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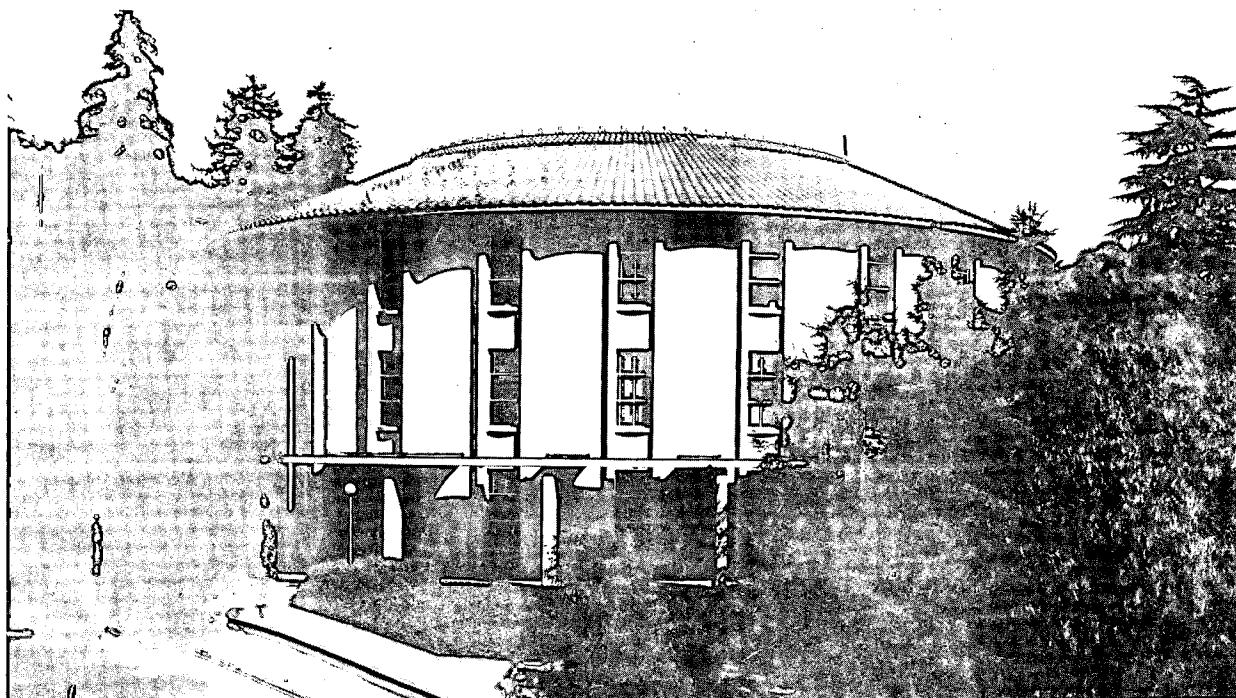
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A STUDY OF CULTURED FIBROBLASTS in vivo USING NMR

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August 1984

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THIS THESIS IS DEDICATED TO MOM AND DAD AND CHRIS

A Study of Cultured Fibroblasts In Vivo using NMR

Gregory Stanislaus Karczmar

Abstract

The goal of this thesis was to study the compartmentation of phosphorylated glycolytic intermediates in intact Chicken Embryo Fibroblasts (CEFs) using ^{31}P NMR at 109 MHz.

Because glycolysis is regulated differently in normal and virally transformed CEFs, NMR experiments were performed on both types of cells.

A technique for maintaining functional cells at high densities in an NMR magnet is described. Dialysis fibers were imbedded in the cell pellet and medium was pumped through the tubing, perfusing the cells with oxygen, glucose and buffer, and removing waste products.

Signals were detected from cytoplasmic inorganic phosphate (P_i), ATP, NAD, NADH, phosphorylcholine and phosphorylethanolamine.

The effect of external glucose on cytoplasmic pools of phosphates was studied. When cells were perfused with glucose-free medium the rate of glycolysis decreased, the amplitudes of the ATP resonances decreased, and the P_i intensity increased. The quantity of NMR-

detectable P_i produced was significantly greater than the quantity of NMR-detectable ATP which was lost. When the amount of glucose in the perfusate was increased from 1 mg/ml to either 10 mg/ml or 40 mg/ml, the amplitude of the signal from P_i decreased, without a corresponding increase in the amplitudes of the ATP resonances. However, experiments with ^{32}P labeled P_i showed that as the concentration of glucose in the medium was increased, the amount of phosphate sequestered in the cells increased. We conclude that there is a pool of P_i which is not detected by high resolution NMR and that the size of this pool increases as the rate of glycolysis increases. These effects were found only in cultured cells; the data for transformed and normal cells were similar.

Longitudinal relaxation times of intracellular phosphates in normal, transformed, and primary CEFs were measured. Relaxation times were significantly shorter in primary cells than in normal and transformed cells. These results demonstrate that relaxation times of phosphates are sensitive to structural and metabolic changes which occur when cells are grown in culture.

Melvin P. Klein

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CHAPTER 1

INTRODUCTION

Intracellular compartmentation, and the role it plays in regulating metabolism, has been the object of a great deal of experimentation and speculation, particularly as it relates to the mechanism of glycolysis (Ottaway, 1980). It is generally believed that the cytosolic concentrations of P_i , AMP, ADP, and ATP play an important role in the control of glycolysis. The cytosolic phosphorylation potential (Veech), the energy charge (Racker), and related thermodynamic variables are the functions of these concentrations which are thought to serve as control points for glycolysis. It has been shown (Veech, Racker) that there are large bound intracellular pools of at least two of these intermediates (ADP and P_i), and it is thought that these bound pools do not participate directly in glycolysis. However it is difficult to detect these bound pools directly, using traditional biochemical techniques. Assays of cell extracts will yield only the total concentration of the intermediates, and will not differentiate between free and bound pools. Experiments with radioactive tracers can sometimes be used to probe for intracellular compartmentation; the effectiveness of this technique depends on the rates at which label equilibrates between various compartments. These techniques require the preparation of cell extracts, and the isolation and purification of metabolites.

NMR studies offer the possibility that compartmentation of

certain metabolites can be studied directly in living systems. We have used NMR to study the compartmentation of phosphorylated metabolites in Chicken Embryo Fibroblasts (CEFs) which are grown in culture. The experiments which are described here indicate that there are bound and free pools of intracellular inorganic phosphate (P_i) in CEFs. Exchange of P_i between these two pools is correlated with the rate of glycolysis. This may affect the thermodynamics of glycolysis by limiting the amount of P_i which is available to phosphorylate ATP. Alternatively, it may be a consequence of the mechanism of glycolysis, and may, for example, reflect the binding of phosphorylated substrates to glycolytic enzymes.

NMR measurements can yield some of the same types of information as more traditional techniques, and in addition, can provide information which is not otherwise accessible. Phosphorous NMR spectra allow us to monitor cytosolic concentrations of ATP, ADP, P_i , and sugar phosphates. In addition the chemical shifts of some of the phosphates, particularly P_i can be used as a measure of intracellular pH. Observations of intracellular pH gradients in living systems (Roberts; Busby), have been used as evidence for the existence of pools of metabolites which are sequestered in separate compartments. Of particular importance for this work is the fact that the transverse relaxation times ($1/T_2$), and therefore the linewidths of NMR signals are sensitive to the correlation times of molecules. In the work presented here, we use NMR to distinguish between bound pools of metabolites, i.e. molecules which have long correlation times, and free pools of metabolites, i.e. molecules which have short correlation

times.

The most enticing part of the NMR experiment is that one can look at biological systems in vivo, while they are functioning normally. The ideal in vivo NMR experiment is done on an unperturbed living system. (In this thesis the term in vivo will refer to cultured cells as well as whole animals, while in vitro will refer to nonliving systems such as cell extracts.)

Over the past ten years, the use of NMR to study living systems has become widespread, but to date it has been very difficult to do meaningful experiments on isolated mamalian cells. This has been due to the difficulty of maintaining cells in the bore of an NMR magnet, and to the difficulty of obtaining mamalian cells in quantities sufficient for an NMR experiment. Early in vivo NMR experiments were done on microorganisms such as yeast. Most of the current work in in vivo NMR currently involves metabolic studies of perfused organs and intact animals. Particular emphasis has been placed on NMR studies of glycolysis and the compartmentation of phosphorylated intermediates in intact muscle (Radda; Busby). Studies of organs in situ suffer from the disadvantage that the NMR signals come from regions which are not well defined. It is difficult to control the shape and size of the sensitive volume of the surface coils which are typically used in such experiments. Even surgically planted solenoidal coils (Koretsky, 1983) suffer from this drawback, albeit to a lesser degree. In experiments on perfused organs and intact animals, the regions which are studied often contain a variety of cells: muscle cells, epithelial cells, fibroblasts, etc., so that the data which are obtained are

difficult to interpret. NMR studies of simpler systems are needed as a basis for the experiments on organs and intact animals. The work on microorganisms cannot adequately fill this need. Thus, it is important that in vivo NMR studies be supplemented by experiments on isolated, live, mamalian cells.

The initial effort of this thesis was directed towards the development of a method for performing NMR experiments on living cells. Because NMR is relatively insensitive, high densities of cells are required in order to obtain adequate signal to noise ratios. At the same time nutrients must be delivered to the cells and waste products must be removed at rates which satisfy the demands of cellular metabolism. Our solution to this problem is described in detail in Chapter 2.

Chicken Embryo Fibroblasts are obtained from the body walls of 10 day old embryos. In culture they grow rapidly, to very high densities. The cells are easily transformed in culture by infection with Rous Sarcoma Virus, a virus which causes cancer in chickens. Cells which are virally transformed in culture form tumours when they are returned to the animal. They provide us with a model for tumours which can be easily manipulated in culture. Because transformed CEFs are prepared in exactly the same way as normal CEFs, we have a well controlled system in which to study the effects of transformation on the mechanism of glycolysis. (In this thesis, for convenience, the terms 'transformed' and 'virally transformed' are used interchangeably.)

A third type of CEF is also available: cells which are taken

directly from the body walls of 10 day old embryos. These cells were studied in the hope that our techniques would be sensitive to changes which occur when cells are grown in culture.

It is well known that metabolism in these cells is extremely sensitive to environmental influences (Bissell, 1981); thus it is to be expected that metabolism under our experimental conditions is different from metabolism in culture, and different from metabolism in the embryo. This problem is addressed in Chapter 3. The rates of oxygen and glucose uptake and lactate production were measured. It was shown that under our experimental conditions, glycolysis was the primary source of ATP. The signals which were detected by ^{31}P NMR were identified, and the dependence of their chemical shifts on pH was determined. The concentrations of NMR-detectable metabolites in the cell pellet was calculated.

Our ^{31}P spectra of intact cells were used to study the response of high energy phosphates to changes in the rate of glycolysis. In Chapter 5, evidence is presented that CEFs contain bound pools of inorganic phosphate, and that there is an exchange between these pools which correlates with the rate of glycolysis. The bound pool is distinguished from the free pool on the basis of its characteristic correlation time.

It was of interest to do these experiments in both normal and transformed cells. It has been shown that in CEFs in culture, as the rate of glucose uptake increases, the levels of the glycolytic intermediates increase, as well as the rate of the production of ATP and lactic acid (Bissell, 1973). Bissell et al have suggested that

the rate limiting step in glycolysis is the rate of transport of glucose across the cell membrane, so that an important regulator of the rate of glycolysis is the availability of extracellular glucose, and the activity of glucose transporters. Transformed cells in culture have a higher number of glucose transporters, and for this reason have a higher rate of glycolysis than do normal CEFs. Thus it might be expected that NMR would detect differences in the response of normal and transformed cells to changes in the concentration of glucose in the medium.

At the end of Chapter 5, experiments are described which suggest that at high rates of glycolysis, two distinguishable intracellular P_i resonances are detected. This may indicate the existence of separate pools of P_i which experience different pHs.

In order to interpret data from the experiments which are described above, it was necessary to make accurate measurements of the longitudinal relaxation times (T_1 s) of the various phosphate resonances. These relaxation times determine the rate at which data can be acquired. At high rates of data acquisition, a knowledge of T_1 is necessary so that the true intensity of each resonance can be calculated. Measurements of these relaxation times are described in the Appendix.

These measurements of longitudinal relaxation times may have intrinsic value aside from their importance for the interpretation of spectra. Damadian (Damadian, 1975) has suggested that relaxation times of cytoplasmic phosphates may be sensitive to physiological differences between normal and transformed cells, and that such

measurements may ultimately yield information about the intracellular environment of phosphorylated molecules. In the work presented in the Appendix, we investigated the possibility that different types of tissues can be distinguished on the basis of intracellular phosphorous relaxation times. In addition, we studied some of the factors which may be responsible for longitudinal and transverse relaxation in vivo.

CHAPTER 2

Introduction to NMR

The purpose of this chapter is to provide the reader with a brief introduction to the concepts and terms which are used in this thesis. The basic parameters which determine the characteristics of the NMR spectrum are introduced. No attempt is made to explain physical principles; for excellent, comprehensive introductions to the theory and practice of NMR the reader is referred to the books by Farrar and Becker, and by Fukushima and Roeder (Fukushima, 1981). NMR will be discussed in terms of the classical analogy, with little reference to quantum mechanics.

2.1 Physical Basis of NMR

NMR spectroscopy takes advantage of the fact that atomic nuclei with an odd number of nucleons (the sum of protons and neutrons) have a magnetic moment and an angular momentum which is proportional to the magnetic moment. When they are placed in a magnetic field (the magnetic field of the spectrometer is commonly referred to as H_0 , and its axis defines the 'z' direction) the nuclei line up along the field, and precess around it with at a frequency which is determined by their gyromagnetic ratio (γ) and H_0 . The precession frequency is referred to as the Larmor frequency and is given in Herz as:

$$\omega_0 = \gamma \cdot H_0$$

(Corrections in ω_0 due to smaller terms are discussed below.)

The gyromagnetic ratio depends on the nuclear magnetic moment and on the spin quantum number. (For example, the spin quantum number, I , for phosphorous is $1/2$.) This means that gamma is uniquely determined for each nucleus.

For a spin $1/2$ nucleus such as ^1H or ^{31}P there are only two allowed orientations with respect to the field. A nuclear spin with a positive moment is in its lower energy state when it is aligned parallel to the H_0 field, and in its high energy state when it is aligned antiparallel to H_0 . The energy difference between these two states is equal to the Larmor frequency. In a population of nuclear spins which is at thermal equilibrium, there will be slightly fewer nuclei in the high energy state than in the low energy state. Thus the spins have a net magnetization, which can be represented by a magnetization vector which precesses about, and is aligned parallel to, H_0 .

2.2 How the NMR Signal is Detected

In practice transitions are induced by applying a magnet field rotating at the Larmor frequency in a plane perpendicular to the static field (i.e, in the xy plane). An RF amplifier generates an alternating current in a coil which is placed around the sample inside the magnet. The resulting field is referred to as the 'applied field', or H_1 . This causes the net magnetization vector to rotate

away from H_0 , about H_1 . An explanation of this phenomenon requires a development of the rotating frame formalism. An excellent discussion of the rotating frame can be found in the book by Fukushima and Roeder (Fukushima, 1981). The angle of the rotation depends on the strength of the applied field, and the length of time for which it is applied (referred to as the pulse length). If the net magnetization vector is rotated 90° from H_0 , we say that a 90° pulse has been applied. The nuclear spin can be rotated by an applied field only if the applied field is rotating at its larmor frequency:

$$\nu_{rf} = \nu_1$$

When the applied magnetic field is turned off, the spins continue to precess freely under the influence of H_0 . If some component of the magnetization has been rotated into the xy plane, the precessing spins can induce a current in a coil which is oriented parallel to H_0 , in the same way that a spinning bar magnet generates a current in the coil of an electrical generator. This signal is called a free induction decay. As its name implies, the signal decays, usually exponentially, with a time constant which is referred to as T_2^* . The reasons for this decay are discussed below. The signal in the coil can be amplified and eventually fed into a computer where a fourier transform is performed in order to separate the various components, and determine their frequencies. A maximum signal is obtained from a 90 degree pulse, which rotates all of the magnetization into the xy plane. No signal is obtained from a 180 degree pulse, which inverts the net magnetization, so that it is antiparallel to H_0 .

2.3 Chemical Shift

The frequency of the signal which is detected will depend to a small but significant degree on the chemical environment of the nucleus. This small correction to ω_0 is referred to as the chemical shift. The chemical shift arises primarily because electrons in molecular orbitals shield the nucleus from the external magnetic field. When a nucleus is shielded by high electron density it experiences a lower effective field, and as a result has a slightly lower precession frequency, than a nucleus which has little shielding. As an extreme example, a proton which is bonded to a carbon atom has a lower Larmor frequency than a bare proton. The chemical shift correction to the precession frequency is usually on the order of a few parts per million. Because the magnitude of the correction depends on the nature of the molecular orbitals, molecules can be identified on the basis of the chemical shifts of their proton, phosphorous, and carbon-13 nuclei.

2.4 Description of the NMR Spectrum

Figure 3, Chapter 3 shows a typical ^{31}P NMR spectrum of cells. It is the Fourier Transform of a free induction decay. Each peak in the spectrum represents a signal which comes from a different chemical species. The spectrum was taken in a 6.3 T magnet in which the Larmor frequency for phosphorous is 109 MHz. On the horizontal axis the

chemical shift is given in parts per million (ppm), i.e. parts per million of the Larmor frequency. An aqueous solution of 0.85% H_3PO_4 is arbitrarily assigned a chemical shift of 0.00 and the difference in resonance frequency between H_3PO_4 and another molecule, such as inorganic phosphate is divided by 109 MHz and multiplied by 10^6 to yield the chemical shift in ppm. The β phosphorous of ATP is less shielded than the other phosphates which are detected, and thus resonates at a higher energy; its signal is said to be 'downfield' from the other resonances.

The integrals of the signals are proportional to concentration. In order to use these integrals to determine the concentration of a metabolite, they must be compared to the integral of a signal from a standard; in this case methylene diphosphonic acid is used as the integration standard.

2.5 PH Dependence of Phosphate Chemical Shifts

The chemical shift of phosphate esters, and especially that of inorganic phosphate, is very sensitive to pH. This sensitivity derives from the fact that a proton draws electron density away from the phosphorous nucleus, leaving it less shielded than it would be if it were unprotonated. In acidic solution the phosphate is, on the average, more protonated than in basic solution, and this shifts the resonance downfield. In basic solution the phosphate has, on the average, a higher negative charge, and the resonance is shifted upfield. In 1972 Moon and Richards (Moon, 1972) demonstrated that the

chemical shift of intracellular inorganic phosphate can be used to measure intracellular pH. The state of protonation of P_i , which has a pK at 6.8, is very sensitive to physiological pH, which ranges from 6.2 to 7.8. This property of phosphate chemical shifts is referred to throughout this thesis.

2.6 Transverse Relaxation

The width of the signals (which are lorentzians) at half height is an important experimental variable. It is determined by the apparent transverse relaxation time (T_2^*) and is sensitive to a number of variables which are determined by the environment of the spin. We will discuss some of these variables briefly, because they figure importantly in our interpretation of our experimental results.

An important property of the chemical shift is that it is anisotropic. This means that the chemical shift for most chemical species varies depending on the orientation of the molecule with respect to the magnetic field of the spectrophotometer. In a powder the signals from a single chemical species are spread out over many thousands of Herz, because the individual molecules orient randomly. When the molecules are free in solution, they diffuse rapidly (with a rotational correlation time of $3 \cdot 10^{-11}$ seconds), through all possible orientations, and the orientation dependent chemical shift takes on its average, or isotropic value. The signal from the powder is difficult to detect because its intensity is distributed over a large bandwidth and disappears into the baseline noise. The signal from a

molecule in solution is much easier to detect because all of its intensity is in a relatively narrow bandwidth.

High resolution NMR spectroscopy detects only small molecules which diffuse freely. Phosphates which are part of large molecules such as DNA, or are bound to surfaces have long correlation times, and give rise to very broad resonances which may not be detected. Thus NMR distinguishes between molecules on the basis of their correlation times. In Chapter 4, we make use of this property to study compartmentation in CEFs.

Paramagnetic metals generate large fluctuating magnetic fields which broaden the resonances of nearby nuclei. This mechanism of transverse relaxation is particularly important for phosphates, which can chelate di and trivalent metal ions such as manganese very strongly. This form of broadening will also be discussed in connection with our results.

Many other sources of line broadening exist. Since they do not enter into this discussion, the reader is referred to Chapter 10 of Abragham (Abragham, 1983).

2.7 The Precision of the Measurement of Chemical Shift is Limited by the Transverse Relaxation Time

The transverse relaxation time for a given resonance determines the precision with which the chemical shift can be determined. For example, if a resonance has a T_2^* of 1 msec then its width at half height will be approximately 1000 Hz and the uncertainty in its

chemical shift will be very large. The resonances in the spectra which are presented here have T_2^* s of approximately 10 msec. The error in the determination of chemical shifts was determined empirically to be ± 8 Hz, or approximately ± 0.07 ppm.

2.8 Longitudinal Relaxation

Another type of relaxation which determines the behavior of nuclear spins is the longitudinal relaxation time, or T_1 . If the magnetization is rotated by the H_1 field so that it is in the xy plane, it will eventually return to its lower energy state, parallel to H_0 . This means that the spins must give up energy which must be absorbed by their surroundings. Since nuclear spin states are isolated from their surroundings it can sometimes take a very long time before there is an interaction which allows them to relax to their ground states; in solids this process can take hours. However, there are many sorts of coupling which can result in efficient longitudinal relaxation; commonly phosphorous nuclei are coupled to nearby protons, or to paramagnetic electrons which can accept the excess energy of the phosphorus. In a system where the mechanisms of relaxation have been well characterized, measurements of T_1 can give us a unique insight into the ways in which the nuclei interact with their environments. Longitudinal relaxation of phosphorous nuclei in living systems will be discussed in detail in the Appendix.

2.9 Dependence of the Signal to Noise Ratio on T_1

When magnetization is rotated by a 90° pulse so that the maximum signal is detected, it returns to its equilibrium orientation (along the z axis) with an exponential time course which has a time constant T_1 .

$$M(t) = M_0(1 - e^{-t/T_1})$$

(M_0 is the equilibrium magnetization, and $M(t)$ is the magnetization after a time t)

Immediately after the 90° pulse, no magnetization remains along the z axis; thus if a second RF pulse is applied immediately after the first (in the simplest case) no signal is detected. If there is a delay of T_1 between the two pulses a signal will be observed as a result of the second pulse which is approximately 63% of the maximum intensity. If there is a delay of $5 T_1$ s between the first pulse and the second pulse, the signal will be over 99% of the maximum intensity. A 'fully relaxed' spectrum is obtained when the delay between acquisitions is long enough to allow complete relaxation of the nuclear magnetization between pulses, that is to say, complete return to the equilibrium magnetization. (The word 'complete' here is used advisedly, and is usually taken to mean that the magnetization has reached greater than 95% of its equilibrium value.) Clearly, T_1 determines the rate at which data can be acquired. If 100 acquisitions are required in order to achieve adequate an signal to noise ratio for a given sample, it will take $100 \cdot 5 \cdot T_1$ to obtain a spectrum: 100 seconds if the T_1 is 0.2 seconds, or 1000 seconds if the T_1 is 2 seconds.

