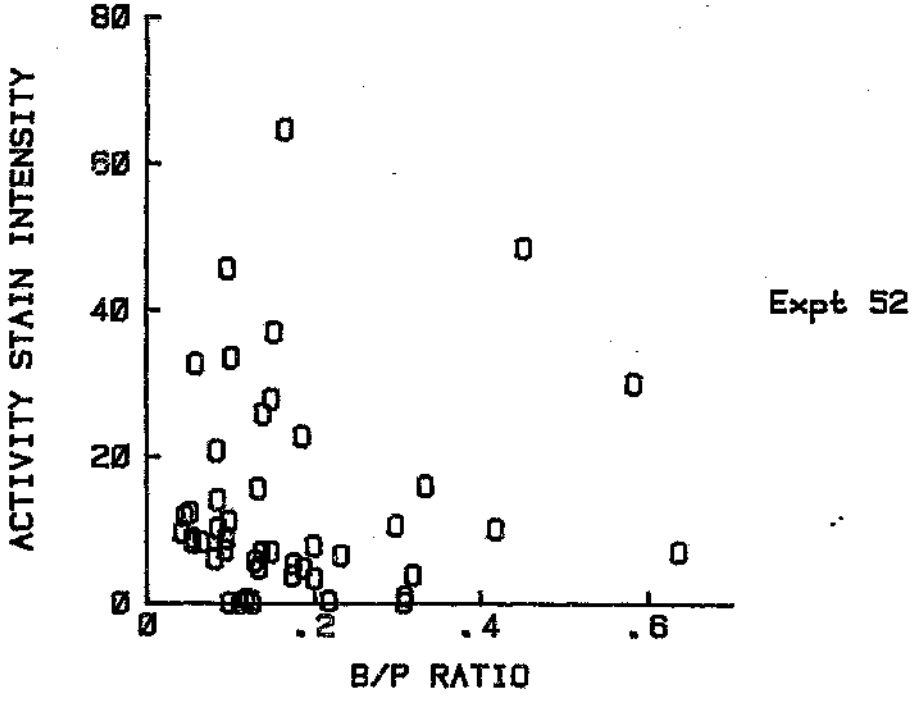




Fig. 20

**Fig.21 ENRIGHT PERIODOGRAMS OF EXTRACT PYRUVATE AND GEL STAIN ACTIVITY VARIATIONS**
(MEL cells in stirred suspension)

These diagrams confirm the high frequency of the LDH activity oscillations and show that several rhythms seem to be involved. The results also indicate that the extract activity fluctuations cannot be entirely explained simply by the isozyme changes although sometimes there is good agreement between the two curves (see also Fig.22). The effect of insulin is considered in a later section.

Cell number = $6.99 \times 10^6$ cells/ml

Window: 15pts
Period calculation interval: 0.05
Threshold: 0.05
Step interval: 0.5

# LACTATE DEHYDROGENASE ACTIVITIES
# EXTRACT PYRUVATE ACTIVITY vs STAIN INTENSITY

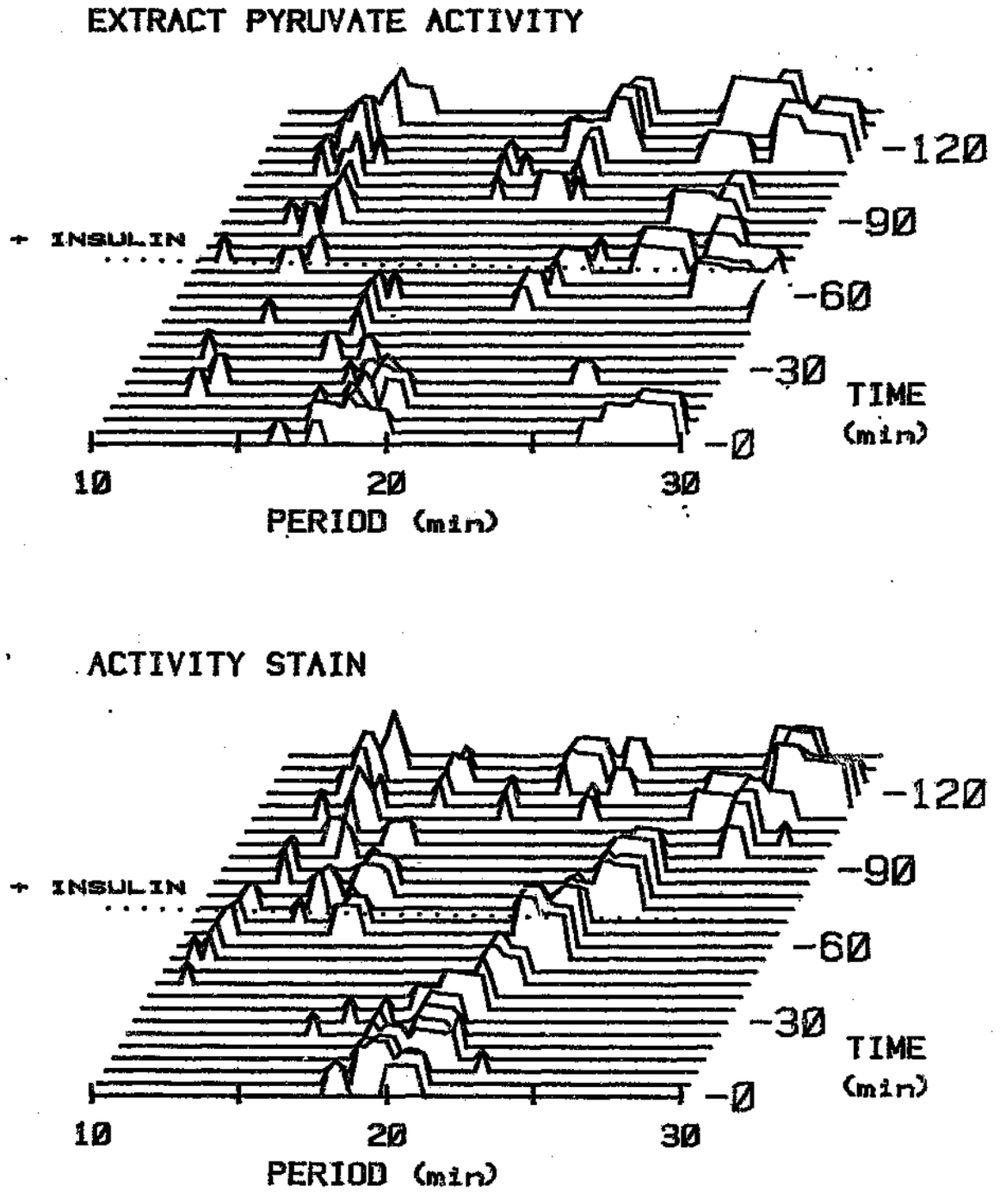



Fig. 21

**Fig.22a COMPARISON OF THE EXTRACT AND ISOZYME BAND ACTIVITY OSCILLATIONS**
(MEL cells in stirred suspension)

For comparison purposes, I superimpose graphs of corresponding oscillations in the activity of the extract (determined kinetically) and the variations in the electrophoretic isozyme band intensity for one experiment. The band intensity values have been scaled down. There are similarities but also distinctions at times. The sampling time here was 1 minute and the diagram shows the high frequency of the variations. In Fig.22b curves are presented were the sampling time was 5 minutes and these show the persistence of the behaviour. Plots of the ratio of the two values are also periodic. The values of the pyruvate activity are scaled so as to superimpose the curves.

Cell number = $4.95 \times 10^6$ cells/ml

57GPY
57PY

LDH ACTIVITY COMPARISON

GEL ACTIVITY STAIN ve EXTRACT PYRUVATE ACTIVITY

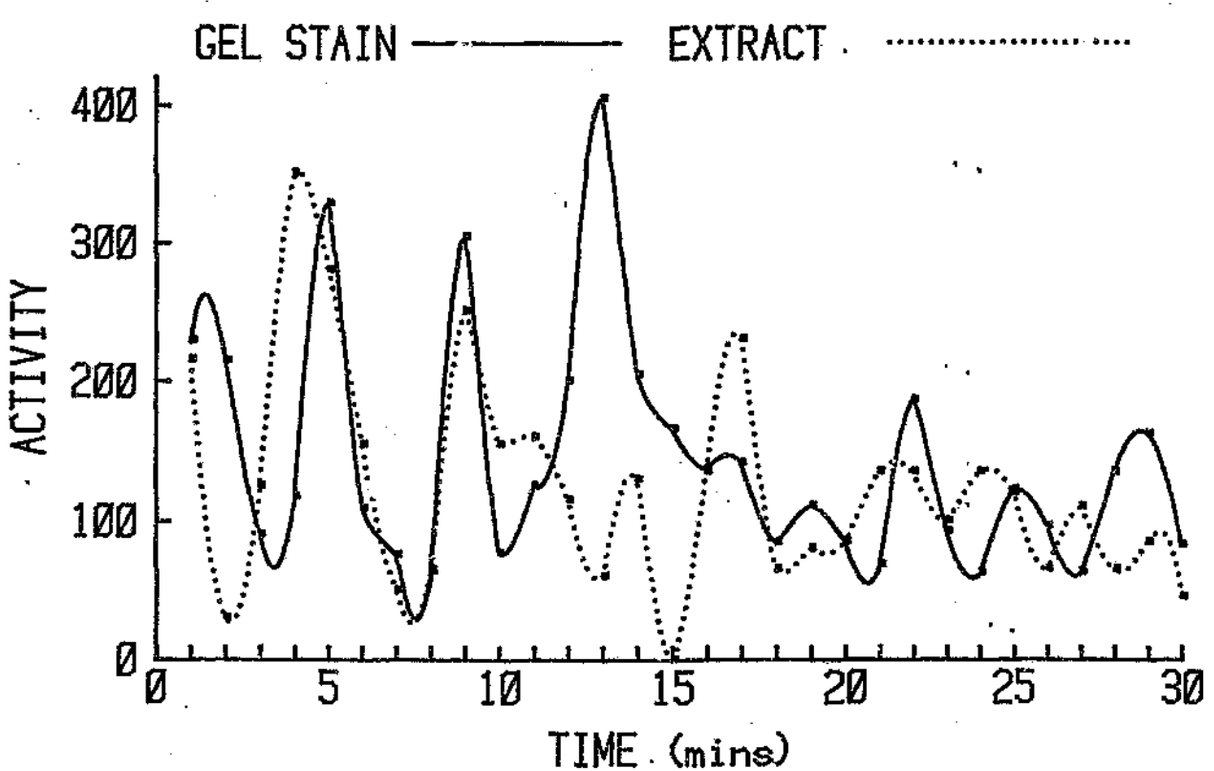



Fig. 22a

**Fig.22b COMPARISON OF THE LDH ACTIVITY OF THE EXTRACTS OF MEL CELLS WITH THE ELECTROPHORETIC BAND INTENSITY**
(cells in stirred suspension)

In this diagram are results corresponding to those given in Fig.22a, but for another experiment. In this case there is virtually no agreement between the two curves even with regard to the phasing of the rhythms. In this case, it seems that the variations in the amount of isozyme plays a minor role in determining the extract activity.

The right hand scale refers to gel stain.

51PY
51GEL

LDH: COMPARISON OF ACTIVITIES

EXTRACT PYRUVATE ve GEL STAIN INTENSITY

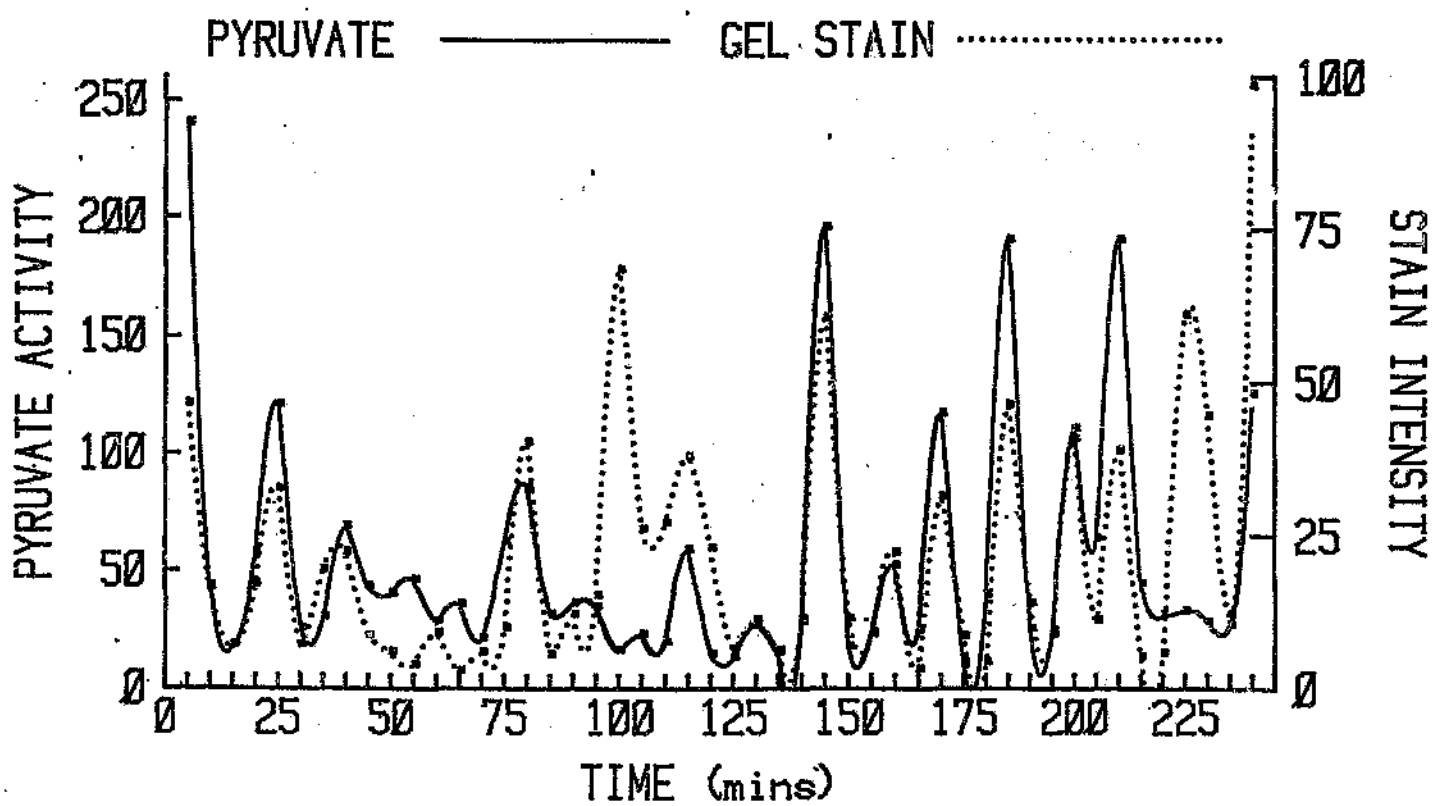



Fig. 22b

IMOVR 12.6.93 CYM 8

**Fig.23 OSCILLATIONS IN THE LDH SPECIFIC ACTIVITY SHOWING THE MODULATION CHARACTERISTICS**
(MEL cells in stirred suspension)

Presented here are variations in the specific activity of the extract sampled at 5 minute intervals for a 3-4 hour period. It can be seen that the specific activity also oscillates and the rhythm shows the same periodic modulation characteristics with regard to period and amplitude of the rhythm. Insulin was added at the time indicated, it appears to modify the modulation pattern, but the effect is somewhat different in the two experiments. This might be the result of differences in the metabolic state including differences in insulin concentration at the begining of the experiment.

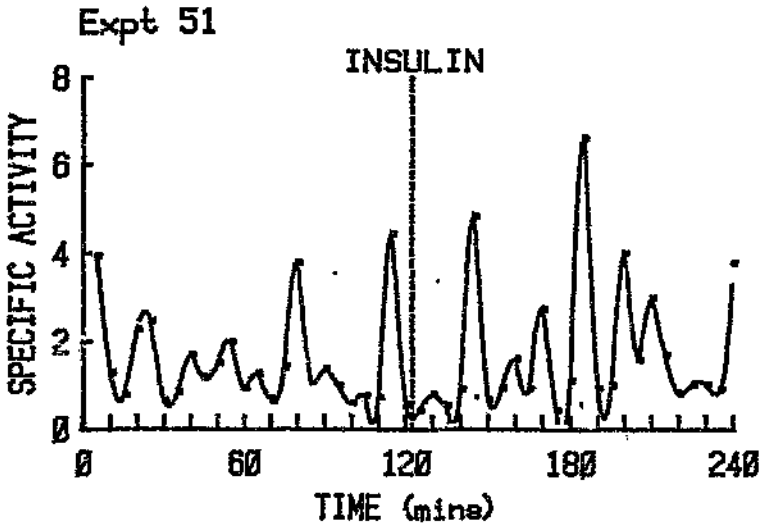
Cell number for a) $1.22 \times 10^6$ cells/ml (exp.51)
b) $1.60 \times 10^6$ cells/ml (exp.52)

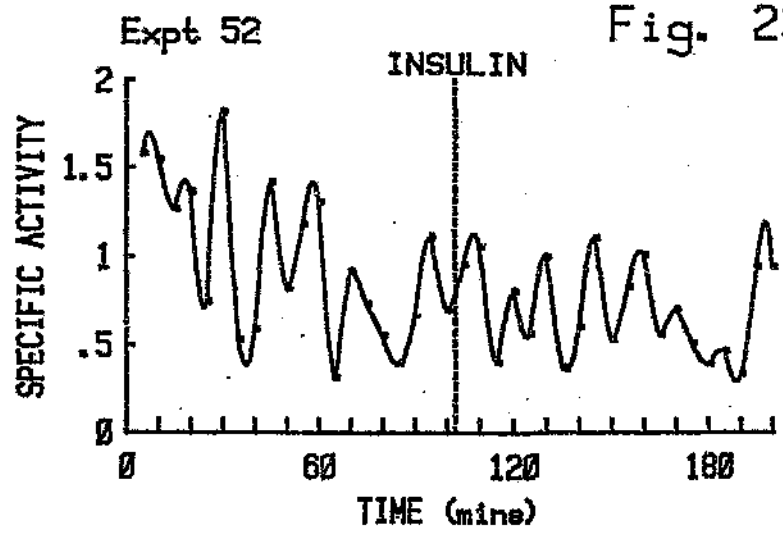
51P/PR

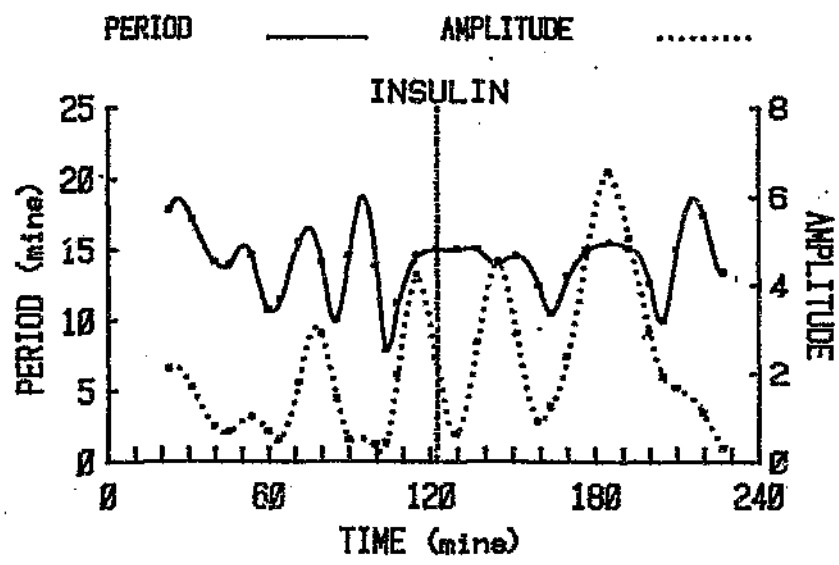
52P/PR

# LDH EXTRACT SPECIFIC ACTIVITY

Fig. 23



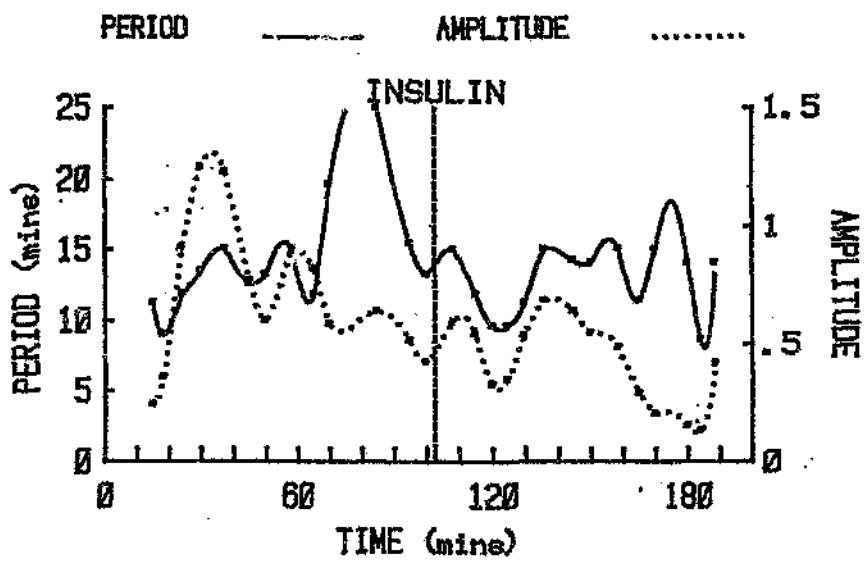



**Fig.24 OSCILLATIONS IN THE RATIO OF THE LDH STAIN INTENSITY TO THE AMOUNT OF EXTRACTABLE PROTEIN** (MEL cells in stirred suspension)

Here one plots the temporal variations in the gel isozyme "specific activity" i.e. the ratio of the intensity or the electrophoretic LDH band to the concentration of protein in the corresponding extract. Once again it appears that the activity stain intensity variations cannot be explained simply in terms of changes in the amount of protein. This was confirmed by running gels using equal amounts of protein rather than equal volumes of extract; oscillatory behaviour is still seen. As with all other oscillations there is evidence (from the lower diagrams) for periodic modulation of both the period and amplitude. Insulin does not appear to have a marked effect.

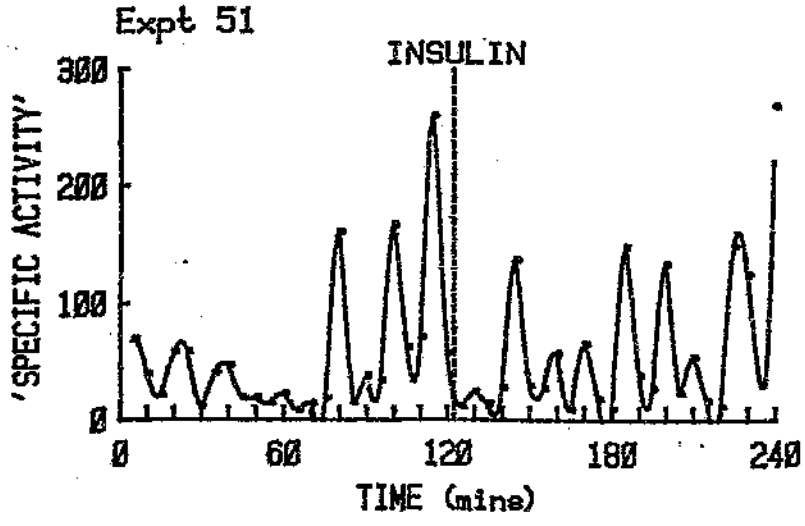
Cell number for a) $1.22 \times 10^6$ cells/ml (exp.51)
b) $1.60 \times 10^6$ cells/ml (exp.52)

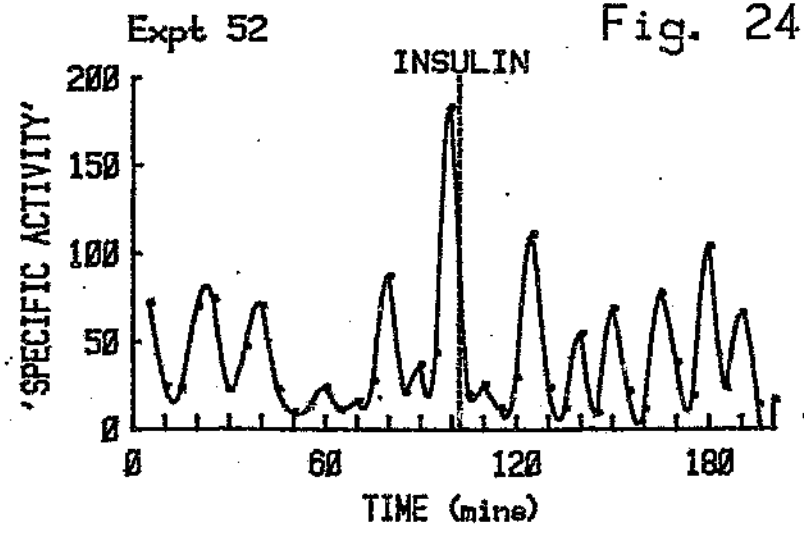
51G/PR

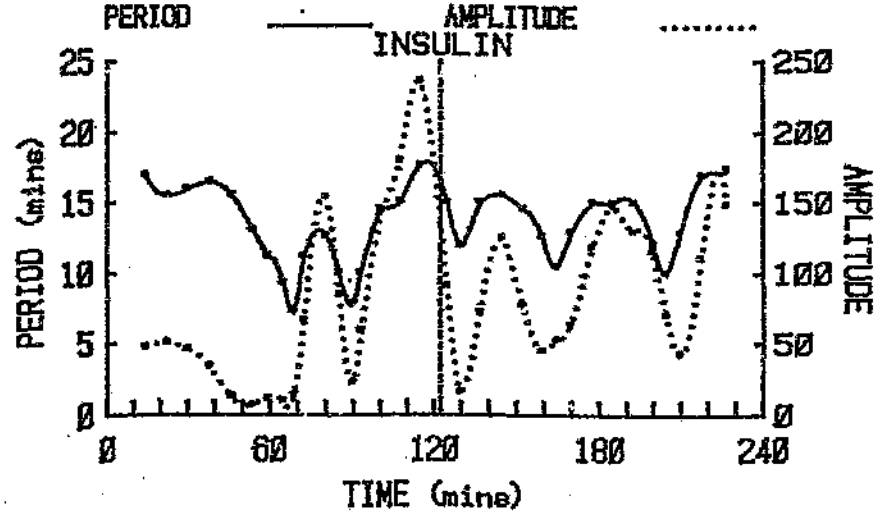
52G/PR

# LDH STAIN INTENSITY/PROTEIN RATIO

Fig. 24



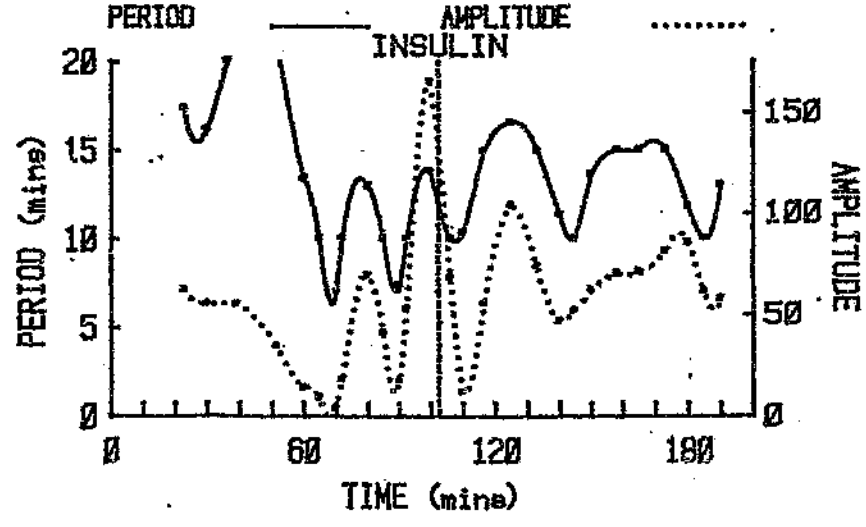



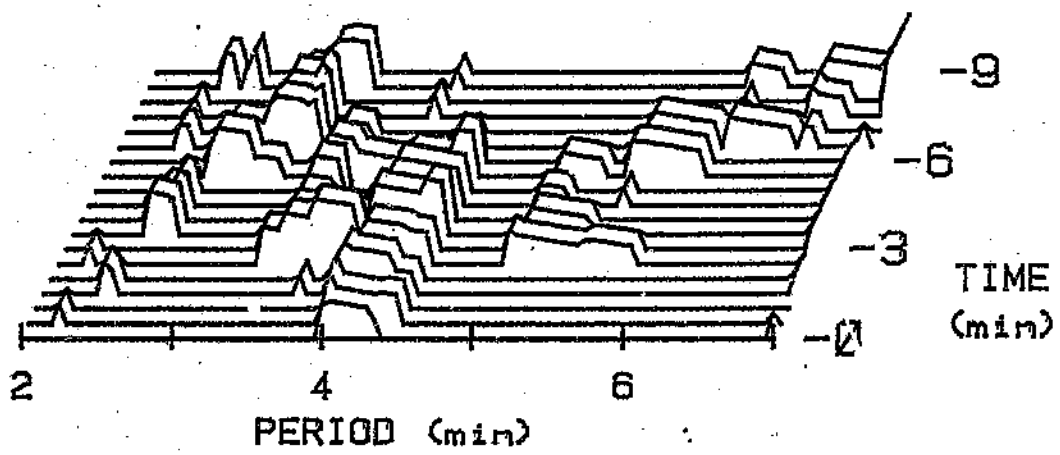
**Fig.25 ENRIGHT PERIODOGRAM OF EXTRACT AND GEL STAIN ACTIVITIES**
(MEL cells in stirred suspension)

This diagram shows Enright periodograms for the actual activities corresponding to the specific activities curves given in Fig.23 and Fig.24 respectively. Some similarities exist as one might expect, since the extract kinetic values will probably be affected by changes in the amount of active isozyme determined electrophoretically. Thus both show evidence for an oscillation with the initial period of about 18 minutes which increases during the period of observation to about 27 minutes. There is also evidence of a common band with a fairly constant period of about 12 minutes, although for the activity stain, this is evident only after the addition of insulin, but it seems to decrease somewhat in frequency. It could thus be a sub-harmonic of the longer period band. However, in the extract the oscillation in this latter region stays fairly constant.