In cases where optimum signal to noise performance is necessary, it is not desirable to wait after each acquisition until the system returns to equilibrium. Greater signal to noise ratio is obtained if the spins are not allowed to relax fully between pulses. For example, experiments are sometimes done using 45 degree pulses with delays between pulses set equal to the T_1 of inorganic phosphate. In this case, the intensity of each FID is relatively weak, because not all of the magnetization is tipped into the xy plane, and because there is not sufficient time for the system to reach thermal equilibrium between acquisitions (that is, the net magnetization vector has not rotated back to the Z axis). For this reason, we say that such a spectrum is obtained under saturating conditions. For a given number of pulses, the amplitudes of the signals obtained under these conditions are not as great as they would have been if a fully relaxed spectrum had been obtained from the same number of acquisitions. However the spectrum obtained under saturating conditions is acquired in a much shorter time than the fully relaxed spectrum, and the amount of signal which is acquired per unit time is much greater under saturating conditions than under fully relaxed conditions. The signal to noise is given by

$$S/N = M_0(1 - e^{-t/T_1}) \sin \theta / t^{.5} (1 - e^{-t/T_1} \cos \theta)$$

where t is the delay between pulses, and θ is the pulse angle.

Although the signal to noise ratio is improved when spectra are taken under saturating conditions, the amplitude of each resonance is no longer directly proportional to concentration; the concentration

can be calculated, using the equation given above, if the T_1 and the pulse angle are known. This relationship is used in the interpretation of data which will be presented here.

CHAPTER 3:

A Device for Maintaining Viable Cells at High Densities for NMR Studies

In recent years there has been increased interest in the use of NMR to study living cells (Hollis, 1980; Radda, 1979). Because the technique is relatively insensitive, cells must be studied at high densities. Thus investigators have developed methods of maintaining dense pellets of healthy, stable cells in a magnet for long periods of time. In early studies of aerobic cells oxygen was supplied from a bubbling apparatus, with no attempt to supply nutrients or remove waste products on a continuous basis (Navon, 1977). Recently, methods have been developed which allow the study of anchorage dependent cells which are on solid supports and are continuously perfused with oxygenated media. Ugurbil et al (Ugurbil, 1980) allowed cells to attach to Cytodex beads (Pharmacia) and perfused the cells with medium while spectra were acquired. Spectra have also been obtained from cells grown in commercially available Vitafiber Units (Amicon) (Gonzalez-Mendez, 1982). A disadvantage of these approaches is that the solid supports occupy most of the radiofrequency coil volume and as a result there is a significant loss in sensitivity. In addition the devices required are not compatible with conventional NMR probes, which are accessible only from the top. Neither technique has been used with cells which are not anchorage dependent.

3.1 Characteristics of the NMR Flow Cell.

We have developed a system which maintains cell suspensions in a steady state at densities necessary for NMR experiments and is directly compatible with conventional probes (Karczmar, 1983). Using hollow fiber dialysis tubing to perfuse the cell pellet with oxygenated medium, we have obtained ^{31}P spectra of secondary chicken embryo fibroblasts and a free living protozoan, *Tetrahymena thermophila*.

Figure 1 is a schematic illustration of our apparatus. A bundle of cellulose acetate fibers occupying approximately one sixth of the sample volume is sealed into the ends of polyethylene tubing using water resistant epoxy (2 ton epoxy, Devco). The NMR sample holder is a glass tube, 13.5 mm I.D., epoxied to a nylon top with inside threads. Three polyethylene tubes enter the sample holder; one carries medium flowing into the fibers, one carries medium leaving the fibers, and one is a vent tube. An O ring is placed around each tube, between two pressure plates. A threaded top screws down on the pressure plates, compressing the O rings and creating a seal at the point where the polyethylene tubes enter the sample holder. At the same time, the bottom pressure plate compresses a fourth O ring creating a seal around the circumference of the sample holder. Dialysis fibers, which are epoxied into the polyethylene tubes, are distributed evenly throughout the two ml sample volume. Nutrient rich medium is pumped through the fibers at a rate of 3 mls/minute by a peristaltic pump. The fibers are semipermeable, allowing molecules

with a molecular weight of less than one thousand Daltons to pass in and out of the cell pellet. Alternatively, Celanese hollow fibers can be used; these fibers have pore sizes on the order of 0.1 microns.

The peristaltic pump generates a pressure difference across the walls of the fibers, causing the level of the fluid in the sample tube to change, even though there are no breaks in the fibers or in the epoxy. If the peristaltic pump is used to force fluid into the fibers, thus producing a positive pressure inside the fibers, the fluid level inside the sample increases. Alternatively if the pump is used to produce a negative pressure inside the fibers, the fluid level in the sample tube decreases. Since changes in the cell density will affect NMR results, we devised several methods for keeping the fluid level constant. The sample tube is hermetically sealed, so that as medium is pumped through the fibers, the fluid level increases until a limiting pressure is reached. The limiting pressure will depend on the flow rate and the number and type of fibers used; our apparatus operates at a pressure of 4 psi. The system reaches equilibrium at this point, and there is no further increase in fluid level or in pressure. To minimize the initial increase in fluid level which occurs before the pressure equilibrates, a tightly fitting nylon plug occupies most of the empty space inside the tube. The equilibrium pressure is reduced considerably when the peristaltic pump is used to equalize the input and output pressures, i.e. to push on the supply tube and pull on the return tube.

When fibers with relatively large pores are used (Celanese Celgard), and the pump maintains a low pressure inside the fibers,

there is a substantial flow of liquid from the cell suspension into the fibers. Because the sample tube is airtight there is a compensating flow of liquid down the vent tube and into the sample tube, maintaining a constant cell density. This arrangement has the advantage of providing a net flow of medium through the pellet.

Another approach is to imbed the cells in an agar/collagen gel which holds the cells in place in the sample tube. The fluid level is then allowed to increase without affecting cell density while excess medium is removed through the vent line. In this mode of operation a pressure seal is not required so that unmodified NMR tubes may be used. Nandi et al have shown that fibroblasts can be induced to grow and form matrix when in a collagen gel. Although we did not try to induce cell growth during our experiments, this technique offers the possibility in future work of studying fibroblasts under conditions which approximate the in vivo environment.

In most of the experiments described in Chapters II and III, Celanese Hollow fibers were used in the perfusion apparatus. Because CEFs are very 'sticky' cells collagen gel was not necessary to hold them in place. Fluid was either pushed or pulled through the fibers, and the pressure on the fibers was adjusted so that the rate of 'percolation' through the cell pellet was 0.3 to 0.5 mls/minute. The pressure sealed apparatus was always used in experiments with transformed cells in order to contain the virus. the eluent from the vent tube was run into a large bucket containing bleach and Zephirin Chloride.

The data in Fig. 2 demonstrate the ability of the fibers to

transport glucose, lactate, oxygen, and protons. The rate of glucose and lactate transport can be as high as 20 mgs/hour. Oxygen reaches equilibrium much more rapidly than the other solutes, presumably because it is carried by water so that the rate of oxygen transport is the same as the rate of flow of water across the fiber membranes. The equilibration of pH is slow compared to that of oxygen (Fig. 2D), indicating that hydronium ions are transported more slowly than water. The rates of transport are strongly dependent on the size of the concentration gradients across the fiber membranes. We maintain large gradients by changing the perfusate at frequent intervals, and bubbling oxygen into the reservoir. When the glucose concentration in the perfusate is kept at its normal level (1 mg/ml) the rates of transport are in excess of what is required to satisfy the needs of our cells; confluent CEFs on culture dishes use 15 ugms of glucose/ 10^6 cells · hour, and they secrete lactate at a similar rate. When the level of glucose in the perfusate is very high it may be necessary to use more fibers in order to remove the lactate which the cells produce. Figure 3 shows the response of the cells as glucose is delivered through the fibers. When the perfusate contains no glucose the cells produce little ATP; when glucose is added to the perfusate the ATP level increases within 20 minutes. Once the maximum ATP level is achieved, it can be maintained for up to 48 hours. The fact that ATP can be made, and that the steady state level is constant for long periods of time, is a partial indication that the cells are functional under our experimental conditions. Further tests of the viability of the cells are discussed in Chapter 3.

Tetrahymena thermophila consume oxygen at high rates (4.2 $\mu\text{moles/min} \cdot \text{gm wet weight}$) and thus provide us with a good test of the fibers' ability to deliver oxygen. The inset to Figure 4 shows the oxygen tension of a suspension of *Tetrahymena* at densities necessary for good sensitivity in an NMR experiment. As an oxygenated solution is pumped through the fibers, the Oxygen level rises from zero and reaches a steady-state within minutes. The spectra in Figure 4 show that ATP levels detected by NMR rise as oxygen is delivered to the cells from the dialysis fibers, reaching levels equal to those obtained by bubbling oxygen directly into the cell pellet.

3.2 Probe

In order to increase the volume of cells which could be used in these experiments a probe was constructed which had a 2 ml sensitive volume. A helmholtz coil 15 mm in diameter and 15 mm in height was used for both excitation and detection of ^{31}P . It was mounted on the inside of a cylinder made of polystyrene (Rexalite). Without a sample the coil had a Q of 120 at 109 MHz, with a cell sample the Q was between 70 and 80. The Q was degraded significantly when the fluid level in the sample tube rose above the upper edge of the copper. Attempts to minimize this effect using the balanced matching arrangement of Koretsky et al (Koretsky, 1983) were unsuccessful. The phosphorous coil was double tuned for deuterium using a 'trap' circuit (Shih, W., 1978). However during most experiments on cells the trap circuit was left disconnected in order to maximize the Q for

phosphorous. During most of the in vivo experiments a proton lock signal was obtained using a remote tuning arrangement. The proton signal was used only for shimming the magnet, and the remote tuning circuit was removed during data acquisition. The body of the probe, and the outer shield, were made of aluminum.

Nonspinning linewidths were typically between 10 and 14 Hz. This was adequate for in vivo experiments where ^{31}P linewidths were between 40 and 60 Hz.

3.3 Culture Conditions

It was necessary to grow cells to very high densities on the culture dishes, in order to obtain enough material for NMR experiments. This requirement created difficulties in the case of transformed cells. Cells are transformed most efficiently when they are dividing, thus a culture does not become fully transformed if the cells reach confluence too early. On the other hand, the cells should not be fully transformed before the culture reaches confluence. As they become more dense and more transformed, their extracellular matrix dissolves, and they come off the surface of the culture dishes. This is not desirable because cells in suspension grow very slowly, are easily lost, and difficult to handle. Ideally, the cells should reach confluence when approximately 95% of the cells are transformed. The amount and type of RSV which is used to transform primary cultures and the composition of the medium can be adjusted so that this result is obtained.

Ten day old chicken embryos were prepared following the procedure of Bissel et al (Bissel, 1973). Primary CEFs were seeded in Medium 199 containing 2% Tryptose Phosphate Broth, 1% Donor Calf Serum, 1% Chicken Serum, and 1 mg/ml glucose (this recipe is referred to by the abbreviation 2:1:1). When transformed cells were desired, virus was added to the primary cells in suspension. Medium was changed after 3 days, and secondaries were seeded on the fourth day. We experimented with a number of different media for secondary cultures of transformed cells. Primary transformed cells were grown as described above. One secondary culture was seeded in 2:2:1+ (the plus indicates that the medium contained 2 mg/ml glucose instead of 1 mg/ml glucose) on day 1, and on day 2, the medium was changed to fresh 2:2:1+. Another secondary culture was seeded in 2:2:1+ and on day 2, the medium was changed to 10:4:1+. A third secondary culture was seeded in 2:6:1+ and on day 2, fresh 2:6:1+ was added. A fourth secondary culture was seeded in 6:6:0+, and the medium was changed to fresh 6:6:0+ on the second day. For four days after seeding the number of cells per 35 mm dish in each of the cultures were counted. The results of this experiment are shown in Table 1. Each measurement was done in triplicate and the standard deviations were within $\pm 10\%$.

We found that cells grown in 2:6:1+ grew to high densities, became extremely transformed, as indicated by morphology, and remained on the dishes for a full four days. Cells grown in 2:2:1+ or 10:4:1+ were well transformed and grew to high densities, but came off the dishes on Wednesday. Cultures grown in 6:6:0+ grew slowly, and did not look highly transformed; they remained flat and elongated on the

Table 1

cells/dish $\cdot 10^{-6}$ (2^0 cells)

		Cell Counts:				Result
Medium: day 1	Medium: day 2	day 1	day 2	day 3	day 4	
2:2:1+ --> 2:2:1+		1.0	2.1	2.7	2.5	off dish, day 3
2:2:1+ --> 10:4:1+		1.0	2.1	2.6	3.3	off dish, day 3
6:6:0+ --> 6:6:0+		1.0	2.0	1.8	2.3	not well transformed
2:6:1+ --> 2:6:1+		1.0	2.1	2.7	3.1	confluent, day 4

Growth rates of transformed CEFs in various media
 Standard deviations are within $\pm 10\%$

dishes.

An experiment was done to determine the type of virus which was best suited to our experiments. Cells were infected with two different titres of the SRD (Schmitt Rubin) or PA (Prague A) strains of RSV. Primary cultures were grown in 2:2:1, and secondary cultures were grown in 2:6:1+ on 60 mm culture dishes. The secondary cells were counted each day for four days after seeding. The cells infected with SRD appeared to come off the dishes individually instead of as a single sheet. As a result large numbers of cells were lost, and the cell count after 4 days was low. The data in Table 2 show that until they reached confluence, normal cells had a growth rate similar to that of transformed cells. Again, experiments were done in triplicate, and the standard deviations were within 10%.

On the basis of these results we chose to grow both normal and transformed secondary cells in 2:6:1+. PA virus was always used to infect cells, but the amount of virus added varied depending on the virus stock which was used. Three embryos were used to seed 32, 100mm culture dishes with approximately 10^6 cells each. After four days these cells were harvested, suspended in 2:6:1+, and plated onto 128, 100 mm dishes. After 48 hours the old medium was removed, and fresh 2:6:1+ was added. Secondary cells were harvested after four days, and approximately $2 \cdot 10^9$ cells were obtained. The volume of the cell pellet after centrifugation at 200G for 4 minutes was between 2.5 and 3 mls.

3.4 Conclusions

Table 2

cells/dish $\cdot 10^{-6}$ (2^0 cultures)

	day 1	day 2	day 3	day 4
Normal	0.9	1.5	3.8	5.1
SRD	0.8	0.4	1.8	3.0
PA	0.9	2.2	4.4	5.0

Effect of virus on cell growth
Standard deviation is within $\pm 10\%$

In conclusion, we were able to maintain cells at high densities for long periods of time in a superconducting magnet. The levels of oxygen and other nutrients were kept at adequate levels and waste products were removed continuously. As a result the cells could be maintained during lengthy NMR experiments. This method is particularly useful for cells whose growth is not anchorage dependent (i.e. protozoa, virally transformed CEFs, lymphocytes etc.) although a combination of the fiber system with cells attached to beads could prove useful for experiments on attached cells.

Figure Legends for Chapter 3

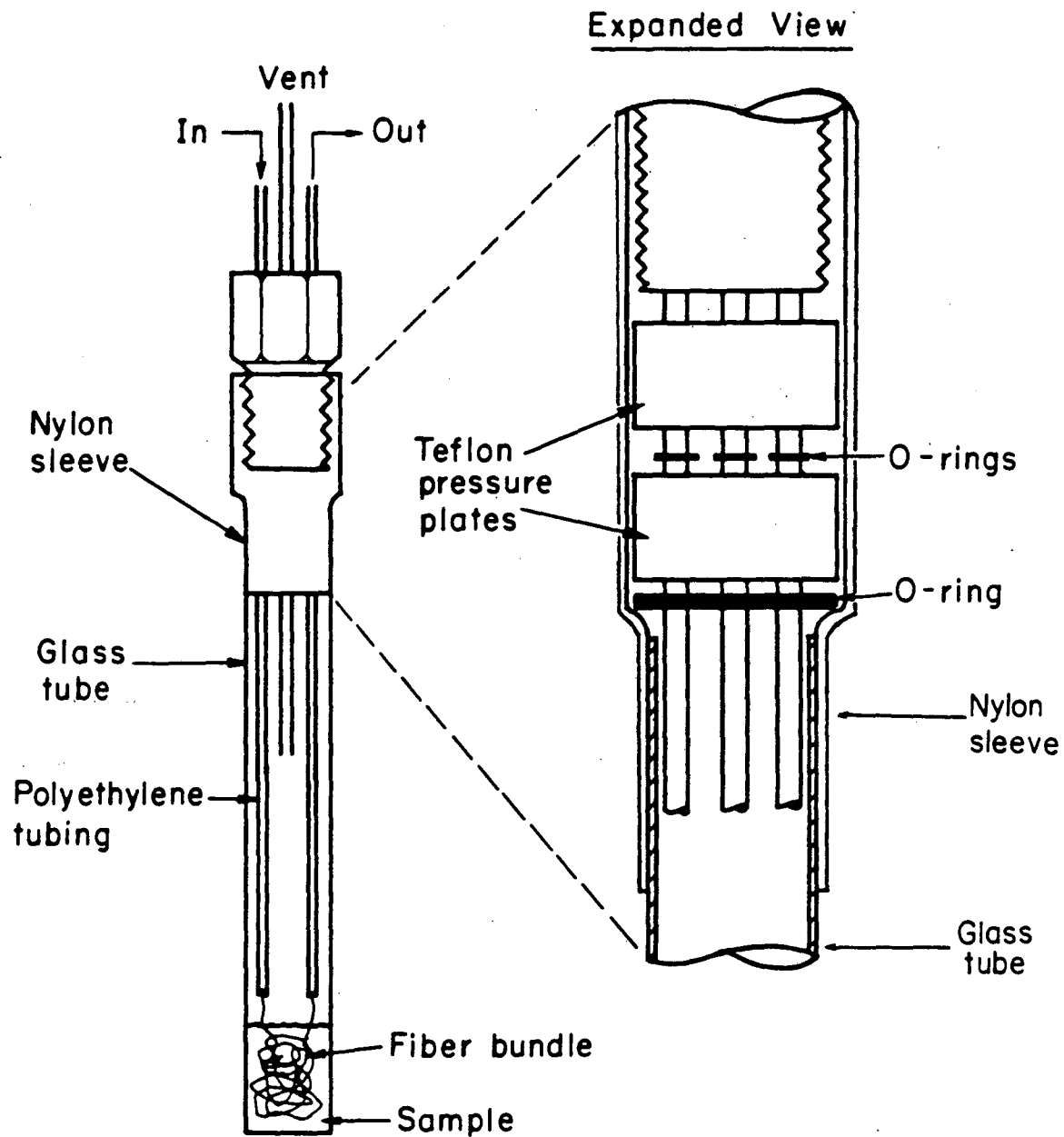
1) The NMR sample holder is a glass tube, 13.5 mm I.D., epoxied to a nylon top. Three polyethylene tubes enter the sample holder; one carries medium flowing into the fibers, one carries medium leaving the fibers, and one is a vent tube. An O ring is placed around each tube, between two pressure plates. A threaded top screws down on the pressure plates, compressing the O rings and creating a seal at the point at which the polyethylene tubes enter the sample holder. At the same time, the bottom pressure plate compresses a fourth O ring creating a seal around the circumference of the sample holder. Dialysis fibers (or Celanese hollow fibers), which are epoxied into the polyethylene tubes, are distributed evenly throughout the two ml sample volume.

2) Transport properties of dialysis fibers: a) Glucose transport; fluid is pumped from a 75 ml reservoir of Medium 199 with 5 mM glucose through fibers in a test tube containing 10 mls of Med 199. Graph shows concentration of glucose in the test tube. b) Lactate transport; fluid is pumped from a 75 ml reservoir of Medium 199 through fibers immersed in 25 mM lactic acid. Graph shows concentration of lactic acid in reservoir. c) Oxygen transport; fluid from an air saturated reservoir is pumped through fibers immersed in a test tube containing 5 mls of O_2 depleted water. Graph shows O_2 tension in the water. d) Equilibration of pH; phosphate buffer at pH 6.8 was pumped through fibers in a test tube with 10 mls phosphate buffer at pH 4.9. Graph shows pH in test tube.

3) Effect of glucose on metabolism of CEFs. Cells were grown as previously described (Bissell, 1973), removed from culture dishes using trypsin and pelleted at 200G. ^{31}P spectra were taken at 109 MHz using 45° pulses and a recycle time of 200 msec. Each spectrum is the average of 3000 free induction decays and an exponential filter of 20 Hz was applied. a) CEFs perfused with glucose free Medium 199. b) 5 mg/ml glucose added to the perfusate. Peak assignments are: 1) phosphorylcholine and phosphorylethanolamine 2) inorganic phosphate 3) YATP 4) α ATP 5) β ATP

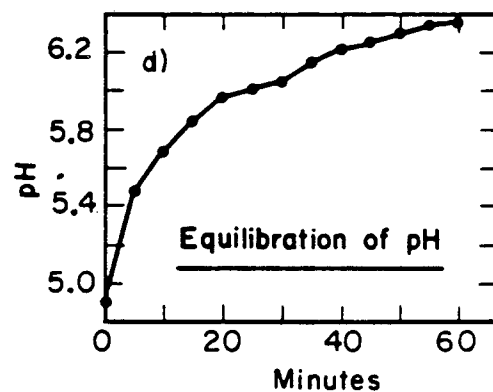
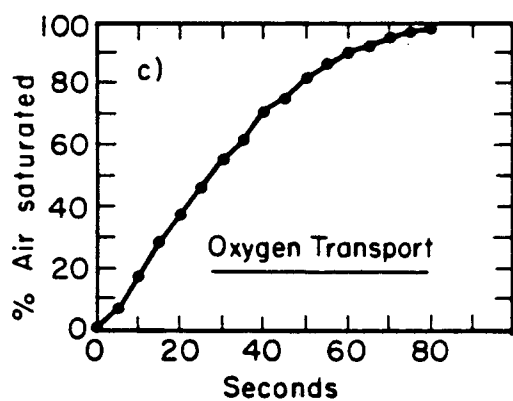
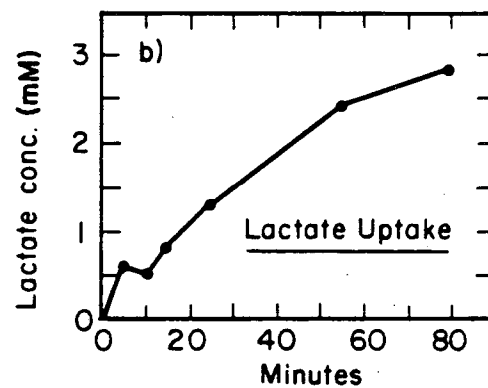
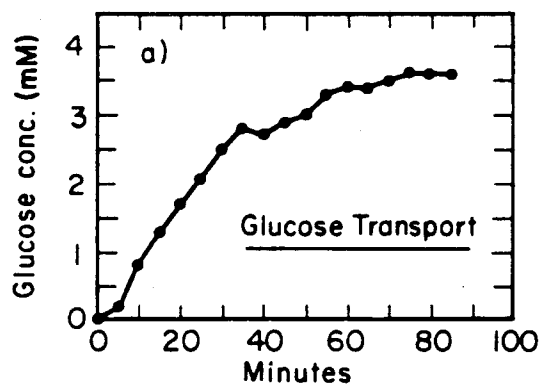
4) Effect of perfusing Tetrahymena with Tris buffer (pH 7.2) equilibrated with 100% oxygen. Tetrahymena thermophila BIV 1868 were grown to mid-log phase at 30 degrees. Then harvested by centrifugation at 200g at 4 degrees. The resulting pellet was transferred into the flow cell for NMR or a duplicate chamber containing both fibers and a Clarke oxygen electrode (YSI). ^{31}P spectra were obtained at 109 MHz at 25 degrees using a 45° pulse and a 206 msec delay between pulses. a) Spectrum obtained before buffer is passed through fibers. b) Spectrum obtained 10 min. after the pump has been switched on. Peaks shown are 1) methylene

diphosphonic acid (pH 9) in an external capillary 2) inorganic
phosphate 3) γ ATP 4) α ATP, NAD/ NADH 5) β ATP



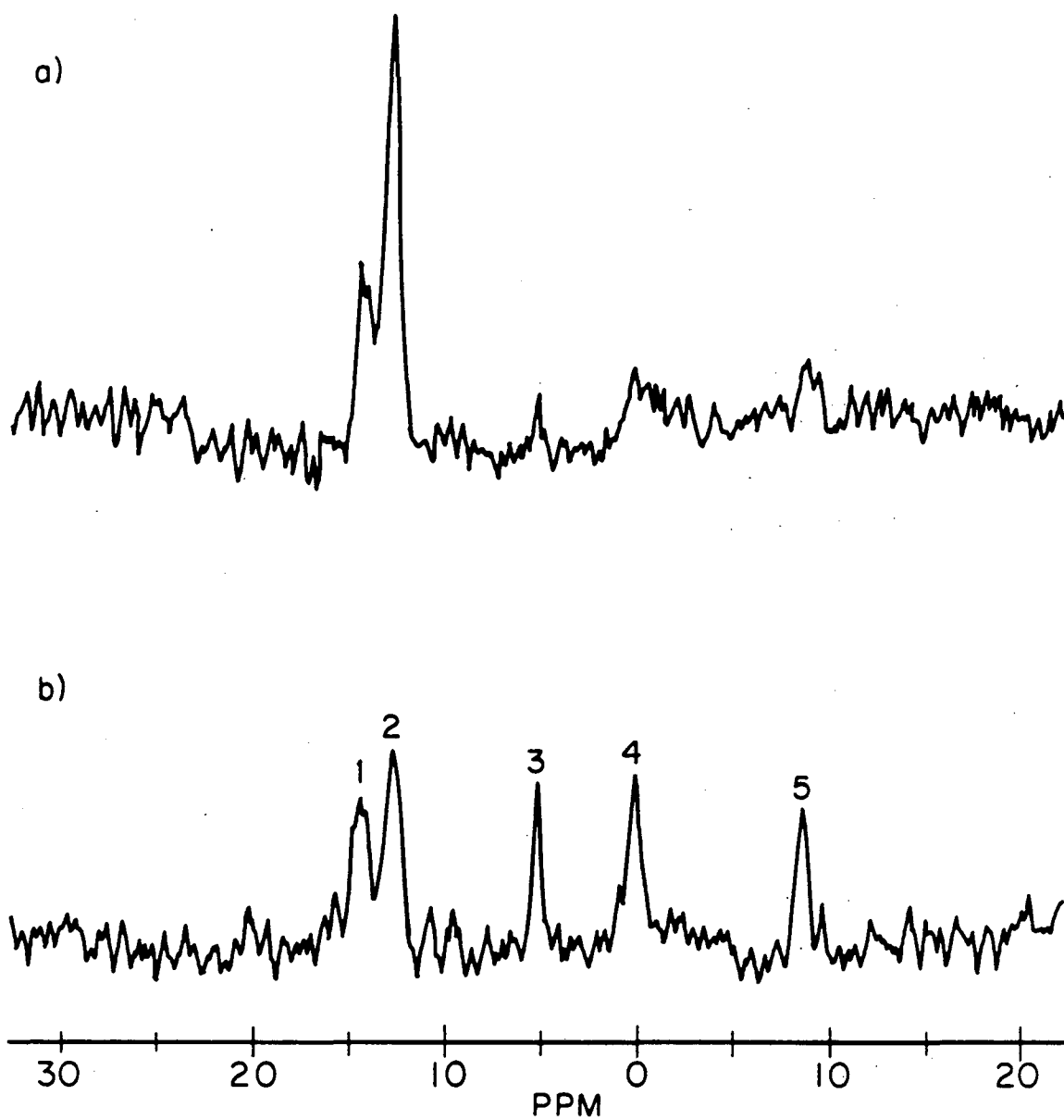
XBL8210-4196

FIGURE 1



XBL8210-4190

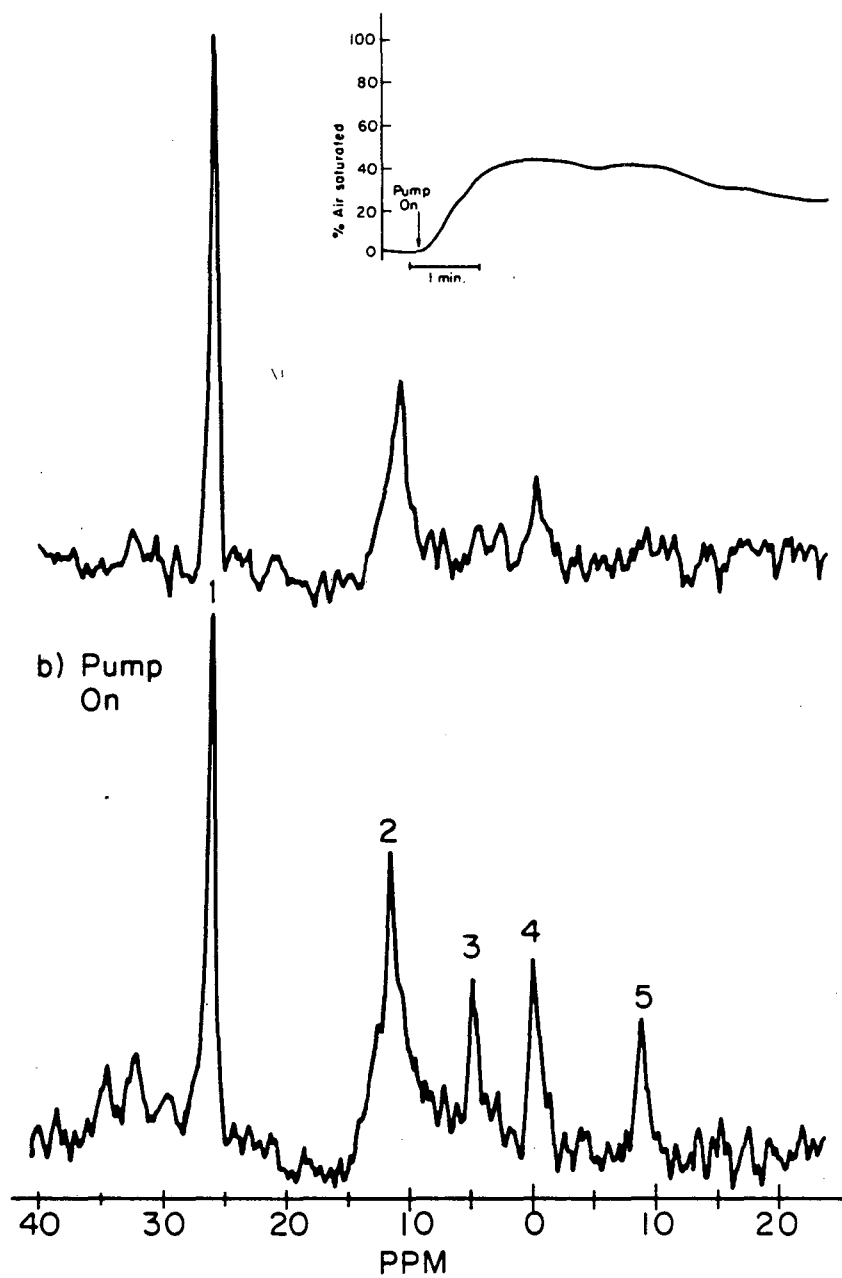
FIGURE 2



XBL 8210-4192

FIGURE 3

a) Pump Off



XBL 8210-4191

FIGURE 4

CHAPTER 4:

Characterization of Metabolism in a Dense Pellet of CEFs

Under normal culture conditions confluent CEFs are very glycolytic; only about 20% of their ATP comes from oxidative phosphorylation, with the rest coming from glycolysis (Bissell, 1972). Confluent fibroblasts on culture dishes produce approximately 10 nanomoles of lactic acid/minute·mg protein. However, we expect that many aspects of cell function are changed under our experimental conditions. Glycolysis is relatively slow in fibroblasts in suspension; Bissell et al have shown that cells in suspension take up glucose at about 40% of the rate of attached cells (Bissell, 1977). Changes in metabolism might also lead to changes in the steady state concentrations of intermediates. In this chapter, some aspects of metabolism in a dense pellet of CEFs are discussed.

4.1 Oxygen Consumption

Using a Clarke Oxygen electrode we measured the O_2 consumption of fibroblasts in suspension. The cells were removed from dishes using trypsin and suspended in Med 199. Five mls of medium containing $5 \cdot 10^7$ cells were placed in an airtight cavity with the electrode and were stirred rapidly. Oxygen consumption was linear with time indicating that during the two minute period required for an accurate reading, the rapid stirring did not cause significant damage to the

cells. In Medium 199 containing no glucose the cells consumed 1.6 nanomoles O_2 /minute·mg protein. Assuming a P:O ratio of approximately 3, and assuming that most of the Oxygen is used to drive the phosphorylation of ATP, this implies that 4.8 nanomoles ATP/minute·mg protein are produced. When the concentration of glucose in the medium is 25 mM, oxidative phosphorylation produces only 0.5 nanomoles of ATP/min·mg protein. Oxygen consumption increases slightly when the medium contains 5.5 mM glucose and 10 mM succinate.

Under the conditions of the NMR experiment however, there is no indication that oxidative phosphorylation occurs. Although oxygen consumption of the cell pellet could not be monitored directly, oxidative phosphorylation could be measured indirectly by observing the response of the cells to 2-deoxyglucose. Deoxyglucose (2DG) is taken up by the cells and is phosphorylated by hexokinase to form 2-deoxyglucose-6-phosphate (2DG6P). A good estimate of the rate at which deoxyglucose is taken up and phosphorylated by the cells can be obtained from the data in Table I. The decrease in the height of the gamma ATP resonance was used as a measure of the decrease in the steady state level of ATP, and the increase in the height of the sugar phosphate resonance gave the concentration of deoxyglucose-6-phosphate in the cell. Within 2 hours 25 nanomoles 2DG6P/mg protein was synthesized. (Protein content of the cells is discussed in the Appendix.) This experiment was repeated several times, with similar results; the time required to reduce the ATP level to zero was always between 1.5 and 2.5 hours. This is also a good estimate of the rate of uptake and phosphorylation of glucose.

Rate of uptake and phosphorylation of Deoxyglucose

Time (minutes)	Height of SP	Correction	Height of YATP	Correction
0	2.9	8.9	2.8	4.6
10	2.3	7.0	2.7	4.5
20	2.6	7.9	2.5	4.2
30	2.5	7.6	2.5	4.2
40	2.5	7.6	2.5	4.2
70	3.5	10.6	2.1	3.5
80	3.7	11.2	1.2	2.0
100	4.8	14.5	1.4	2.3
120	5.4	16.4	0	0
135	5.8	17.6	0	0

The spectra from which these data were obtained were taken under saturating conditions. The corrections for saturation were made on the basis of a T_1 for the PME resonance of 3.0 seconds, and a T_1 for the YATP resonance of 0.7 seconds.

Deoxyglucose competes with glucose for hexokinase, limiting the amount of glucose which can enter the glycolytic pathway. Hexokinase is further inhibited by the high concentration of 2DG6P which builds up inside the cells. The inhibition of glycolysis can be followed by monitoring the intracellular pH of cells which are phosphorylating 2DG6P. This is done by measuring the chemical shifts of P_i and 2DG6P. (The dependence of phosphate chemical shifts on intracellular pH is discussed in Chapter 2.) The cytoplasm becomes more alkaline because of a decrease in the rate of lactic acid production. Table 2 shows the chemical shifts of the phosphate monoesters, ATP, and P_i resonances before, during, and after addition of deoxyglucose. Cells were perfused initially with Medium 199 containing 10 mg/ml glucose, and then with medium containing 1 mg/ml glucose, and 5 mg/ml deoxyglucose. The P_i signal shifts from -2.02 ppm before 2DG is added, to -2.48 ppm after 2DG. This implies an intracellular pH shift from 6.8 to 7.1.

The chemical shifts given in Table 2, and all subsequent tables, are assigned errors of .07 ppm. This error was determined empirically. It was found that when cells were perfused with Med 199 over a period of 12 hours, the chemical shift of each resonance was constant to within .07 ppm. If the chemical shift of each resonance was measured from the same spectrum a number of times, the accuracy was ± 0.05 ppm. The accuracy with which chemical shift can be determined is primarily limited by the widths of the resonances, which are between 40 and 80 Hz. There may also be small changes in chemical shift due to variations in the pH of the cell pellet.

The ATP which is used to phosphorylate deoxyglucose can only be

Table 2

	PME	P _i	α ATP	β ATP	γ ATP
Before 2DG	4.24 $\pm$.07	2.02 $\pm$.07	-9.88 $\pm$.07	-18.70 $\pm$.07	-4.85 $\pm$.07
During 2DG uptake	4.35 $\pm$.07	2.15 $\pm$.07	10.00 $\pm$.07	18.55 $\pm$.07	4.95 $\pm$.07
After 2DG	4.51 $\pm$.07	2.48 $\pm$.07			

The chemical shifts of phosphorylated intermediates (in ppm) are monitored as the cells take up 2DG.

replaced by oxidative phosphorylation, which can use substrates other than pyruvate, i.e. glutamine, succinate, etc. If inorganic phosphate were used to phosphorylate ATP via oxidative phosphorylation, the amplitude of the inorganic phosphate resonance would decrease. In our experiments there is no change in the amplitude of the P_i resonance as the cells produce 2DG6P. Figure 1a shows that while the intensity at 4.6 ppm increases as Deoxyglucose-6-phosphate is formed there is no change in the amplitude of the P_i resonance. In fact the amplitude of the 2DG6P resonance is equal to the sum of the intensities of the ATP resonances which disappear. Figure 1b illustrates that 2DG6P can be synthesized even when very little P_i is present.

In one experiment we found that 20 mM KCN had no affect on the steady state level of ATP, or on the intracellular pH.

Thus it appears that although cells in a dilute suspension use a small amount of Oxygen, no oxygen is used in our dense pellet of cells. All of the ATP which we detect must be a direct product of glycolysis.

4.2 Production of Lactic Acid

Lactic acid production was monitored during the NMR experiments.

Eluent from the vent tube, as well as from the fibers was collected and the lactic acid concentration was measured using a standard enzymatic assay. With no glucose in the medium, no lactic acid could be detected in the eluent. At 1 mg/ml glucose, the cells produce 420 nanomoles of lactic acid/minute or 1.7 nanomoles lactic acid/minute·mg

protein. Since the production of 1 molecule of lactic acid requires the hydrolysis of 1 molecule of ATP and the phosphorylation of 2 molecules of ATP, the overall ATP turnover rate due to glycolysis is 5 nanomoles ATP/minute·mg protein. When the perfusate contained 40 mgs/ml glucose 2.5 nanomoles lactic acid/minute·mg protein were produced. This is a much smaller increase than would be seen for cells on dishes, where the rate of lactate production increases by a factor of 4 as glucose concentration increases from 0.55 mM to 5.5 mM (Bissell, 1973).

4.3 CEFs Were Metabolically Intact Under Our Experimental Conditions

The data presented here are a good indication that the cells were metabolically intact. The cells were able to breakdown ATP and regenerate it, and they produced lactic acid. In addition they were able to phosphorylate 2-deoxyglucose, and 2-deoxyglucose inhibited glycolysis as expected.

Trypan Blue exclusion tests were done regularly on cell samples during NMR experiments. Over 90% of the cells were able to exclude Trypan Blue, indicating that the cell membranes were intact. Another indication that the membranes were intact was the fact that the intensities of signals from ATP, PE, PC, and P_i could be kept constant for 2 days.

When cells were replated on culture dishes at reasonable densities (approximately 10^6 cells/ dish) following NMR experiments,

they were able to attach to the dishes and grow normally for 24 hours. After 24 hours the cultures were overrun by yeast; it proved to be very difficult to keep the perfusion apparatus sterile.

The data presented here constitute strong evidence that the cells were intact and that the apparatus of glycolysis was operating normally during our NMR experiments.

4.4 Identification of Resonances

Identification of the various signals comes in part from preparation of extracts and comparison with published spectra. Figure 2 shows a spectrum of fibroblasts which are perfused with Medium 199 containing 40 mgs/ml glucose and a spectrum of a perchloric acid extract of the same cells at pH 7.4 made immediately after 'a' was obtained. Notice that several signals are resolved in the extract which are not distinguishable in vivo.

The resonances which are assigned to α ATP, β ATP, and γ ATP should more properly be labeled as nucleotide triphosphate resonances. It is not easy to distinguish between ATP and UTP with high resolution ^{31}P NMR. However the primary nucleotide triphosphate in these cells has been identified, by other methods, as ATP (Rubin, 1974).

Figure 3 shows an extract in which some ADP is present. The β ADP phosphate appears as a downfield shoulder on the γ ATP resonance. After chelex treatment (Figure b) it is resolvable as a separate resonance. The α ADP phosphate is also more clearly distinguishable from the α ATP phosphate. The concentration of ADP in this particular preparation was

unusually high, about 20% of the concentration of ATP. This may be because these cells were perfused for 20 hours with medium which did not contain phosphate. This data is discussed in a later section and is shown here in order to demonstrate that ATP and ADP signals are resolvable in extracts only after treatment with chelex.

The sensitivity of the chemical shifts of phosphates and phosphate esters to pH can be used as an aid in the identification of resonances, and was particularly helpful for the identification of resonances in the phosphate monoester (PME) region. The results of a titration of a cell extract are shown in Table 3; these data will be referred to frequently in the discussion which follows. It is likely, *a priori*, that a portion of the intensity in the PME region comes from glycolytic intermediates because they are known to be present in the cytosol of cells on culture dishes in concentrations which should be detectable by NMR (Rubin, 1974). However, when fructose-6-phosphate, 2-phosphoglycerate, and fructose-1,6-diphosphate were added to cell extracts it was found that they did not titrate with either of the two unknown phosphate monoesters. The chemical shift of glucose-6-phosphate is at the upfield edge of the PME(1) resonance and may appear in vivo as a small shoulder on PME(1). This point is discussed further in the next section. Fructose-6-phosphate has approximately the same chemical shift as PME(1) at pH 8.8 and 7.3, but is 0.3 ppm downfield from PME(1) at pH 5.0. Data published by Gadian et al (Gadian, 1979) indicate that 2-phosphoglycerate (2PG) should resonate approximately 0.5 ppm downfield from PME(1), at the same frequency as PME(2). But the chemical shift of 2PG in our extracts is 0.25 ppm

Table 3: Extract Titration

pH	Pi	PME(1)	PME(2)	alpha	beta	gamma
8.75	-3.40	-4.45	-3.91	9.47	19.08	4.67
7.75	-3.19	-4.36	-3.86	9.98	19.14	5.01
7.35	-2.94			10.26	20.05	5.11
7.13	-2.73	-4.20	-3.73			5.49
6.91	-2.38					
6.7	-2.00	-4.00	-3.51			
6.50	-1.81	-3.88	-3.32	10.08	20.00	5.77
5.09	-0.69	-1.91	-1.23	10.08	20.91	8.30

The chemical shifts of the phosphorous resonances were measured as extracts were titrated. The error in the determination of chemical shifts was ± 0.07 ppm.

downfield from PME(1) and about 0.25 ppm upfield from PME(2). Slight differences between the chemical shift in our extract and the shift reported by Gadian are probably the result of the interaction of the 2PG with small amounts of metal ions such as Mg^{+2} which are present in the cells.

Evanochko et al (Evanochko, 1983) have identified phosphorylcholine (PC) and phosphorylethanolamine (PE) in extract and in vivo spectra of fibrosarcoma. Navon et al (Navon, 1978) have found PE and PC in a lymphoid cell line. On the basis of their observations, we added these compounds (obtained from Sigma Chemical Company) to our extract preparations and measured their chemical shifts at pH 8.4 and 5.0. Figure 4a shows a spectrum of an extract at pH 8.4. The spectrum of the extract after addition of PE and PC (obtained from Sigma Chemical Co.) is shown in Figure 4b. Figure 4a also contains an overlay which shows the signals from the commercial compounds which were added to the extract. The chemical shifts of PME(1) and PME(2) at pH 5.0 were -1.91 and -1.23, respectively, while the chemical shifts of PE and PC were -1.96 and -1.25. At pH 8.4, PME(1) and PME(2) resonated at -4.40 ppm and -3.91 ppm respectively, while PE and PC resonated at -4.43 ppm and -3.90 ppm. Clearly PE and PC have the same chemical shifts as the unknown resonances to within 0.05 ppm.

Identification of a metabolite on the basis of phosphorous chemical shift alone is not satisfactory. Identification of proton resonances from the same metabolite would make the assignment more certain. However, as demonstrated by the spectrum shown in Figure 5

assignment of proton resonances will be difficult. Interpretation of the proton spectra is complicated by the presence of 10 mM glucose. Nonetheless, three resonances which could be assigned to PE and PC are present. We also hope to develop enzymatic assays for these compounds. For the present the two resonances are identified as PE and PC.

The PME region can be further studied by determining the chemical shift of DG6P in vivo. This will be the same as the chemical shift of glucose-6-phosphate in vivo. Figure 1 shows spectra taken before and after addition of deoxyglucose to the perfusate. In these spectra the two sugar phosphate peaks at 3.9 and 4.4 ppm are not well resolved, probably because the spectra were obtained under saturating conditions. As 2DG is taken up, P_i shifts from 1.98 ppm to 2.46 ppm indicating a shift in cytoplasmic pH from 6.7 to approximately 7.0. From titration of extracts (Table 3) we know that the PME resonances at pH 7.1 are at 4.26 ppm and 3.79 ppm. The resonances shift only 0.2 ppm upfield as the pH of the extract changes from 6.7 to 7.1. In fact, as is discussed below, the PME resonances do not shift at all in vivo. Therefore the positive signal in the difference spectrum at 4.86 ppm must be due primarily to 2DG6P. In the spectra in Figure 6, the PME region is not well resolved, but it is clear that there is little overlap between the 2DG6P resonance and PME(1) and PME(2). We can conclude that the chemical shift of G-6-P in vivo as well as in vitro is slightly upfield from PE and may be responsible for a small upfield shoulder on the PE signal.