Window: 14pts
Period calculation interval: 0.05
Threshold: 0
Step interval: 0.5

# LACTATE DEHYDROGENASE ACTIVITIES
# EXTRACT PYRUVATE ACTIVITY vs STAIN INTENSITY

EXTRACT PYRUVATE ACTIVITY




ACTIVITY STAIN INTENSITY

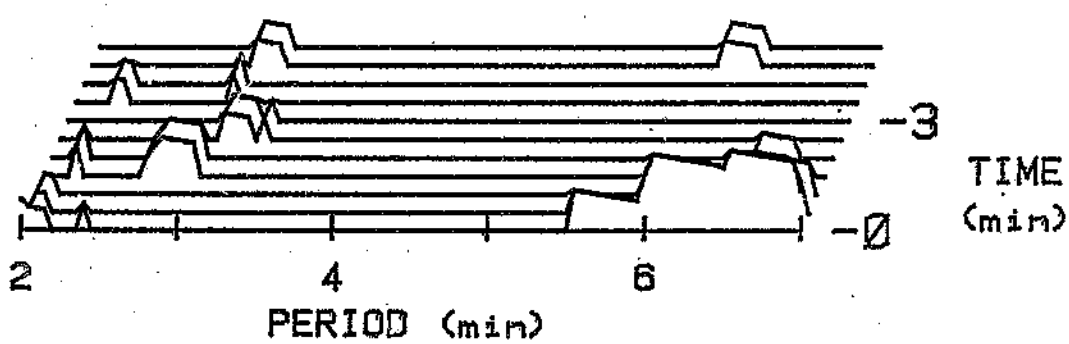



Fig. 25

**Fig.26 HIGH FREQUENCY OSCILLATIONS IN THE EXTRACT LDH PYRUVATE ACTIVITY: COMPARISON FOR TWO EXPERIMENTS** (MEL cells in stirred suspension)

For comparison purposes in this diagram the oscillations in the extract pyruvate activity (kinetic) values are shown for two experiments both show the high frequency nature of the rhythm with apparent period of about 3-4 minutes.

Cell number for a) $7.25 \times 10^6$ cells/ml (exp.53)
b) $7.09 \times 10^6$ cells/ml (exp.54)

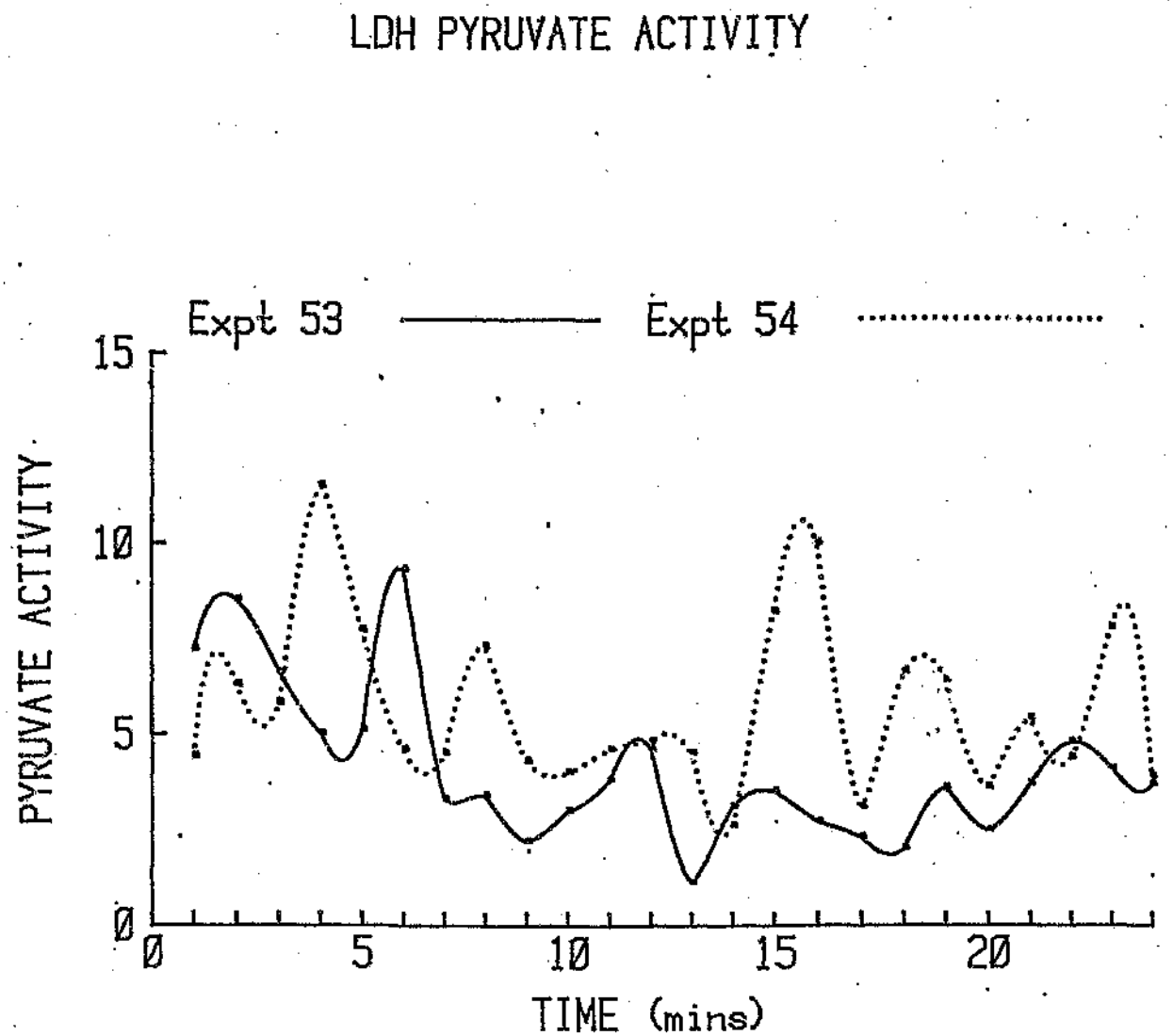



Fig. 26

53PY
54PY

12.6.93 CYN 4

**Fig.27 COMPARISON OF THE LDH ACTIVITY IN EXTRACTS OF MEL CELLS WITH THE ELECTROPHORETIC BAND INTENSITY**
(cells in stirred suspension)

This diagram shows that at times there is good agreement between the extract and the corresponding gel activity stain fluctuations but at other times for example between about 80-120 minutes there are quite marked distictions (compare with the next diagram). The lack of agreement between the two curves, whether it occurs at intervals as here, or over a longer time period (as in the next diagram), indicates that several factors can regulate the LDH system.

The right hand scale refers to gel stain.

LDH: COMPARISON OF ACTIVITIES

EXTRACT PYRUVATE vs GEL STAIN INTENSITY

Fig. 27

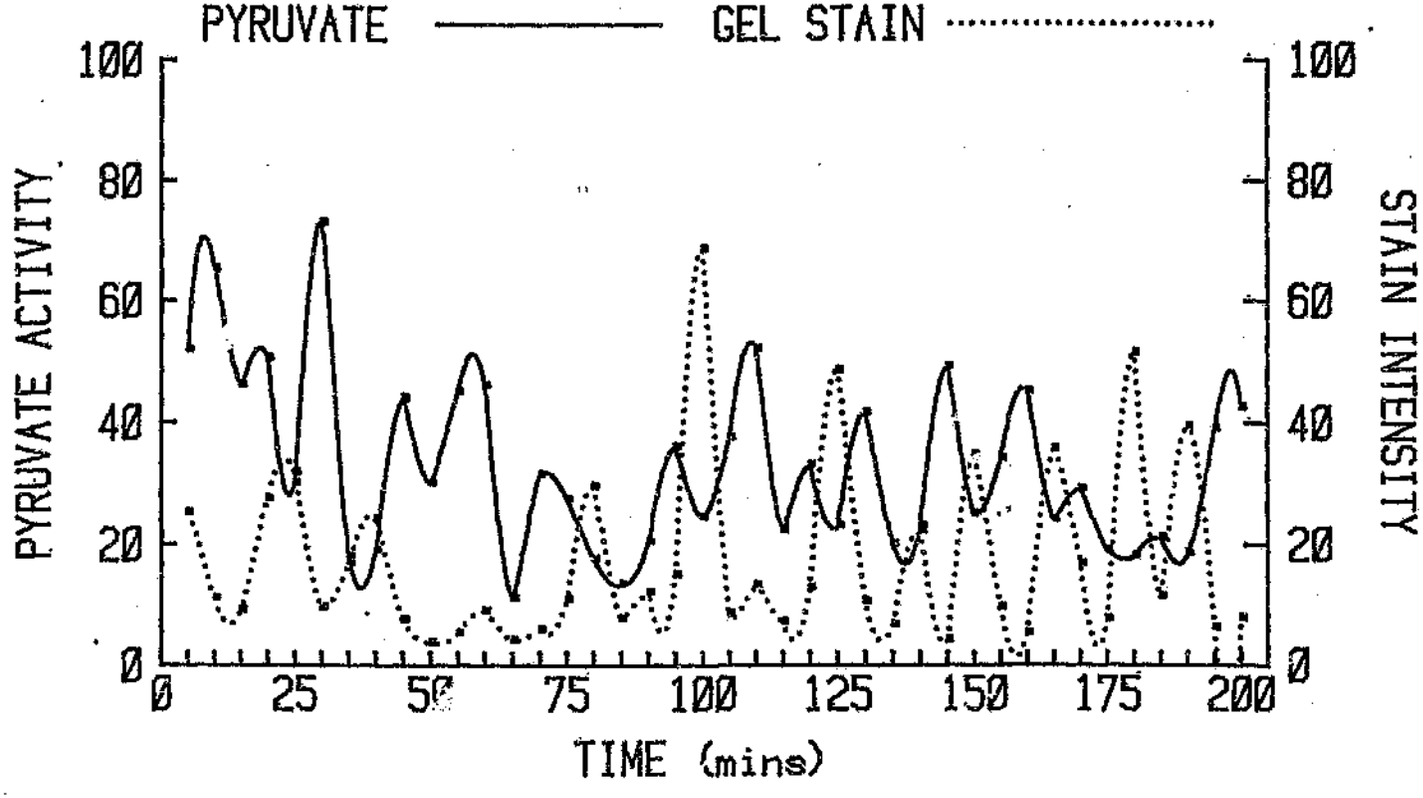


**Fig.28 OSCILLATIONS IN THE RATIO OF THE LDH PYRUVATE ACTIVITY AND CORRESPONDING GEL BAND INTENSITY** (MEL cells in stirred suspension)

As indicated in the text, the variation in the pyruvate activity of extracts can not be explained by the changes in the amount of active isozyme (as determined by electrophoresis). To confirm this view, four plots are given here for the ratio of the two activities, from which it can be seen that the ratio also oscillates irrespective of whether a 5 or 1 minute sampling time is used. The apparent period seems to be roughly 3 times the sampling interval in each case, supporting the view that aliasing is present in at least the 5 minute sampling data (the latter values are multiplied by 100). Birhythmicity and trirhythmicity can be seen.

51P/G
52P/G
57PY/G
58PY/G

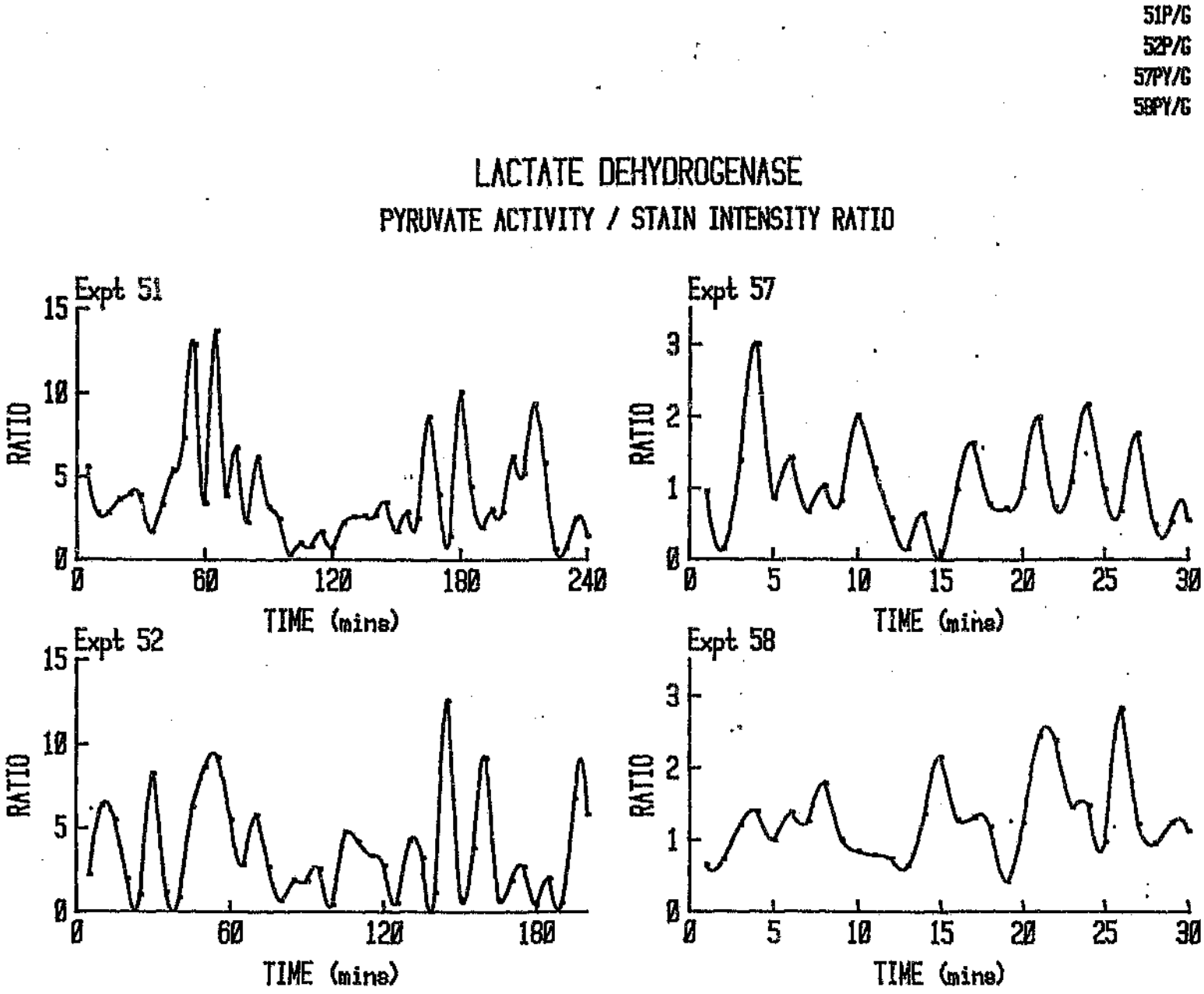



Fig. 28

**Fig.29 OSCILLATIONS IN THE APPARENT ISOZYME PATTERN OF THE EXTRACT AS DETERMINED KINETICALLY (B/P RATIO)**
(MEL cells in stirred suspension)

In a number of studies (see text) Gilbert has used the B/P rate ratio as a measure of the apparent isozyme pattern and shown that the values oscillate in a number of cells which exhibit several LDH isozymes. Here it is shown that this is also true for MEL cells yet they only exhibit one isozyme. Moreover, the amplitude is very high, of the order of the maximum expected for human cells, and the period of the rhythm (2-4 mins) is much lower than previously reported. Again, as for all other oscillations discussed in this thesis, there is evidence for periodic modulation of the period and amplitude of the oscillations. (see fig.31 for a comparison of the B/P variations with the activity stain rhythm). The limiting values of B/P ratio for murine LDH are not known but the human limits are approximatelly 0.2 - 0.8 (Lax found 1972). The upper limit is not likely to be greater than 1 because that would mean that alpha-ketobutyrate would be a preferred substrate.

Cell number for a) $7.25 \times 10^6$ cells/ml (exp.53)
b) $7.05 \times 10^6$ cells/ml (exp.54)

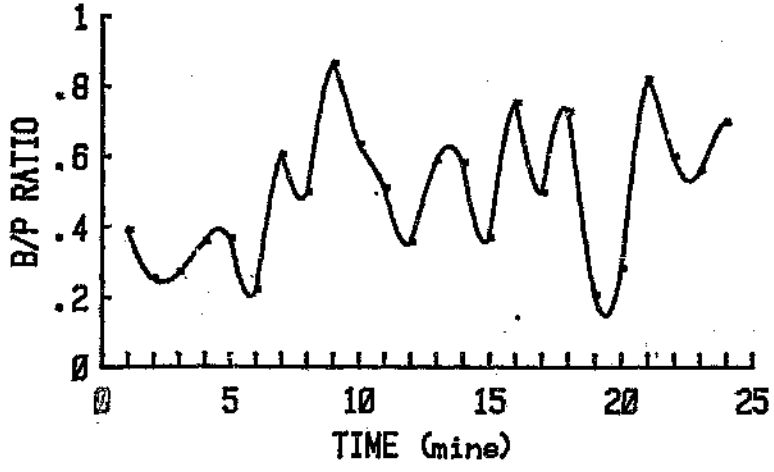
53B/P

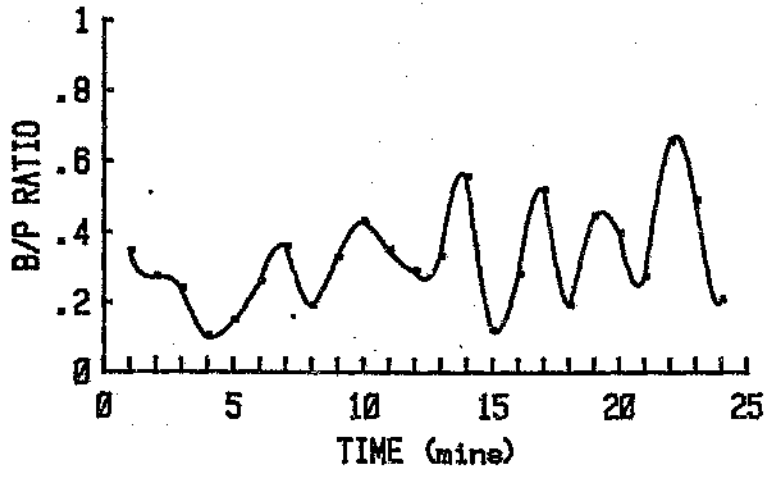
54B/P

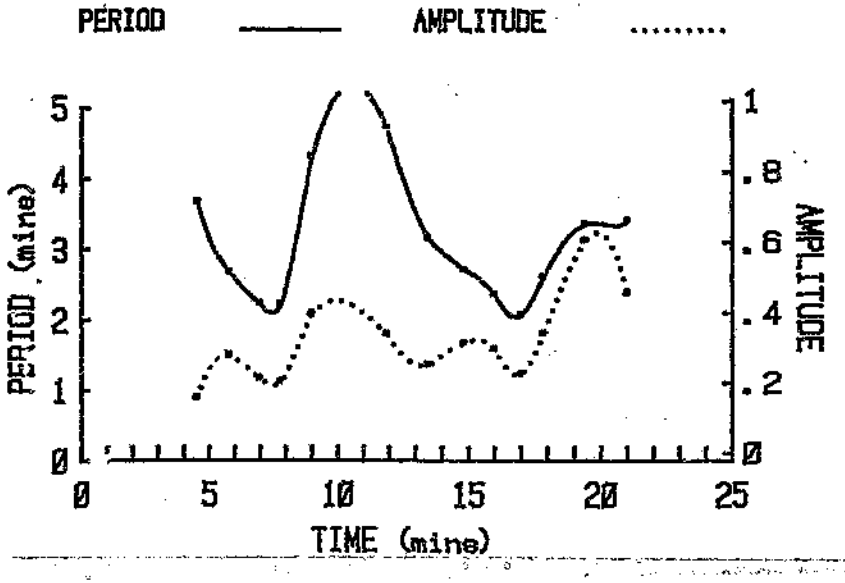
# LACTATE DEHYDROGENASE: MEL CELLS

## APPARENT ISOZYME PATTERN

Fig. 29



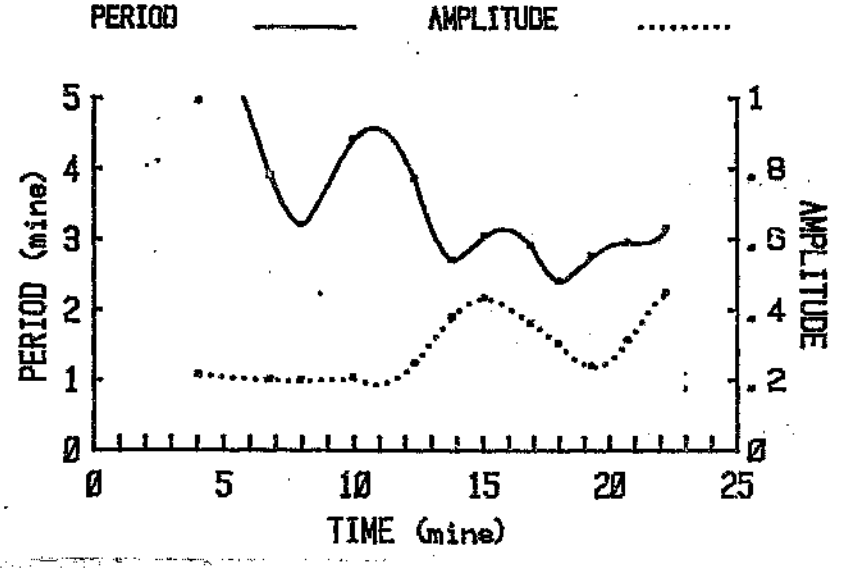



**Fig.30 ENRIGHT PERIODOGRAM OF THE HIGH FREQUENCY B/P RATIO OSCILLATION**
(MEL cells in stirred suspension)

This diagram confirms the high frequency nature of the B/P rhythm given in the last figure. There appear to be several oscillations involved but the picture is complicated by the apparent presence of harmonics.

Window: 15pts
Period calculation interval: 0.05
Threshold: 0.05
Step interval: 0.5

Expt. 54B/P

MEL

SAMPLE INTERVAL 1 mins
CALC INTERVAL .Ø5
WINDOW 15 pts
STEP INTERVAL .5
THRESHHOLD .Ø5
SCALE FACTOR 2

# LDH B/P RATIO

ENRIGHT PERIODOGRAM
PEREN3.7 CYNS MAY 83

major periods

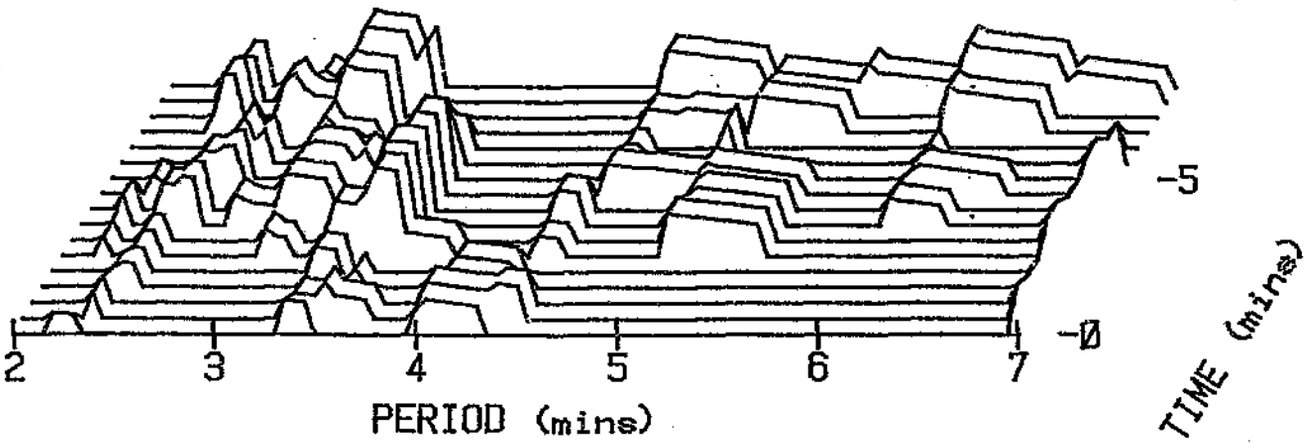



Fig. 3Ø

**Fig.31 COMPARISON OF THE B/P OSCILLATION WITH THE CORRESPONDING RHYTHM OF THE AMOUNT OF ACTIVE ISOZYME**
(MEL cells in stirred suspension)

Only one isozyme of LDH was detected in MEL cells (see Fig. 17) and therefore one might expect the B/P ratio to be constant. As shown here, very marked variations occur in the ratio. In order to see if the B/P variation correlates with the changes in the actual amount of the active isozyme, the two curves are overlaid in this diagram. It shows that they are quite distinct. The differences are probably due to the action of regulators present in the extract which change the specificity of the isozyme.

Cell number = $1.60 \times 10^6$ cells/ml

LDH GEL ACTIVITY -- ISOZYME PATTERN

52GEL
52B/P

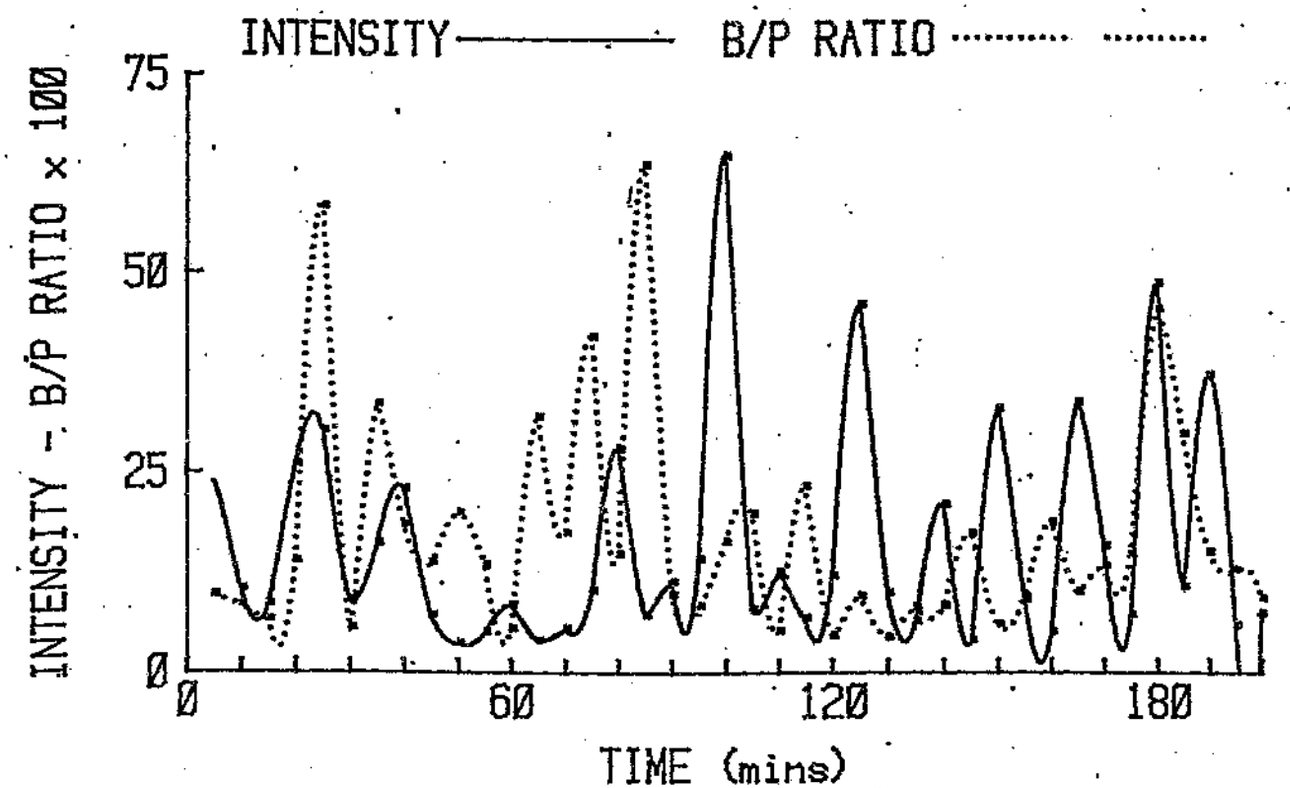



Fig. 31

12.6.88 CYN 4

**Fig.32 COMPARISON OF THE TWO LDH RATE RATIOS**

As indicated in Fig.31, the B/P oscillation cannot be accounted for by the rhythm in the amount of active isozyme. The B/P oscillation could be due to periodicity in the amount of an alpha-ketobutyrate dehydrogenase in the cells, therefore the LDH isozyme pattern was also checked by determining the ratio of the rates toward two different pyruvate concentrations. Where a single isozyme of LDH is present, the ratio should be constant, but as can be seen in this diagram, this ratio also oscillates. Moreover, the oscillation is distinct from that in the B/P ratio. These results seem to indicate the presence of regulators in the extract which affect both the kinetics and the specificity of the single isozyme of LDH. A separate keto-butyrate dehydrogenase seems unlikely as the B/P ratio never significantly exceeds unity.

# LDH APPARENT ISOZYME PATTERN

## COMPARISON OF RATE RATIOS

B/P vs Py.3/Py.7

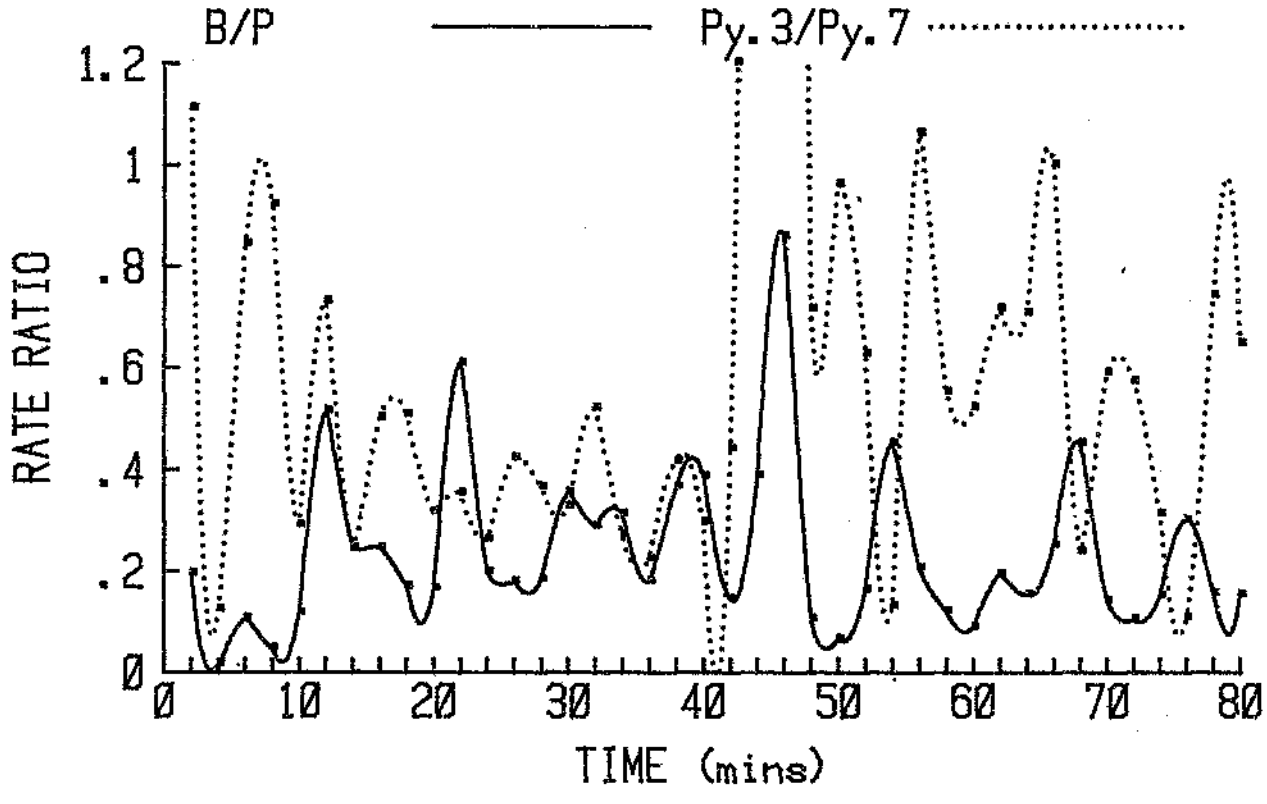
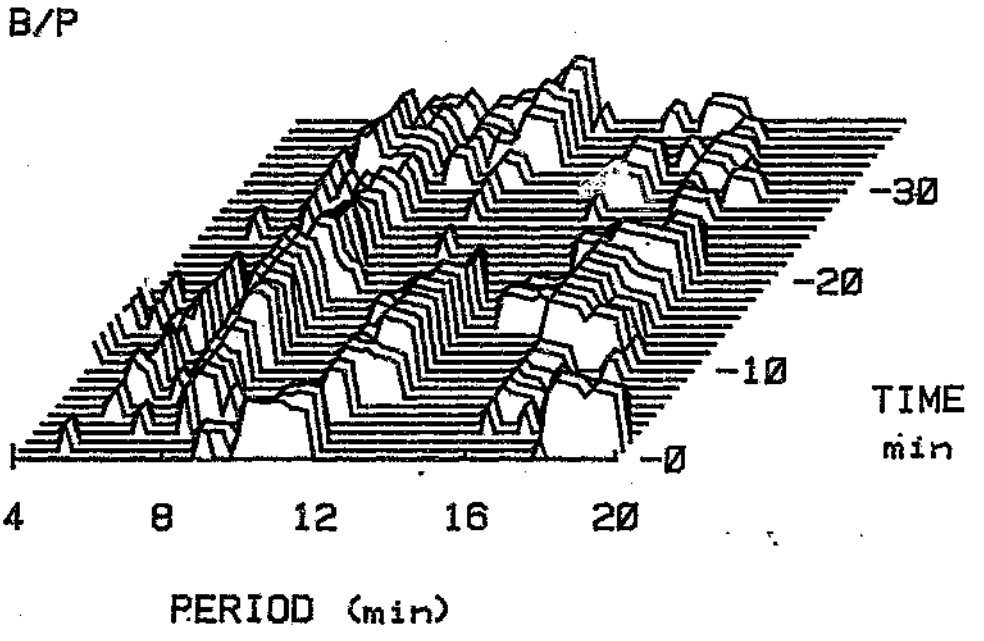


**Fig.33 ENRIGHT PERIODOGRAMS FOR THE TWO RATE RATIOS**
(MEL cells in stirred suspension)

These periodograms confirm the distinction between B/P oscillation and the rhythm for the rate ratio for the two pyruvate concentrations. There seems to be some correlation in the 15-18 minute region but marked difference in the very high frequency periodicity (4-8 minute).

Window: 20pts
Period calculation interval: 0.1
Threshold: 0.05
Step interval: 0.5

# LACTATE DEHYDROGENASE
# ENRIGHT COMPARISON OF RATE RATIOS



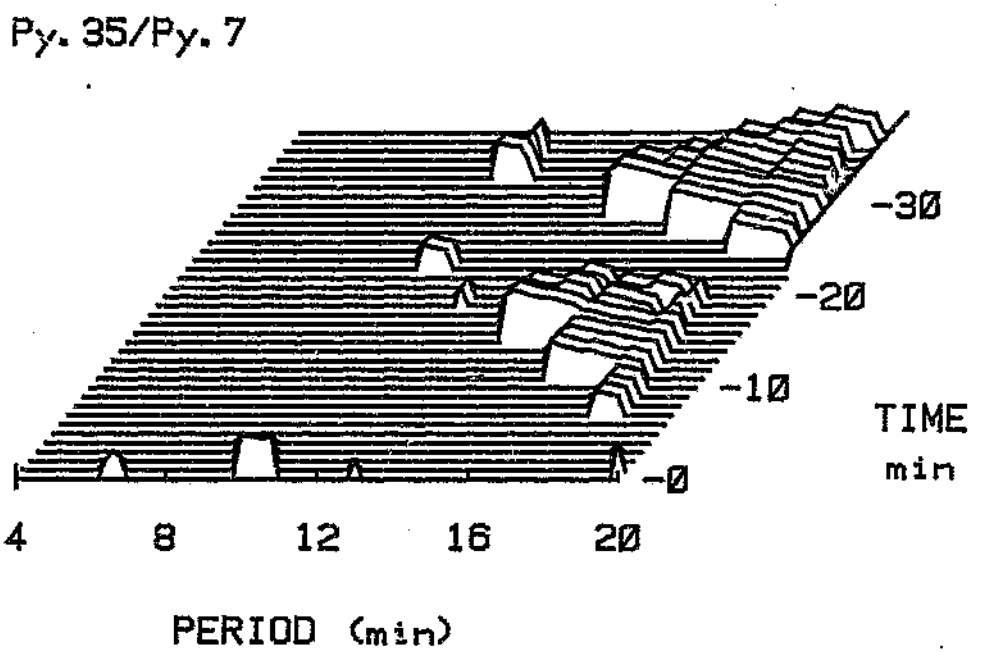
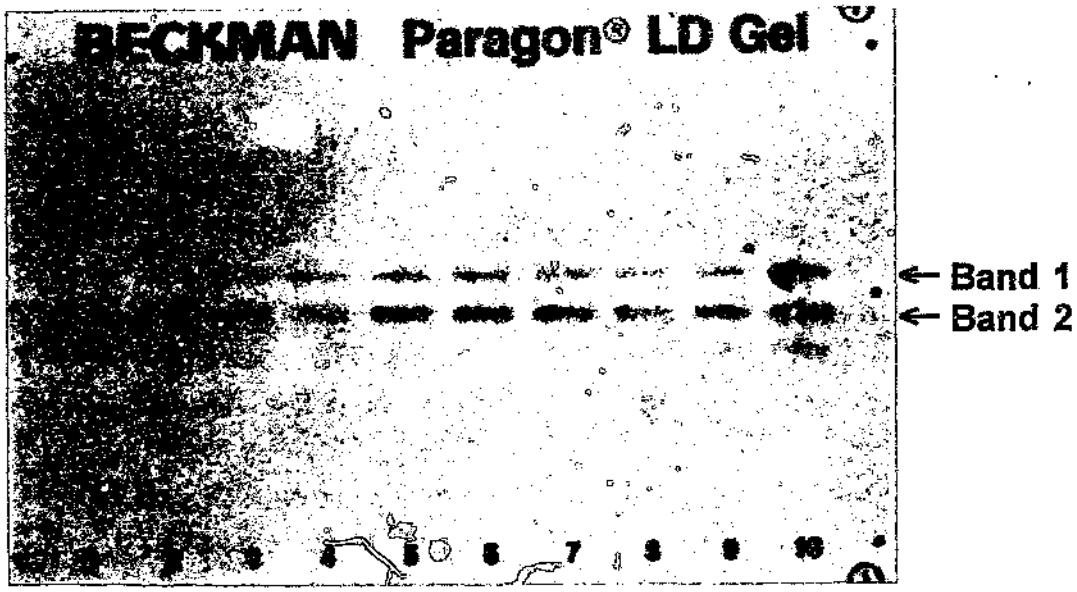




Fig. 33

**Fig.34 VARIATIONS IN THE LDH ISOZYME BAND INTENSITIES FOLLOWING ELECTROPHORESIS OF EXTRACTS OF HL-60 CELLS: SAMPLING INTERVAL 5 MINUTES**
(cells in stirred stirred suspension)

As can be seen here the human leukaemic cell line HL-60 exhibits three LDH isozymes, two major bands and one minor band. It is also obvious from the two photographs (for two different experiments) shown here, that the amounts of the active isozymes fluctuate with time. The two major bands, marked 1 and 2, were selected for further study. Laser scanning densitometry shows that, as with MEL cells, the stain intensities of both isozymes oscillate as shown in the following diagrams.



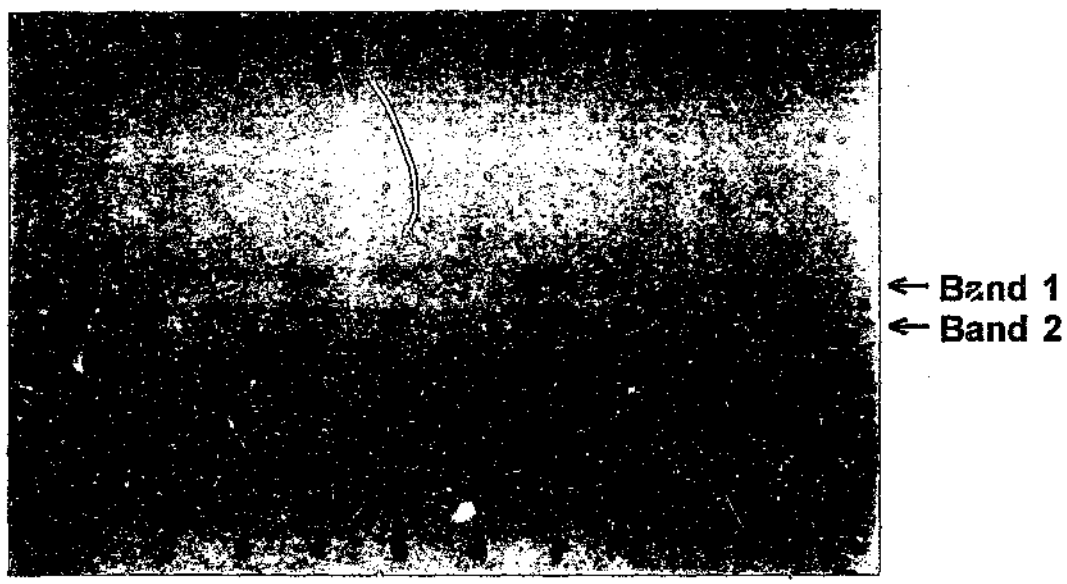




Fig. 34

**Fig.35a,b OSCILLATIONS IN THE LDH ISOZYME PATTERN IN HL-60 CELLS**
(cells in stirred suspension)

As seen in Fig.34 three LDH isozymes can be detected in HL-60 cells, this diagram shows the oscillations in the intensity of the two major bands, obtained in two experiments. As can be seen, although there seems to be some correlation between the phasing of the two oscillations (peak values occuring at the same time), there are marked differences in the intensities of the two bands at different times. This suggests that the isozymes are being regulated by at least two factors, one of which is common and one of which acts independently on individual isozymes.

Cell number = $7.8 \times 10^6$ cells/ml

63G1

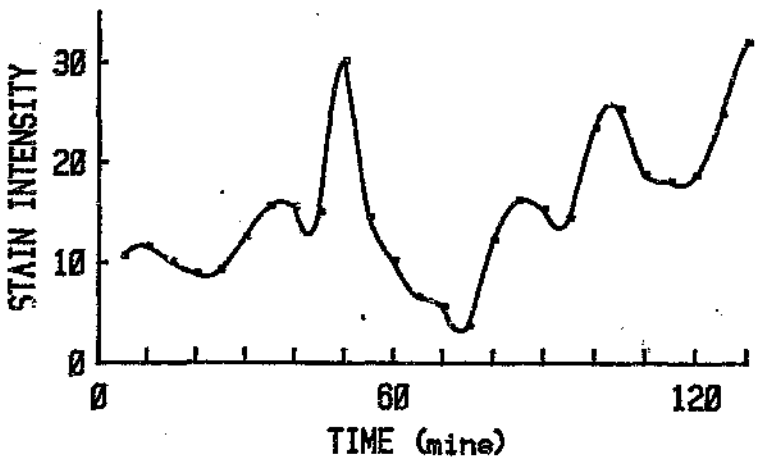
63G2

# LACTATE DEHYDROGENASE: HL-60 CELLS

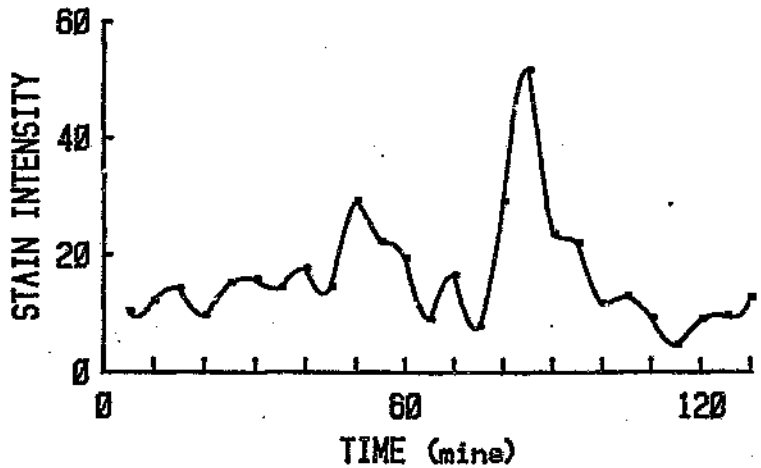
ELECTROPHORESIS ACTIVITY STAIN

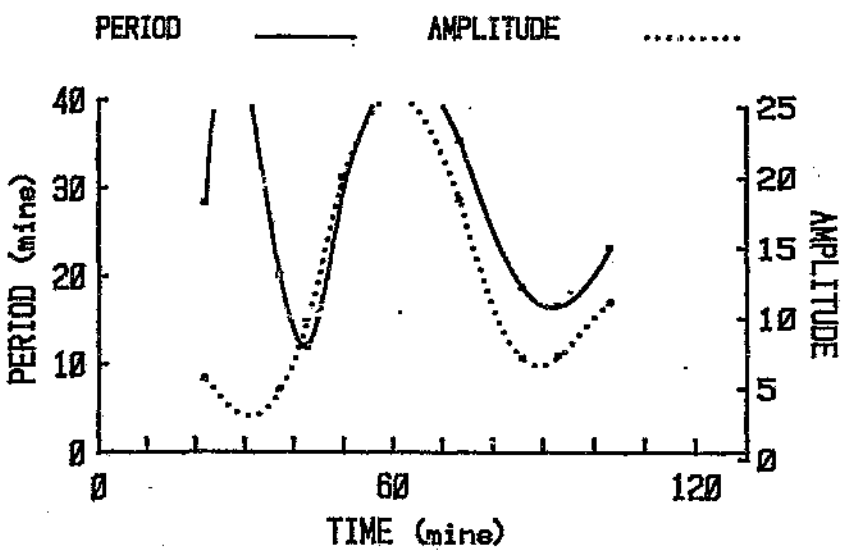
Fig. 35a

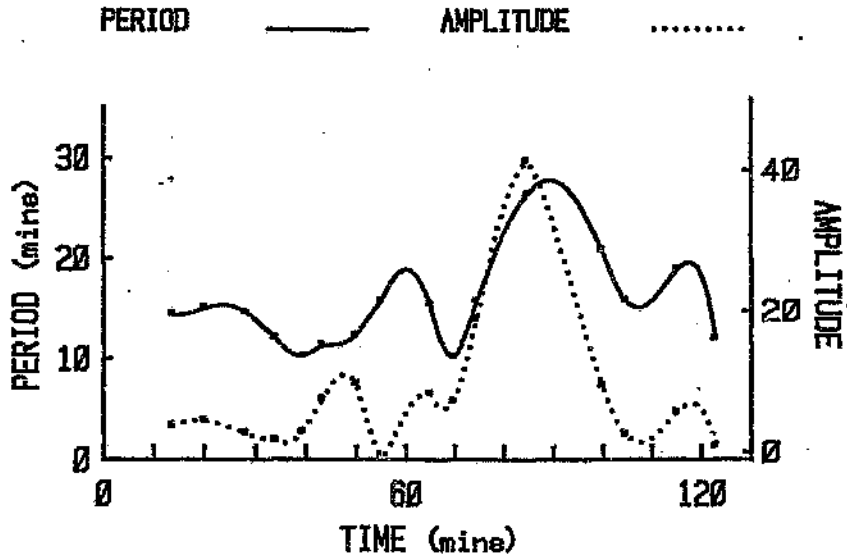
ISOZYME 1




ISOZYME 2



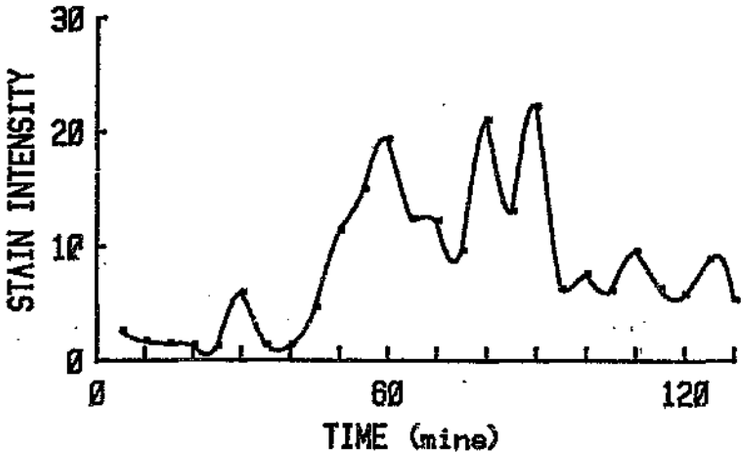
65G1

65G2

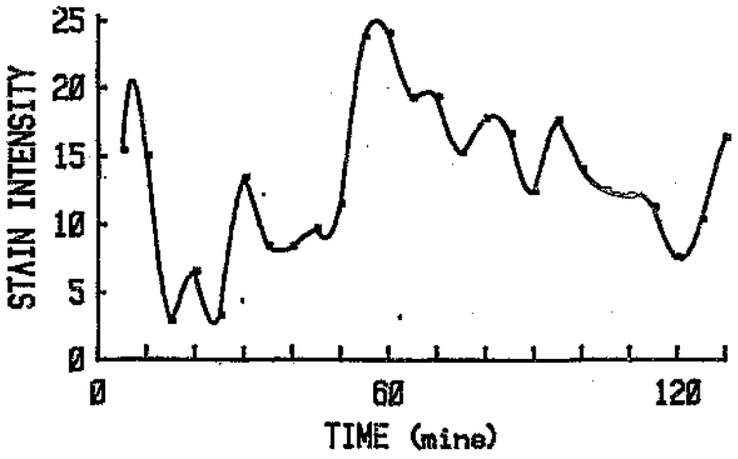
Fig. 35b LACTATE DEHYDROGENASE: HL-60 CELLS

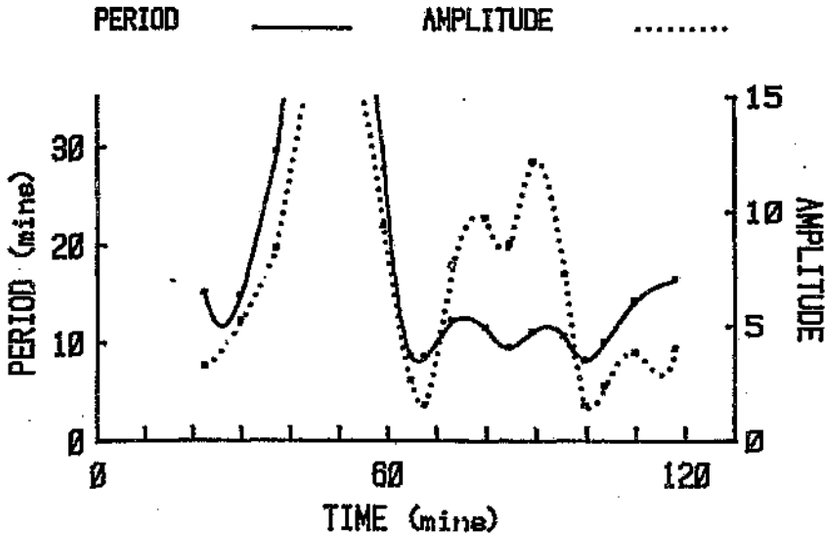
ELECTROPHORESIS ACTIVITY STAIN

ISOZYME 1




ISOZYME 2



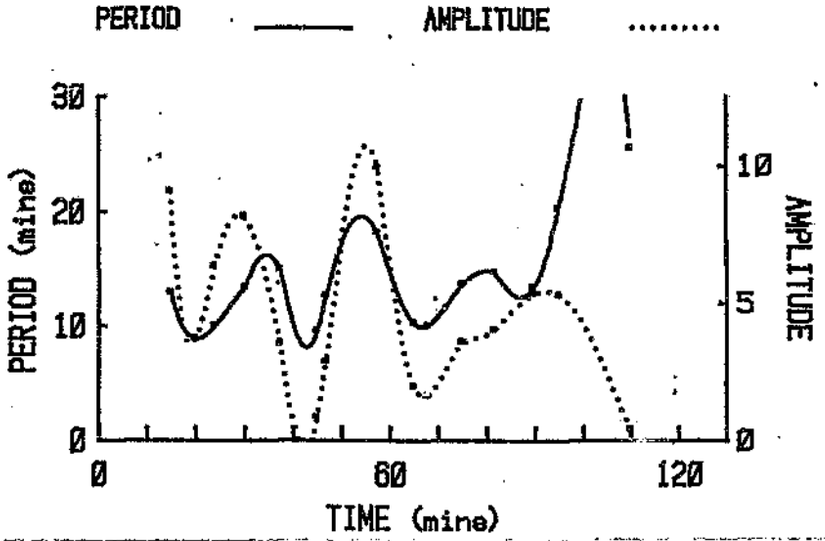



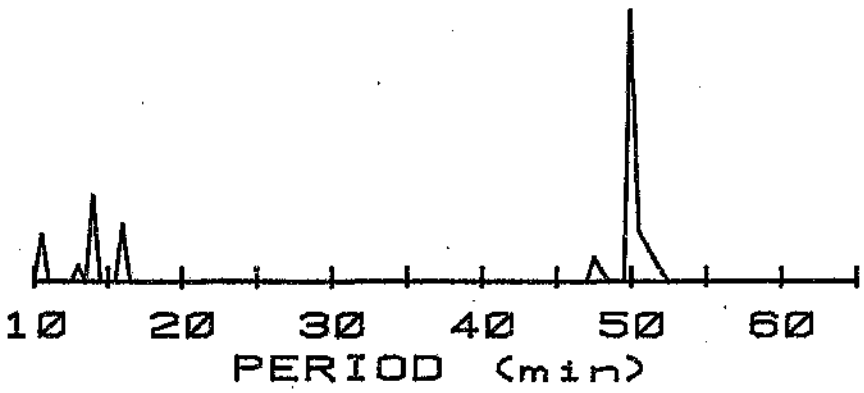
**Fig.36 ENRIGHT PERIODOGRAMS FOR THE TWO ISOZYME OSCILLATIONS IN HL-60 CELLS FOR TWO DIFFERENT EXPERIMENTS**
(cells in stirred suspension)

Overall Enright periodograms (using all values in the data set instead of the window method) are given here for the individual isozyme oscillations for the two experiments described in Fig.35a,b. In confirmation of the conclusions given there, the rhythms for band 1 differ from those of band 2 in both experiments. The actual periods for the individual isozymes involved are not the same in the two experiments, presumably due to slightly different conditions. However, this diagram confirms that the two major isozymes (at least) are being regulated independently. This view is supported by plots of the ratio of the intensities of the two bands (Fig.38) and plots of the difference between the intensities of the two bands (not shown).

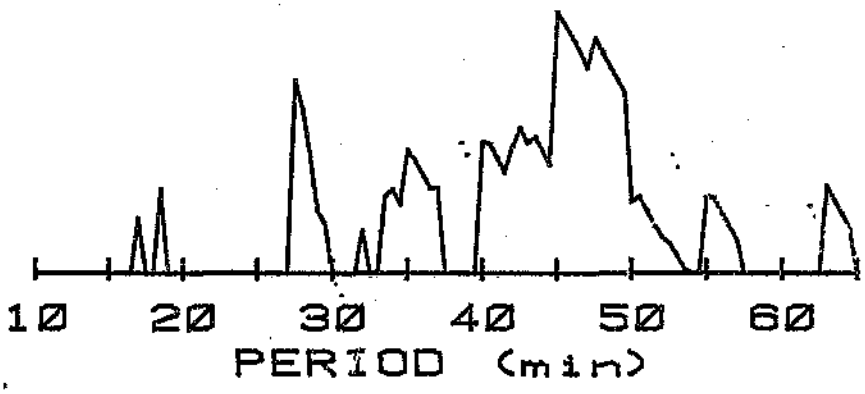
Cell number for a) $7.8 \times 10^6$ cells/ml (exp.63)
b) $7.2 \times 10^6$ cells/ml (exp.65)

# LACTATE DEHYDROGENASE HL-60
# ISOZYME BAND ACTIVITY STAIN

BAND 1
Expt 6




BAND 2
Expt 6




BAND 1
Expt 6

BAND 2
Expt 6

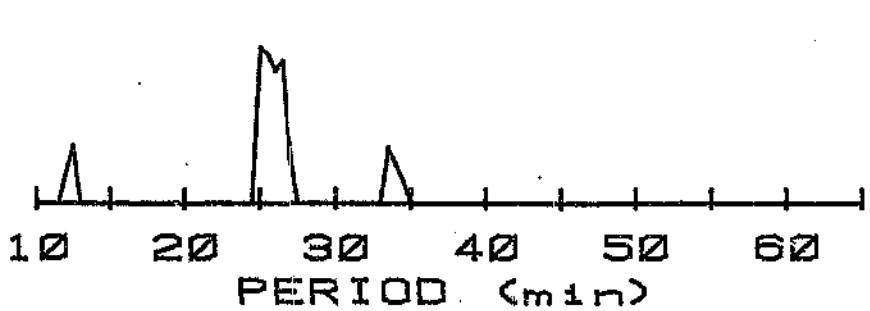



Fig. 36

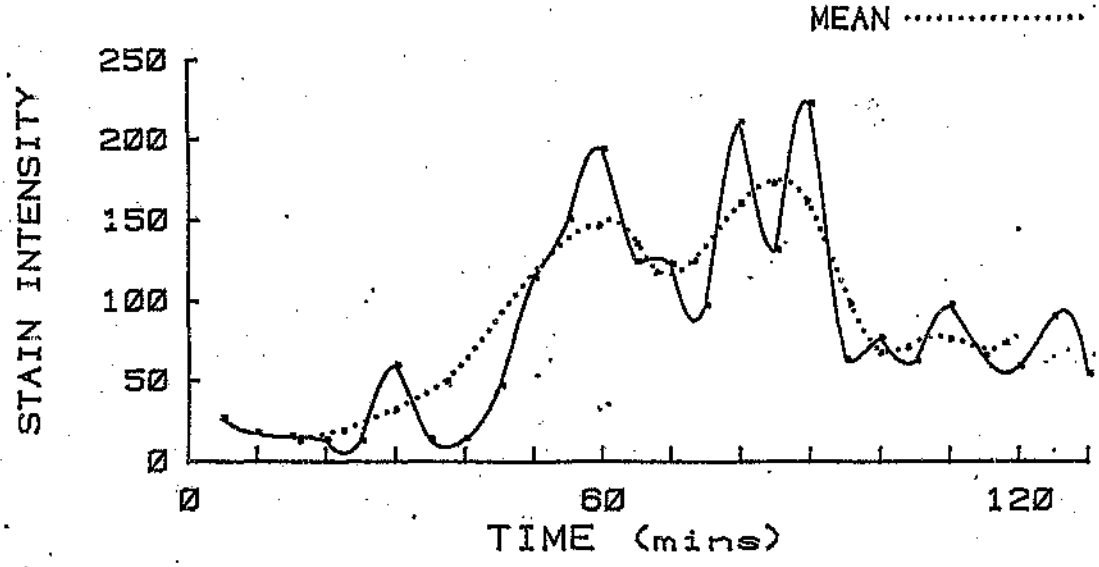
**Fig.37a,b PER AMP PLOTS FOR THE INDIVIDUAL LDH ISOZYME OSCILLATIONS IN HL-60 CELLS** (MEL cells in stirred suspension)

As can be seen from these plots for two experiments the oscillations for the individual isozymes show the same modulation characteristics as all other oscillations and the modulation pattern differs for the two isozymes further supporting the view that they are independently regulated.