4.5 The Effect of PH on Chemical Shifts In Vivo

In 1972 Moon and Richards (Moon, 1972) demonstrated the sensitivity of the chemical shifts of intracellular phosphorylated metabolites, particularly inorganic phosphate, to intracellular pH. They were able to determine the pH of the cytoplasm of red blood cells from the chemical shift of the P_i resonance. The signals from CEFs do not titrate in the same way in the extract as in vivo. Table 3 shows that in extracts between pH 7.8 and 5.1, the chemical shifts of all of the phosphates, with the exception of the β ATP resonance are sensitive to pH. We can perform a 'pseudo titration' in vivo by changing the amount of glucose which is available to the cells. As more glucose is added to the perfusate, the rate of glycolysis increases and the rate of lactic acid production increases. As a result, the cytoplasm becomes more acidic. Table 4 shows data which was obtained from cells as the intracellular pH changed from relatively acidic to relatively basic and then back to more acidic. Because of the linewidths of the signals, only changes in chemical shifts of 0.1 ppm or greater are considered significant.

It is clear that in vivo only the inorganic phosphate signal is sensitive to pH. As the cells are perfused with 1 mg/ml glucose the chemical shift of P_i is constant at 2.0 ppm. When glucose is removed from the medium the P_i resonance shifts as much as 0.7 ppm downfield. When glucose concentration in the medium is increased to 10 mg/ml, the chemical shift of P_i returns to 2.0 ppm. The amplitude of the P_i resonance decreases at the higher glucose concentration (this effect

TABLE 4

IN VIVO TITRATION

P_i	PME(1)	PME(2)	α ATP	β ATP	γ ATP
-2.04	-4.33	-3.93	9.92	18.77	4.92
-1.95	-4.23	-----	9.98	18.67	4.98
-1.98	-4.33	-3.93	9.96	18.67	4.99
-2.03	-4.33	-3.93	9.91	18.80	4.96
-2.03	-4.23	-3.93	9.97	18.82	4.97
-2.43	-4.33	-3.93	9.88	18.77	4.87
-2.59	-4.23	----	9.97	18.63	5.01
-2.67	-4.33	-3.93			
-2.66	-4.33	-3.93			
-2.43	-4.33	----	9.83	18.85	4.96
-2.08	-4.33	-3.93	9.90	18.70	4.98
-2.43/-1.83	-4.33	-3.93	9.93	18.73	4.91
-2.63/-1.83	-4.33	-3.93	9.93	18.73	4.91
-2.6/-1.8	-4.33		9.93	18.73	5.00

The chemical shifts of the various resonances were determined as the pH of the cell pellet was changed by changing the amount of glucose in the perfusate. The chemical shifts are given in ppm, and the error in the chemical shifts is ± 0.05 ppm.

will be discussed in a later section), and at very low P_i concentrations, a distribution of P_i chemical shifts is detected. The distribution ranges from 0.6 to 0.8 ppm. This is indicative either of heterogeneity among the cell population, or a distribution of intracellular pHs.

Why are the chemical shifts of the other phosphates sensitive to pH in extracts, but not in vivo? There are several possibilities. The phosphates bind metal ions to varying degrees, and metal ion binding affects their chemical shifts. The metal ion content of extracts may be very different from that of the cytoplasm in vivo. Metal ions which were not in the cytoplasm in the intact cell, for example, Ca^{+2} or Mg^{+2} may be dissolved in the extract buffer. Other metal ions which may have been present in the cytoplasm in the intact cell may be lost or diluted in the extract. Molecules which compete with cytoplasmic phosphates for metal ions and which were not dissolved in the cytoplasm in vivo may be present in the extract. In fact the former possibility appears more likely. At physiological pHs the beta ATP phosphate has a chemical shift of about 20.0 ppm in extracts, but is shifted downfield to 18.8 ppm in vivo. The gamma ATP has a chemical shift of 5.2 to 5.7 ppm at physiological pH's in extracts, but is shifted downfield to 5.0 ppm in vivo. This shift would be expected if extracts contained less than the cytoplasmic concentration of Mg^{+2} . When the gamma and beta ATP phosphates are 99% complexed with Mg in vivo their titration curves are broadened and their apparent pKs are lower, so that the inflection points occur at lower pHs rather than in the middle of the physiological range

(Roberts, 1981; Gadian, 1979). In extracts, where the concentration of Mg is lower their chemical shifts are very sensitive to pH between pH 6.5 and 7.5.

PME(1) and PME(2) may be affected similarly by magnesium and other metal ions, but if these two resonances are due to PE and PC, they should bind metal ions very weakly. Thus it is puzzling that they do not respond to changes in pH in the same way in vivo as they do in extracts. The pKs of PE and PC (6.0) (Shulman, 1978) are lower than the pK of inorganic phosphate (6.8); their state of protonation, and thus their chemical shifts would not be as sensitive as that of P_i in the physiological pH range. Nonetheless, the extract data leads us to expect some shift in vivo as a function of pH. The fact that we do not see this suggests that the PE and PC are at a pH different from that of the cytoplasm.

4.6 Concentrations of Phosphorylated Compounds

Concentrations of phosphorylated compounds were determined by comparing integrated intensities of the various signals with that of the MDPA standard. Concentrations in intact cells perfused with Medium 199 containing 1 mg/ml glucose at 25 degrees are shown in Table 5. They are compared to values obtained for cells on dishes using enzymatic methods. No differences were found between normal and virally transformed cells.

Under our experimental conditions concentrations of sugar phosphates are much lower than in cells in culture, and the

Table 5
METABOLITE CONCENTRATIONS

	NMR	ENZYMATIC ASSAY*
Sugar Phosphates	-	6.7
PE	12±2	-
PC	12±2	-
ATP	6.8±1.0	18.0
ADP	0.0	1.5
AMP	0.0	0.88
Pi	15.0±0.5	-
NAD/NADH	7.8±1.0	-

Metabolite levels are given in nanomoles per mg protein. The NMR data were obtained by comparing the integrated area of each signal with that of the signal from a 100 mM MDPA standard in a sealed capillary. Standard deviations were determined empirically, based on 5 experiments during which cells were perfused with Medium 199 at 25°C.

* H. Rubin and D. Fodge, From "Control of Proliferation in Animal Cells", pp 801-816, 1974, Cold Spring Harbor

concentration of ATP is a third of that cells in culture. We find significant concentrations of phosphorylethanolamine and phosphorylcholine. These compounds have not previously been found in CEFs. It is possible that they are difficult to detect using radioactive isotopes because their turnover rate is slow, and thus they are labeled slowly. They commonly function as intermediates in the synthesis of phospholipids (Lehninger, 1975)

4.7 Conclusions

On the basis of these experiments we conclude that all of the ATP we observe comes from glycolysis. The almost complete inhibition of oxidative phosphorylation under our experimental conditions may be due to the fact that the cells are not stimulated to grow in the absence of serum, especially when they have been removed from their matrix (Bissell). In addition, cells on culture dishes become more glycolytic at high densities (Bissell, 1972). In the discussion of the regulation of glycolysis which follows, it should be kept in mind that the Crabtree and Pasteur effects should not be operative under our experimental conditions.

The rate of glycolysis, calculated from the rate of production of lactic acid is only 17% of the rate in cells which are attached to culture dishes. However it is still high enough so that the cells' pool of ATP turns over completely every 3.5 minutes; the cells under our conditions are metabolically active.

The concentrations of phosphorylated metabolites under our

experimental conditions are significantly different from the concentrations in cells on culture dishes. In particular, we detect very little signal from the sugar phosphates which are intermediates in glucose metabolism. We find high concentrations of phosphorylcholine and phosphorylethanolamine; these compounds have not previously been found at such high concentrations in CEFs.

There were no metabolic differences which we could detect between normal and virally transformed cells.

Figure Legends for Chapter 4

Figure 1a: ATP is used to phosphorylate 2DG, but no P_i is used. A is a spectrum of cells before perfusion with 2DG, B is a spectrum taken after perfusion with 2DG. C is the difference spectrum, B-A. The large positive signal in the difference spectrum is due primarily to 2DG6P. Assignments are 1) PE and PC; 2) P_i ; 3) γ ATP; 4) α ATP; 5) β ATP; 6) 2DG6P; LB=15 Hz, TM=6

Figure 1b: Deoxyglucose can be phosphorylated even when the concentration of free P_i is very low. This experiment demonstrates clearly that the 2DG6P is downfield from the PME region. As the cells take up 2DG, the 2DG6P resonance is clearly resolvable. These spectra were taken under saturating conditions: 90 degree pulse, 600 msec delay, LB=30 Hz. The chemical shifts in ppm of the 'phosphate monoester' signals are given on the figure.

Figure 2a: Preparation of extracts: A) cells in vivo, B) perchloric acid extract, C) After chelex treatment. Assignments are: 1) phosphorylcholine and phosphorylethanolamine; 2) γ ATP; 3) α ATP; 4) β ATP; 5) phosphorylethanolamine; 6) phosphorylcholine; 7) P_i ; LB=20 Hz, TM=6

Figure 2b: EDTA added to chelex treated extract, solution filtered through 0.25 μ m filter: expansion of PME region. LB=10 Hz

Figure 3: ADP was sometimes resolved in extracts: A) perchloric acid cell extract, B) extract after treatment with chelex. Assignments are: 1) PE; 2) PC; 3) P_i ; 4) γ ATP; 5) α ATP; 6) NAD and NADH; 7) UDPG; 8) β ATP; 9) β ADP; 10) α ADP

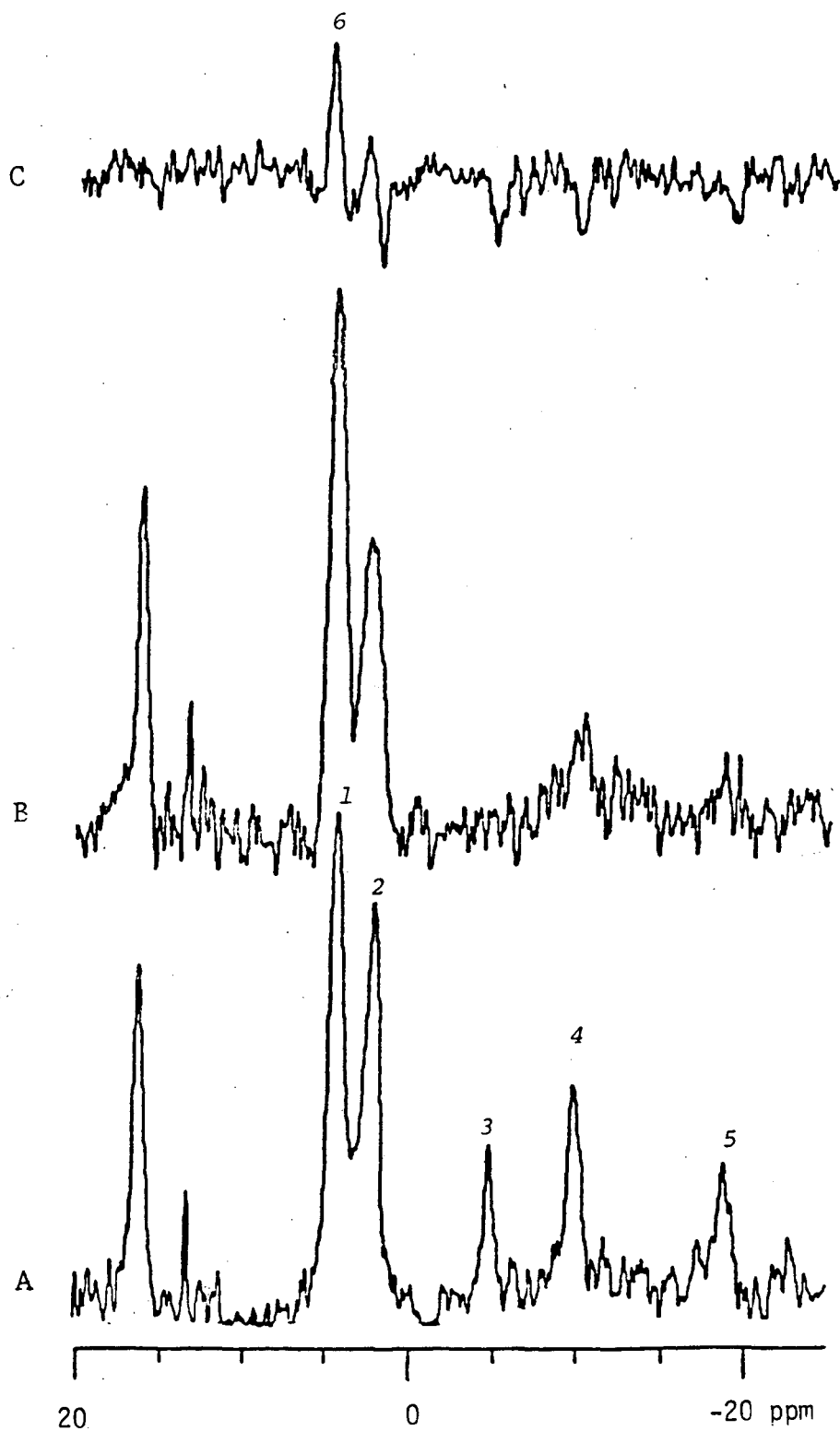
Figure 4a: Spectrum of cell extract before addition of PE and PC. The registered plot shows the position of signals from PE and PC added to same extract. LB=5 Hz, TM = 8

Figure 4b: Spectrum of cell extract shown in 'a', after addition of PE and PC.

Figure 5: Proton spectrum of a cell extract. Assignments are as follows; 1) H_2O ; 2) primarily glucose; 3) Possibly PE and PC; 4)

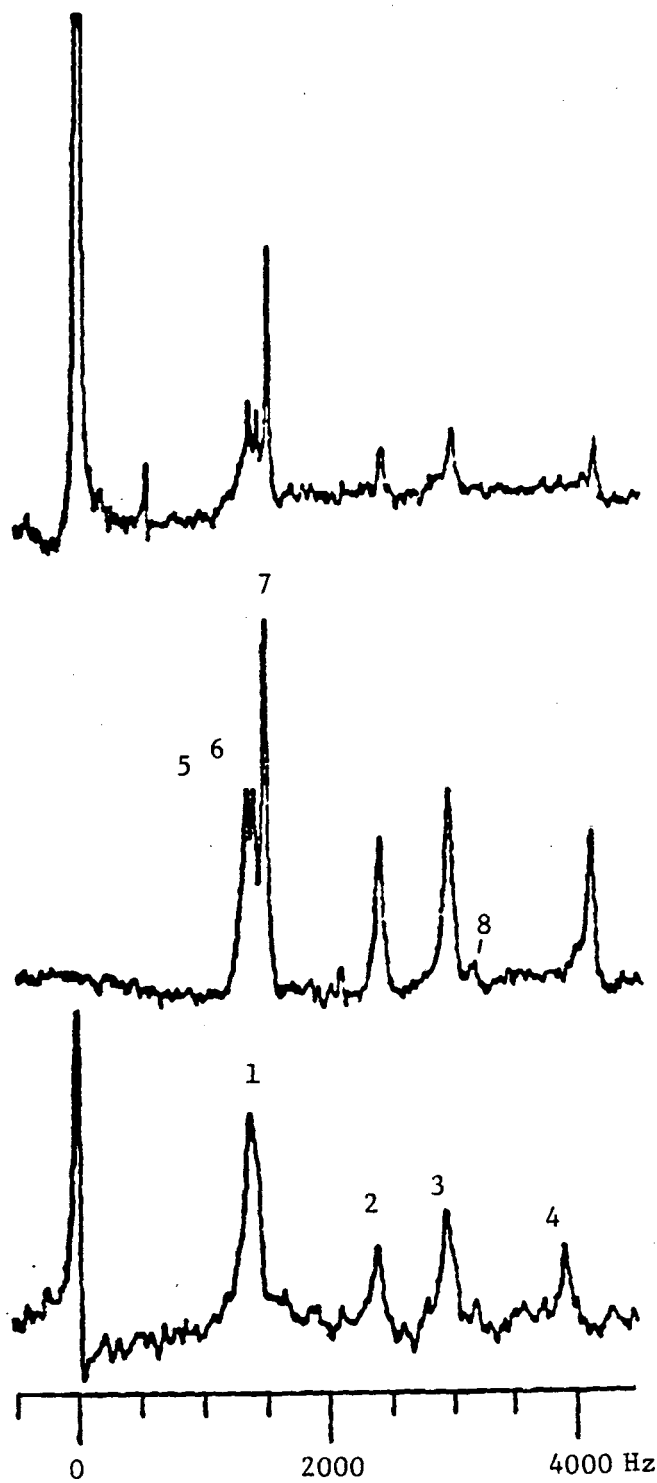
lactate

Figure 6: Chemical Shift of 2DG: A) Cells without 2DG, B) During 2DG uptake, C) After 3 hours of perfusion with 2DG, D) C-A. Chemical shifts are as follows: a) 4.64 ppm; b) -4.92 ppm; c) -9.92 ppm; d) -18.74 ppm. Assignments are as follows: 1) PMEs; 2) P_i ; 3) α ATP; 4) β ATP; 5) γ ATP; 6) 2DG6P. LB=20 Hz, TM=2



XBL 849-4023

FIGURE 1



XBL 849-4024

FIGURE 2A

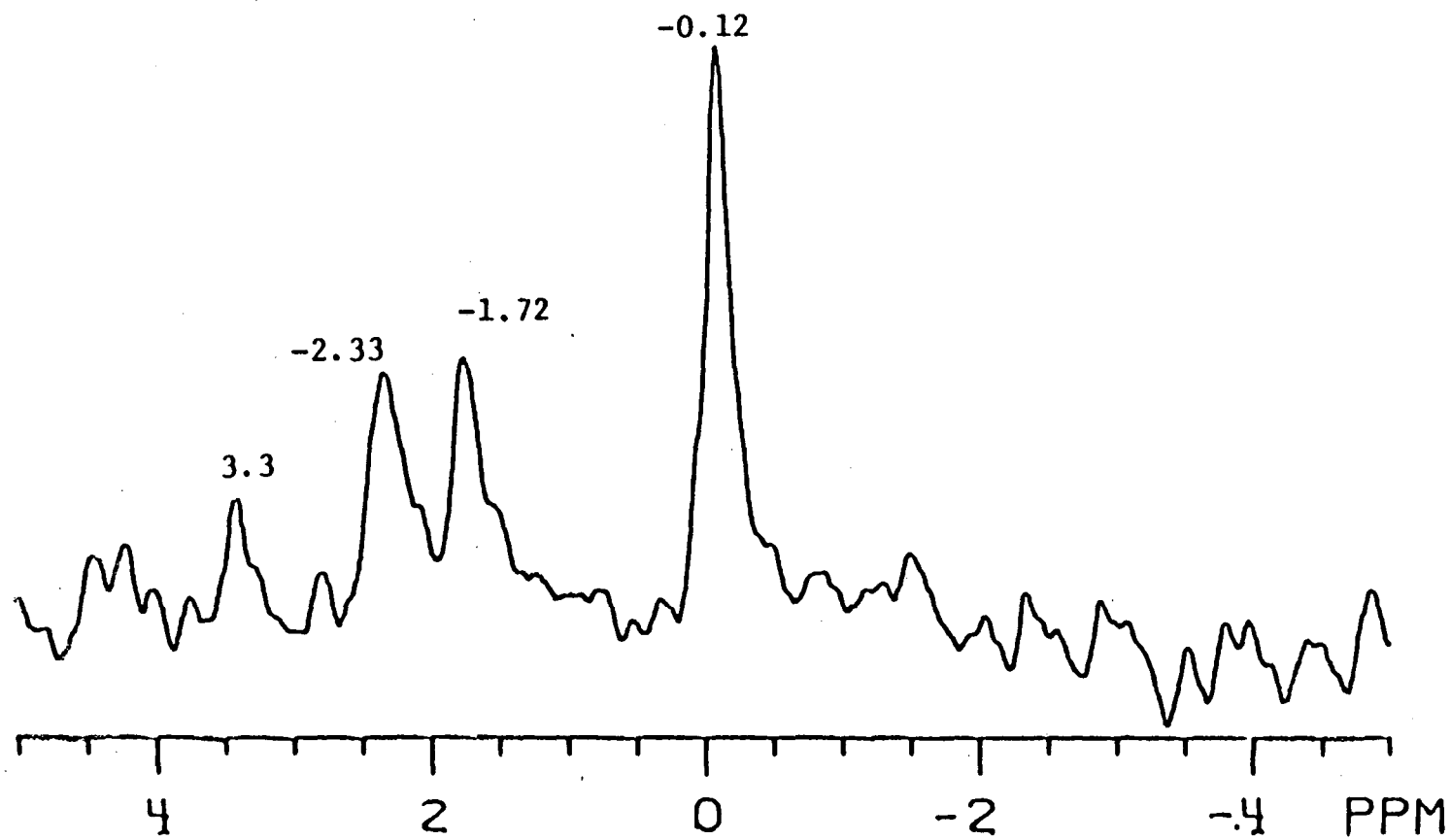
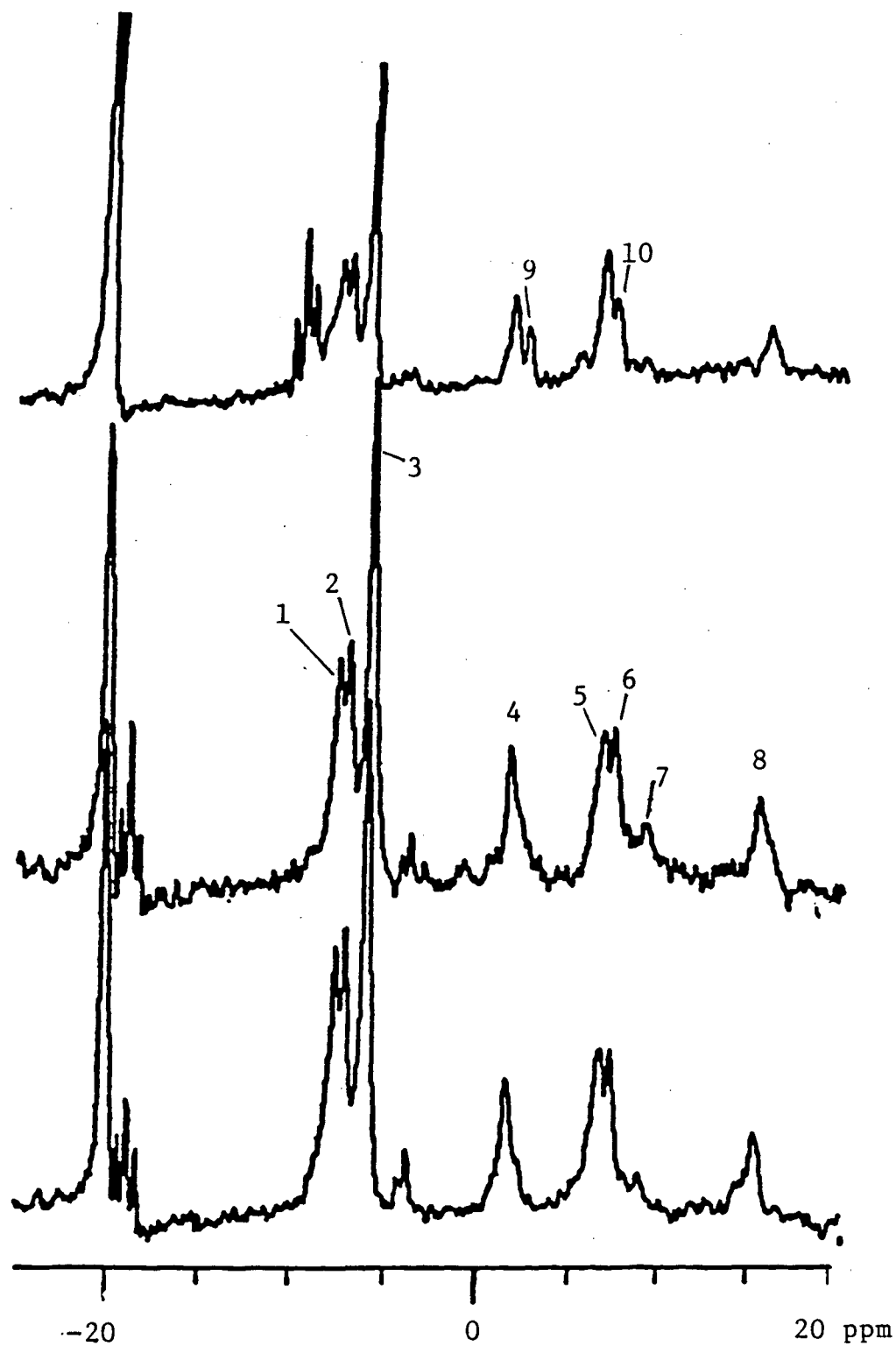