LACTATE DEHYDROGENASE HL-60

ELECTROPHORESIS ACTIVITY STAIN

BAND 1



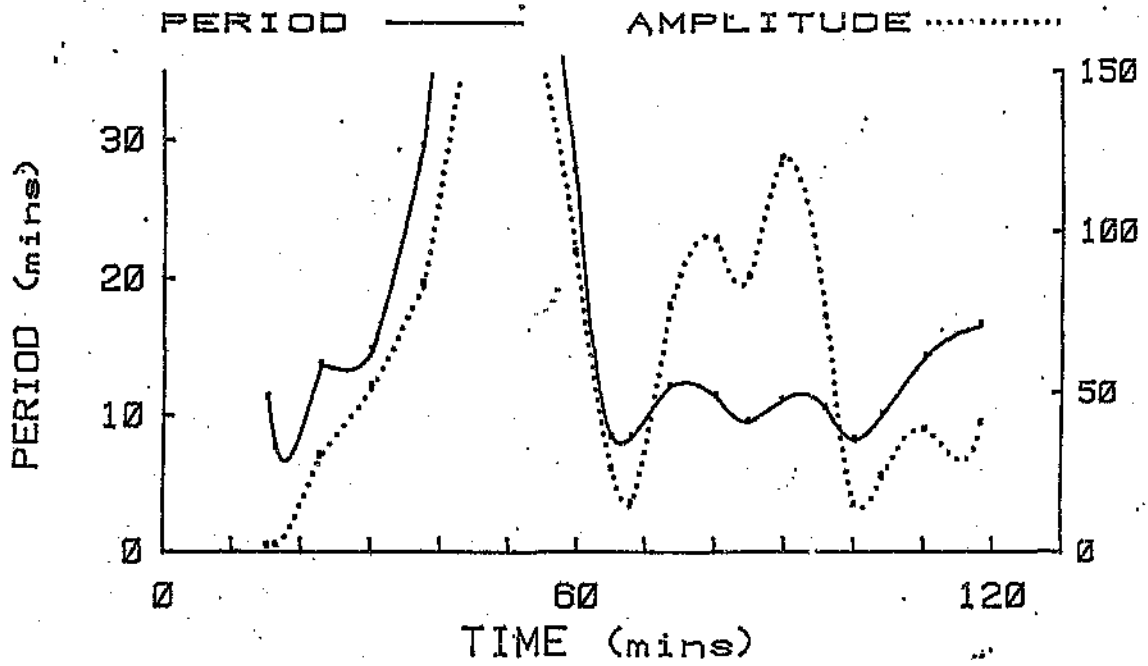
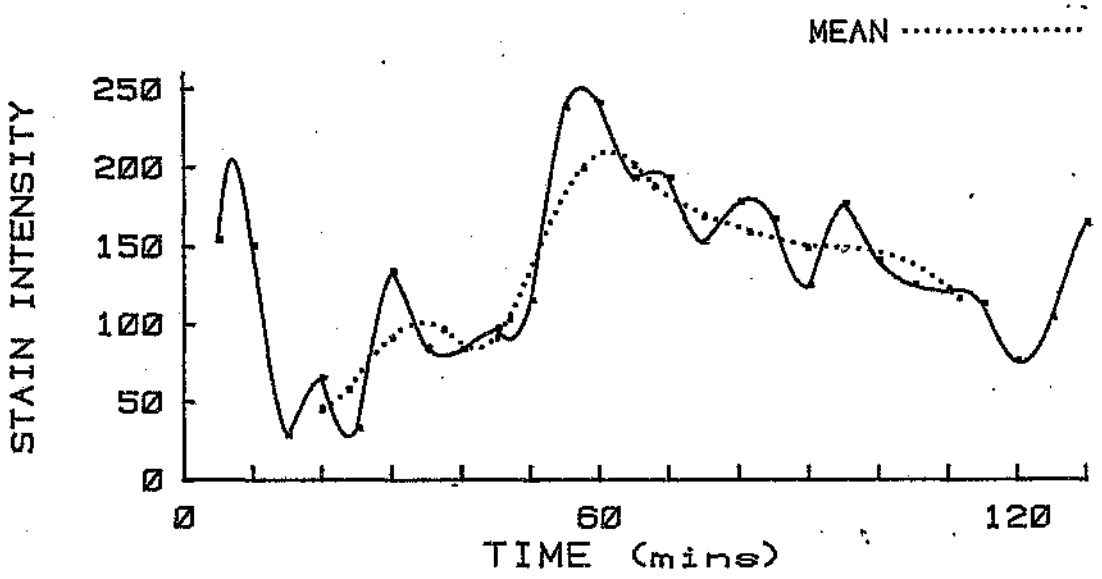




Fig. 37a

LACTATE DEHYDROGENASE HL-60

ELECTROPHORESIS ACTIVITY STAIN

BAND 2



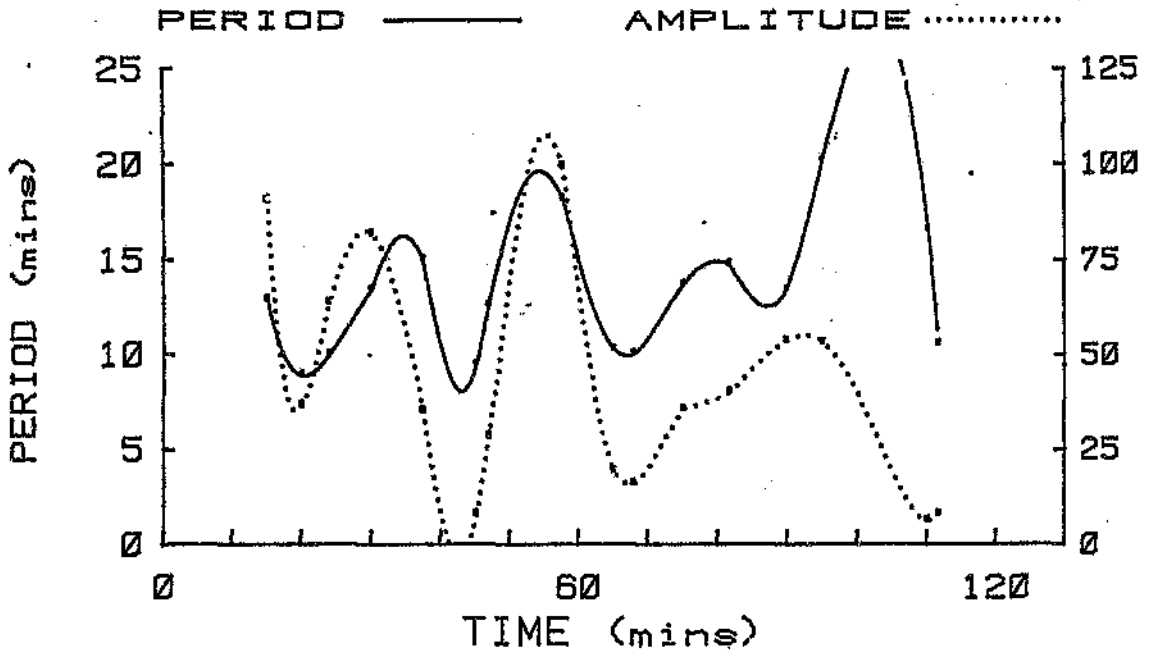




Fig. 37b

**Fig.38 OSCILLATIONS IN THE ISOZYME 1/ISOZYME 2 RATIO FOR LDH IN HL-60 CELLS**
(cells in stirred suspension)

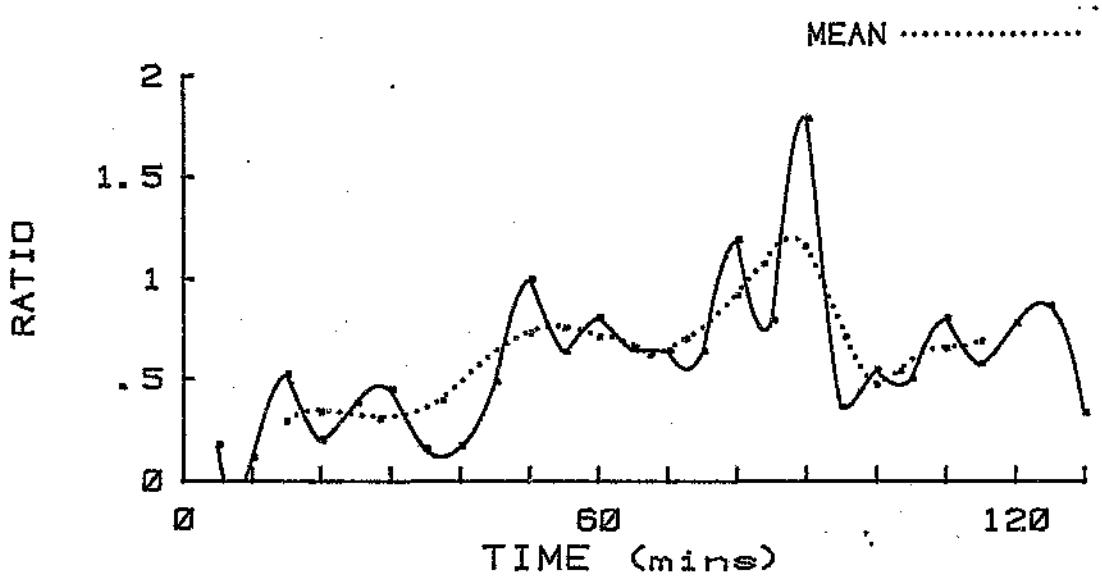
In further confirmation of the conclusion that the two major LDH isozyme bands in HL-60 cells are independently regulated, given here are plots of the ratio of the intensities for two experiments. As can be seen the ratio also oscillates and appears to be modulated with regard to period and amplitude, as with all other oscillations studied. Although there are differences in the mean values for the ratios, the period (10-12 minutes) is the same in the two experiments.

Cell number = $7.2 \times 10^6$ cells/ml

# LACTATE DEHYDROGENASE HL60

## ELECTROPHORESIS ACTIVITY STAIN

## BAND1/BAND2 RATIO



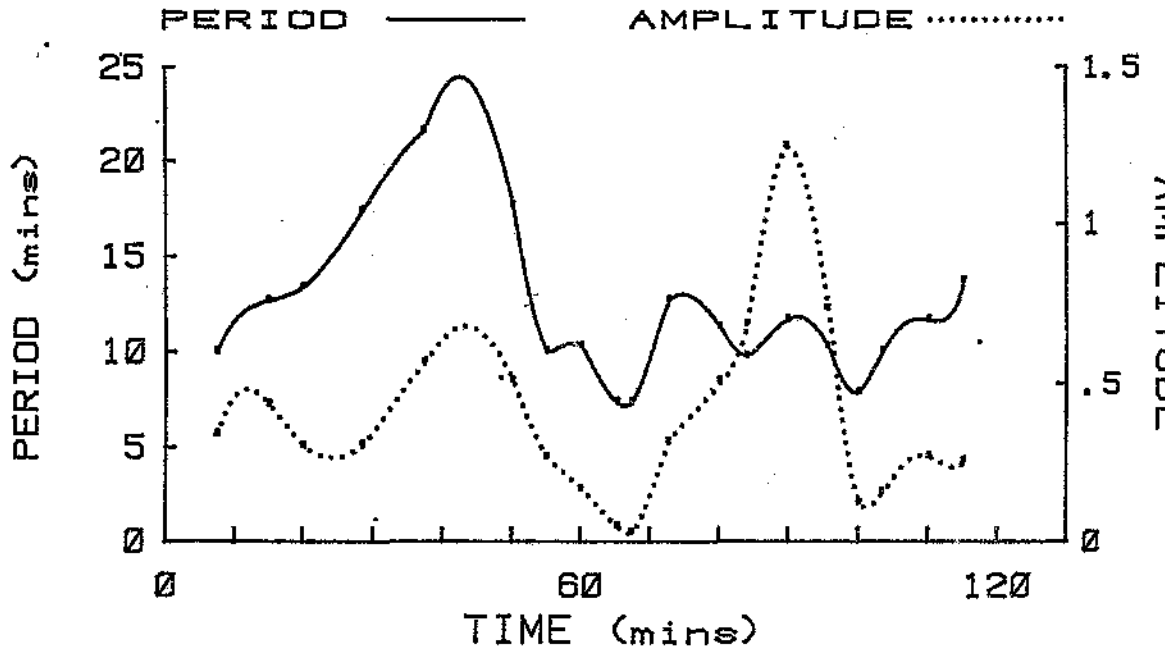



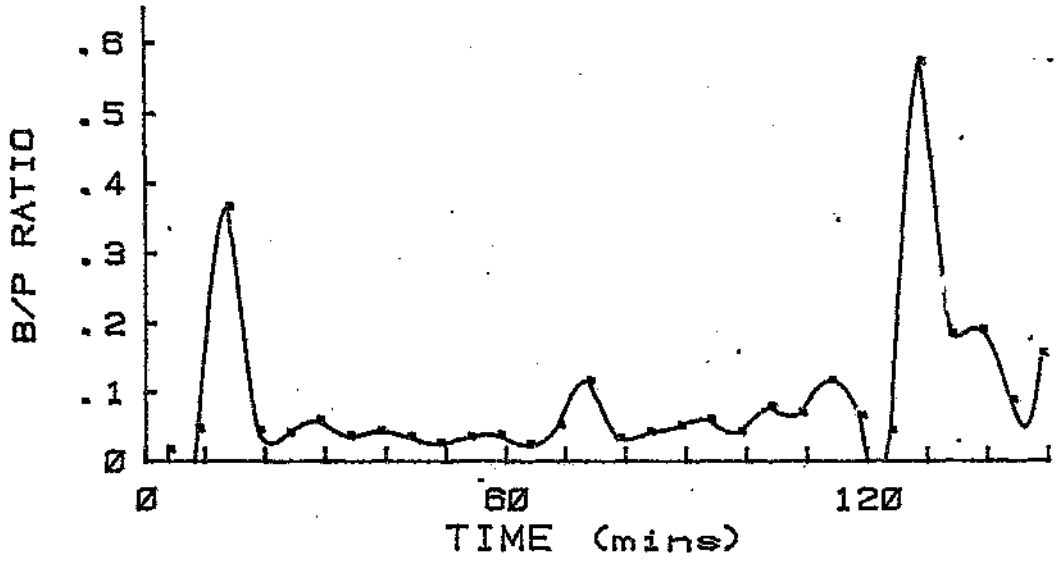

Fig. 38

**Fig.39 VARIATIONS IN THE EXTRACT B/P RATIO FOR HL-60 CELLS**
(cells in stirred suspension)

Oscillations also occur in both the pyruvate activity (not shown) and the B/P ratio in extracts of HL-60 cells. In this experiment the amplitude of the B\P fluctuations is small at first but then increases in magnitude. One possible explanation is that the system is being rhythmically modulated by at least two components having different frequencies, so that the phasing(s) between them gradually change. The maximum value of the B/P ratio is less than for MEL cells where only one isozyme is seen.

Cell number = $7.80 \times 10^6$ cells /ml

# LDH B/P RATIO HL60



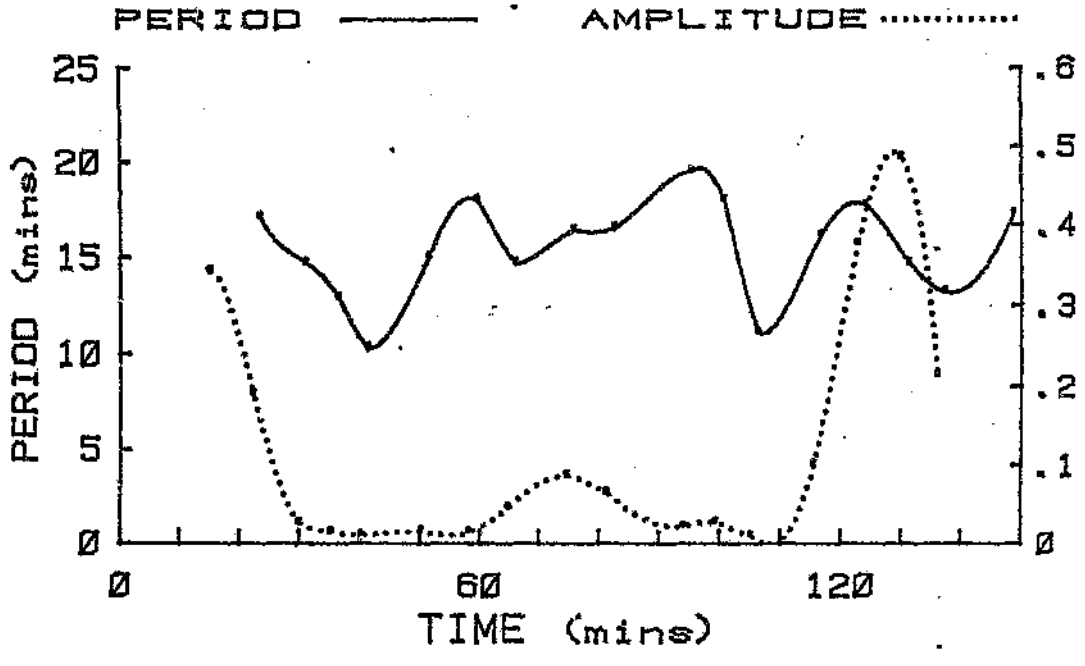



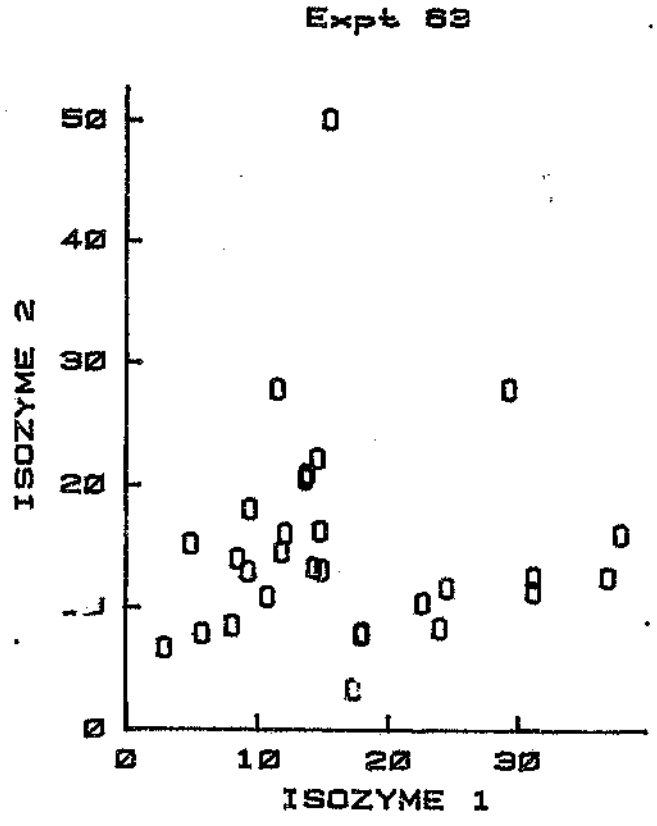

Fig. 39

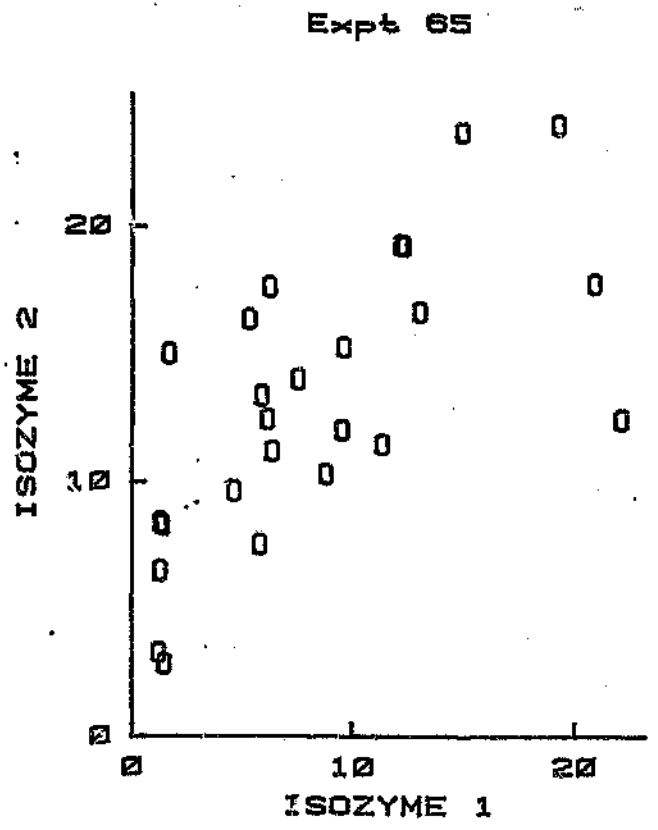
**Fig.40 PHASE PLANE PLOT OF THE VARIATIONS IN THE AMOUNTS OF TWO ACTIVE ISOZYMES IN HL-60 CELLS** (cells in stirred suspension)

In this diagram the activity stain intensity for one LDH isozyme is plotted against the corresponding value for isozyme 2. If the ratio was constant the values should fall on a straight line (positive slope), indicating an in-phase relationship. However, no particular relationship is evident, from which we tentatively conclude that the phase between the two oscillations is varying because of the difference in the frequencies of the two oscillations. The involvement of multiple oscillations would also yield a complex pattern.

# PHASE PLOTS
# LDH ISOZYMES IN HL-60 CELLS

Fig. 40



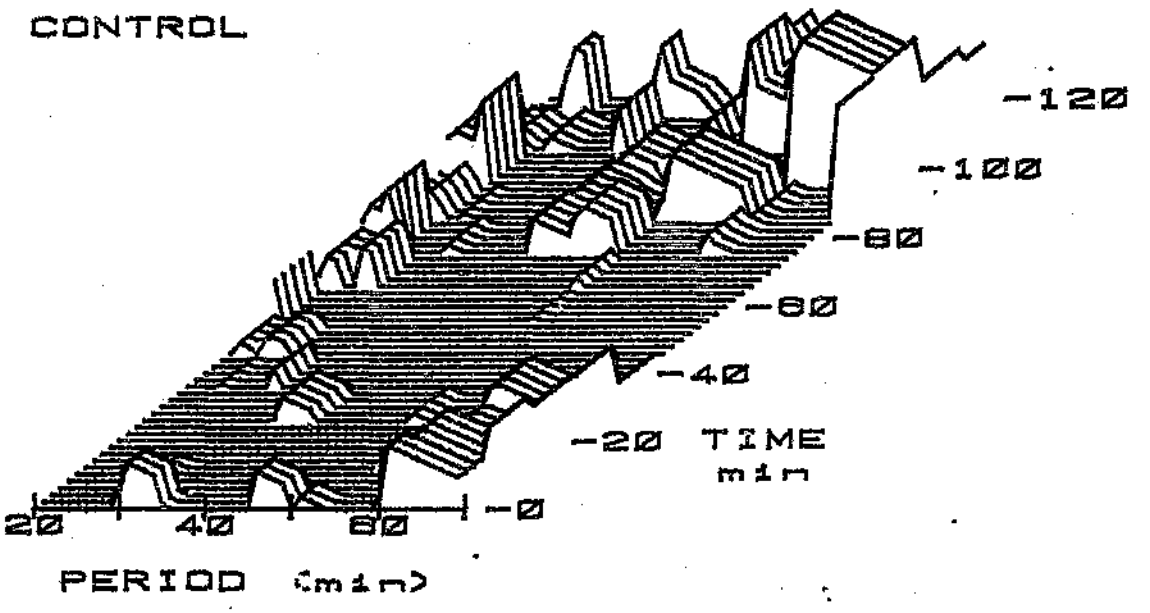
# ACTION OF INSULIN

**Fig.41 INSULIN STIMULATION OF PHOSPHORYLATION POTENTIAL OSCILLATIONS: PROTEIN X**
(MEL cells in stationary monolayer)

This diagram gives Enright periodograms for the oscillation in the level of the 81KD phosphoprotein (protein X), for extracts of MEL cells in the absence or presence of insulin. The latter was added at time zero minutes while an equal volume of buffer was added to the control culture. The stimulating effect of insulin can be seen affecting several oscillations, although a contribution from the harmonics probably exists. Corresponding changes for 63kD phosphoprotein (protein Y) are given in Fig.42. The effect may be partly due to changes in the periods of some oscillations.

(The phosphorylation potential results, including the effect of insulin are discussed more fully in an article in press (Ferreira et al 1994a).

PROTEIN X



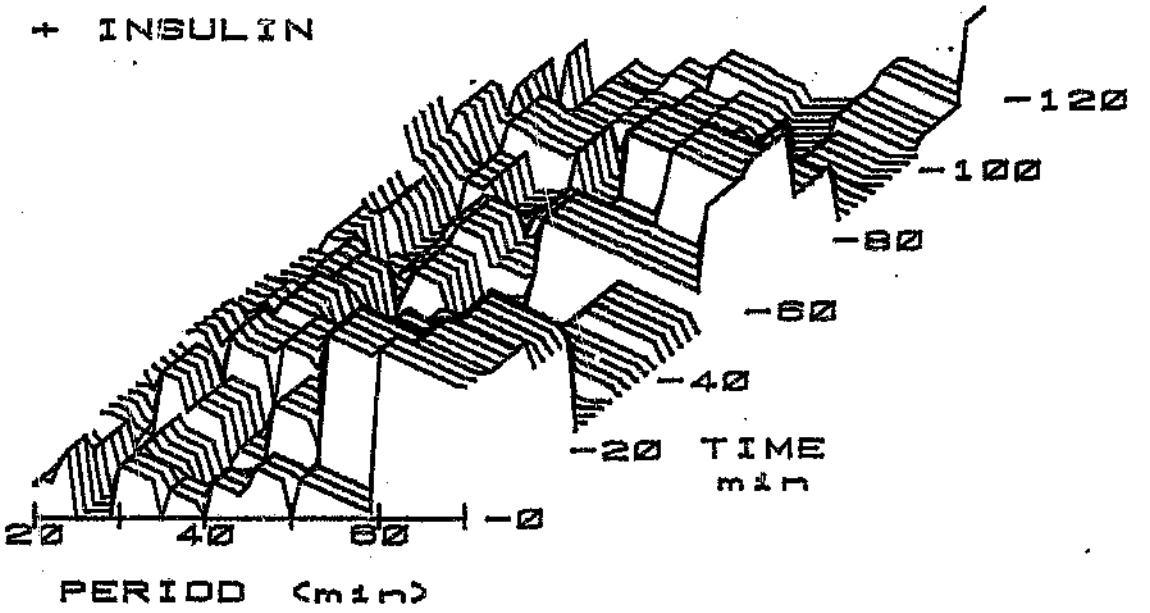
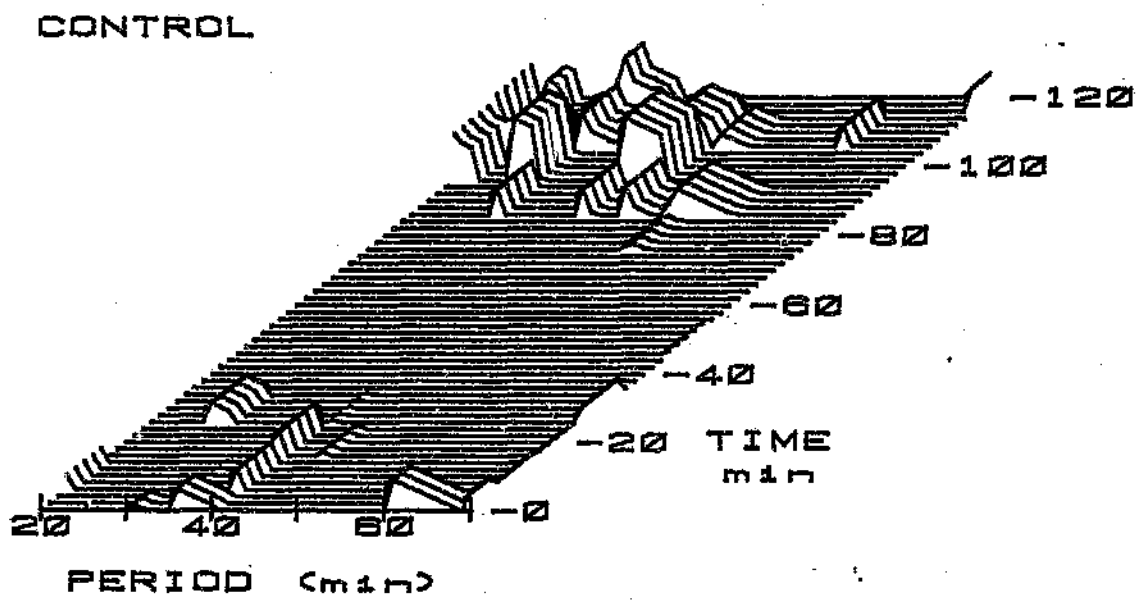




Fig. 41

**Fig.42 INSULIN STIMULATION OF PHOSPHORYLATION POTENTIAL OSCILLATION: PROTEIN Y**
(MEL cells in stirred suspension).

This diagram gives Enright periodograms for the oscillation in the intensity of the 63kD phosphoprotein (protein Y), for extracts of MEL cells in the absence or presence of insulin. The latter was added at time zero minutes while an equal volume of buffer was added to the control culture. It is evident that the hormone also stimulates the oscillations affecting the phosphorylation potential for protein Y. There is evidence for the presence of bursts of some oscillations in the control, after the addition of the hormone there seems to be continuous activity.

PROTEIN Y



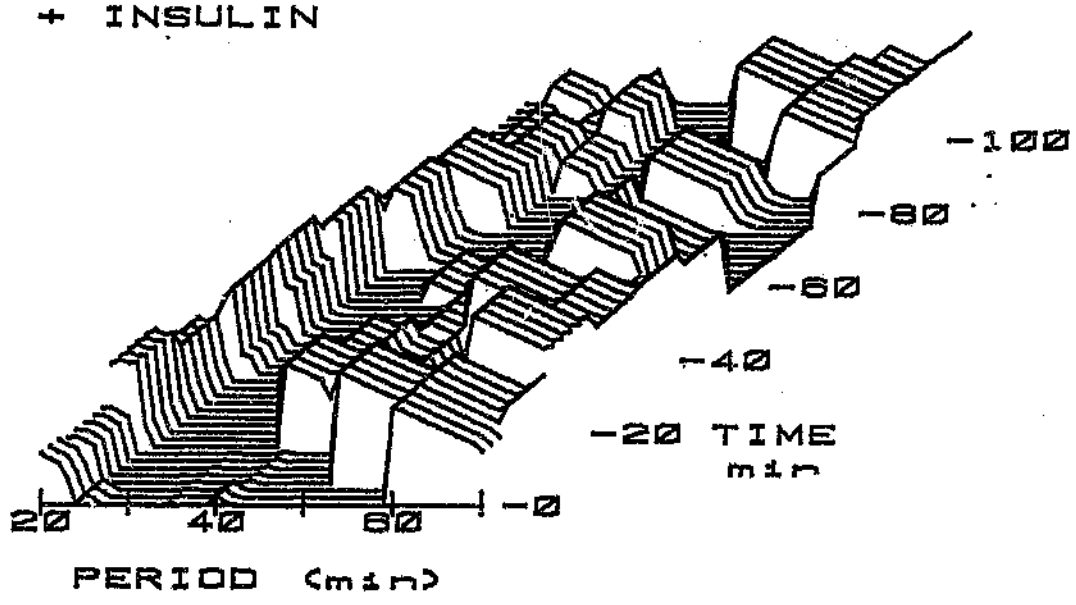
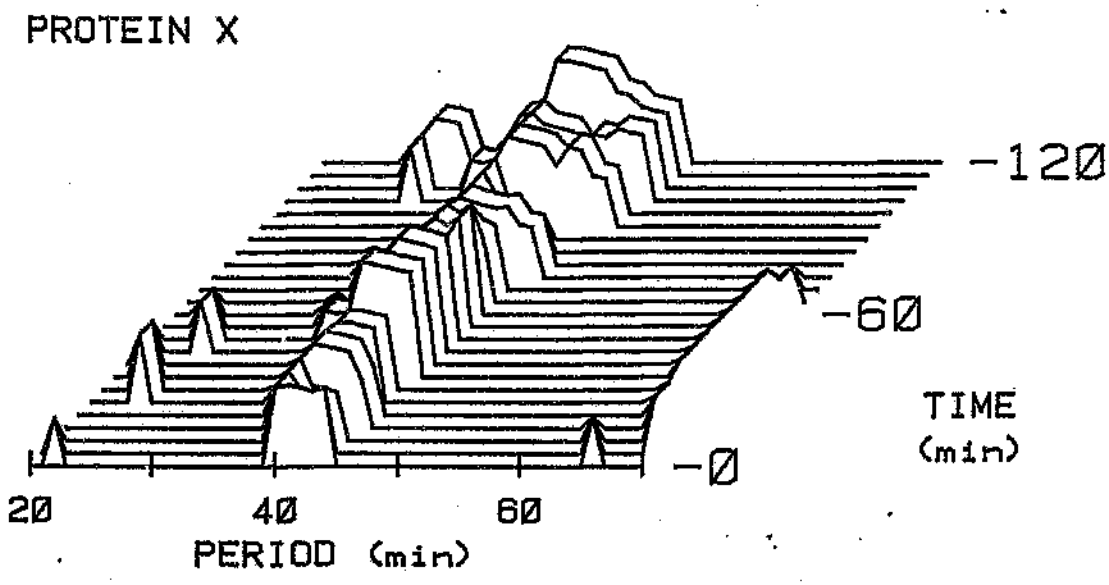




Fig. 42

**Fig.43 COMPARISON OF THE OSCILLATIONS IN THE PHOSPHORYLATION POTENTIAL FOR PROTEIN X AND PROTEIN Y IN INSULIN TREATED MEL CELLS**
(cells in stationary monolayer)

This diagram gives Enright periodograms for variations in the phosphorylation potential for protein X and protein Y in another experiment. It can be seen that for these insulin treated cells, the major oscillations for the protein X and protein Y differ in period.

# PROTEIN PHOSPHORYLATION POTENTIAL
# INSULIN TREATED MEL CELLS



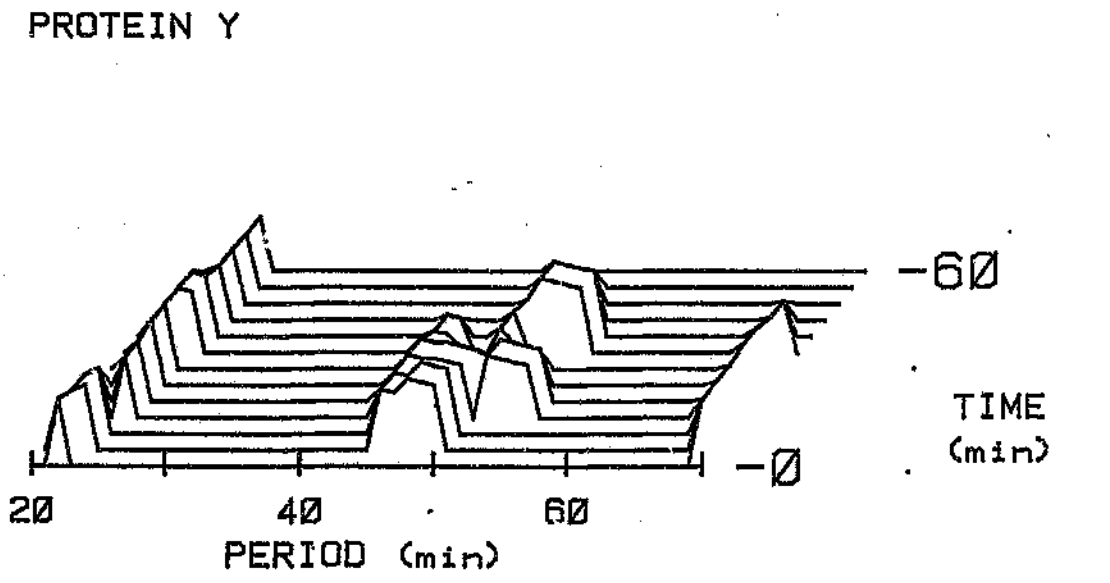




Fig. 43

**Fig.44 THE IMMEDIATE EFFECT OF INSULIN ON PHOSPHOTYROSINE PHOSPHATASE OSCILLATIONS IN MEL CELLS**
(cells in stationary monolayer)

This diagram shows oscillations in the enzyme activity for two control experiments and also for corresponding cultures to which insulin was added at time zero. The initial cultures were split into two groups of subcultures, which were arbitrarilly assigned to control and treated groups. Insulin was added to one group of cultures and an equal volume of buffer added to the other (see appendix IX). Although there are amplitude differences for the two control experiments, the frequencies are similar, around 10 minutes, and both show evidence for double periodicity (discussed in the phosphotyrosine phosphatase section). The results for the insulin-treated cultures are almost identical but the oscillation period is now about a 25 minutes, only a trace of the double periodocity is evident in one experiment.

The continuous spectrophotometric assay was used for all the studies reported here.

The right hand scale refers to experiment 41.

Cell number for a) $5.9 \times 10^6$ cells/ml (exp.40)
b) $6.1 \times 10^6$ cells/ml (exp.41)

PHOSPHOTYROSINE PHOSPHATASE MEL CELLS
IMMEDIATE EFFECTS INSULIN

Fig. 44

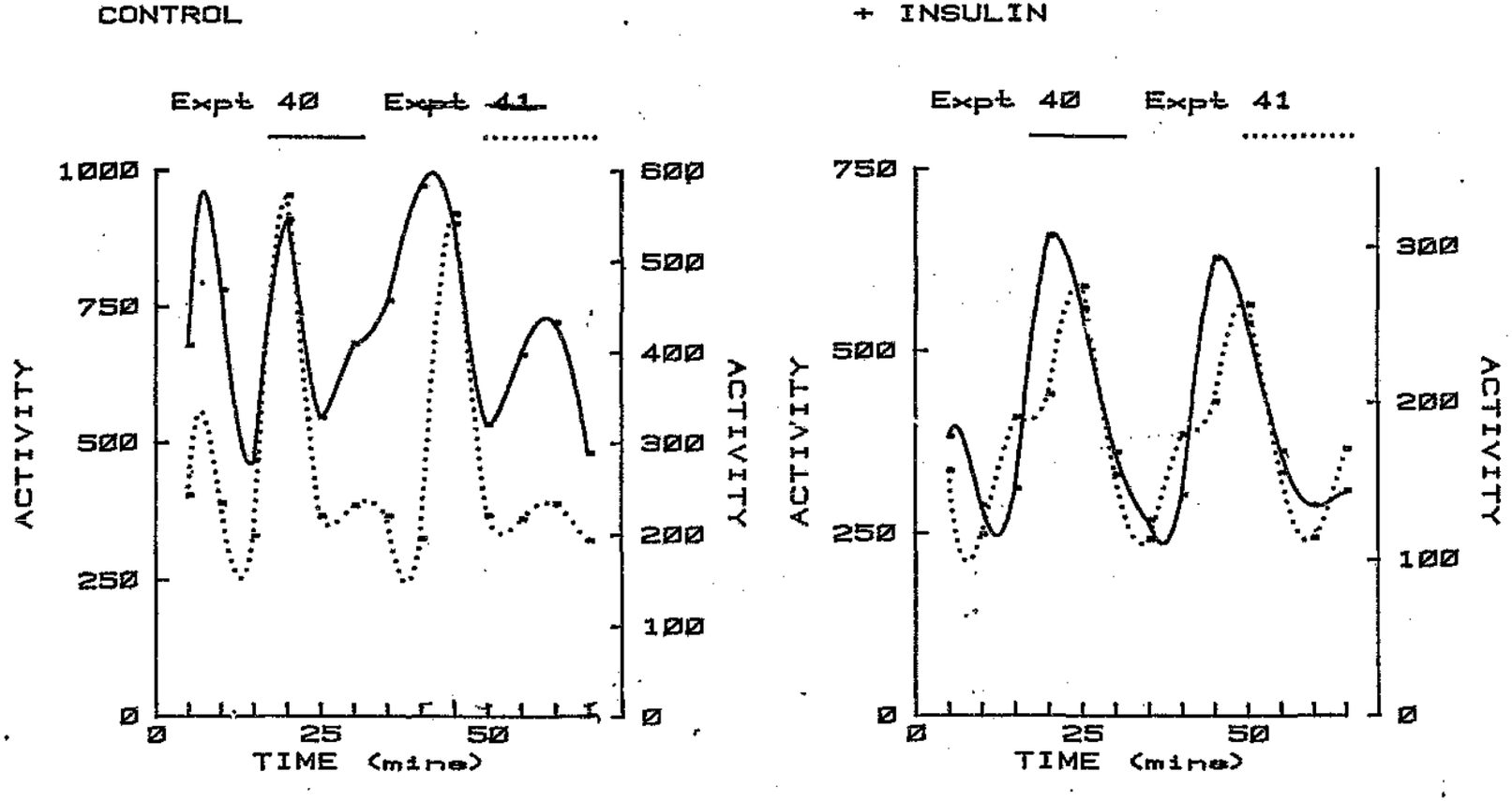


**Fig.45. THE EFFECT OF INSULIN ON PHOSPHOTYROSINE PHOSPHATASE ACTIVITY OSCILLATIONS IN MEL CELLS**
(cells in stationary monolayer)

This diagram shows the activity oscillations for the enzyme for two experiments (using the same initial MEL cell suspension) for the control and insulin treated series of cultures, 36 and 48 hours after treatment with the hormone. At 36 hours the amplitude of the oscillation in the hormone treated cells is considerably lower but the mean level is much higher: there is evidence for the existence of multiple periodicity in both. At 48 hours the amplitudes are more similar for the control and insulin treated cells, but the mean level was higher, possibly because further replication had been initiated by the hormone (unfortunately the cell numbers were not measured at that time). The double periodicity is still marked in the control culture but less in the treated cells.

The right hand scale refers to insulin treated cells.

Initial cell number for
a) $6.2 \times 10^6$ cells/ml (exp.43)
b) $5.2 \times 10^6$ cells/ml (exp.44)

PHOSPHOTYROSINE PHOSPHATASE MEL CELLS ACTIVITIES

Fig. 45

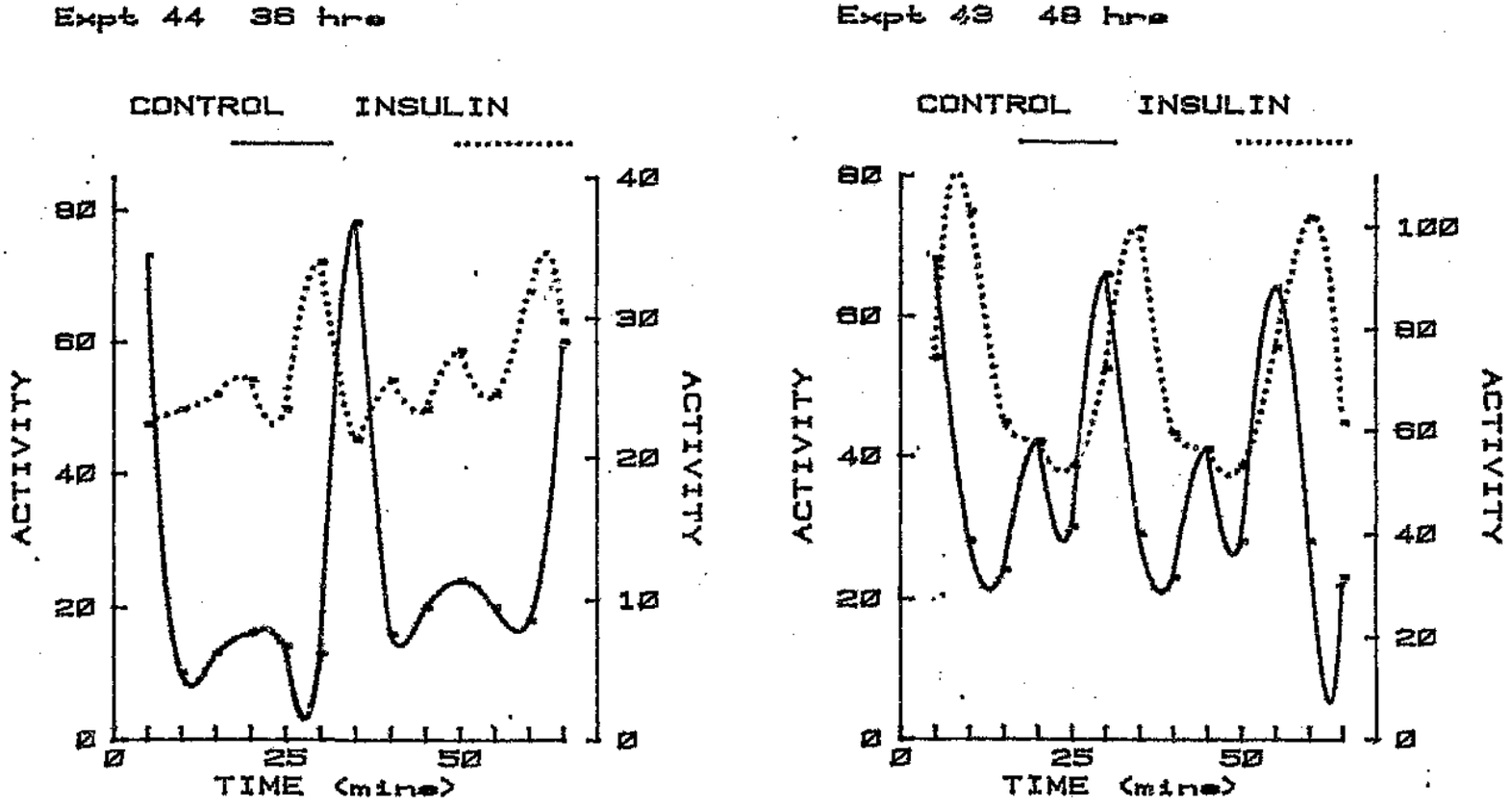


**Fig.46 IMMEDIATE EFFECTS OF INSULIN ON THE PHOSPHOTYROSINE PHOSPHATASE SPECIFIC ACTIVITY OSCILLATION**
(MEL cells in stationary monolayer)

In previous diagrams (Fig.44 and Fig.45) the oscillations shown were for the activity of the enzyme, no correction being made for the amount of protein in the particular extract. As discussed elsewhere, there is an oscillation in the amount of extractable protein which complicates interpretation of specific activity fluctuations but examples are given here for two experiments. Comparison of the insulin treated cells with the corresponding control indicates that the hormone rapidly alters the period, mean and waveform but has less effect on the amplitude. Thus from visual inspection the period increases from about 13 minutes to around 20 minutes (the number of values is too small for per amp plots).The multiple periodicity seen in the controls is lost, there is a slight drop in the mean specific activity in both cases.

Cell number for a) $5.90 \times 10^6$ cells/ml (exp.40)
b) $6.10 \times 10^6$ cells/ml (exp.42)

PHOSPHOTYROSINE PHOSPHATASE MEL CELLS
SPECIFIC ACTIVITIES IMMEDIATE EFFECTS

Fig. 46

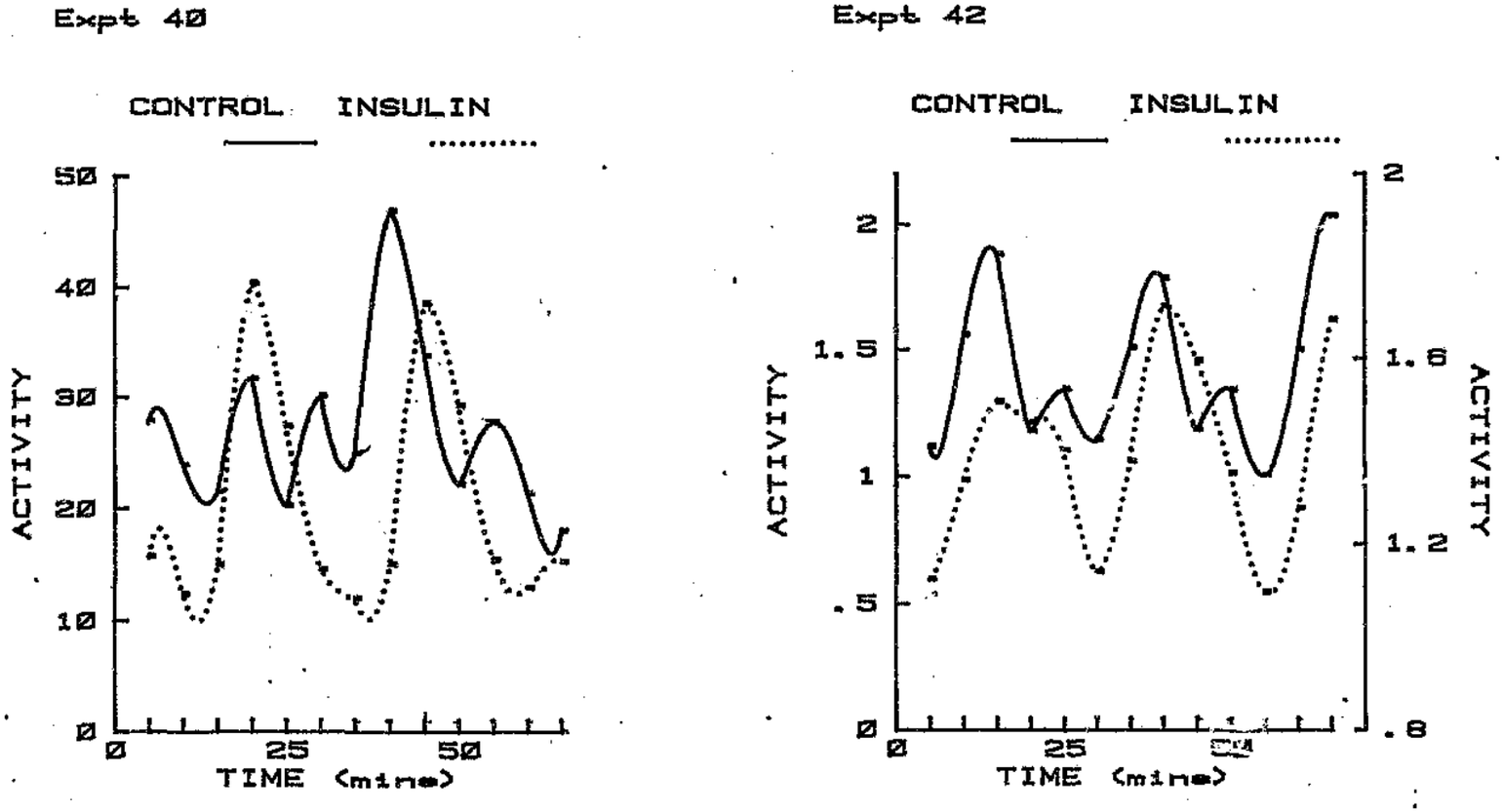


**Fig.47 THE COMBINED EFFECTS OF INSULIN AND HMBA ON PHOSPHOTYROSINE PHOSPHATASE ACTIVITY OSCILLATIONS**
(MEL cells in stationary monolayer).

This diagram shows oscillations in the activity of control cultures and those treated with both insulin and HMBA, immediately after adding the agents (or buffer to control cultures) and 48 hours later. In both cases the agents decrease the amplitude of the oscillation and slightly increase the mean. Double periodicity can be seen in both control cultures but the waveform of the oscillation in the treated cells is more complex, this contrasts with the affect of insulin alone. It is more difficult to estimate the period of the oscillation in the treated cells because of the complex waveform, but the frequency obviously remains high, again in contrast to the effects of insulin alone. This result supports the original idea that insulin and HMBA may have antagonistic action on the cells.

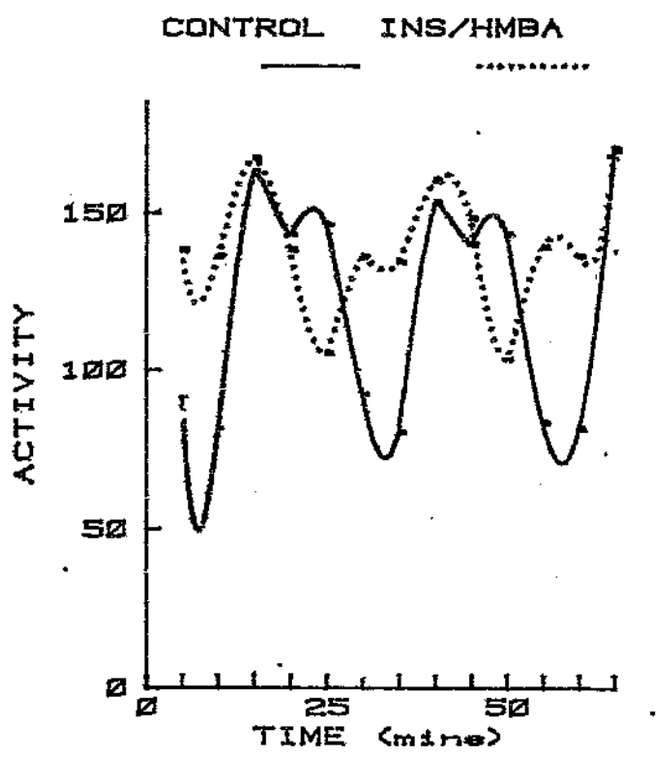
The right hand scale refers to the agents treated cells.

Cell number for a) $6.10 \times 10^6$ cells/ml (exp.42)
b) $6.20 \times 10^6$ cells/ml (exp.43)

PHOSPHOTYROSINE PHOSPHATASE MEL CELLS
COMBINED ACTION INSULIN/HMBA

Fig. 47

Expt 42 IMMEDIATE EFFECTS




Expt 43 48 hrs

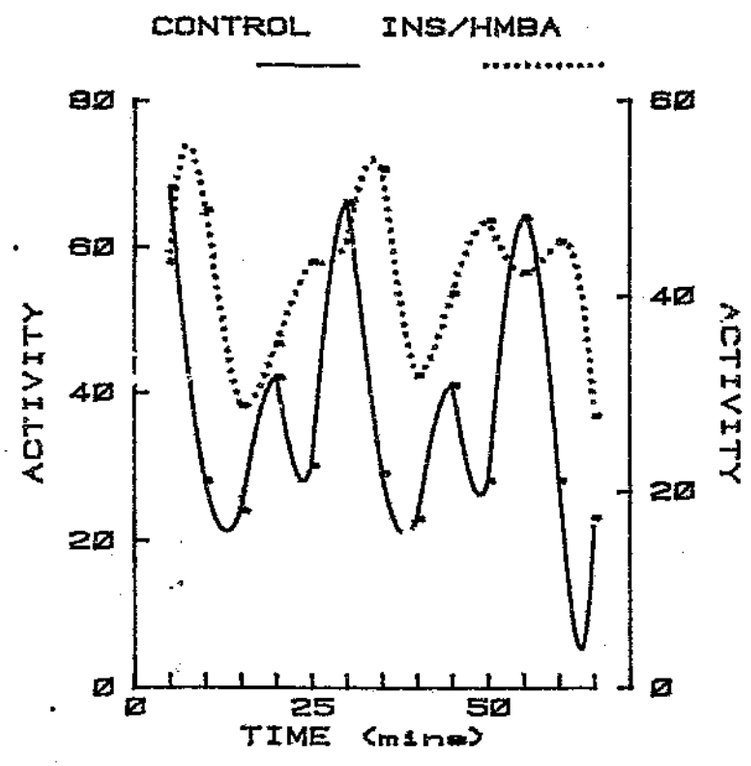


**Fig.48 EFFECT OF INSULIN ON THE OSCILLATION IN THE PYRUVATE ACTIVITY OF LDH**
(MEL cells in stirred suspension)

Insulin has no obvious effect on the pyruvate activity oscillation as judged from the time plots of the raw data. This Enright periodogram shows that several rhythms contribute toward the observed periodicity. Insulin might have caused a drift in the periods of one or two rhythms but this effect could have been spontaneous. It should be born in mind that any action of the hormone on long period rhythms would not be detected because of the short duration of these experiments.

Cell number = $1.60 \times 10^6$ cells/ml

Expt. 52PY
MEL SUSPENSION

SAMPLE INTERVAL 5 mins
CALC INTERVAL .1
WINDOW 26 pts
STEP INTERVAL .5
THRESHHOLD .05
SCALE FACTOR 2

# LDH PYRUVATE ACTIVITY

+ INSULIN
ADDED AT 37.5mins

major periods

ENRIGHT PERIODOGRAM
PEREN3.7 CYN3 MAY 99

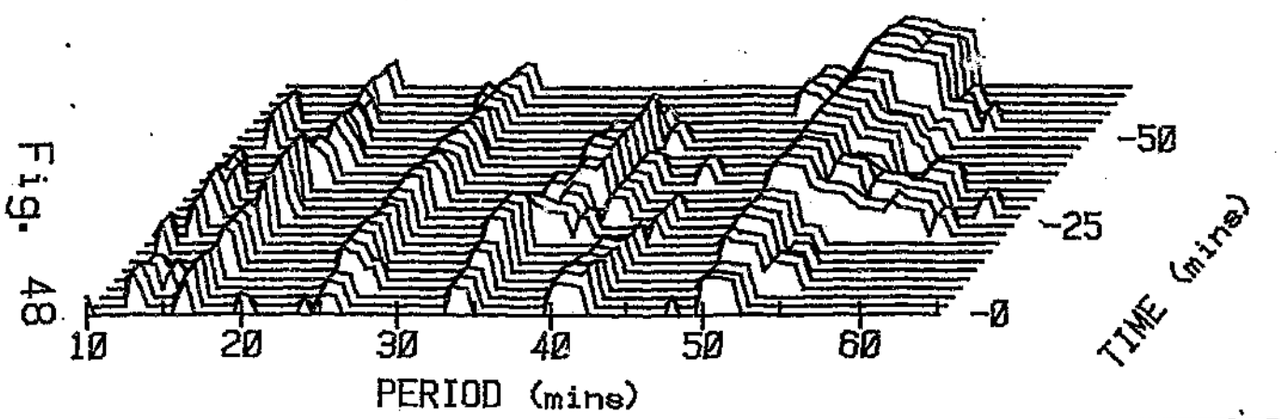


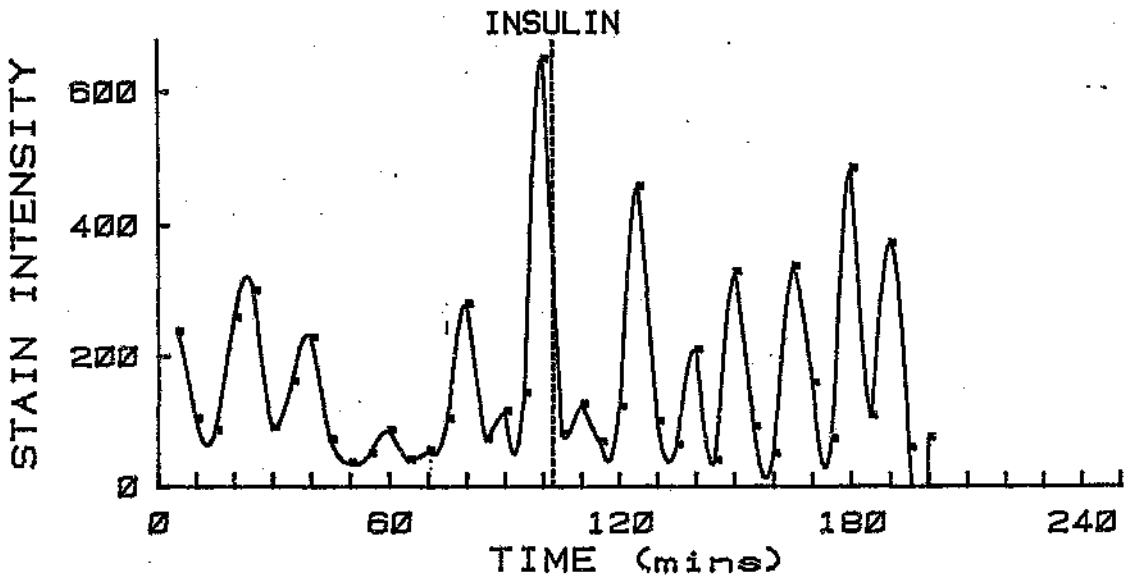

Fig. 48

**Fig.49 EFFECT OF INSULIN ON OSCILLATION IN THE AMOUNT OF ACTIVE LDH ISOZYME IN MEL CELLS** (cells in stirred suspension)

From this diagram it would seem that insulin has a rapid effect on the two modulating rhythms. There also seems to be an increase in the mean and the amplitude of the modulating rhythm. Although the wave forms of the two modulating rhythms seem distinct before the addition of the hormone, they are similar afterwards. This might be due to a synchronizing effect of insulin.