FIGURE 2B

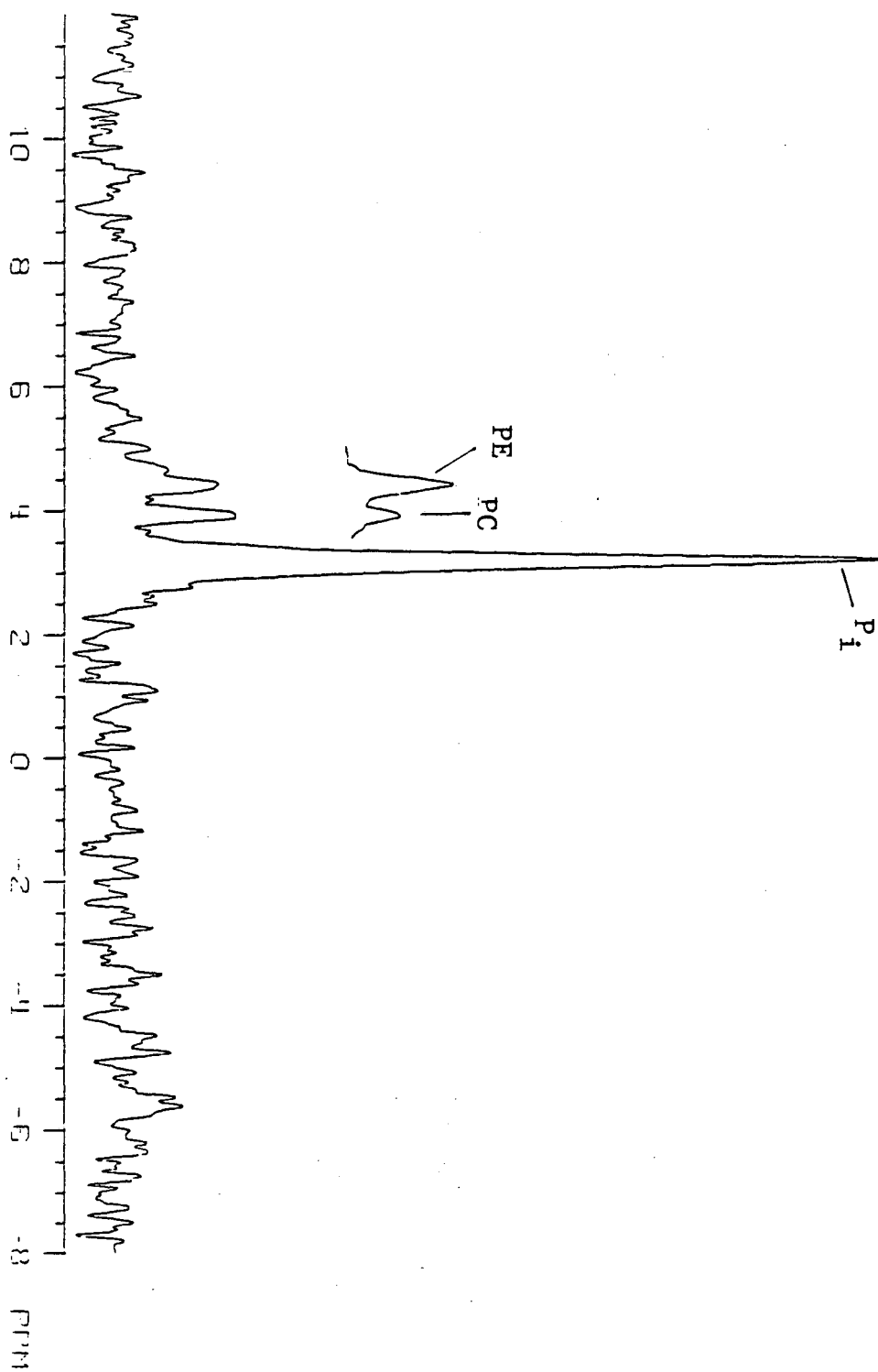
Perchloric acid extract after chelex and 0.45 micron filter

XBL 849-4004



XBL 849-4022

FIGURE 3



XBL 849-4003

FIGURE 4A

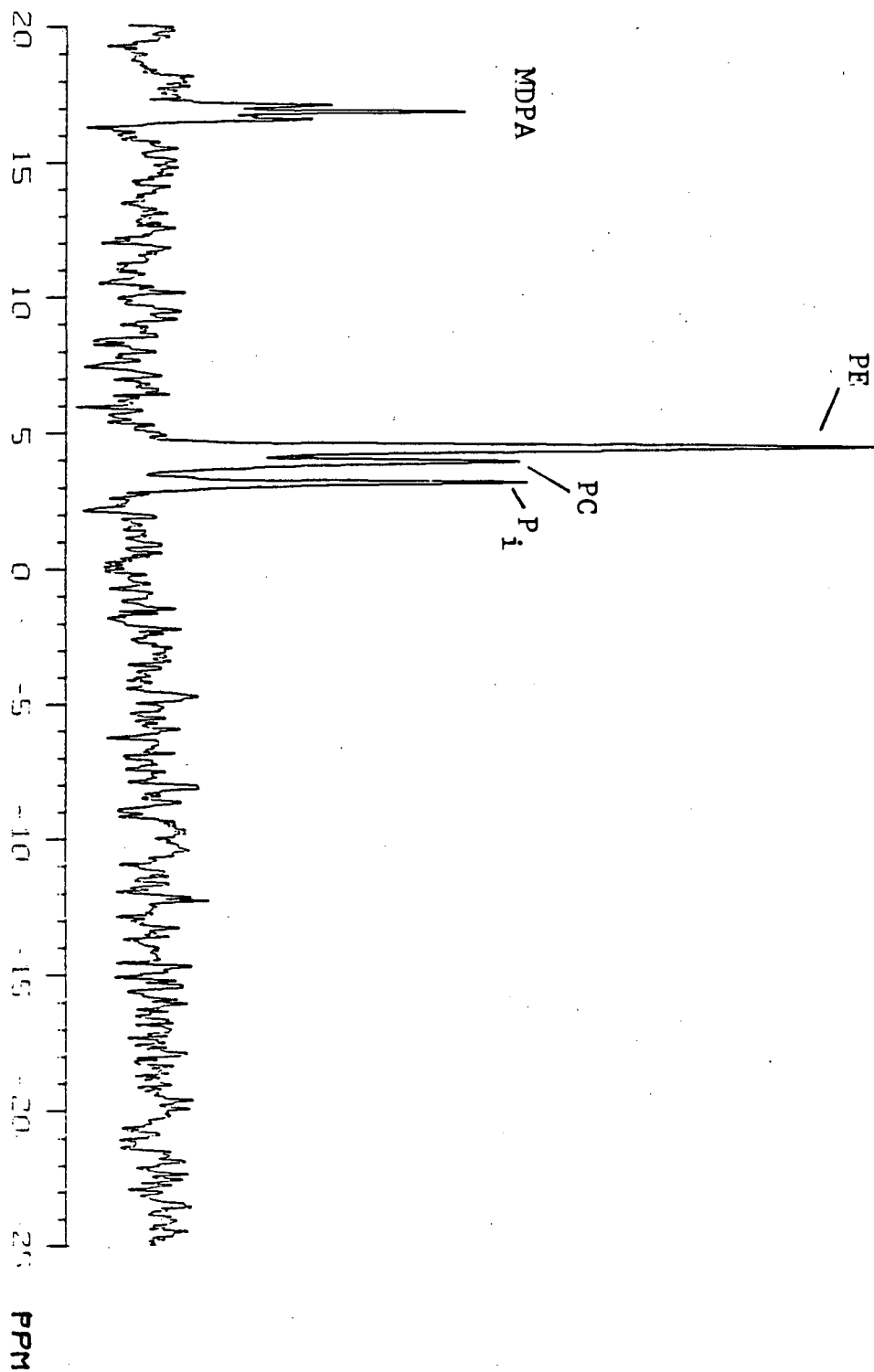


FIGURE 4B

XBL 849-4002

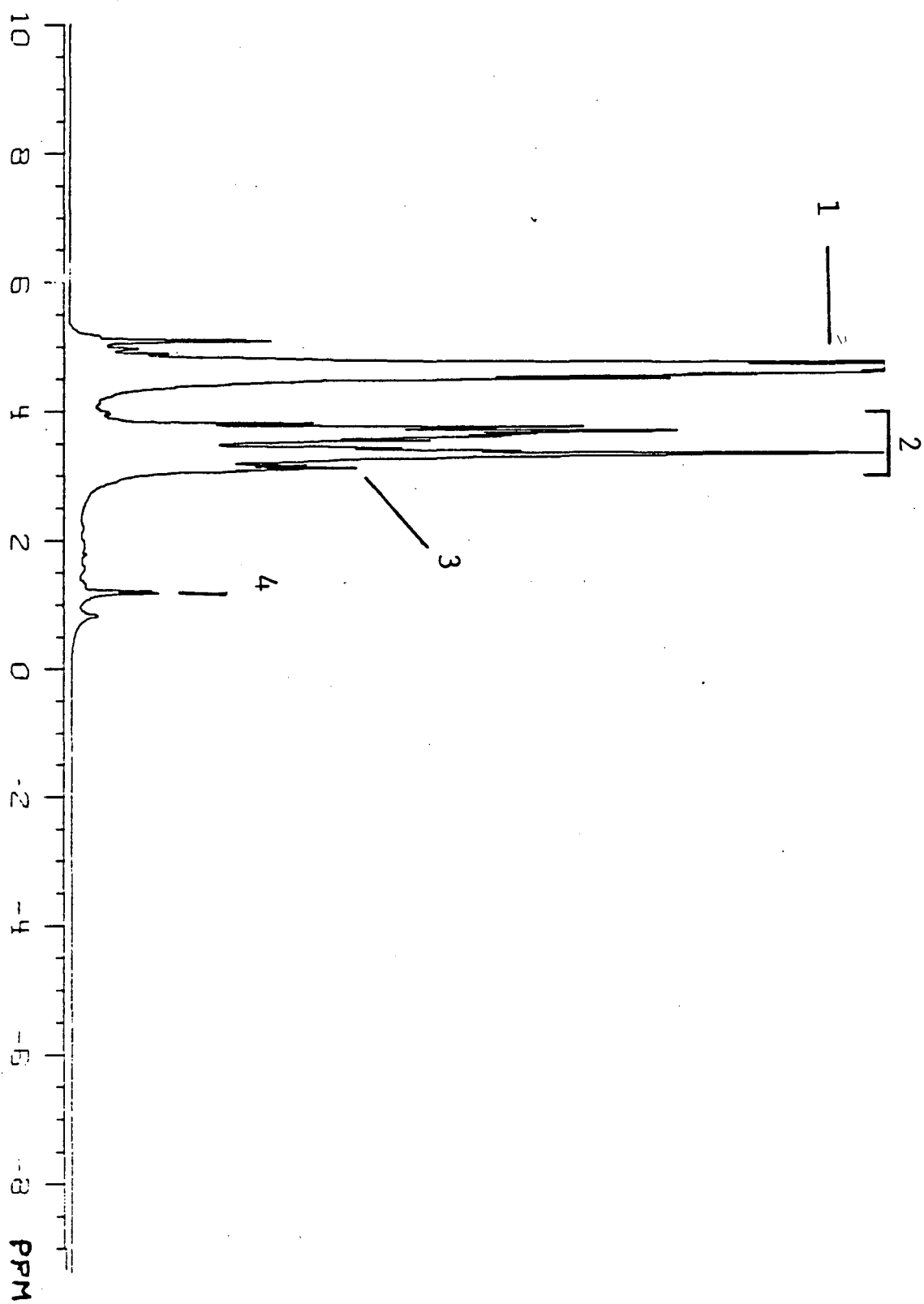
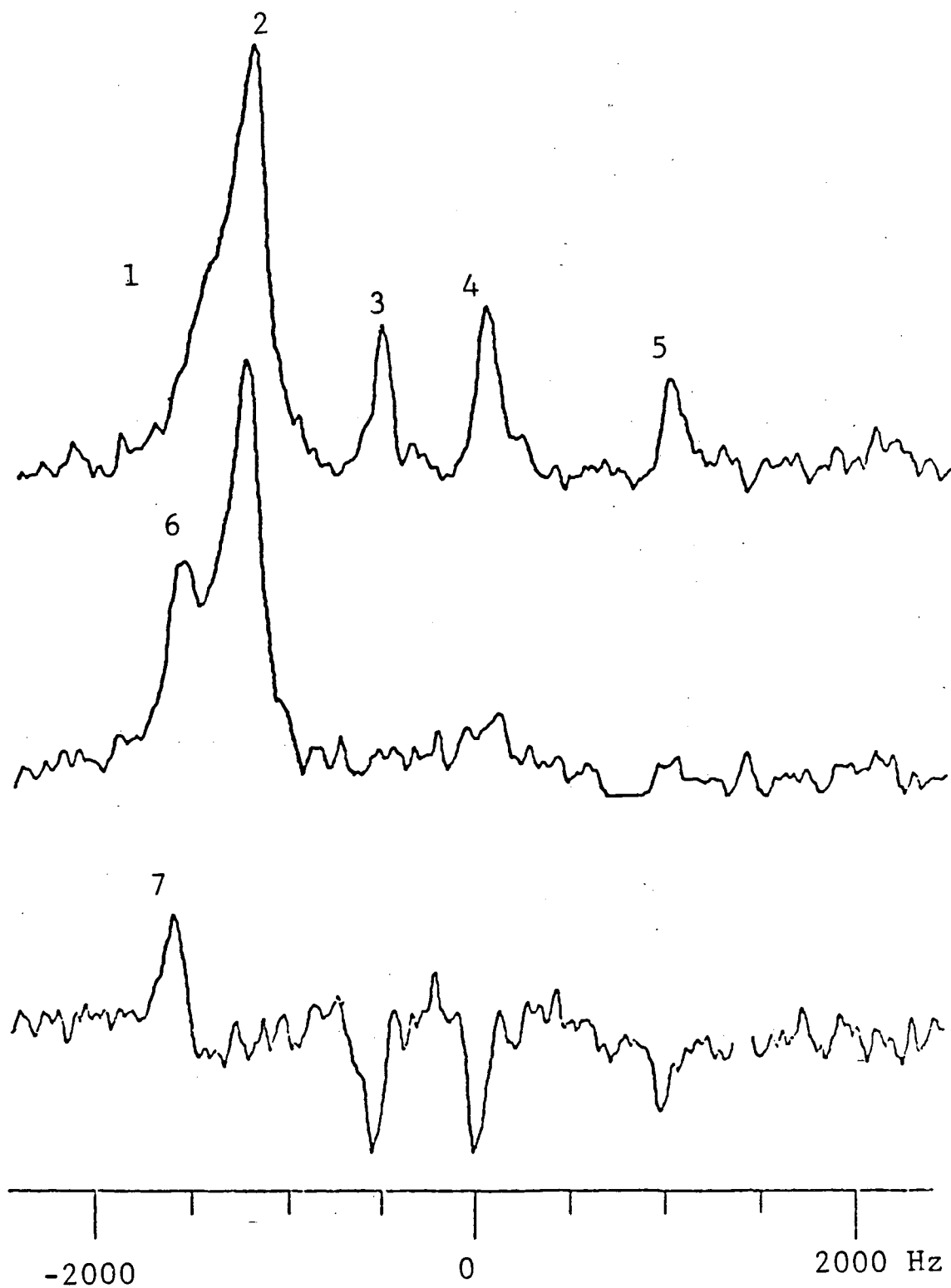


FIGURE 5

XBL 849-4001



XBL 849-4020

FIGURE 6

CHAPTER 5

Evidence of Intracellular Compartmentation

In this chapter, we make use of the ability of NMR to distinguish between molecules on the basis of their correlation times to study compartmentation of phosphorylated intermediates in vivo.

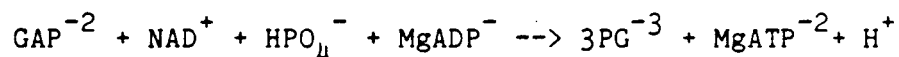
5.1 Background: Evidence of a Role for Compartmentation in Regulation of Metabolism

Ottaway and Mowbray (Ottaway, 1977) have discussed a large body of work which suggests that the apparatus of glycolysis is compartmented. Glycolytic enzymes have been shown to be localized in various parts of the cell. All of the hexokinase in brain is found in the mitochondrial fraction (Cell Reg). In skeletal and cardiac muscle, much of the Hexokinase is bound to mitochondria. Aldolase and glyceraldehyde phosphate dehydrogenase (GAPDH) bind strongly to red cell membranes, and phosphofructokinase, aldolase, and GAPDH have all been reported to bind to some extent to sarcoplasmic reticulum. On the basis of these data Ottaway and Mowbray discuss the possibility that glycolysis takes place to some extent on intracellular surfaces. Reactions which take place on surfaces can be much more efficient than

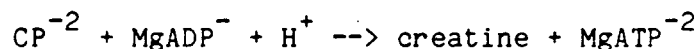
those which take place in three dimensions. The substrates can be 'guided' into the enzyme active sites, and diffusion-limited rates can be greatly increased.

The idea that glycolysis occurs on intracellular surfaces is at odds with the more traditional view that only substrates and enzymes which are dissolved in the cytosol participate in glycolysis. This view is supported most directly by the fact that all of the glycolytic enzymes are soluble in water at their physiological concentrations. In addition, the experiments of Veech (Veech, 1978) and others indicate that the activities of at least some phosphorylated metabolites are the same in extracts as they are in vivo.

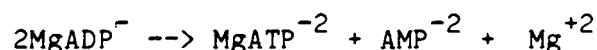
These workers measured tissue contents of the substrates of glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, creatine kinase, and myokinase in rat liver. These enzymes catalyze the synthesis of 3-phosphoglycerate from glyceraldehyde-3-phosphate:



the phosphorylation of creatine:



And the synthesis of ATP from ADP:



The activity of these enzymes is high enough relative to their flux to insure that they are near equilibrium. In rat liver and red blood cells the concentrations of all the substrates with the exception of

ADP are very close to the equilibrium concentrations for these reactions. (It is believed that large amounts of ADP are sequestered in the mitochondria so that the cytosolic ADP concentration is only 1/20 of the total.) This implies that the activity of these substrates in vitro is very close to their activity in vivo, a result which would not be expected if they were bound to surfaces inside the cell. The fact that three different kinases have MgADP^{-2} and MgATP^{-2} as common substrates, and all four reactions are close to equilibrium, implies that the different enzymes all have access to a common pool of these substrates. This can be most easily explained if there is no microcompartmentation, and if the enzymes and substrates are dissolved in the cytoplasm.

In the past five years NMR studies have tended to support the view that compartmentation plays an important role in certain aspects of metabolism. The most straightforward experiments compare concentrations of metabolites measured in vivo by NMR with concentrations determined from assays of cell extracts. Freeman et al (Freeman, 1982) have found that spectra of the intact kidney show only 25% of the ADP and P_i which is seen in extracts. Similar findings have been reported in muscle (Ackerman, 1980), where no ADP is observed, and the concentration of P_i detected is much less than that which is found in extracts prepared using a freeze clamping technique. It is assumed that the discrepancy between NMR and biochemical data derives from the fact that some of the metabolites are sequestered in a bound form inside the cell.

Radda et al (Radda, 1984) have monitored high energy phosphates

in human skeletal muscle. During exercise, PCr is broken down and the concentration of P_i increases. When the muscle is rested the amplitude of the P_i signal decreases with a half time of 1.6 minutes. Although this P_i is used to phosphorylate creatine, the time constant for the reappearance of PCr is 5.3 minutes. There is an intermediate period during which a significant fraction of the signal is lost. Radda et al suggest that the P_i which is not detected is sequestered in the mitochondria, perhaps as the calcium salt. Gradually it is used to phosphorylate ADP, which in turn phosphorylates cytosolic creatine.

NMR has also been used to measure steady state rates of catalysis in vivo. These rates can be used to calculate activities of the enzymes and their substrates, and these activities can be compared to activities measured in vitro. Discrepancies between in vivo and in vitro activities may be the result of compartmentation. Simpson et al have measured the rate of equilibration of the methyl groups of lactate and pyruvate in erythrocytes (Simpson, 1982). Since both lactate and pyruvate are transported rapidly across the cell membrane, it is possible to control the intracellular concentrations by controlling the extracellular concentrations. If this is done only the activities of the lactate dehydrogenase and its cofactors NAD and NADH are experimental variables. The authors find that the activity of NADH in vivo, as measured from the rate of catalysis, is only 10% of the total extractable activity. They suggest that most of the NAD and NADH in the cell is bound to proteins, and that the concentration of free NAD and NADH is the rate limiting factor in the oxidation of lactate in the intact cells.

The observation of intracellular pH gradients is further evidence of compartmentation in vivo. Busby et al (Busby, 1978) used ^{31}P NMR to study the rat vastus lateralis muscle. They found that in the absence of oxygenation, the P_i resonance broadens. Since the linewidth is much greater than would be predicted on the basis of the T_2 , these authors suggest that the intracellular pH is not uniform. Incubation of the muscle with acetate buffer at pH 5.2, which rapidly penetrates the cell membrane, results in splitting of the P_i resonance into two components. This implies that P_i exists in two compartments inside the cells, one which is accessible to acetate, and one which is not. Podo et al (Podo, 1982) have observed selective broadening of the G6P resonances in insulin treated rat diaphragm muscle. Their results support the hypothesis that there is compartmentation of glycolytic intermediates and glycolytic enzymes within the cell.

As is clear from this brief review of the literature, the evidence for a role of compartmentation in regulating metabolism is indirect. NMR experiments may yield less ambiguous data than that which is available through the use of other techniques. NMR experiments on isolated cells are of particular interest because they provide the simplest systems in which compartmentation can be studied. We turn now to a consideration of data obtained in this laboratory which imply that compartmentation of high energy phosphates occurs in CEFs at high rates of glycolysis.