Cell number = $1.60 \times 10^6$ cells/ml

# LDH ELECTROPHORESIS



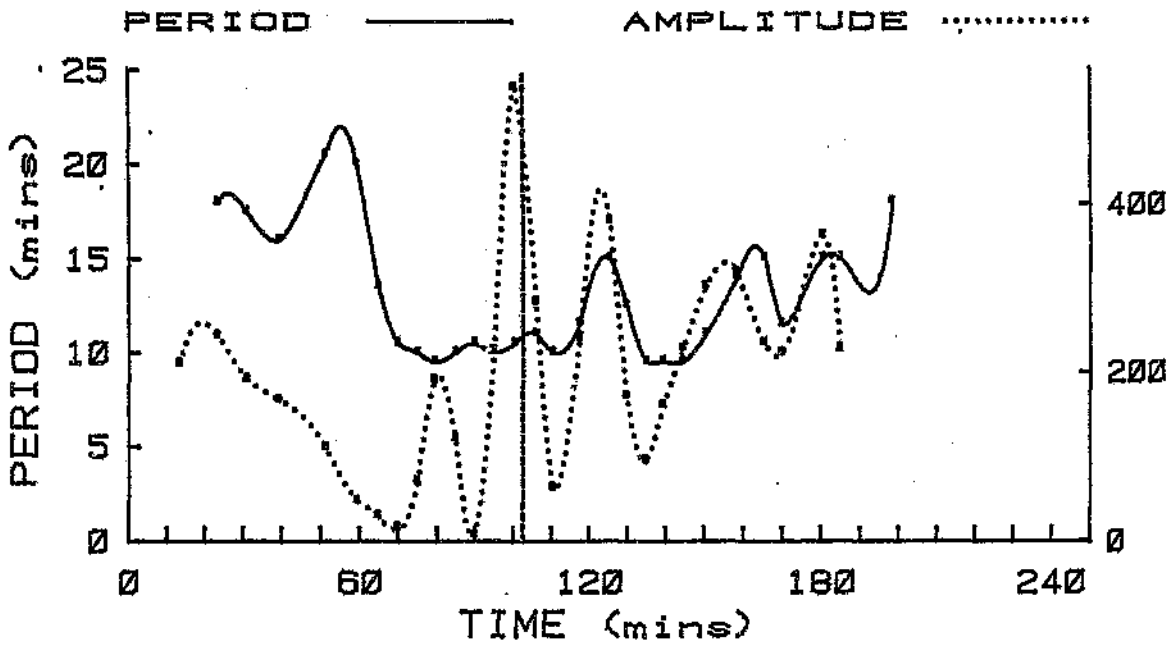




Fig. 49

**Fig.50 ENRIGHT PERIODOGRAM FOR THE ACTIVE LDH ISOZYME OSCILLATION: EFFECT OF INSULIN ON MEL CELLS**
(cells in stirred suspension)

This diagram also suggests that the hormone influences this rhythm more than that of the pyruvate activity of the extracts. However, in contrast to the last diagram this one indicates an enhancement of low frequency components. The period of some of the high frequency oscillations may be affected, some appear to be increasing in period during the initial stages, which makes interpretation difficult.

Cell number = $1.00 \times 10^6$ cells/ml

Expt. 50G
MEL

SAMPLE INTERVAL 5 mins
CALC INTERVAL .1
WINDOW 20 pts
STEP INTERVAL .5
THRESHHOLD .05
SCALE FACTOR 1

# LDH STAIN INTENSITY

+ INSULIN
ADDED AT 72.5mins

major periods

ENRIGHT PERIODOGRAM
PERIOD.6 CYNI Nov 82

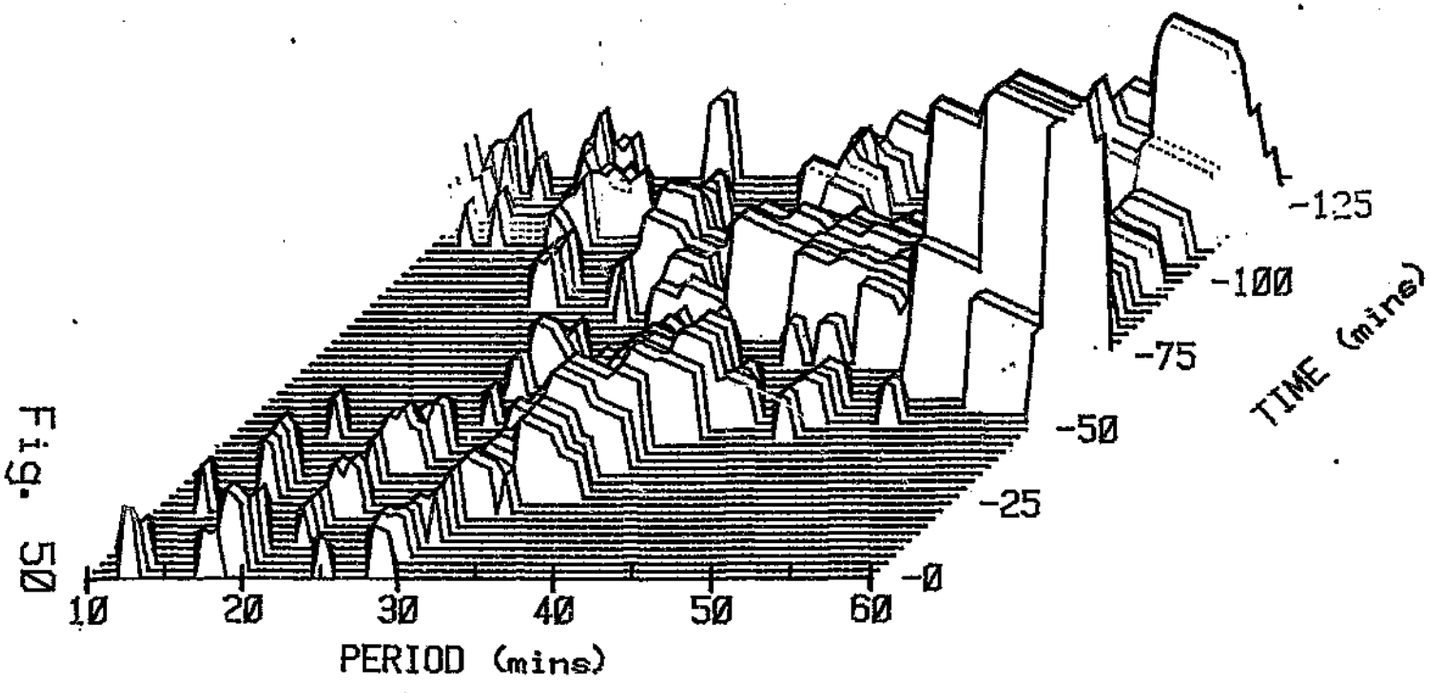



Fig. 50

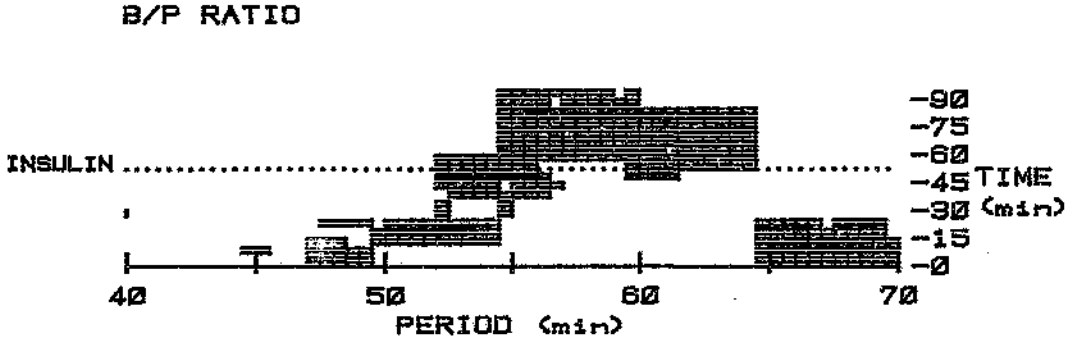
**Fig.51 ACTION OF INSULIN ON LDH ACTIVITIES AND APPARENT ISOZYME PATTERN: ENRIGHT PLAN PLOTS** (MEL cells in suspension)

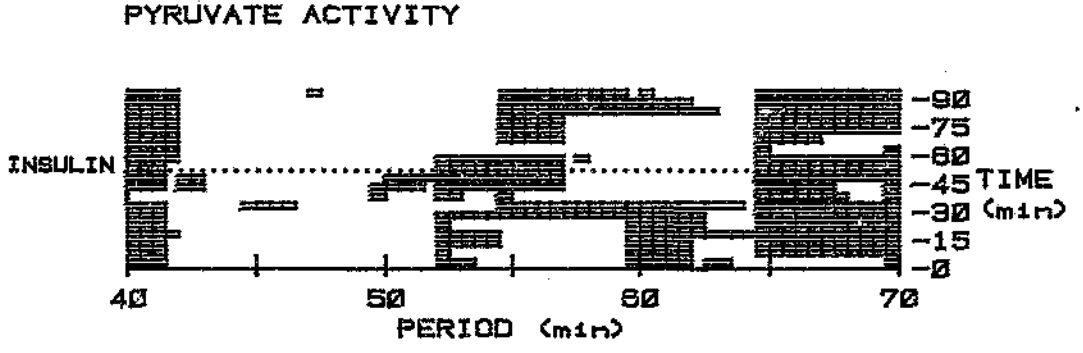
In the experiment concerned here, insulin appears to give rise to the appearance of isozyme oscillations with periods between 55-65 minutes and either inhibits those with periods 65-70 minutes and 50-55 minutes or changes their frequency. Alternatively it may have stabilised the rhythm with a period varying from about 46-55 minutes.

Although there ~~is~~ are some similarity in the pyruvate activity periodogram, the response differs from those seen in the B/P plot. The periodogram for their ketobutyrate activity oscillations are quite distinct from the isozyme rhythms. This would seem to support the view (see Fig.32) that kinetics and specificity of LDH are modulated by distinct factors.

Cell number = $1.23 \times 10^6$ cells/ml

# LACTATE DEHYDROGENASE
# ENRIGHT PLAN PLOTS



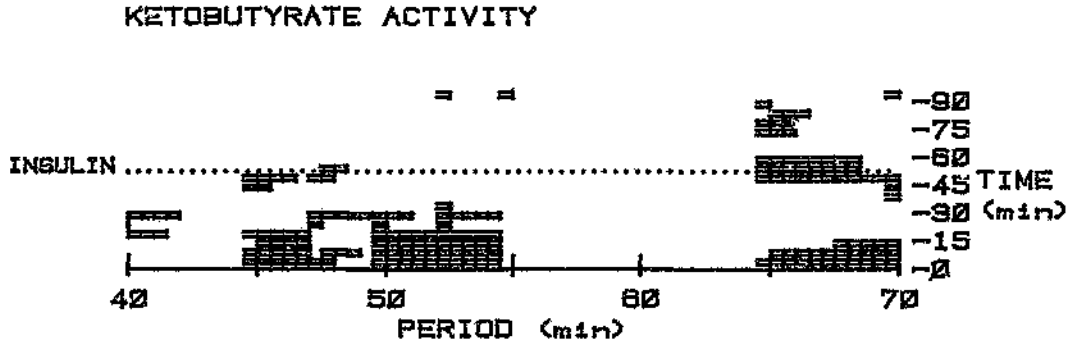




Fig. 51

**Fig.52a,b EFFECTS OF INSULIN ON THE ISOZYME BAND INTENSITY AND ISOZYME "SPECIFIC ACTIVITY": INVERSE PERIODOGRAMS**
(MEL cells in stationary monolayer)

These diagrams obtained from two different experiments show periodograms for the oscillation in the stain intensity (a) without "correction" for the protein level and (b) with this "correction" (the activity stain "specific activity"). Both diagrams show the same effects, these are:

(i) the occurrence of several dominant rhythms (and harmonics thereof which initially are all gradually increased in frequency)

(ii) after the addition of insulin the pattern of behaviour appears to stabilize

(iii) both diagrams show the simultaneous existence of a broad band of frequencies at the beginning of the plots which exist only for a short duration of time. Such behaviour (seen also in other parameters) may be due to the existence of transient chaotic behaviour [see text].

Cell number for a) $1.60 \times 10^6$ cells/ml (exp.52)
b) $1.22 \times 10^6$ cells/ml (exp.51)

52

SAMPLING INTVL 5 mins
WINDOW 20 pts
CALC INTERVAL .1
STEP INTERVAL .5
THRESHOLD .12

# INVERSE PERIODOGRAM
# LOH STAIN INTENSITY
# INSULIN
# ADDED AT 50 mins
MAJOR COMPONENTS

INV3D.4 CYN 3 14.5.93
52GEL CYN 3
18.5.93

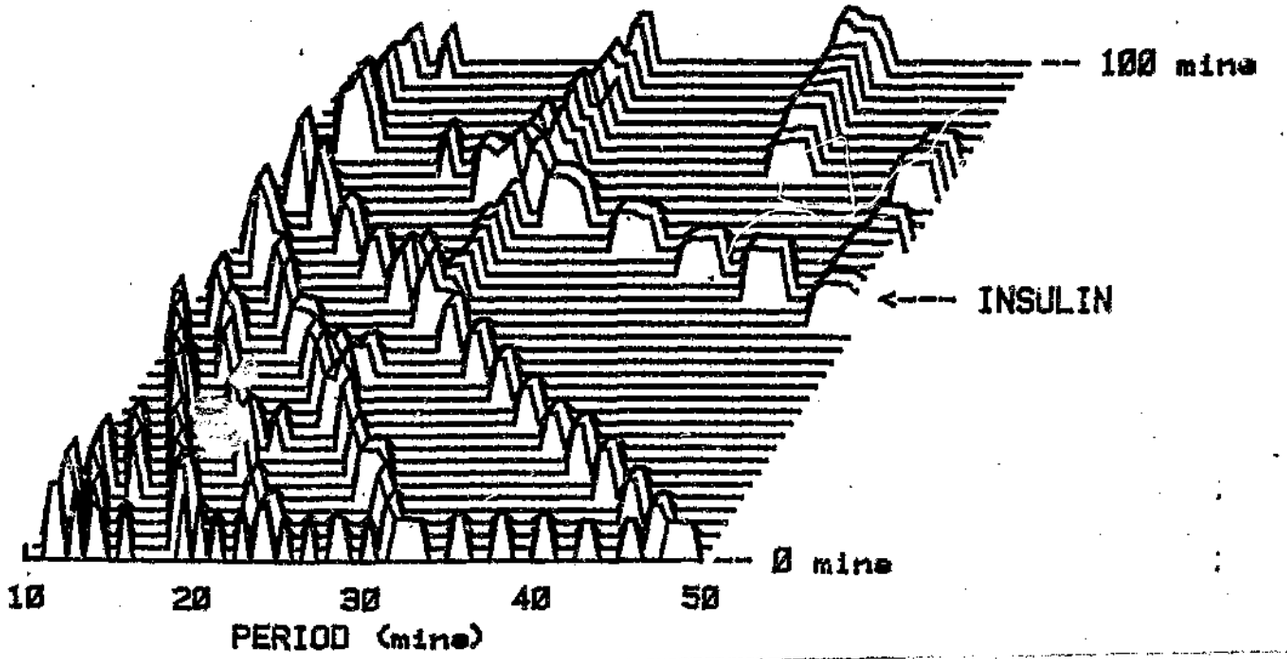



Fig. 52a

51

SAMPLING INTVL 5 mins
WINDOW 20 pts
CALC INTERVAL .1
STEP INTERVAL .5
THRESHOLD .12

# INVERSE PERIODOGRAM
# LDH GEL/PROTEIN
# INSULIN
# ADDED AT 70 mins

MAJOR COMPONENTS

INV3D.4 CYN 3 14.5.93
51G/PR CYN 3
15.5.93

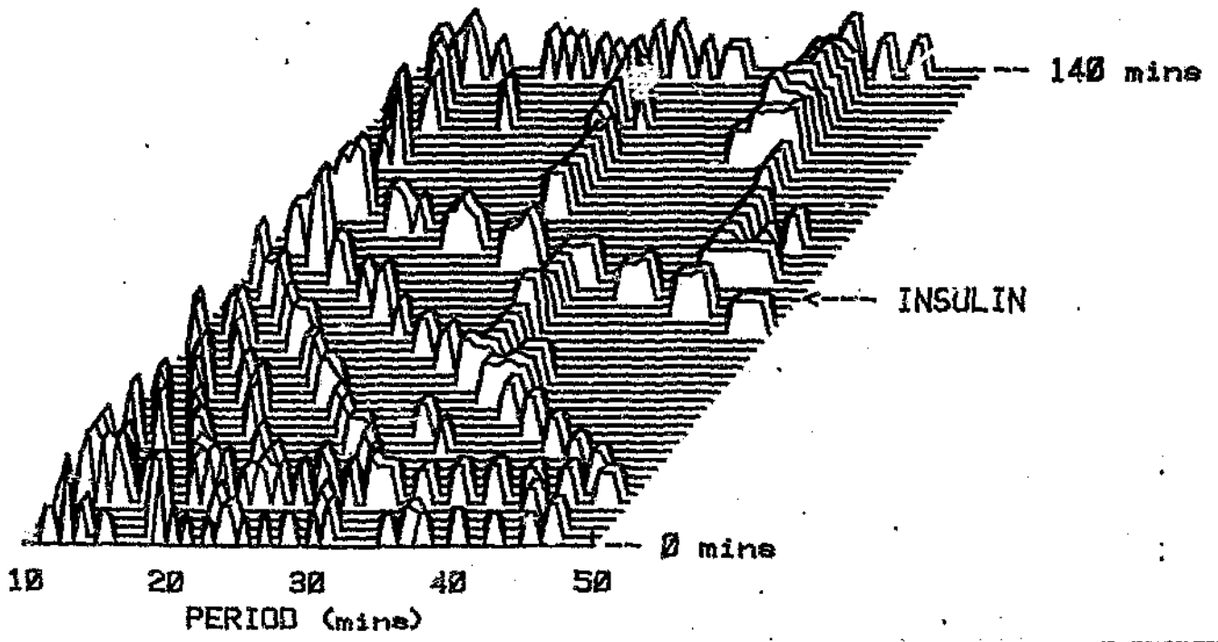



Fig. 52b

**Fig.53 ENRIGHT PERIODOGRAM: EFFECT OF INSULIN ON THE B/P RATIO OSCILLATION IN MEL CELLS**
(cells in stirred suspension)

The results shown here seem to suggest that the hormone increases the period of the oscillations with initial period of 45-50 minutes, and decreases the period of oscillations having periods of 65-80 minutes. However, the changes appear to be initiated before the point of addition of insulin. Since the two rhythms (bands) have similar period after the addition of the hormone, the possibility seems to exist that they mutually synchronise with one another. After a short delay the long period rhythms reappear but are enhanced in amplitudes.

Cell number = $1.22 \times 10^6$ cells/ml

ENRIGHT PERIODOGRAM
peran3.2 CYN7 FEB 94

# LDH B/P RATIO
## ENRIGHT PERIODOGRAM

Expt. 51BP
MEL

SAMPLE INTERVAL 5 mins
CALC INTERVAL .1
WINDOW 24 pts

STEP INTERVAL .5
THRESHHOLD .05
SCALE FACTOR 1

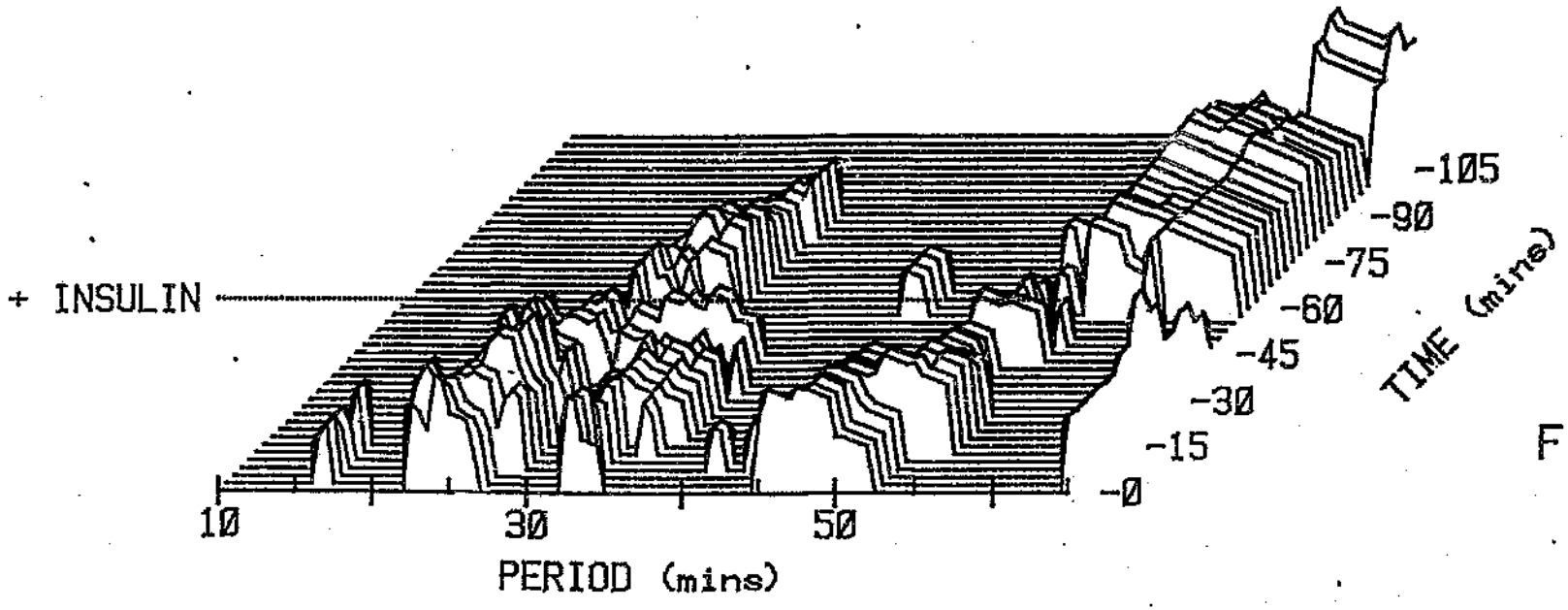



Fig. 53

**Fig.54 THE EFFECT OF INSULIN ON THE EXTRACTABLE PROTEIN OSCILLATION: ENRIGHT PERIODOGRAM** (MEL cells in stirred suspension)

This diagram could be taken to indicate that insulin affects the frequencies of certain oscillations contributing towards the extractable protein rhythms, enhancing some and inducing long period oscillations. The results may be compared with the effect of the hormone on the B/P ratio oscillation shown in Fig.53. More extensive analysis of these effects is given in the article in press (Ferreira et al 1994b).

EXTRACTABLE PROTEIN

ENRIGHT PERIODOGRAM

MEL SUSPENSION

SAMPLE INTERVAL 5 mins
CALC INTERVAL .1
WINDOW 20 pts
STEP INTERVAL .5
THRESHHOLD .05
SCALE FACTOR 2

+ INSULIN
ADDED AT 72.5mins

major periods

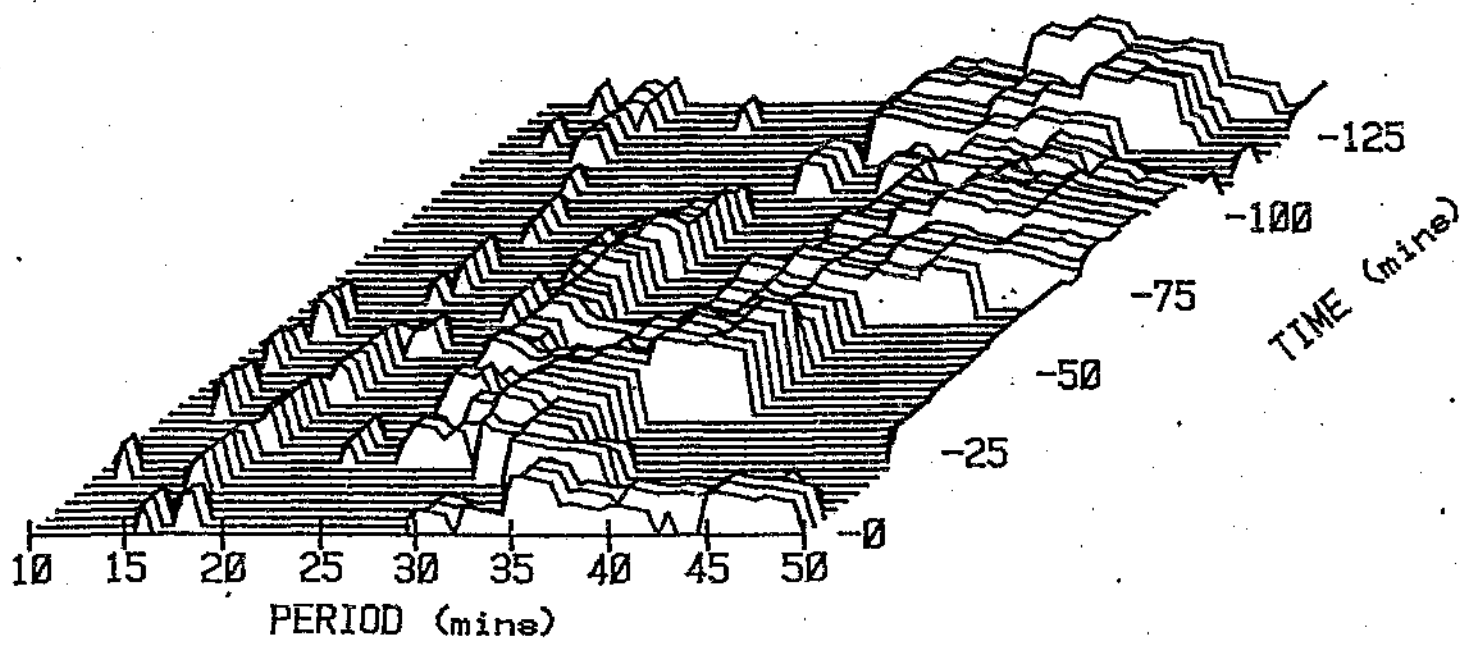



Fig. 54

**Fig.55 INVERSE PERIODOGRAM FOR THE EXTRACTABLE PROTEIN OSCILLATION: EFFECT OF INSULIN**
(MEL cells in stationary monolayer)

These periodiograms are presented in support of the view that insulin appears to

(i) induce additional oscillations in some parameters, and

(ii) stabilize period drift (compare with Fig.52a and Fig.52b).

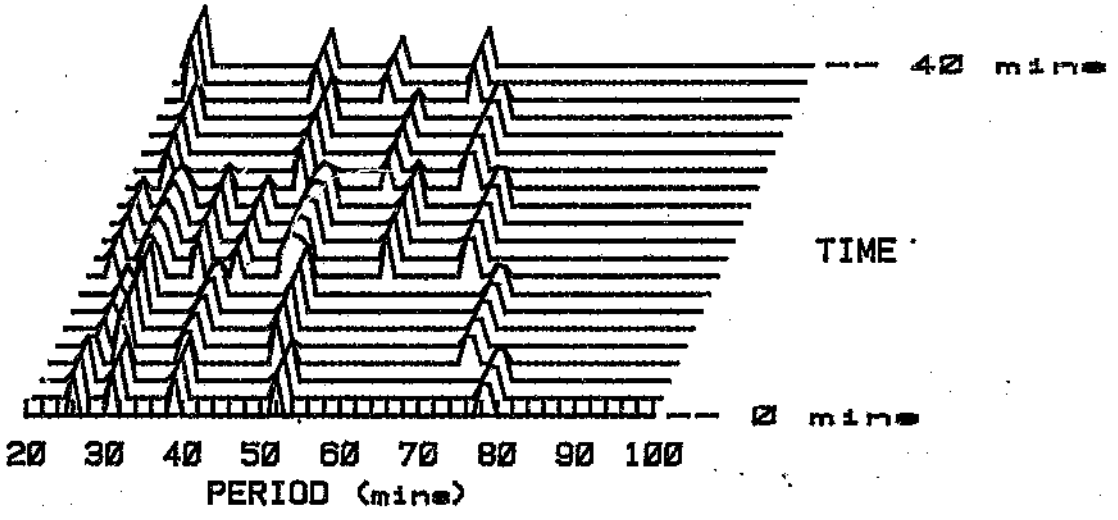
Additional bands appear in the insulin treated cells (some may be harmonics) but all are constant in period. The frequencies may differ slightly from the rhythms in the controls.

n.b: the step interval used in these diagrams is fractional, which emphasizes the pattern of behaviour by repeating each curve several times. However, this procedure tends to suggest discrete shifts where the period is changing with time, whereas in actual fact the variation is smooth (compare with upper diagram of Fig.55).