5.2 Effect of Changes in Glucose Concentration: Starved Cells

ATP levels decreased quickly when the cells were perfused with medium which did not contain glucose. Figure 1 shows amplitudes of the three ATP resonances, as a function of the time elapsed after glucose was removed from the perfusate. As the cells were starved, the amplitudes of the three resonances decreased in parallel. The α ATP resonance was the largest of the three and did not decay to zero. This is because there is additional intensity in the -10 ppm region due to NAD and NADH. When the cells had used all of their ATP a broad NAD/NADH signal remained, giving rise to integrated intensity at later times. The fact that the intensities of the β and γ ATP resonances were equal throughout the experiment indicates that the level of free ADP was less than 0.7 nM/mg protein. Although, as discussed in Chapter 3, attempts were made to insure that the growth conditions were identical for all the cells which were used in NMR experiments, the time required to hydrolyze the entire pool of ATP varied widely between different batches of cells. This may have been due to variability in the glycogen content of the cells.

The phosphate which was hydrolyzed from ATP remained in the cell as inorganic phosphate and the amplitude of the inorganic phosphate resonance increased. However, the amount of phosphate detected by NMR was not constant. Figure 2 is a spectrum of transformed cells which were perfused with Medium 199 containing 1 mg/ml glucose. Spectrum 2b was obtained after perfusion for 60 minutes with glucose-free medium. These are fully relaxed spectra, as are all other spectra which will be discussed, unless otherwise indicated. The difference spectrum: 2b - 2a is shown in Figure 2c. There is a 30% increase in the amplitude

of P_i , but no significant decrease in the amplitude of the ATP resonances. Figure 3 compares a block averaged spectrum of the same cells, obtained over a two hour period while the cells were perfused with Medium 199 containing 1 mg/ml glucose (3a), with a spectrum acquired between 3 and 4 hours after the beginning of perfusion with glucose-free medium. After 3 hours the cells were producing much less lactic acid, and the intracellular pH was more alkaline, thus the P_i resonance shifted from -1.8 ppm to -2.5 ppm. As a result the P_i resonance in the difference spectrum is biphasic. The steady state free ATP concentration had not yet changed. The sum of the amplitudes of all signals which were detectable (referred to from now on as 'total phosphate intensity') increased by 18% after 4 hours of perfusion with glucose-free medium.

In Figure 3d, a spectrum obtained after 5 hours of perfusion with glucose free medium is subtracted from 3a. The steady state level of ATP had decreased significantly at this point, and negative ATP peaks appear in the difference spectrum.

This effect was also observed in normal CEFs. Figure 4 shows a plot of the total phosphate intensity as cells were perfused with medium which did not contain glucose. (Total phosphate intensity is the sum of the integrals of all the resonances which are detected.) The lower points represent the ATP signals. In order to improve S/N, the spectra were taken under saturating conditions, using a 45 degree pulse and a 500 msec delay between pulses. The true equilibrium magnetizations of the various resonances were determined using the formula described in Chapter I, assuming a T_1 for P_i and the PMEs of

2.2 seconds, and a T_1 for the ATP resonances of 0.8 seconds. In fact, as is reported in the Appendix, the measured T_1 s for P_i , PE, and PC are somewhat longer than 2.2 seconds, and the T_1 s for the ATP resonances are somewhat shorter. Had the measured T_1 s been used to calculate amplitudes, the change in total phosphate intensity would have been even greater than that shown in Figure 4.

Figure 5 shows data from a third experiment in which normal CEFs were perfused with Medium 199 without glucose. Spectrum 5a was acquired while cells were perfused with medium containing 1 mg/ml glucose, and 5b was acquired after 4 hours of perfusion with glucose-free medium. The difference spectrum (5c) is also shown. Again the increase in the P_i amplitude was significantly greater than the decrease in the ATP amplitudes. A net hydrolysis of ATP resulted in an increase in intensity, not only at 1.74 ppm, where inorganic phosphate resonates at pH 6.7, but also at 3.0 ppm. This new intensity is at a chemical shift which is characteristic of many of the glycolytic intermediates. After 5 hours of perfusion with glucose-free medium the decrease in the ATP level, shown in Figure 6, is comparable to the increase in P_i . Table 1 summarizes data from three experiments in which the total phosphate signal detected by NMR increased as cells were perfused with glucose free medium.

Perfusion of cells with glucose-free medium did not result in damage to the cells. When glucose was added to the perfusate the ATP signals returned to their original levels in all cases. In addition, Trypan Blue exclusion tests indicated no decrease in the number of viable cells after 'starvation'.

5.3 Error Analysis

The statistical significance of these data can be demonstrated in two ways. As discussed previously, when cells are perfused with Med 199 for periods of up to 18 hours, the standard deviation in the total phosphate intensity is 5%. Any change greater than 5% which occurs after perfusion with glucose-free medium has begun is of experimental interest. A less empirical measure of significance can also be used. We expect, a priori, that an increase in the amplitude of the inorganic phosphate resonance results from a hydrolysis of ATP. A positive signal in the difference spectrum at the chemical shift of inorganic phosphate should be compensated by three negative peaks, each one third the size of the positive signal, and having the widths of the α , β , and γ ATP signals. It is conceivable that while the positive P_i signal would be clearly visible, the three negative ATP signals would disappear into the noise. Figure 7 is constructed by subtracting a spectrum obtained at 1 mg/ml glucose from a spectrum obtained at 0 mg/ml glucose. In Figure 7b three simulated ATP signals are shown, each one third of the total increase in P_i intensity. In Figure 7d these simulated ATP signals are added to 7a. The simulated ATP signals have a S/N of 4:1 and are clearly visible. We conclude that if the increase in the P_i resonance had been compensated by a decrease in ATP, negative ATP peaks would have been clearly resolvable in the difference spectrum. Since they were not we conclude that the

increase in P_i intensity has some other origin.

5.4 Effect of Increase in Glucose on Embryonic Cells

As discussed in Chapter 2, when the concentration of glucose which is delivered to cells is increased from 0 mg/ml to 1 mg/ml the steady state level of ATP increases, and the steady state level of inorganic phosphate decreases. This is expected, as the cells take up more glucose the rate of glycolysis increases, and the inorganic phosphate pool is used to phosphorylate ADP. But glucose concentrations higher than 1 mg/ml did not greatly increase the steady state concentration of ATP.

Figure 8a is a spectrum of primary CEFs perfused with medium 199 which contained 1 mg/ml glucose. The glucose concentration was increased to 40 mg/ml for six hours, and spectrum 8b was acquired. As shown in the difference spectrum (b-a) there was no significant change in the ATP level. The inorganic phosphate signal broadened and shifted upfield, indicating that the cytoplasmic pH had decreased. At the same time an increased concentration of lactic acid was found in the eluent from the cell pellet. The rate of glycolysis increased, although the steady state level of ATP remained constant. The same result was obtained when glucose concentration in the perfusate was increased to either 10 mg/ml or 20 mg/ml.

These very high glucose concentrations were used in order to insure that the cells in the dense pellet had access to as much glucose as do cells in culture. At 40 mg/ml glucose, there is a

significant change in the osmolarity of the cell pellet; the concentration of glucose in the cell pellet was measured to be 150 mM. As a result it is expected that the volume of the cells would decrease by some 30%. However the osmotic stress did not damage the cells. After eight hours of perfusion with 40 mg/ml glucose the only change in the ^{31}P spectra was the shift of the P_i resonance. Trypan Blue exclusion tests indicated that over 90% of the cells were viable.

In addition, osmotic stress induced in other ways caused no change in the total phosphate intensity in the NMR spectra. The two spectra shown in Figure 9 were obtained before and 4 hours after the addition of 40 mg/ml fructose to the perfusate. The perfusate also contained 1 mg/ml glucose. The fructose appeared to induce a decrease in the steady state level of ATP and an increase in ADP. In spectrum b there is very little intensity at the βATP position, and the amplitude of the P_i resonance is increased slightly. However there was no change in total phosphate intensity, or in the intensity of the αATP and phosphate monoester signals. Trypan blue exclusion tests indicated that over 90% of the cells were intact.

When cultured cells were perfused with Medium 199 which did not contain phosphate, and the concentration of glucose in the perfusate was increased from 1 mg/ml to 40 mg/ml there was a change in pH from 6.8 at 1 mg/ml to 6.6 at 40 mg/ml, but no change in PE, PC, or ATP levels (see Figure 10). This is very similar to the result obtained for primary cells, and very different from the result obtained in cultured cells, which is described below. On the basis of only one experiment it would be imprudent to speculate on the role of

extracellular phosphate in the regulation of glycolysis. But clearly this result indicates that the osmotic gradient did not affect the spectral parameters which are of interest here. The cells were stable for at least 18 hours during perfusion with phosphate-free medium which contained 40 mg/ml glucose.

5.5 Effect of Increased Glucose on Normal and Transformed Cells

The amount of phosphorous detectable by NMR in cultured cells decreased as the concentration of glucose in the perfusate was increased. This effect was particularly evident in transformed cells. Figure 11 is the spectrum of transformed cells which were equilibrated with Medium 199 containing 1 mg/ml glucose. The concentration of glucose in the perfusate was then increased to 40 mgs/ml and spectrum b was acquired. The difference spectrum, 11c, shows that there were significant losses of intensity in the inorganic phosphate resonance, and the α and YATP resonances. Although most of the PME region was unaffected, there was a loss of intensity at about -4.0 ppm. The loss ATP was transient; ATP levels returned to normal after two hours. However, the P_i intensity was not recovered.

A similar effect was seen when the concentration of glucose in the perfusate was increased from 1 mg/ml to 10 mgs/ml. Figure 12 shows spectra of transformed cells at 1 mg/ml and 10 mg/ml glucose, and the difference spectrum. There was a decrease in the amplitude of the P_i resonance and small decreases in the amplitudes of the ATP resonances. In this case there was no change in the phosphate

monoester region. Again, the ATP resonances returned to their original amplitudes after about two hours.

The same effect was seen in normal cells. Figure 13 shows the difference between spectra taken before and after the glucose concentration in the perfusate was increased from 1 mg/ml to 10 mg/ml. The decrease in the amplitude of the inorganic phosphate resonance was not offset by the increase in the amount of ATP which is detected. In order to improve the signal to noise ratio for the ATP resonances, data was collected using a 45° pulse, and a 0.5 second delay between acquisitions. The four resonances in the difference spectrum were integrated, and corrections based on the T_1 of the P_i and the three ATP phosphates were made according to the formula given in the Appendix. The measured T_1 for P_i is approximately 3.2 seconds, and the measured T_1 for ATP is approximately 0.5 seconds. However, in order to make the difference between the corrected amplitudes of the positive ATP signals and the negative P_i signals as small as possible, T_1 s of 2.0 seconds for P_i and 0.7 seconds for ATP were used. With these substitutions we find that there is a 14% decrease in the amount of phosphate which is detected. This of course is a conservative estimate.

Figure 14 shows a spectrum of normal CEFs taken as the cells were perfused with 1 mg/ml glucose (A), and a spectrum taken during perfusion with 40 mg/ml glucose (B) (these are relaxed spectra). There was a decrease in the intensity of the P_i resonance, but no change in the ATP, PE, or PC signals. Thus there was a significant (approximately 34%) decrease in the concentration of NMR-detectable

phosphates, due primarily to a loss of P_i .

In three experiments, we found that an increase in the concentration of glucose in the perfusate from 1 mg/ml to 40 mg/ml caused significant decreases in the amount of phosphate which was detected. In four experiments, an increase in the concentration of glucose in the perfusate from 1 mg/ml to 10 mg/ml or from 0 mg/ml to 10 mg/ml caused smaller, but still significant decreases in the pool of detectable phosphate. In all cases there was a large decrease in the amplitude of the inorganic phosphate resonance which was not compensated by an increase in the steady-state level of ATP. The intensity in the phosphate monoester region remained constant. In two of the experiments a transient decrease in the amplitudes of the ATP resonances was observed. In all cases, an increase in the rate of glycolysis was indicated by an increase in the concentration of lactic acid in the eluent, and a decrease in the cytoplasmic pH.

5.6 Discussion of NMR Data

The data which are discussed above are summarized in Table 1, and the data from 5 experiments are shown in figures 15a - 15d. The total phosphate intensity and the ATP intensity at various glucose concentrations are plotted. In all cases the total phosphate intensity detected could be modulated by changes in the concentration of glucose in the perfusate.

There are several reasonable explanations for the changes in the NMR - detectable pool of phosphates described here. One

TABLE 1

MODULATION OF NMR-DETECTABLE PHOSPHATE:
SUMMARY OF RESULTS

EXPERIMENT IDENTIFICATION	MAXIMUM DECREASE IN TOTAL PHOSPHATE	CHANGE IN [GLUCOSE] (MG/ML)
A) CH111 (NORMAL CELLS)	25%	1 mg/ml to 40
B) CH812 (NORMAL CELLS)	30%	1 to 40
C) CH92 (NORMAL CELLS)	18%	1 to 10
D) CTR84 (TRANSFORMED CELLS)	34%	0 to 40
E) CTR69 (TRANSFORMED CELLS)	29%	1 to 10
F) CH35 (NORMAL CELLS)	14%	0 to 10
G) CH317 (NORMAL CELLS)	13%	0 TO 10

MAXIMUM DECREASE IN TOTAL PHOSPHATE DETECTED WHEN THE CONCENTRATION
OF GLUCOSE IN THE MEDIUM IS INCREASED

EXPERIMENT IDENTIFICATION	MAXIMUM INCREASE IN TOTAL PHOSPHATE	CHANGE IN [GLUCOSE] (MG/ML)
A) CH113 (NORMAL CELLS)	14%	1 mg/ml to 0
B) CH35 (NORMAL CELLS)	80%	1 to 0
C) CTR84 (TRANSFORMED CELLS)	18%	1 to 0

MAXIMUM INCREASE IN TOTAL PHOSPHATE DETECTED WHEN THE CONCENTRATION
OF GLUCOSE IN THE MEDIUM IS DECREASED*

* ERROR IN THE DETERMINATION OF TOTAL PHOSPHATE IS 5%

possibility is that at high glucose concentrations, intensity which was lost in the P_i resonance was transferred to signals from some seven or eight phosphorylated glycolytic intermediates. As discussed earlier in this chapter, the concentrations of these intermediates are known to increase significantly as the rate of glycolysis increases. In addition, one might expect an increase in the steady state level of glucose-1-phosphate, an intermediate in glycogen formation. If the chemical shifts of these intermediates were different enough, it is probable that the seven or eight separate signals would have very low signal to noise, and would not be detected. The chemical shifts of these intermediates are given by Gadian (Gadian, 1979). At pH 6.8, they all fall within a 2 ppm bandwidth, with the exception of phosphoenolpyruvate. Their frequency distribution is narrow relative to their linewidths, which would be on the order of 0.5 ppm. In fact, the concentration of glucose-6-phosphate is at least 10 times higher than any other glycolytic intermediate, so that most of the phosphate in the glycolytic pathway should appear in one peak at 4.4 ppm. Thus an increase in the cytosolic concentrations of all of the glycolytic intermediates would lead to a detectable increase in intensity in the sugar phosphate region. Instead, intensity between -5 ppm and -3 ppm remains constant. Consequently it is not likely that the missing phosphate can be accounted for by changes in the concentrations of cytosolic glycolytic intermediates (i.e intermediates which are NMR detectable).

We are left with two alternatives; either there is an exchange of phosphates between the intracellular space and the extracellular

space, or an exchange between NMR-visible and NMR-invisible pools inside the cell. In order to distinguish between these two possibilities we studied the flow of ^{32}P labeled inorganic phosphate across the cell membrane as a function of extracellular glucose concentration.

5.7 Uptake of ^{32}P Inorganic Phosphate

Medium 199 containing 1.25 nanoCuries of ^{32}P labeled inorganic phosphate per ml was added to secondary CEFs which had been confluent for 24 hours. The medium 199 contained either no glucose, 1 mg/ml glucose, 10 mg/ml glucose, or 40 mg/ml glucose. At two minutes, seven minutes, fifteen minutes, thirty minutes, and sixty minutes, the 'hot' medium was removed and the cells were washed twice with cold Hank's buffer and then dissolved in 0.1% SDS. The counts in the cell extracts are shown in Table 2 and in graphic form in Figure 16. At the early time points there were no striking correlations between the amount of glucose in the medium and the amount of label taken up, although cells at higher glucose concentrations tended to take up slightly more label. At the 60 minute time point however, the amount of label in the cells increased monotonically as a function of the amount of glucose in the medium. Almost twice as much label was taken up at 40 mg/ml as at 0 mg/ml.

The rate of leakage of P_i from the cells was studied as a function of the amount of glucose in the medium. Cells were equilibrated in Medium 199 which contained 1.25 nanocuries/ml for 4

TABLE 2

UPTAKE OF LABELED ^{32}P

CPM (average of 3 measurements)

TIME (min)	0 mg/ml	1 mg/ml	10 mg/ml	40 mg/ml
2	540±250	2100±500	2700±500	3100±500
7	2360±100	1940±100	2280±500	2410±50
15	4700±500	5270±150	4730±50	6110±700
30	13000±200	12940±200	11280±800	12350±100
60	17720±2000	21450±200	24960±3000	31100±3000

hours. The 'hot' medium was washed off of the cells and 'cold' medium containing 0, 1, 10, or 40 mg/ml glucose was added. At the various times shown in Tables 3a and 3b an aliquot of medium was collected, the cells were washed with cold Hank's buffer, and extracted with 0.1% SDS. The label which had leaked (or been transported) from the cells into the medium was counted, as well as the amount of label which remained in the cells. As shown in the Tables 3a and 3b, the amount of glucose in the medium had no significant effect on the rate of leakage of label, although at 30 minutes, and 60 minutes, cells at the higher glucose concentrations may have retained slightly more label than cells in glucose-free medium. This result is in qualitative agreement with the work of Jullien et al (Jullien, 1984). These authors showed that cells in medium with approximately 2 mg/ml glucose take up 30% to 50% more $^{32}\text{P}_i$ than cells in glucose-free medium over a 30 minute period.

5.8 Discussion

As the amount of glucose in the medium is increased cells on dishes take up more label, and the amount of label they retain remains constant or declines slightly. The amount of phosphate in the cells is increasing significantly. We assume that cells in suspension behave in a similar manner. While NMR experiments detect a decrease in the concentration of cytosolic phosphates, the total concentration of phosphates inside the cells is increasing. We do not know at this

TABLE 3A
LEAKAGE OF ^{32}P INTO MEDIUM

Time (min)	CPM (average of 2 measurements)			
	0 mg/ml	1 mg/ml	10 mg/ml	40 mg/ml
5		2450±20	1720±20	1140±100
15	5290±500	4280±400	4670±100	4490±300
30	5310±100	5710±1000	3640±400	5270±600
60	14250±2000	12460±1200	13380±1200	13450±50

TABLE 3B
AMOUNT OF ^{32}P REMAINING IN CELLS

Time (min)	0 mg/ml	1 mg/ml	10 mg/ml	40 mg/ml
0	73000±3000	" "	" "	" "
5	?	49340±4000	44360±2000	?
15	58470±10000	47000±4000	55170±5000	51100±1100
30	?	41010±3000	38370±4200	46430±1000
60	33520±8000	36270±2000	36380±5000	37060±2000

point where the excess phosphate is stored, but we do know that it is not in small molecules which move freely in the cytoplasm.

Since we know the specific activity of $^{32}\text{P}_i$ in the medium, we are able to calculate that when the concentration of glucose is 1 mg/ml approximately 80 nanomoles P_i /hour·mg protein enter the cells. Let us assume that the cells are in a steady state under these conditions, so that the leakage rate is also equal to 80 nanomoles/mg protein. At 10 mg/ml glucose, the rate of uptake of phosphate is 15% higher than at 1 mg/ml glucose, while the rate of leakage remains the same. As a result, the cells accumulate phosphate (probably in a variety of forms) at a rate of 12 nanomoles/mg protein·hour. At 40 mg/ml glucose, the cells accumulate 70 nanomoles/mg protein·hour. The sum of all phosphates detectable by NMR is 80 nanomoles/mg protein. Thus if the phosphate taken up by the cells at high glucose concentrations went into the cytosolic, NMR detectable pools, the intensity of the resonances would have nearly doubled every hour. Since this was not observed, it appears that phosphate taken up from the medium entered relatively immobile pools.

If extracellular P_i which is taken up by the cell enters bound pools relatively rapidly, then a reasonable hypothesis is that free intracellular phosphate undergoes a similar exchange. The results described here indicate that such an exchange does take place, and that it is modulated by the rate of glycolysis.

5.9 Conclusions and Interpretation

We have presented evidence that there is compartmentation of phosphates in CEFs. The amplitude of the inorganic phosphate resonance decreased at high rates of glycolysis, and increased at low rates of glycolysis. There were no corresponding changes in the amplitudes of the other resonances. In addition, we have shown that significant amounts of inorganic phosphate are taken up by the cells when there is a high concentration of glucose in the medium, but that this increase in the level of intracellular phosphate is not detectable by NMR. Our explanation of these results is that there is exchange between bound intracellular pools of phosphates and the intracellular pool of cytosolic, inorganic phosphate. When glycolysis is stimulated by an increase in the concentration of glucose in the medium, cytosolic inorganic phosphate enters the bound pool. It may be used to phosphorylate bound pools of glycolytic intermediates, or it may remain as inorganic phosphate. When the rate of glycolysis slows down or stops, because the medium contains no glucose, the size of the bound pool decreases, and inorganic phosphate is returned to the cytosol.

As the rate of glycolysis increases, the sizes of pools of phosphorylated glycolytic intermediates increase significantly. Bissell et al (Bissell, 1973) have shown that when glucose concentration in the medium increases from 0.55 mM to 5.5 mM the concentration of fructose diphosphate increases by a factor of five. If there is a similar increase under our experimental conditions the amount of phosphate involved in glycolysis becomes significant. In addition the rate at which inorganic phosphate is turned over by the

various glycolytic enzymes increases. It is in part to supply this demand that there is an increase in the rate of uptake of inorganic phosphate. The resonances from these sugar phosphates are not detected by NMR and in fact the amount of detectable phosphate decreases. This phosphate must be sequestered in bound form inside the cell.

It is reasonable to suggest that these intermediates are sequestered near glycolytic enzymes so that they can be processed efficiently. One explanation for our results is that the phosphorylated intermediates are bound to the enzymes themselves, but that the enzymes are dissolved in the cytoplasm. The correlation times of these large macromolecules would be so long that resonances from the phosphates would be broadened, and would not be detected. Bloch et al (Bloch, 1970) have shown that in rabbit muscle, a significant fraction of glycolytic intermediates may be bound to enzymes. In particular, these authors find that the concentration of the active sites of GAPDH in rabbit muscle is 1.6 mM. The total concentration of all glycolytic intermediates in rabbit muscle is about 6 mM. Thus this enzyme alone has significant binding capacity relative to the concentration of substrates with which it is in equilibrium. A number of other enzymes, particularly aldolase and tryptose phosphate isomerase are present in a variety of cells and tissues at concentrations above 1 mM (Ottaway, 1977). Levels of glycolytic enzymes are particularly high in white muscle: there are a total of 72 mg/gm of tissue in rabbit muscle, 55 mg/ml in yeast. No similar data is available for CEFs.

It is also possible that the missing phosphate is sequestered on

intracellular surfaces such as mitochondrial membranes, the cytoplasmic membranes, or cytoskeletal proteins. Exchange between these bound pools of inorganic phosphate and phosphate esters and cytoplasmic pools of inorganic phosphate could regulate the rate of glycolysis. When cells were perfused with glucose-free medium, the concentration of free cytosolic phosphate increased before the ATP level decreased significantly. As a result, the free energy for the production of ATP (as determined by cytosolic concentrations of reactants) decreased. Thus ATP was maintained at a higher concentration than it would have been, had the amount of free phosphate remained constant. When the rate of glycolysis increased, we observed the opposite effect. A decrease in the size of the free P_i pool results in an increase in the free energy for phosphorylation of ATP. The 'cytosolic phosphorylation potential' has been proposed by many investigators (Erecinska, 1977; Veech, 1979; Racker, 1983) as an important regulatory factor in glycolysis and respiration.