INVERSE PERIODOGRAM

EXTRACTABLE PROTEIN

CONTROL




INSULIN

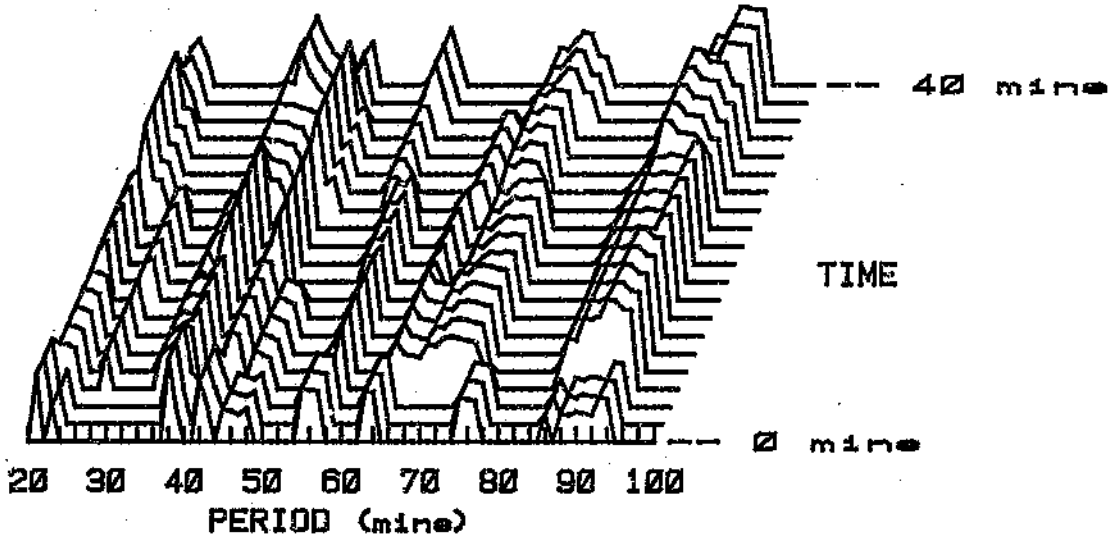



Fig. 55

# ACTION OF HMBA

**Fig.56 THE EFFECT OF HMBA ON THE PROTEIN X PHOSPHORYLATION POTENTIAL OSCILLATION**
(MEL cells in stationary monolayer)

This diagram shows the immediate effects of HMBA on the pattern of behaviour and those observed 36 and 72 hours later. In these figures, the results have not been corrected for the protein level of the various extracts. It would seem that in the short term the inducer of differentiation decreases the mean level and alters the waveshape of the oscillation and possibly slightly affects the period. After 72 hours there is a partial recovery of waveform (i.e. double periodicities are again evident as seen in the corresponding control, also determined at 72 hours). However the mean value is now considerably lower, from this diagram alone it is not clear if the difference in the mean at 72 hours is due to inhibition of replication. The amplitude of the oscillation also seems to recover somewhat by 72 hours. It would thus seem that HMBA has a rapid but transient effect on the phosphorylation potential which may be involved in the induction process.

Cell number for a) $3.3 \times 10^6$ cells/ml (exp.90)
b) $3.2 \times 10^6$ cells/ml (exp.91)
c) $3.3 \times 10^6$ cells/ml (exp.93)

# PROTEIN X PHOSPHORYLATION
# AUTORADIOGRAPH STAIN INTENSITY




Fig. 56

**Fig.57 THE EFFECT OF HMBA ON THE OSCILLATION IN PHOSPHOTYROSINE PHOSPHATASE ACTIVITY**
(MEL cells in stationary monolayer)

This diagram shows the immediate effects of HMBA on the phosphotyrosine phosphatase oscillation and also the pattern observed at 36 and 72 hours later. The inducer has a rapid effect on the amplitude, mean and waveform of the oscillation (although double periodicity is still evident) and there is possibly a slight effect on the period and phase. At 36 hours the response of the mean and amplitude are still evident, and the waveform is more complex. At 72 hours the mean level is considerably lower but the amplitude, waveform and period are much the same as the control at time zero, although the pattern of behaviour for the corresponding control at 72 hours is decreased in amplitude. However these results are not "corrected" for protein and the lower mean at 72 hours may reflect inhibition of replication by HMBA. Compare these results with the action of HMBA on protein phosphorylation (Fig.56). It appears that both phosphorylation and dephosphorylation processes are rapidly but temporarily affected by the inducer.

Cell number for a) $4.3 \times 10^6$ cells/ml (exp.30)
b) $4.6 \times 10^6$ cells/ml (exp.31)
c) $7.0 \times 10^6$ cells/ml (exp.34)

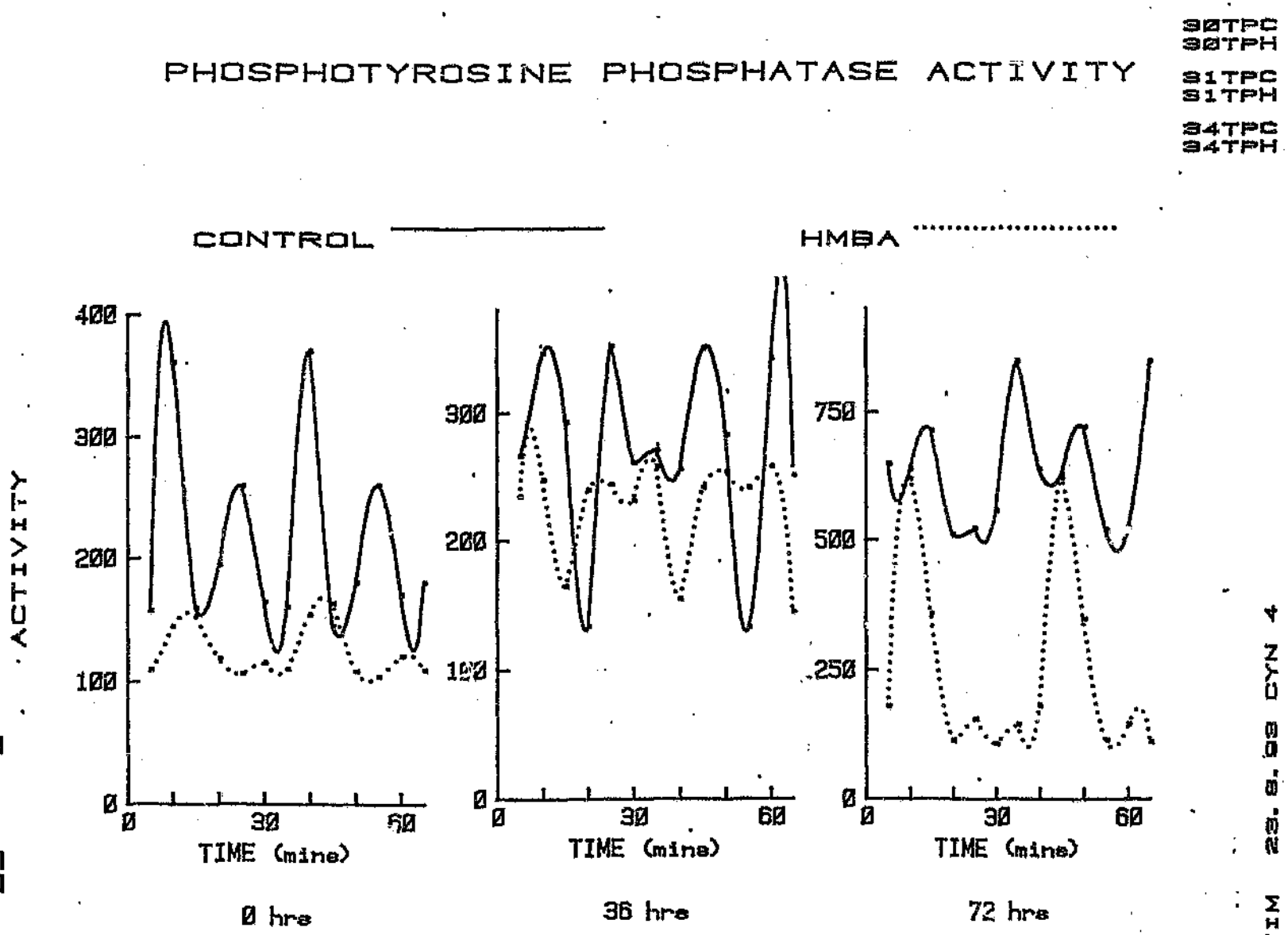

PHOSPHOTYROSINE PHOSPHATASE ACTIVITY
CONTROL
HMBA
ACTIVITY
TIME (mins)
TIME (mins)
TIME (mins)
0 hrs
36 hrs
72 hrs
90TPC
90TPH
91TPC
91TPH
94TPC
94TPH
VRTIM 28.8.98 CYN 4

Fig. 57

**Fig.58 THE EFFECT OF HMBA AT 72 HOURS ON THE OSCILLATION IN THE LDH ISOZYME**
(MEL cells in stationary monolayer)

Unlike the phosphorylation and dephosphorylation results, at 72 hours there is no difference in the mean level for this oscillation compared with the corresponding control. The amplitude of the oscillation is about the same. There appears to be a phase-shift compared with the control but this is uncertain. Double periodicity waveform is evident in the treated cultures, whereas the control seems to exhibit tri-periodicity. The cell number was not determined at 72 hours but if it was lower in treated cultures than in the control (because of an inhibition of replication), one might expect the mean level of this enzyme to be lower as with the phosphotyrosine phosphatase and phosphorylation potential values.

LACTATE DEHYDROGENASE MEL CELLS

ELECTROPHORESIS: ACTIVITY STAIN

HMBA 72 hrs

66GC
66GH

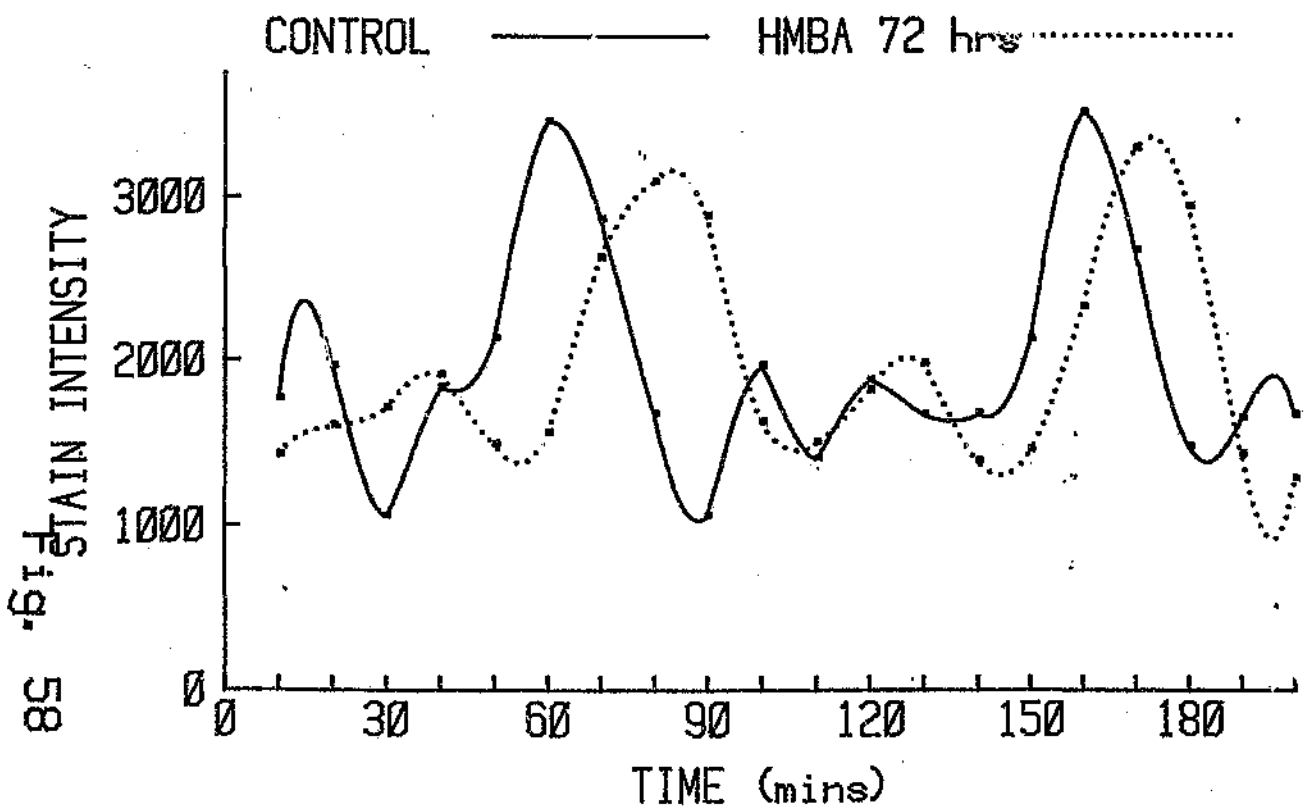



Fig. 58

12.6.93 CYN 4

**Fig.59 EFFECT OF HMBA ON THE EXTRACTABLE PROTEIN OSCILLATION IN MEL CELLS**
(cells in stationary monolayer)

From these results it would appear that the HMBA also has a rapid effect on this rhythm but the duration of the experiment was too short for particular effects to be distinguished. There is possibly a transient effect on the period giving rise to a temporary shift in phase relative to the control. Initially, the mean level appears to be lowered somewhat. At later times (36 and 72 hours), there is an increase in the mean, which does not seem to be explicable in terms of an inhibition of cell replication. There is possibly a slight difference in the period when compared with the control.

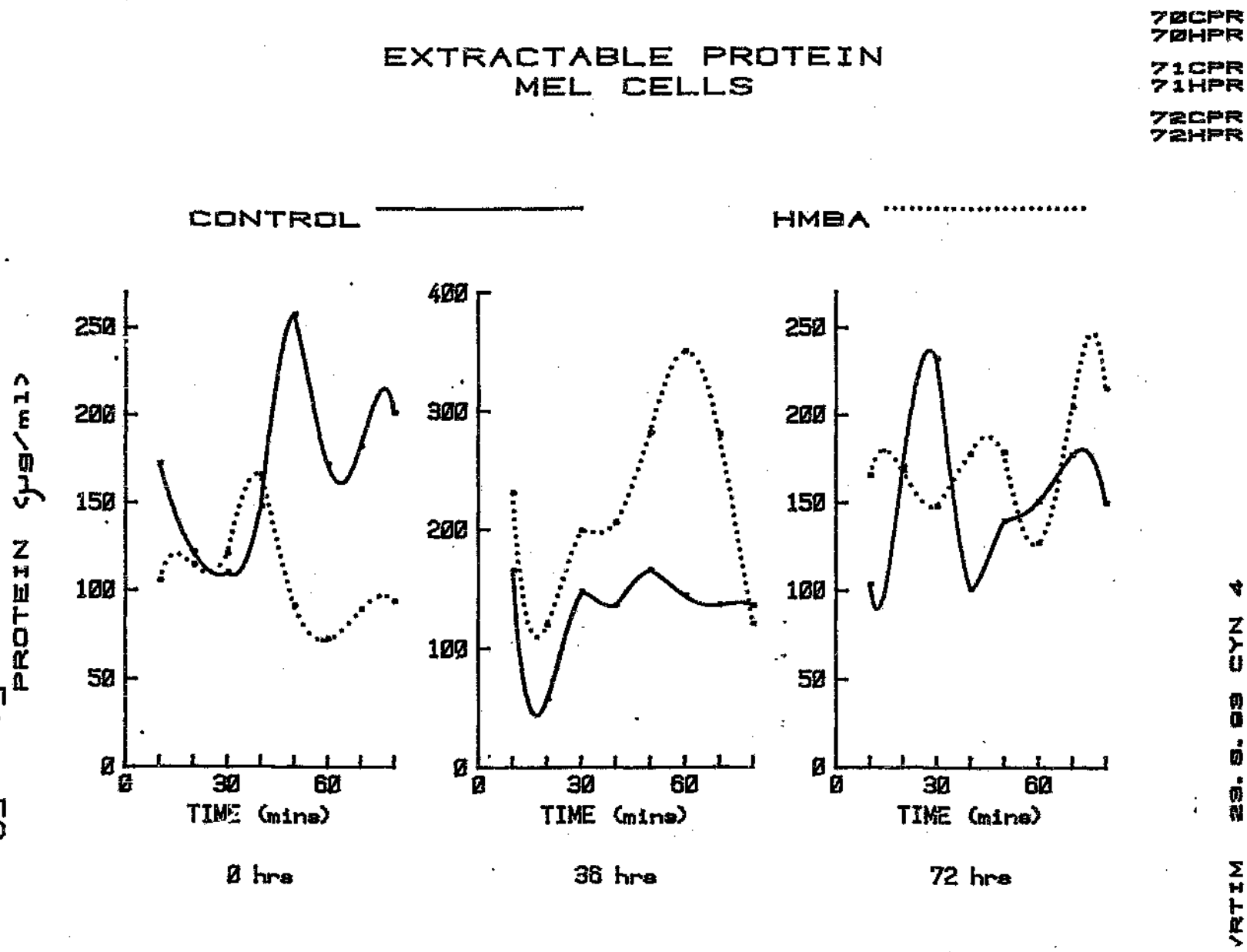

EXTRACTABLE PROTEIN
MEL CELLS
70CPR
70HPR
71CPR
71HPR
72CPR
72HPR
CONTROL
HMBA
PROTEIN (µg/ml)
250
200
150
100
50
0
400
300
200
100
0
30
60
TIME (mins)
0 hrs
36 hrs
72 hrs
VRTIM 23.5.93 CYN 4

Fig. 59

# CELL FREE SYSTEM

**Fig.60 OSCILLATIONS IN THE LDH PYRUVATE ACTIVITY IN CELL FREE EXTRACTS OF MEL CELLS**

Crude extracts of MEL cells were centrifuged at low speed with the intention of attempting to see if phosphorylation and dephosphorylation of LDH could be detected which might thus account for the oscillation in the amount of active isozyme. Determination of the pyruvate activity at intervals suggested that low amplitude oscillation were occuring even in these extracts. ATP increases the amplitude of the fluctuations suggesting that the low amplitude in the untreated cells was due to depletion of the endogenous ATP. These results lead us to question the results of many studies including some of the present results on cell extracts where oscillations may be occuring and are affecting the reactions being observed.

COPY
COPY

MEL CELL EXTRACT
LDH PYRUVATE ACTIVITY

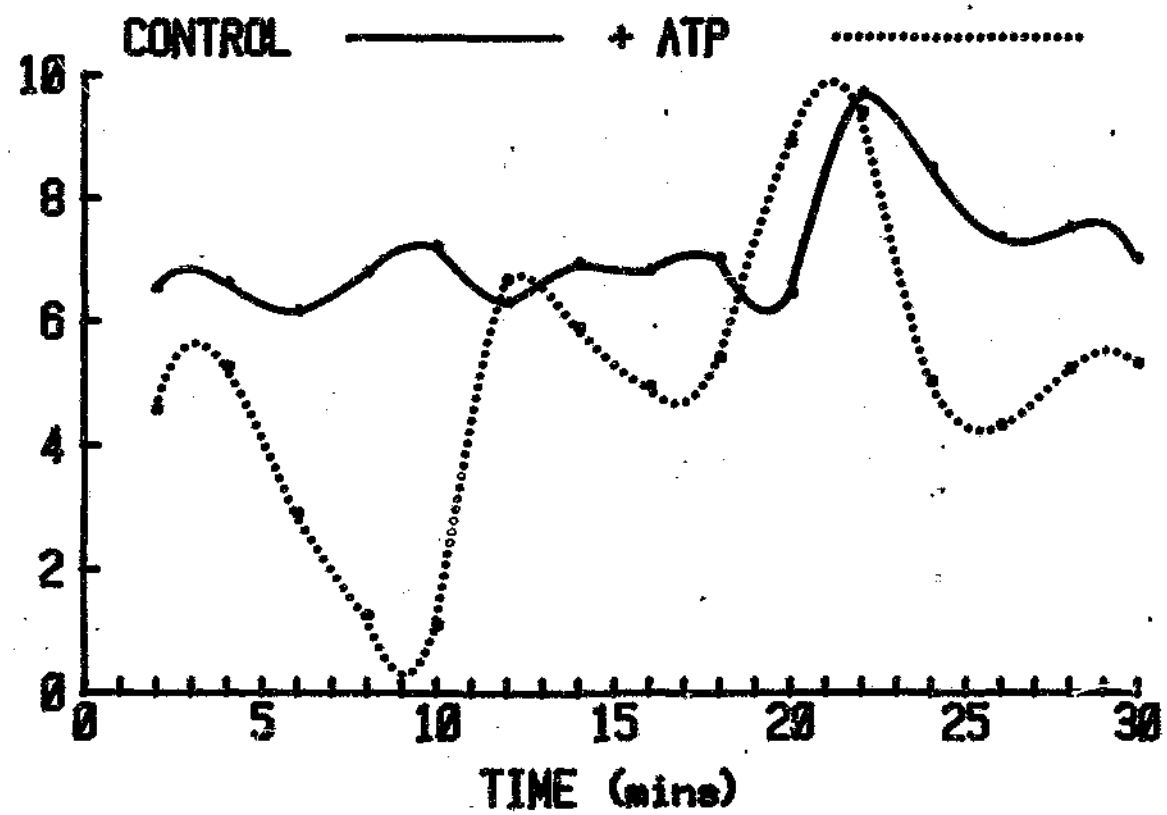



Fig. 60

12.6.83 CTW 4

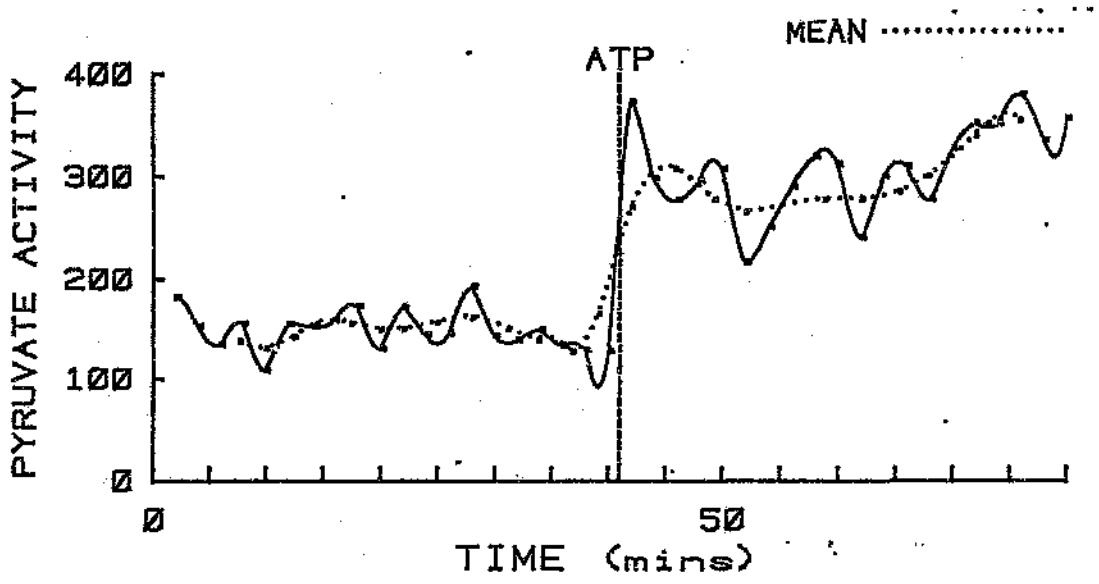
**Fig.61 LDH ACTIVITY OSCILLATION IN THE CELL AND PARTICLE FREE EXTRACTS OF MEL CELLS**

In the original experiment (Fig.60) on the cell free system, the extracts presumably contained mitochondria and other organelles. For the experiment shown here, extracts were prepared as for experiment described in Fig.60 and then centrifuged at high speed (40,000g for 60 minutes) to remove the particles before sampling was started. In this case ATP was added halfway through the experiment as indicated. It can be seen from this figure that low amplitude oscillations were still occured, the period varying in the range of 4-6 minutes before addition of ATP. Thereafter the period increased to 6-12 minutes and the amplitude also increased, both in agreement with the results of the original experiment although here the effects were gradually lost after 30 minutes, presumably due to utilisation of ATP. However, a discrete increase in the mean was retained.

LACTATE DEHYDROGENASE MEL CELLS

CELL AND PARTICLE FREE SYSTEM

PYRUVATE ACTIVITY



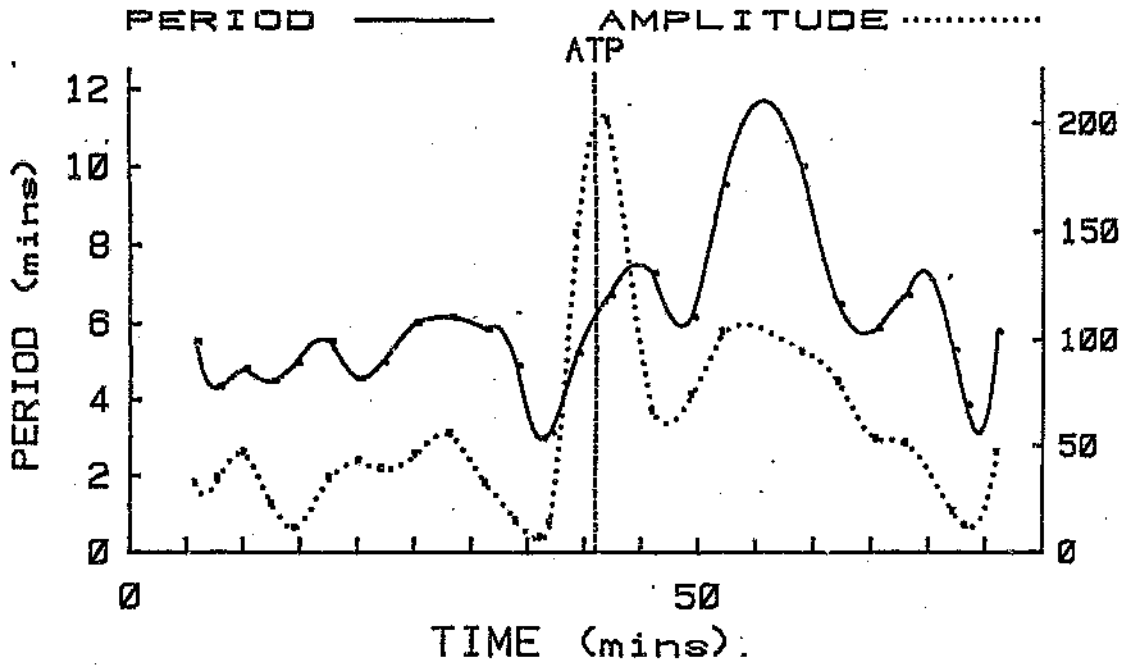
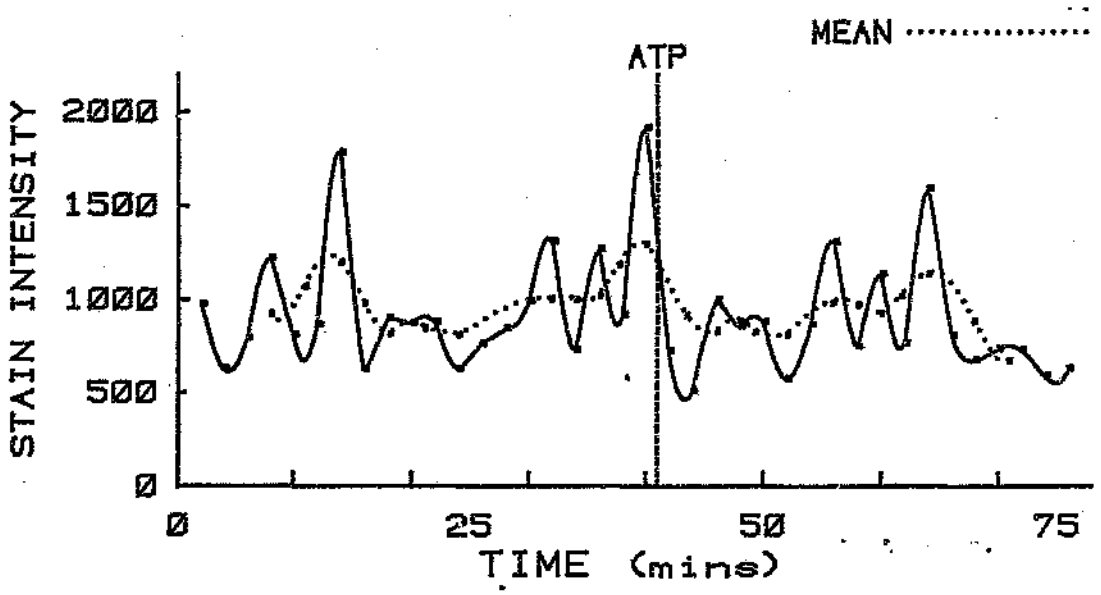




Fig. 61

**Fig.62 OSCILLATION IN THE AMOUNT OF ACTIVE LDH ISOZYME IN CELL AND PARTICLE FREE EXTRACT OF MEL CELLS**

Samples of the extracts used for the experiment described in Fig.61, were frozen down immediately in liquid nitrogen and when convenient these were thawed and immediately electrophoresed. As can be seen in this diagram oscillations in the amount of active isozyme still occurred in the cell and particle free system, but in this case ATP had little obvious effect on the period, amplitude or mean of the oscillation. The agent may have altered the frequency of the period modulating rhythms (the larger amplitude change in the latter at about 25 minutes is artifactual and arises because of a shoulder rather than a well defined peak in the original plot (top diagram).

# LDH: CELL FREE SYSTEM
## ELECTROPHORESIS: ACTIVITY STAIN



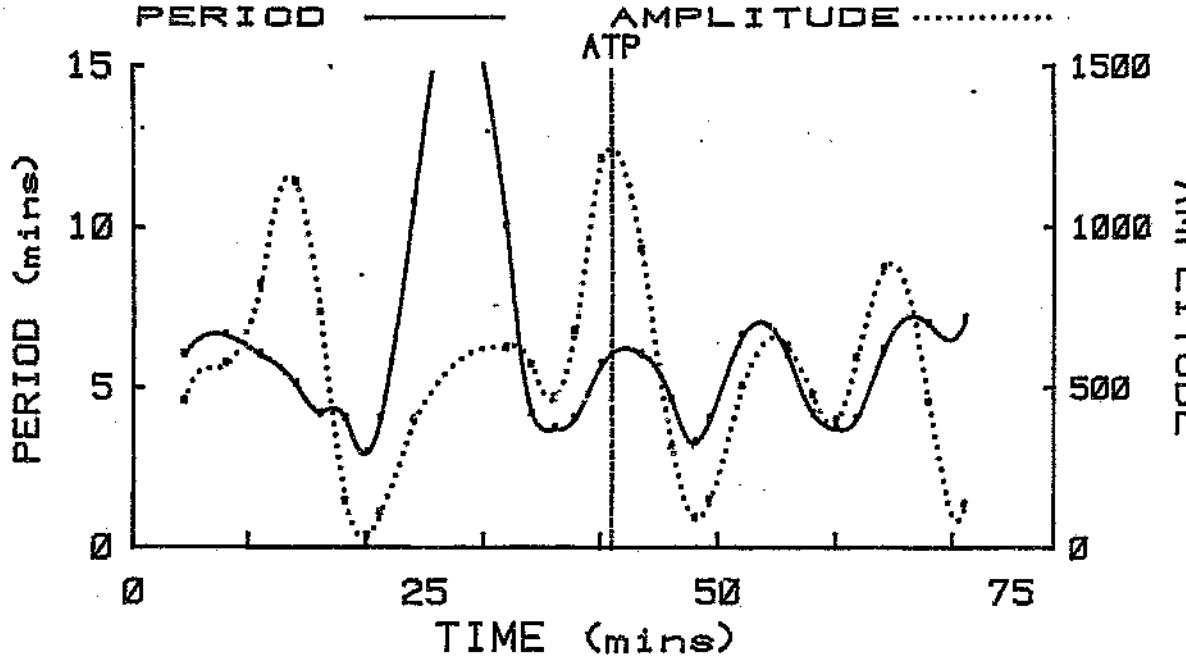




Fig. 62

**Fig.63 ENRIGHT PERIODOGRAM FOR THE LDH PYRUVATE ACTIVITY OSCILLATION IN CELL AND PARTICLE FREE SYSTEM: EFFECT OF ATP**

This diagram confirms the existence of a high frequency oscillation in the pyruvate activity of the extract and that ATP stimulates the rhythm and increases the period slightly. It is possible that some components were depleted during the longer centrifugation period used to prepare this extract.

Expt. 75PY
MEL
SAMPLE INTERVAL 2 mins
CALC INTERVAL .05
WINDOW 24 pts
STEP INTERVAL .5
THRESHHOLD 0
SCALE FACTOR .25

PYRUVATE ACTIVITY
CELL AND PARTICLE FREE SYSTEM

ENRIGHT PERIODOGRAM
PERENG.B CYNS OCT 83

ATP
ADDED AT 17mins

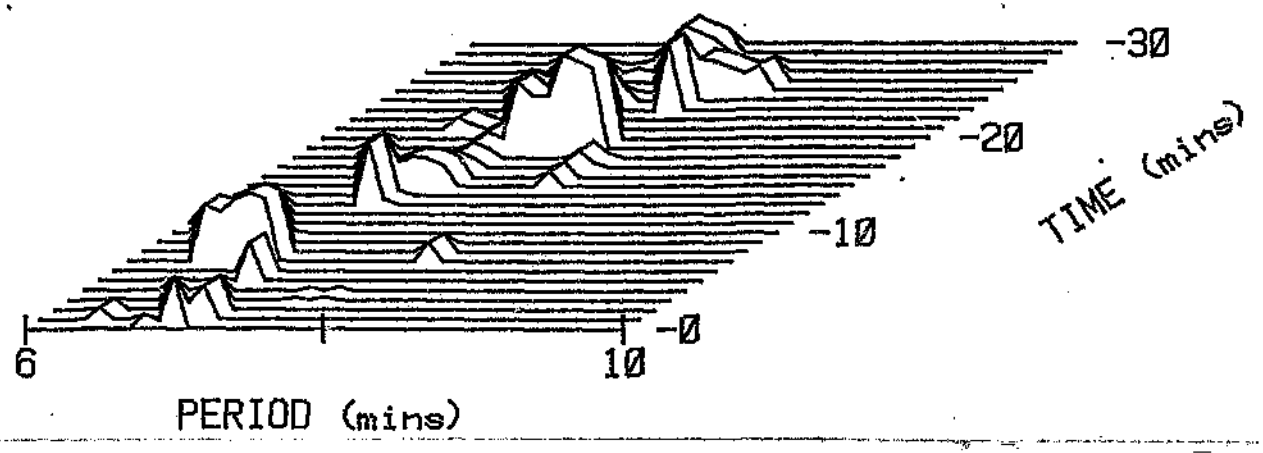



Fig. 63

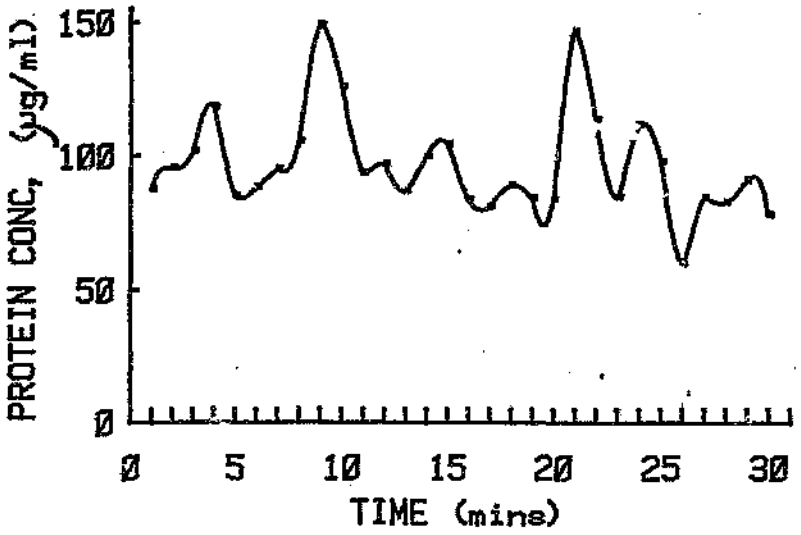
# EXTRACTABLE PROTEIN

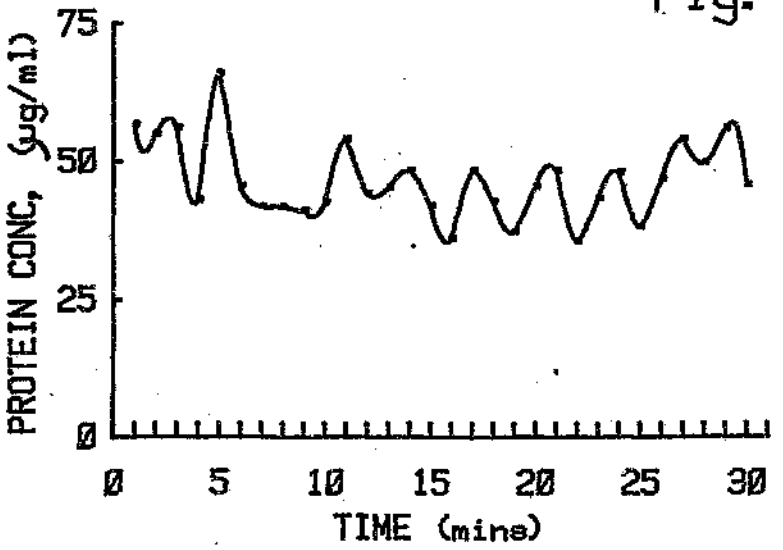
**Fig.64 EXTRACTABLE PROTEIN RHYTHM IN MEL CELLS**
(cells in stationary monolayer)

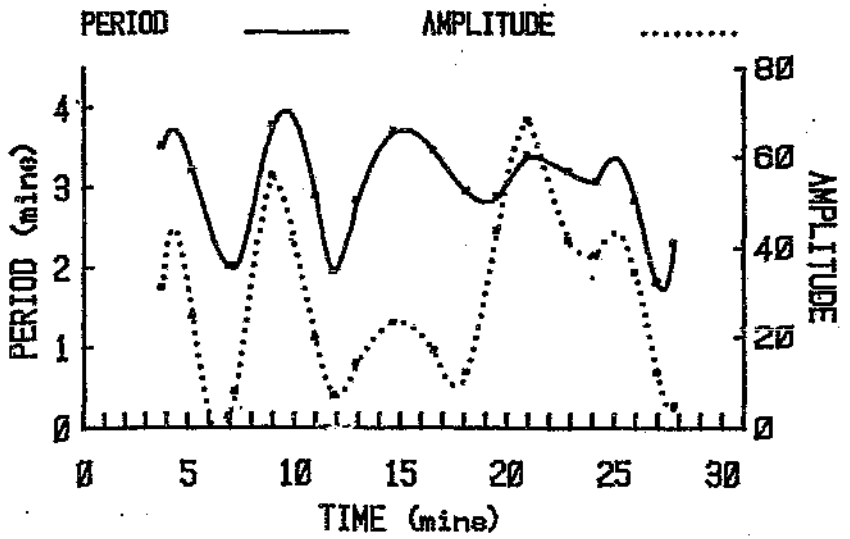
As indicated in the text, this oscillation has been reported to occur in a number of cells: in this and the following diagrams it is shown that it is also seen in MEL and HL-60 cells, not previously described. The oscillation is observed in all experiments carried out but it is shown that it is of a higher frequency than hitherto reported and that it appears to be periodically modulated with respect to period and amplitude as for all other rhythms. These conclusions may be drawn from the per amp plots given here.

# EXTRACTABLE PROTEIN RHYTHM IN MEL CELLS

Fig. 64



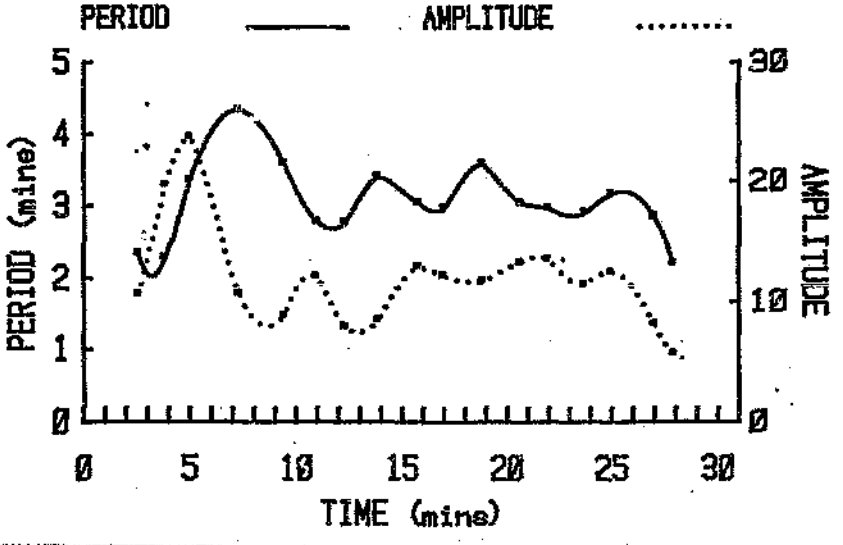



**Fig.65 EXTRACTABLE PROTEIN RHYTHMS IN TWO PARALLEL MEL CELL EXPERIMENTS**
(cells in stationary monolayer)

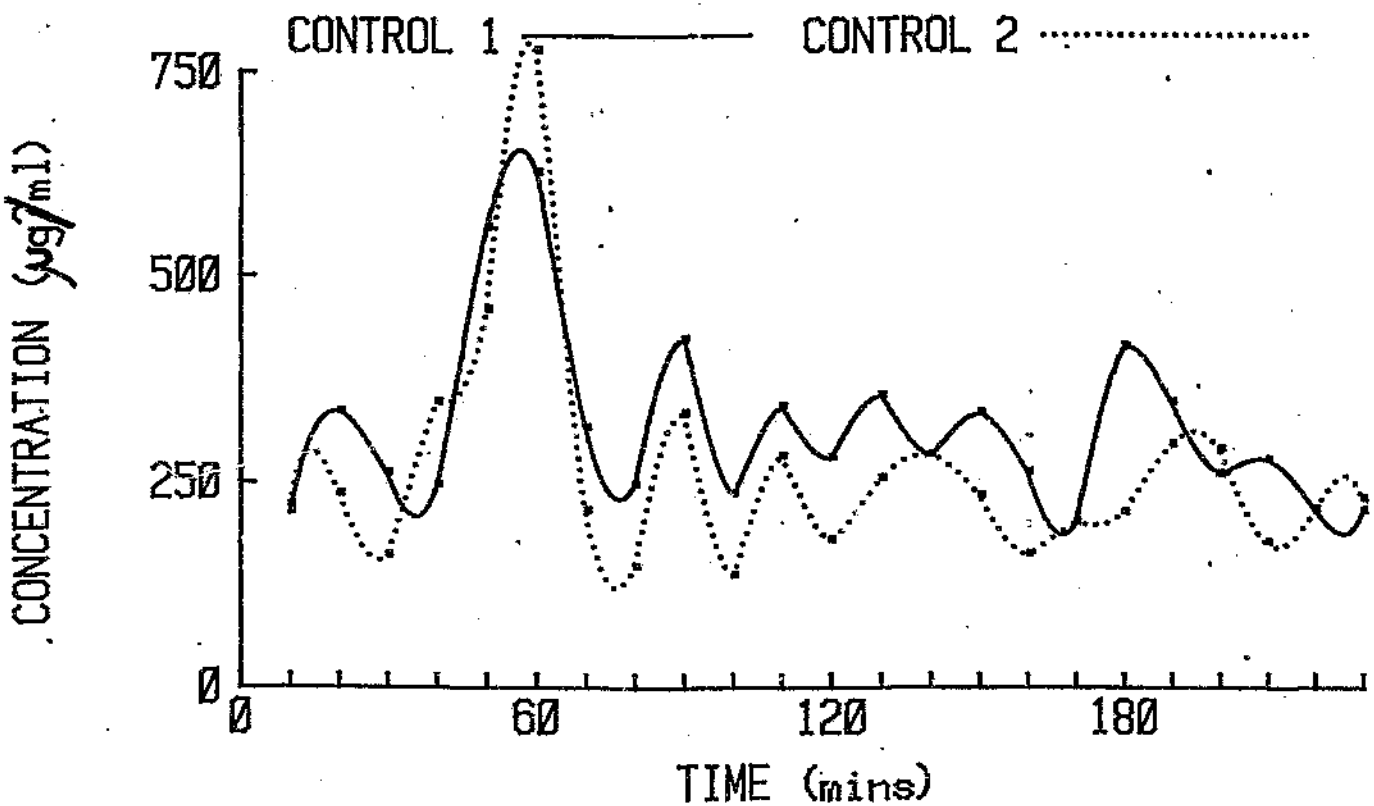
This diagram shows the protein oscillation in two sets of cultures in parallel experiments. Marked similarity between the two is evident, thus confirming for example, that the large peak at 50-60 minutes is real. It should be remembered that in the monolayer studies cultures are arbitrarily assigned to one set of cultures or to the other and these are selected at random at the time intervals. One would not expect to get agreement like this unless what happens in one culture is also taking place in the others.

Cell number for a) $1.28 \times 10^6$ cells/ml (exp.24C1P)
b) $1.38 \times 10^6$ cells/ml (exp.25C1P)

Fig. 65

EXTRACTABLE PROTEIN
PARALLE$L CULTURES

24C1P
25C1P



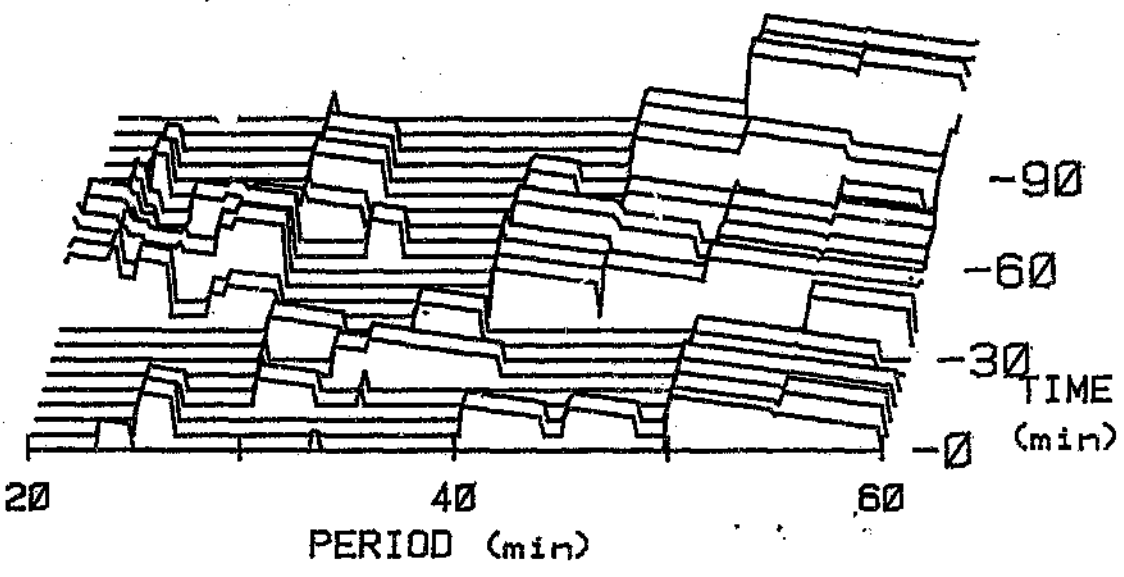

2.6.88 CYN 4

*Fig.66* **ENRIGHT PERIODOGRAM FOR THE EXTRACTABLE PROTEIN OSCILLATION IN PARALLEL CULTURES OF MEL CELLS**
(cells in stationary monolayer)

This diagram gives Enright periodograms for the two oscillations presented in Fig.65. It shows that, despite the close similarities of the two, there are differences detectable by this analysis. Periodograms of a number of oscillations discussed in the thesis suggest the existence of low frequency rhythms (periods greater than 60 minutes), but the short duration of most experiments does not allow us to be dogmatic about them.

# EXTRACTABLE PROTEIN OSCILLATION
# ENRIGHT PERIODOGRAMS

CONTROL 1




CONTROL 2

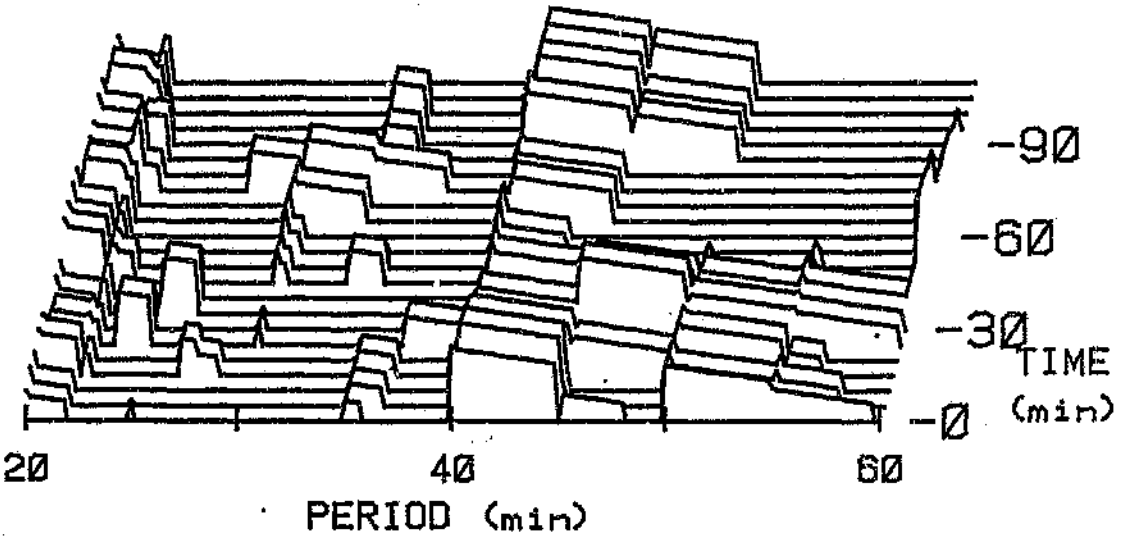



Fig. 66

**Fig.67 EXTRACTABLE PROTEIN OSCILLATION IN HL-60 CELLS**
(cells in stirred suspension)

This diagram confirms that the oscillation occurs in HL-60 cells as well and exhibits the same characteristics with regard to the apparent modulation of the period and amplitude. Although the sampling time is longer here it seems probable that the oscillation period is as short as the rhythm in MEL cells.

Cell number = $7.80 \times 10^6$ cells/ml

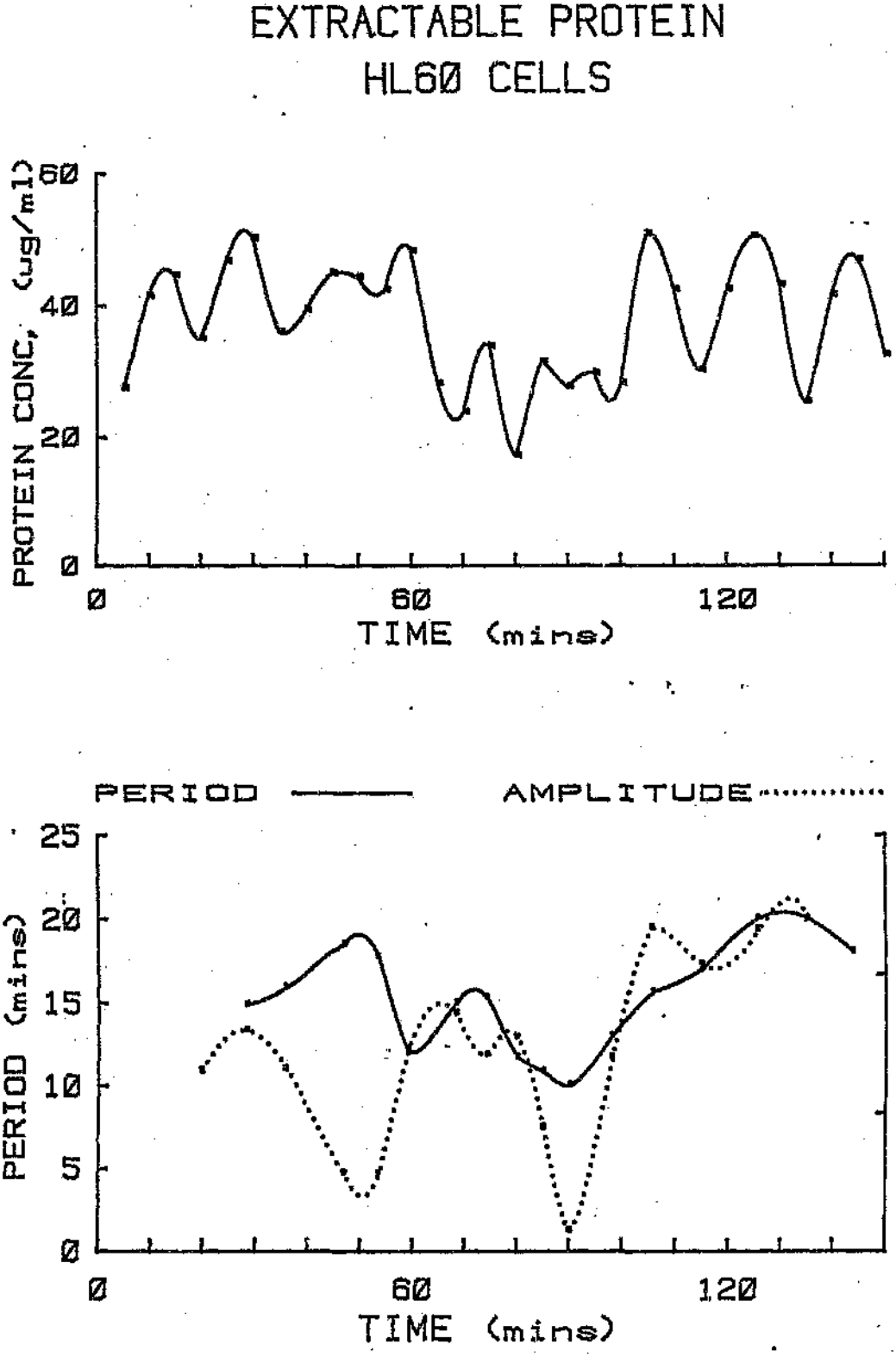

EXTRACTABLE PROTEIN
HL60 CELLS
PROTEIN CONC, (ug/ml)
60
40
20
0
0
60
120
TIME (mins)
PERIOD
AMPLITUDE
PERIOD (mins)
25
20
15
10
5
0
30
20
10
0
AMPLITUDE
0
60
120
TIME (mins)

Fig. 67

# DISCUSSION

Scientists feel that it is not correct
to introduce subjectiveness.

The student of time series
cannot be purists in that sense.

He can make the primary data available
and explain how he has treated that data.

Anyone who disagrees with what he has done
can carry out his own analysis !!!

*from: TIME SERIES ANALYSIS*

by M.G. KENDALL

# CHAPTER IV

# DISCUSSION

## GENERAL COMMENTS

One of the major roles of a biochemist is to provide insight into biological and pathological enigmas. As indicated, the most important outstanding biological problems of cell behaviour, replication, differentiation and ageing, all involve highly dynamic aspects of cell behaviour - they are all time-dependent phenomena and this indicates the need to determine not only the various reactants concerned but also to discover the kind of kinetics responsible for the time dependencies.

A continuous dynamic state can exist only as a result of repetitive disturbances of external origin, or, sustained self-generated behaviour, that is, arising within the system itself. While it is critical to distinguish between oscillations having their origins within the cell and those arising as a result of periodic external signals (zeitgebers) which force internal reactions to follow a similar time course, it seems evident that most observed rhythms are endogenous. However, interaction between all existing rhythms must always be taken into account when considering the overall characteristics and behaviour of

the cell. Unfortunately one is unable to conclude that the observed rhythms exists in each and every cell in the population but certainly one can expect similar variations to exists in each cell otherwise no periodicity would be detected. However, for simplicity in the discussion it will be assumed that essentially the same pattern of behaviour is occurring in each cell.

The various problems can be considered on their own and without reference to the nature of the constituents taking part. Such an approach may actually aid the identification of those components involved in specific processes, as those responsible should have to follow the particular time course necessary to explain the phenomenom considered. This was the case with the cyclins once it was accepted that the cell cycle was a rhythmic process (see Introduction). Time keeping is frequently accounted for in terms of biological clocks (in particular, oscillatory behaviour) possibly because it is believed that cellular rhythms are constant in characteristics, although this thesis, for example, shows that this is not necessarily the case.

It has been suggested by Goodwin, 196.: Gilbert (1968, 1969, 1984a) that the underlying dynamics must be oscillatory and the latter proposed that, under particular steady state conditions, cells have characteristic patterns of temporal organisation. It followed that altered cell properties and both differentiation and transformation involve changes in the dynamic behaviour of cellular control systems, that

is, in the pattern of cellular oscillations. The results presented here have an indirect bearing on various facets of cell biochemistry.

Biochemical periodicities in cells were first reported many years ago but for most of the intervening time such reactions were regarded merely as curiosities. In the last decade greater attention has been paid to rhythmic processes (i) as the number of examples increased markedly (Edmunds, 1988, Berridge and Rapp, 1979), (ii) as the widespread nature of their occurence was confirmed and (iii) as it became understood that oscillatory behaviour offered explanations for a range of important biological phenomena. Additional energy has come from the discovery of new physiological rhythms and the realisation that some of these, at least, may arise within certain individual cells rather than as a result of interactions between distinct tissues. The melatonin/pineal gland system is an example (e.g., Binkley et al, 1978).

In these experiments I have detected, or confirmed, rhythmic variations in the phosphorylation potential of two different proteins (Ferreira et al, 1994a); in the activities of phosphoamino acid phosphatases; in the amount of extractable protein (Ferreira et al 1994b); in the LDH activity; in the amount of active LDH; in the apparent and actual isozyme pattern. Each of these rhythms show exactly the same kind of behaviour (and have similar frequencies) suggesting the same underlying processes. The phosphoamino acid phosphatase experiments are too short in duration to be sure but it seems likely that

they too will exhibit the same general characteristics, namely the periodic modulation of the frequencies, mean levels and amplitudes of the primary rhythms. Indeed, studies of the modulating rhythms show that they too are modulated in similar fashion. The phosphatase oscillation frequency is likely to be even higher than shown here, otherwise there could be little dynamic balance between the forward and reverse phosphorylation reactions. In fact it seems highly probable that all of the rhythms have even higher frequencies.

If one is interested in studying the effects of agents on oscillatory processes, it is generally preferred to examine them when they are in a steady state, otherwise it can be extremely difficult to separate the effects of the agent from the steady state. However, it is impossible to know when the cells are in a true steady state unless one monitors them continuously, possibly for a prolonged period, before hand (Gilbert 1974, 1984). Even then, the continual sampling may influence the observations, and either prevent a steady state being reached, or, it may produce a steady state which differs from that which would exist if the cells were not monitored (Gilbert, personal communication). On the other hand, in the steady state several rhythms may be affecting the parameter of interest but, being co-ordinated in time, they may appear as one. Therefore the advantage of studying cells when they are not in a steady state is that it may be possible to determine whether or not several regulators or processes are involved. Being concerned with this aspect of cellular dynamics, no

particular attempt was made to give cells time to reach a steady state before adding insulin or HMBA although, as the biological effects of the latter do not become evident until 72 hours after adding the agent, some cultures examined had time to stabilise to some extent. However, if differentiation was induced they could not be in a true steady state. Nevertheless, in this case it is noteworthy that the period and amplitude modulating rhythms are more similar than is usually the case which could be due to synchronisation of the different rhythms involved.

The present results thus support the contentions (Gilbert 1968, 1974. 1984) that differentiation and hormone action (intracellular signalling) occur through changes in the pattern of temporal organisation of oscillatory processes in the cells.

## DETECTION OF OSCILLATORY BEHAVIOUR

Although periodograms ideally require windows which are long enough to cover a large number of cycles of an oscillation, it seems that they can nevertheless be used for much shorter time intervals. In fact, long windows may not be suitable at all when, as is often the case here, the frequency of an oscillation changes with time (see Figs.21,50,52a,52b,68 for example). On the other hand, none of the periodograms show evidence for rhythmic modulation of the period but this may be because of the accompanying modulation of the amplitude or because the process is too rapid for the windows used.

## THE PERIODS OF THE OSCILLATIONS

Because the rhythms are so irregular (see later) it is difficult to give the periods of the oscillations with any certainty, especially as they often seem to vary with time and are not single rhythms. One can estimate a range of periods from the Per-amp plots and one can decide whether or not the periodicities are of higher frequency than previously believed, but in each case, the possibility exists that the true frequency is even higher than the lower values determined here. In view of the fact that agents can affect the periods it is also evident that the frequencies are not fixed but depend on the actual conditions used for the experiments.

In order to determine the true period of an oscillation which is sampled in a discrete manner, it is necessary to make at least two determinations per cycle of the rhythm. From other studies (Lax 1972; Gilbert 1974b), and from this one as well, it is evident that interpretation of rhythmic changes can be markedly dependent on both (a) the ratio of the sampling interval to the real period of the underlying oscillation, and (b) by the timings of the sampling relative to the phase of the periodicity. If the sampling time is too long then interpretation of frequency , amplitude and phase of the oscillation can be incorrect; this picture can deteriorate further if the cells are not at steady state and the frequency is changing. Unpublished work (Gilbert) shows that an agent can appear to decrease the period of the rhythm whereas, in fact, the reverse is true. The situation becomes more complex still when more