The bound intracellular phosphates should be soluble in cell extracts. Unfortunately we were unable to achieve a good correlation between the amplitudes of signals in our extracts and the amplitudes in vivo. This was due primarily to the fact that these cells were difficult to obtain in large quantities and we were not able to refine our extraction procedure sufficiently to allow the detection of a 10 to 40% increase in the amplitudes of signals in extracts relative to their amplitudes in vivo. In Figure 2, for example, the amplitude of the inorganic phosphate peak in the extract is larger than the amplitude in vivo, but without a large number of successful extracts

which could be used as controls it is impossible to say that the increase was not due to hydrolysis of phosphate esters during the extraction procedure.

The increase in total phosphate intensity which was observed when the cells were perfused with glucose-free medium was reversible. The total phosphate intensity could be returned to its original level by perfusion with 10 mg/ml or 40 mg/ml glucose. Attempts to recover the phosphate intensity which was lost during perfusion with 10 and 40 mg/ml glucose were only marginally successful. After exposure to high concentrations of glucose for four to six hours the intracellular pH remained low and the free P_i concentration remained low or zero for as long as perfusion could be continued. This may have been due to a build up of glycogen which could be used when the external supply of glucose was removed. However it is of interest that in some of the experiments (see, for example Figures 15a, 15c, and 15d) the total phosphate intensity returned to its original level or began to increase even though the glucose concentration was still high.

5.10 ADP

ADP was seldom detected in our experiments. As discussed in section 4.3, even when glucose was removed from the perfusate and the steady state level of ATP decreased significantly, the concentration of ADP did not reach a level which we could detect. The ADP concentration is kept low due to the activity of adenylate kinase which catalyzes the reaction:



The AMP formed is dephosphorylated and converted to inosine (Veech, 1979; White, 1974) pulling the adenylate kinase reaction strongly towards ATP. As a result, very little ADP is formed, even when detectable ATP disappears completely. Although this was not studied systematically it is interesting to note that ADP was detected in two experiments: one in which cells were perfused with medium which did not contain inorganic phosphate, and one in which cells were perfused with medium containing 40 mg/ml fructose.

5.11 Comparison of Primary and Cultured Cells

In both normal and transformed cells grown in culture the amount of phosphate detectable by NMR was modulated by the rate of glycolysis, but this effect was not seen in normal cells. We have not investigated the reasons for this difference but it may be a consequence of some well defined metabolic and structural changes which occur when cells are grown in culture. It is almost universally true that cells which are grown in culture are more glycolytic than their in vivo counterparts (Bissell, 1981). These changes are sometimes associated with a decrease in the number of mitochondria (Meister, 1983), and increases in the activities of glycolytic enzymes

(Rubin, 1974).

Other differences between primary and cultured cells could cause changes in metabolism. Primary cells and confluent, cultured cells are distributed differently in the cell cycle. A confluent culture consists almost entirely of fully grown cells in the G_0 phase of the cell cycle. In a confluent culture of transformed cells the cells continue to divide, but do so at a greatly reduced rate. Embryonic cells however will be randomly distributed across the cell cycle; this implies that there will be great variability in rates of ATP turnover, and thus in the regulation of glycolysis. In addition, the protein content of CEFs may be greater in cells which are grown in culture. This point will be discussed in the Appendix.

5.12 PH Heterogeneity

Measurements of intracellular pH from ^{31}P chemical shifts can be used to search for compartmentation. It is sometimes inferred, on the basis of chemical shift data, that two different pools of metabolites exist at two different pHs, and thus must be confined to two distinct intracellular compartments. As was discussed in Chapter 3, interpretation of such data is complicated. Changes in the chemical shift of one phosphorylated metabolite which might be attributed to a change in the pH of an intracellular compartment could also be attributed to the selective binding of a metal ion by that metabolite. Although we did not study intracellular pH gradients systematically, some of our data suggest that different intracellular environments can

be detected by NMR at high rates of glycolysis.

Figures 19 and 20 show spectra in which, as the rate of glycolysis increases, a new signal appears in the region between the PME signals and the P_i signal. This new signal does not appear in spectra of extracts, and thus must be due either to inorganic phosphate or one of the phosphate monoesters. It is most simply assigned to an inorganic phosphate resonance from the medium. It would usually be hidden by the much larger signal from cytoplasmic phosphate, but would be resolved when the large signal shifts upfield. According to this hypothesis, a pH gradient of approximately 0.6 units is developed across the cell membrane at high rates of glycolysis. However, there are two problems with this interpretation. First, under these conditions, the pH of the medium in the cell pellet is about 6.2, due to the large amounts of lactic acid which are produced by the cells and picked up by the perfusate. As the cytoplasmic P_i resonance shifts, the resonance from P_i in the perfusate should shift as well, and should resonate much farther downfield than the new signal at 2.7 ppm. In one experiment where the P_i concentration in the medium was increased to 3 mM the external P_i resonance appears as a shoulder which is barely resolvable, approximately 0.2 ppm upfield from the large cytoplasmic P_i resonance. By contrast, the resonance which appears in Spectra 8a and 8b is a full part per million upfield from the cytoplasmic P_i resonance. Thus it is possible that it represents intracellular P_i which is at a higher pH than the bulk of the P_i . Alternatively, it may represent PE or PC which is shifted downfield. As discussed above the chemical shifts of the PMEs do not

respond to changes in cytoplasmic pH, but this signal may represent a small population of PME's which are in an environment which becomes more acidic as the rate of glycolysis increases.

Figure Legends for Chapter 5

Figure 1: Plot of amplitude of α , β , and YATP resonances when glucose is removed from the perfusate.

Figure 2: Effect of starvation on transformed CEFs: A) Cells perfused with 1 mg/ml glucose, B) Cells perfused with glucose-free medium, C) A-B. LB= 30 Hz, TM=6

Figure 3: Effect of starvation on transformed cells. Block averaged spectrum (A) taken during perfusion with 1 mg/ml glucose subtracted from block averaged spectrum taken after 3 - 4 hours of perfusion (B) with glucose-free medium.

Figure 3d: Spectrum obtained after 5 hours of perfusion with glucose-free medium subtracted from 10a.

Figure 4: Effect of starvation on normal cells: the total phosphate intensity (the sum of the integrals of all phosphate signals) is plotted as a function of time and glucose concentration (+). ATP intensity (o) is also plotted. The vertical bars represent the estimated errors in the integrals. Horizontal bars represent time over which data is acquired. A) cells are perfused with 1 mg/ml glucose, B) cells are perfused with glucose-free medium C) cells are perfused with 10 mg/ml glucose.

Figure 5: Effect of starvation on normal cells: A) Spectrum taken while cells are perfused with 1 mg/ml glucose, B) Cells have been perfused with glucose-free medium for four hours, C) B-A. The chemical shifts of the signals are given in the figure in ppm. LB=30 Hz, TM=4

Figure 6: From same experiment as Figure 5: Perfusion of cells with glucose-free medium for 6 hours results in a significant decrease in [ATP].

Figure 7: Figure A shows the change in the NMR spectra of transformed cells which occurs when cells are 'starved'. A spectrum obtained while cells were perfused with medium containing 1 mg/ml glucose is subtracted from a spectrum obtained during perfusion with glucose-free

medium. Figure B is a simulation of 'A', and also shows three simulated ATP signals. These represent the intensity which would be seen if the increase in the amplitude of the P_i signal were accounted for by a decrease in ATP. Figure C shows B-A and demonstrates that the decrease in ATP would have been detectable with a signal to noise ratio of 4:1. Figure D is a simulation of what the difference spectrum would have looked like if the 'missing' phosphate had come from hydrolysis of NMR-visible ATP.

Figure 8: Effect of 40 mg/ml glucose on primary CEFs: A) Cells are perfused with 1 mg/ml glucose, B) Cells are perfused with 40 mg/ml glucose, C) B-A. LB = 40 Hz, TM = 3.

Figure 9: Effect of osmotic gradient: perfusion with 40 mg/ml fructose. A) Cells perfused with 1 mg/ml glucose, B) Cells perfused with 40 mg/ml fructose, C) B-A. LB=40 Hz, TM=6

Figure 10: Effect of 40 mg/ml glucose on normal cells perfused with phosphate-free medium: A) Cells perfused with phosphate-free medium with 1 mg/ml glucose, B) Cells perfused with phosphate free medium containing 40 mg/ml glucose, C) B-A. LB=40 Hz, TM=6

Figure 11: Effect of increased glucose concentration on transformed cells: A) cells are perfused with Medium 199 with 1 mg/ml glucose, B) glucose concentration is increased to 40 mg/ml, C) the difference spectrum, B-A.

Figure 12: Effect of 10 mg/ml glucose on transformed cells: Spectrum A is taken while cells are perfused with Medium 199 containing 1 mg/ml glucose, Spectrum B is obtained after glucose concentration is increased to 10 mg/ml, and C is the difference B-A.

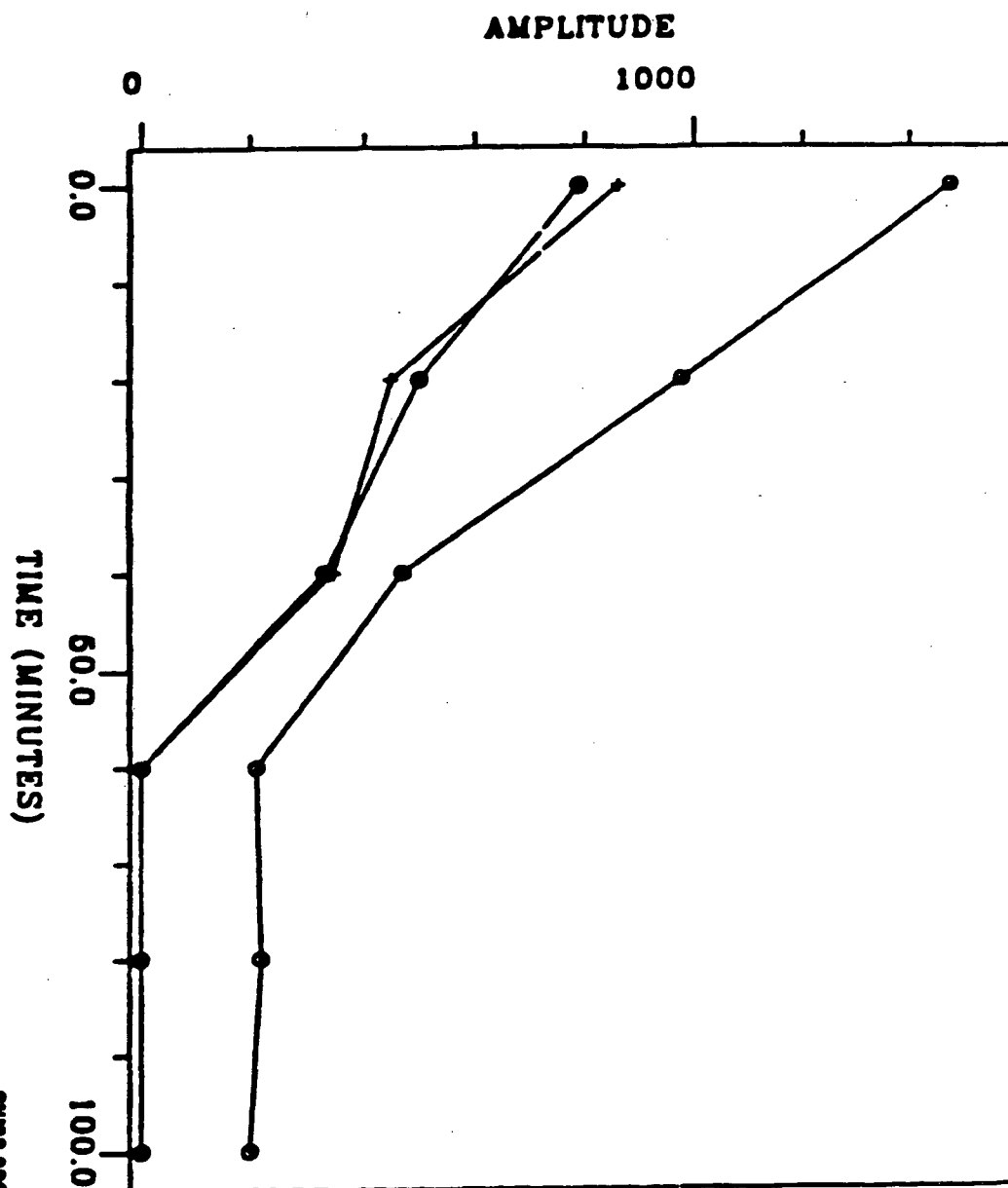
Figure 13: Effect of 10 mg/ml glucose on normal cells; the difference between spectra obtained before and after the glucose concentration in the medium was increased from 1 mg/ml to 10 mg/ml is plotted. The integrals of the signals in the difference spectrum are shown in the table. The spectra are obtained under saturating conditions: 90 degree pulse, 500 msec delay,. The integrals corrected for the effect of saturation are also shown.

Figure 14: Effect of 40 mg/ml glucose on normal cells: A) Cells perfused with medium containing 1 mg/ml glucose, B) Cells perfused with medium containing 40 mg/ml glucose, C) B-A. Spectra are obtained under saturating conditions: 90 degree pulse, 7 sec delay, LB=40 Hz, TM=6.

Figure 15a-d: Plots of total phosphate and ATP at various glucose concentrations: see explanation for Figure 11.

Figure 16: Plots of CPMs taken up by CEFs on dishes. The activity in the cells was measured at 5 different times and 4 different glucose concentrations.

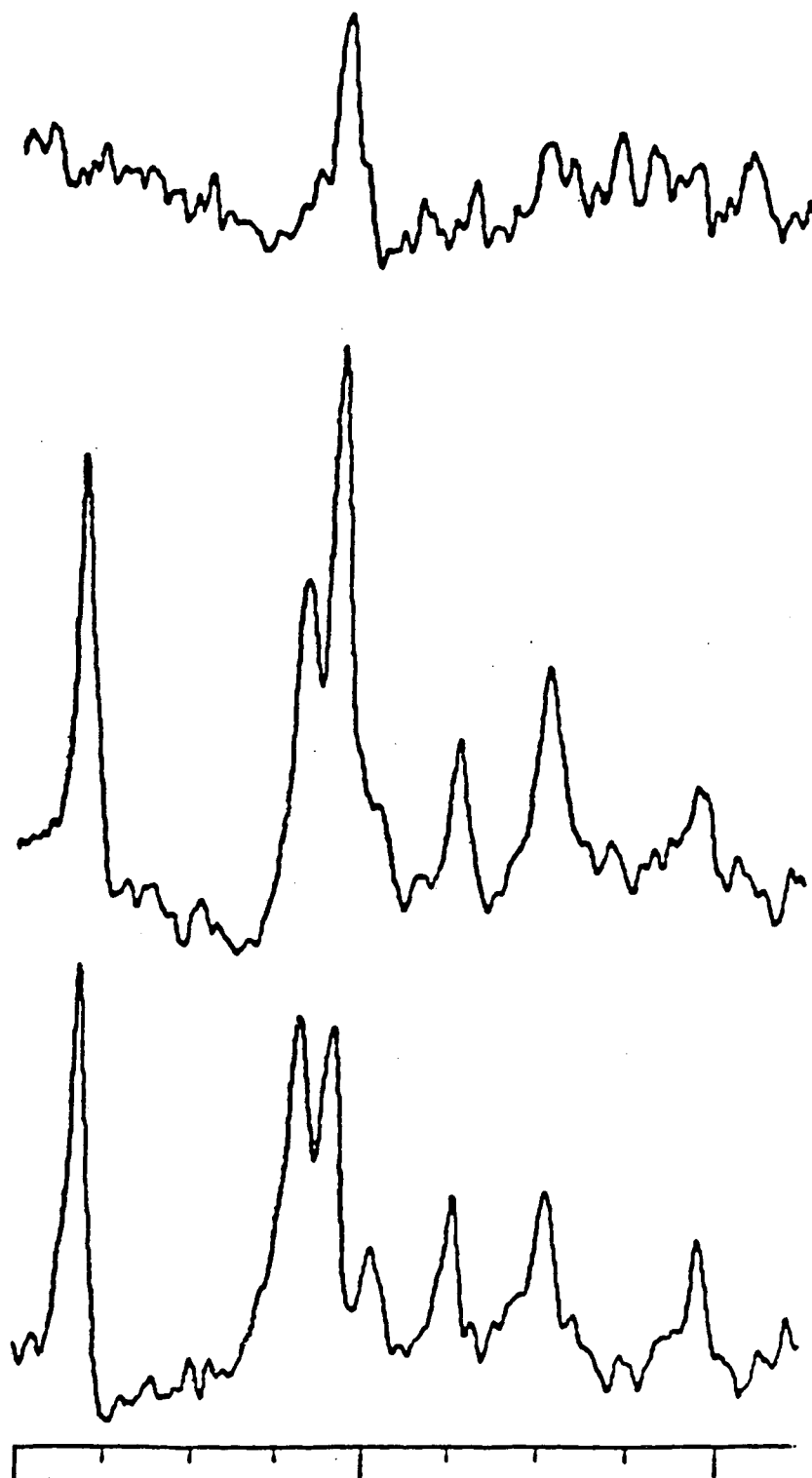
Figures 17, 18: At high rates of glycolysis a new signal is sometimes resolved at approximately 2.7 ppm: A) cells perfused with 1 mg/ml glucose, B) cells perfused with 10 mg/ml glucose. LB=15 Hz, TM=6



XBL 849-3994

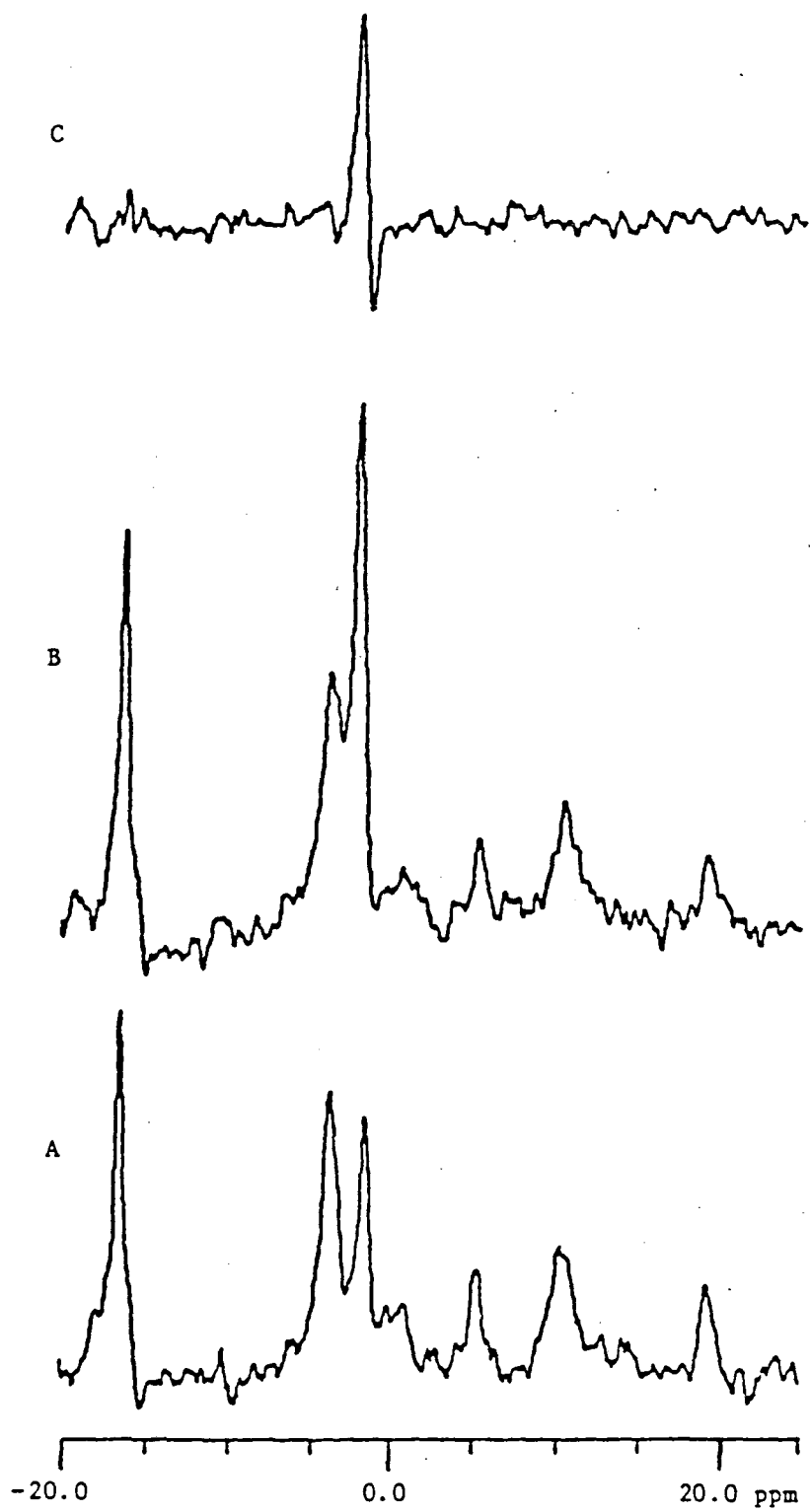
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FIGURE 1



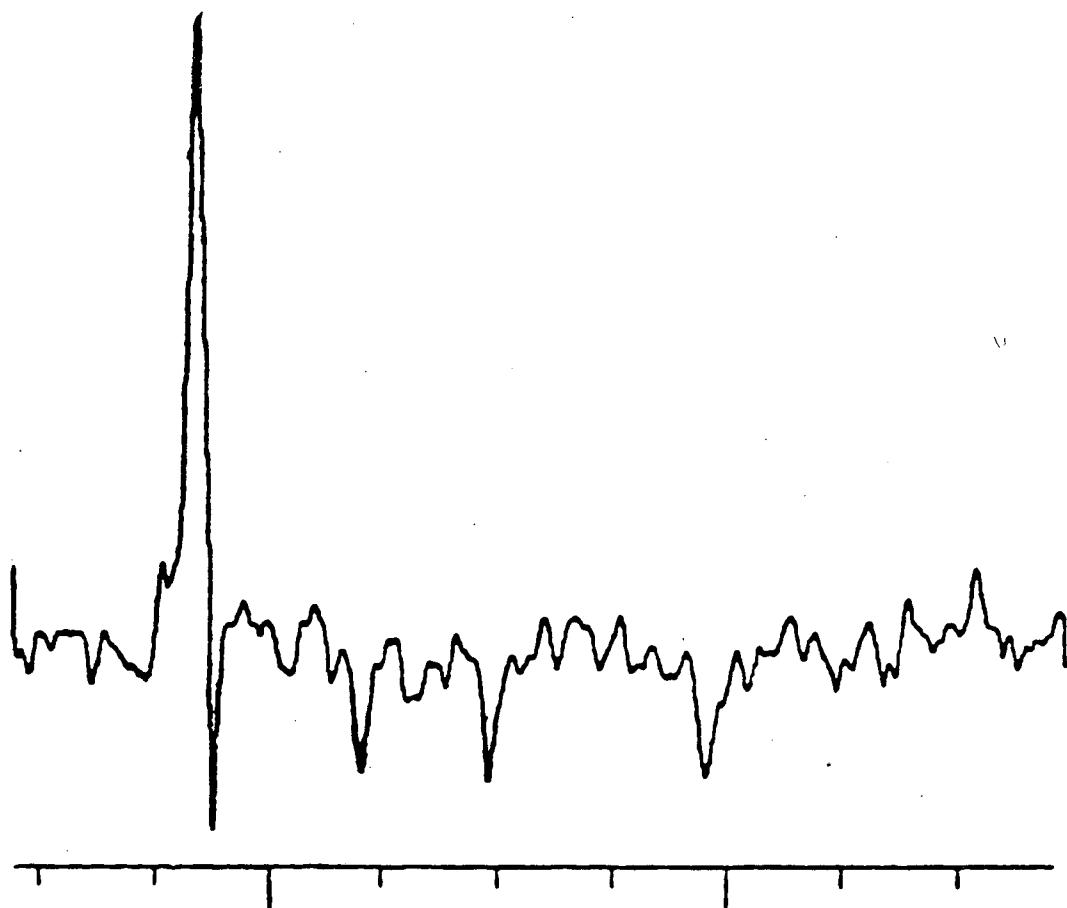
XBL 849-4019

FIGURE 2



XBL 849-4018

FIGURE 3



XBL 849-4021

FIGURE 3D

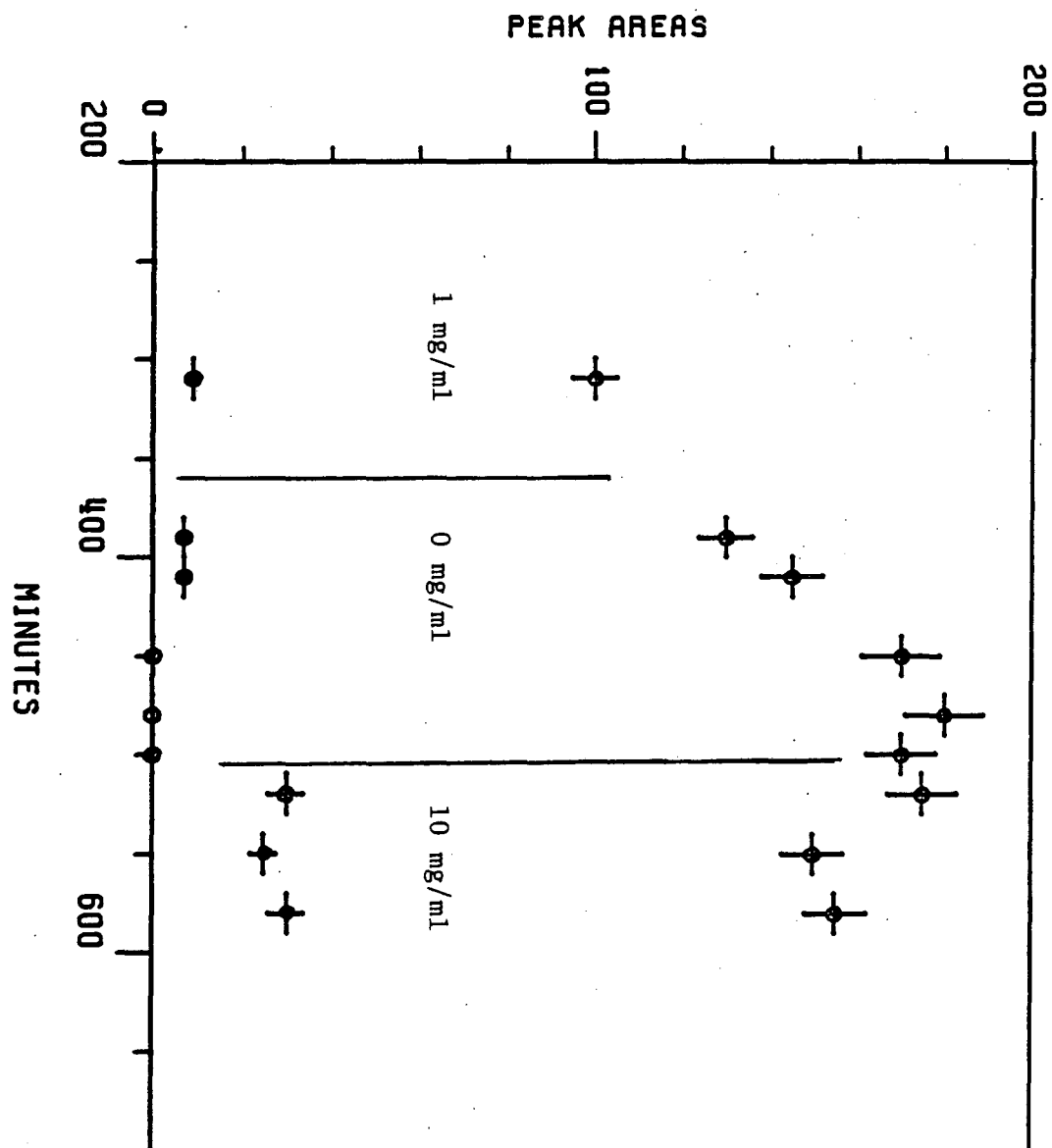
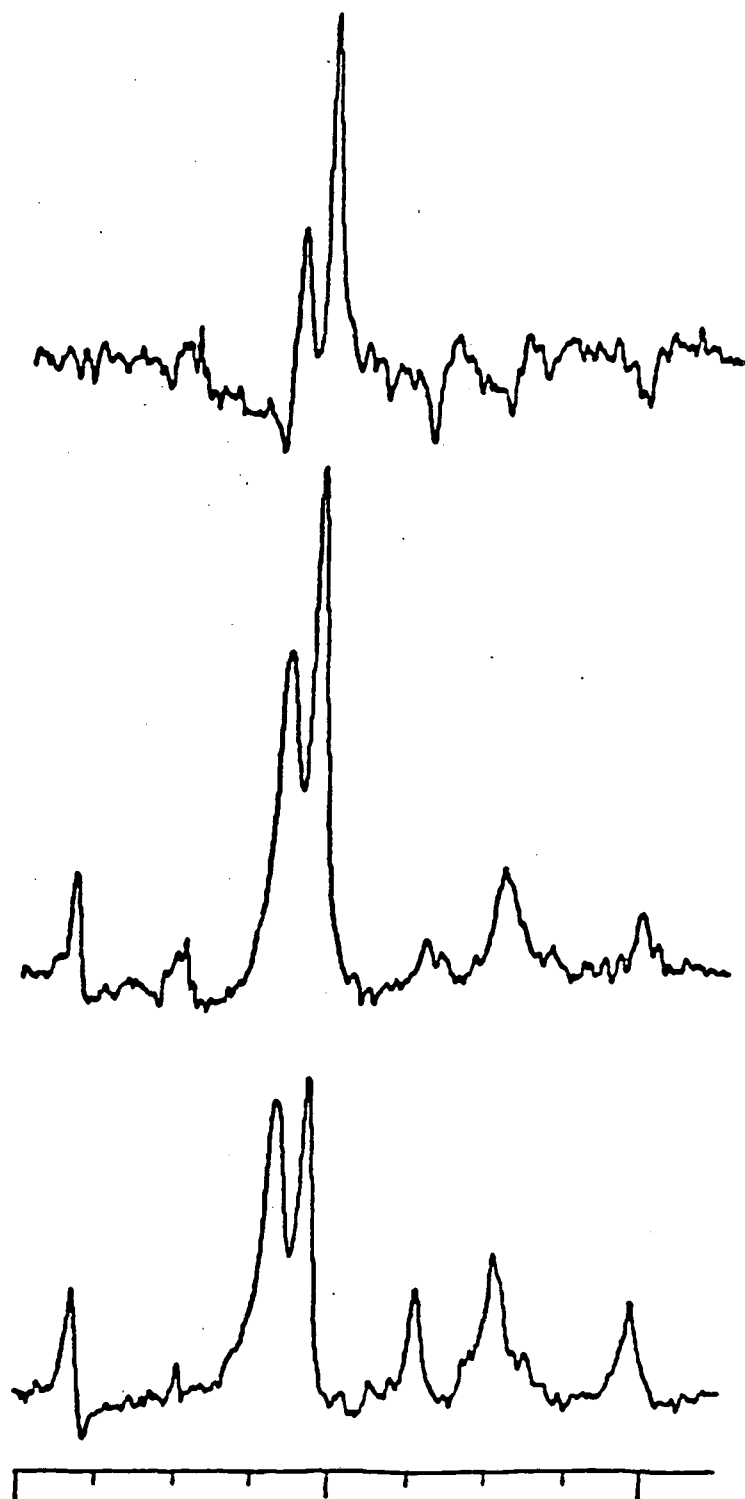
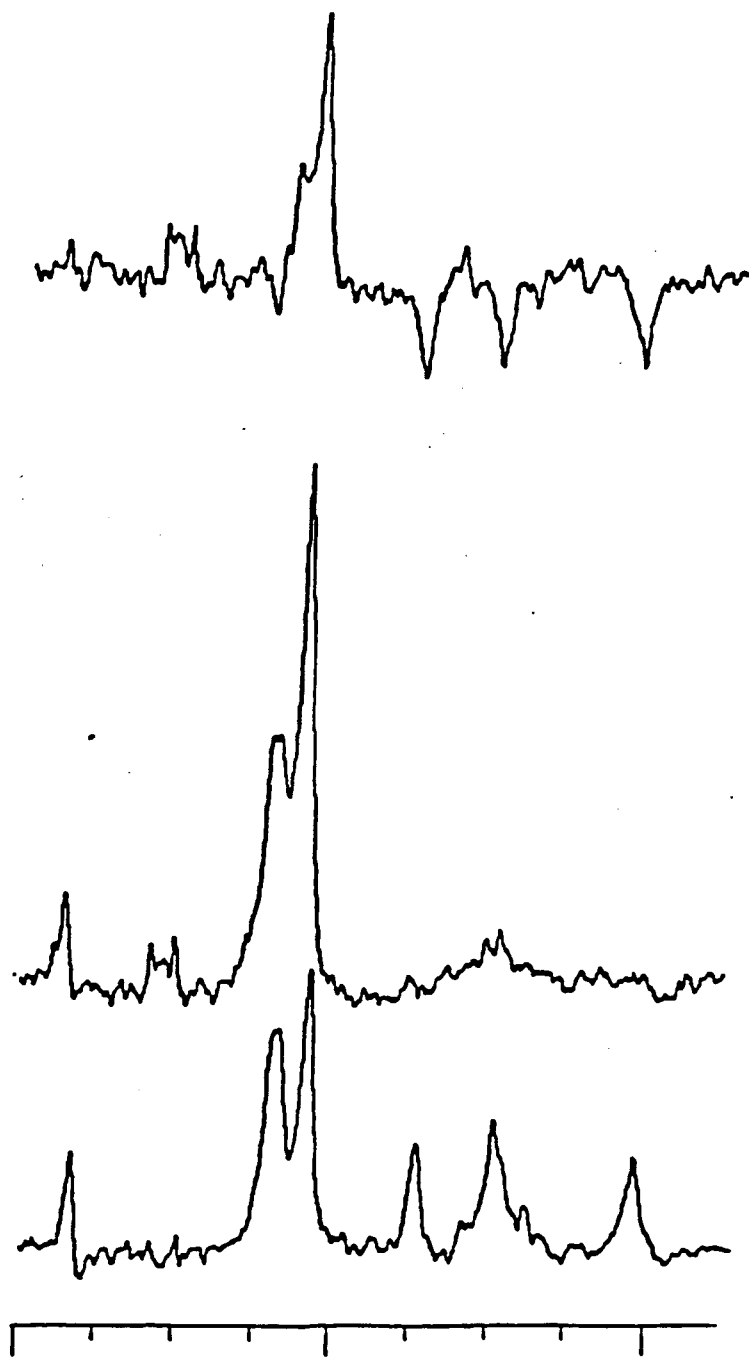


FIGURE 4



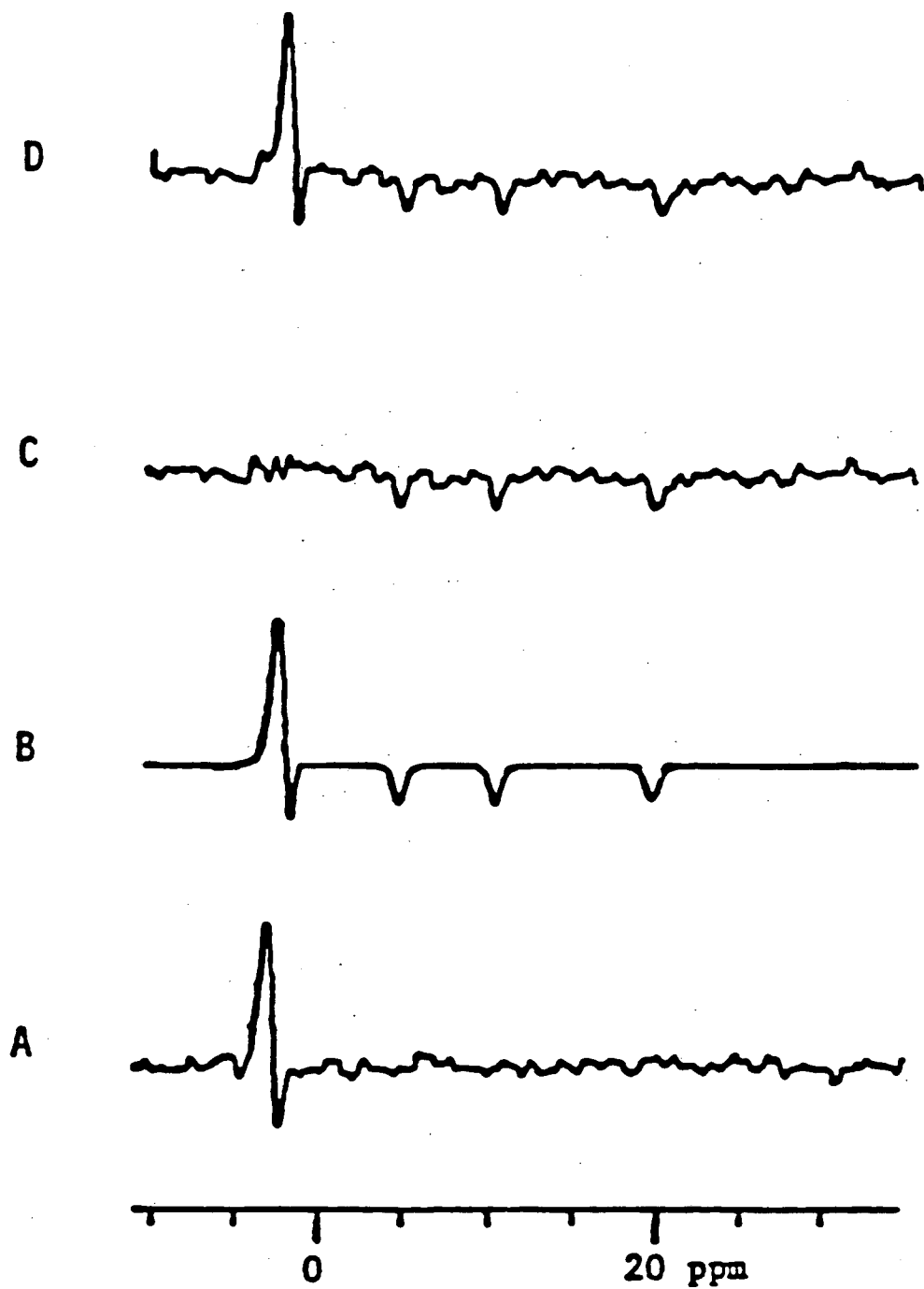
XBL 849-4017

FIGURE 5



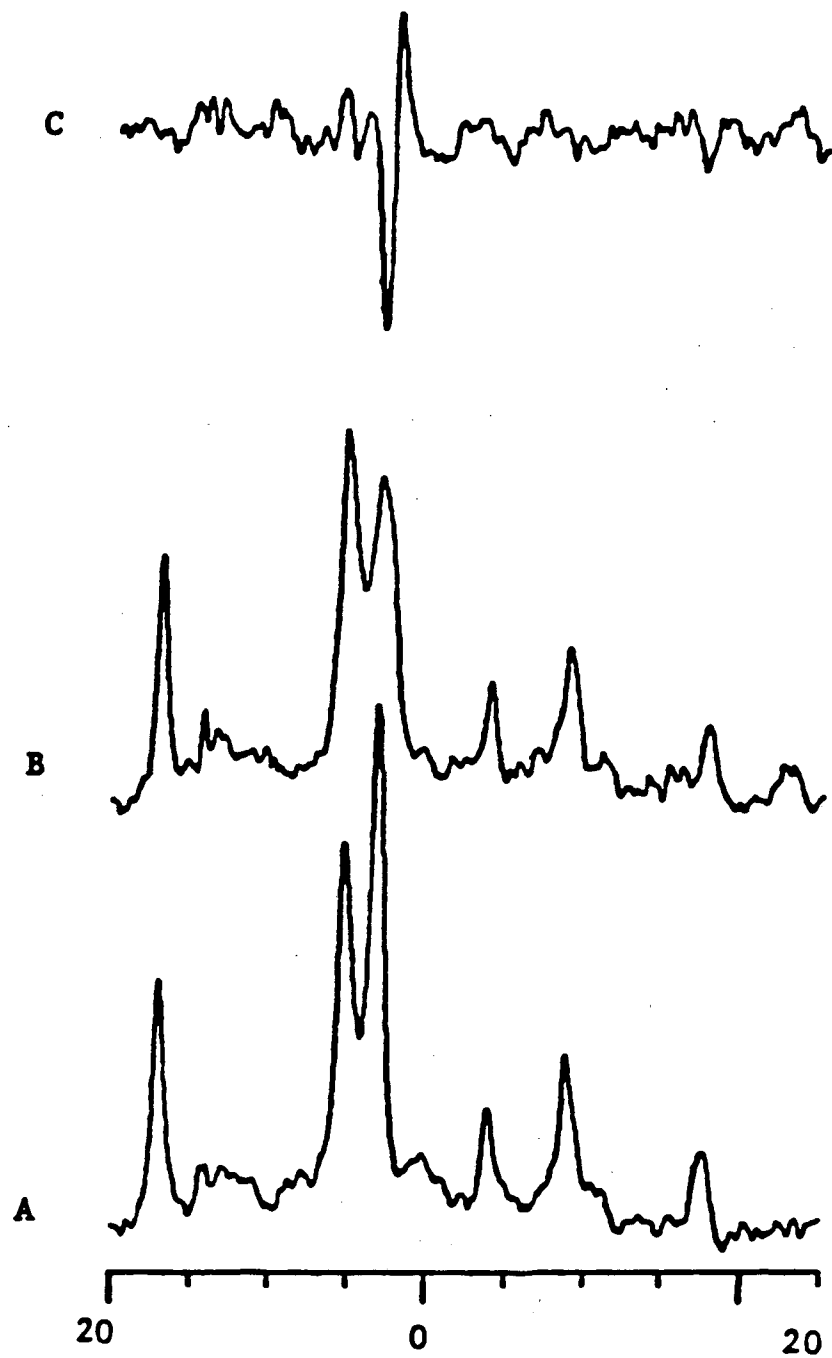
XBL 849-4016

FIGURE 6



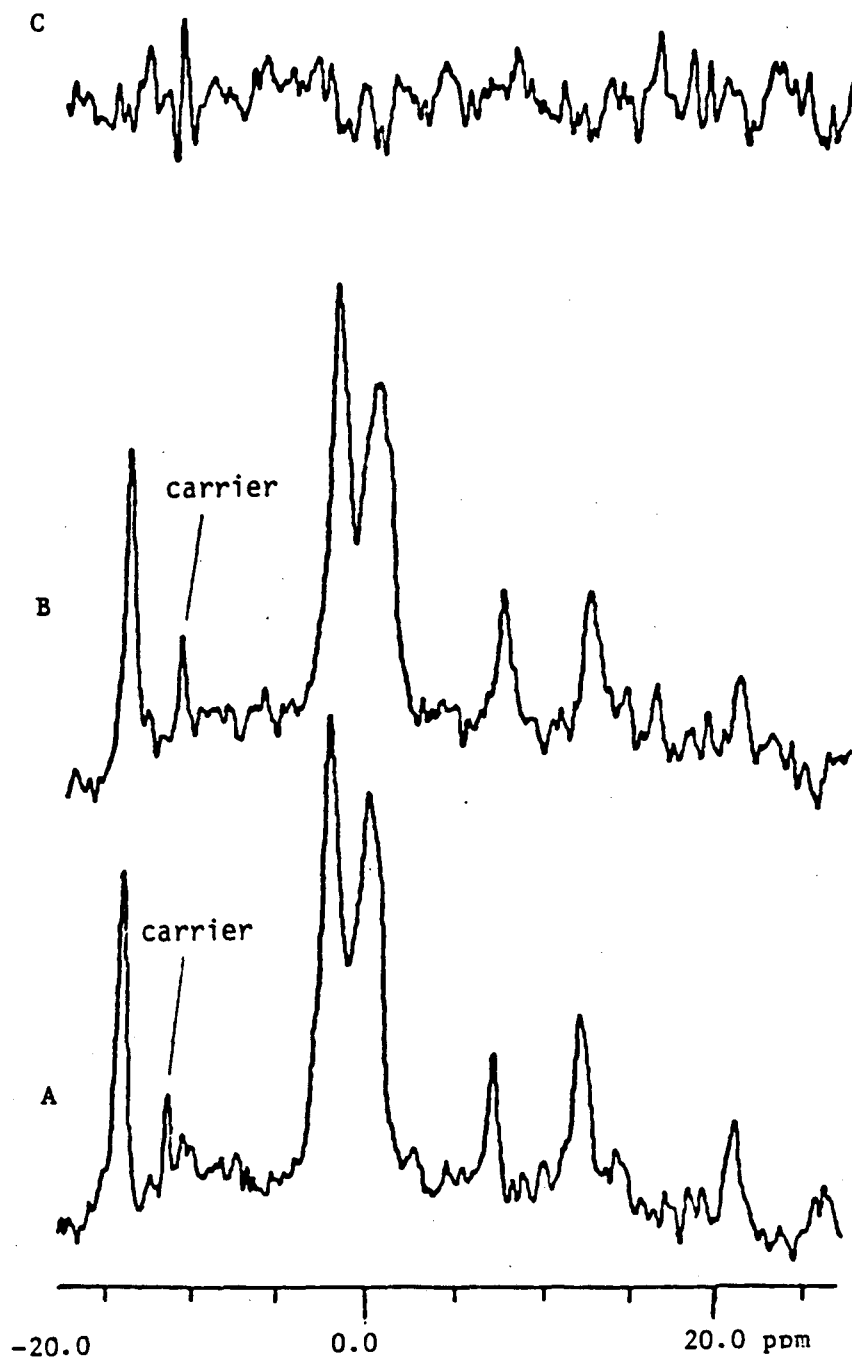
XBL 849-4015

FIGURE 7



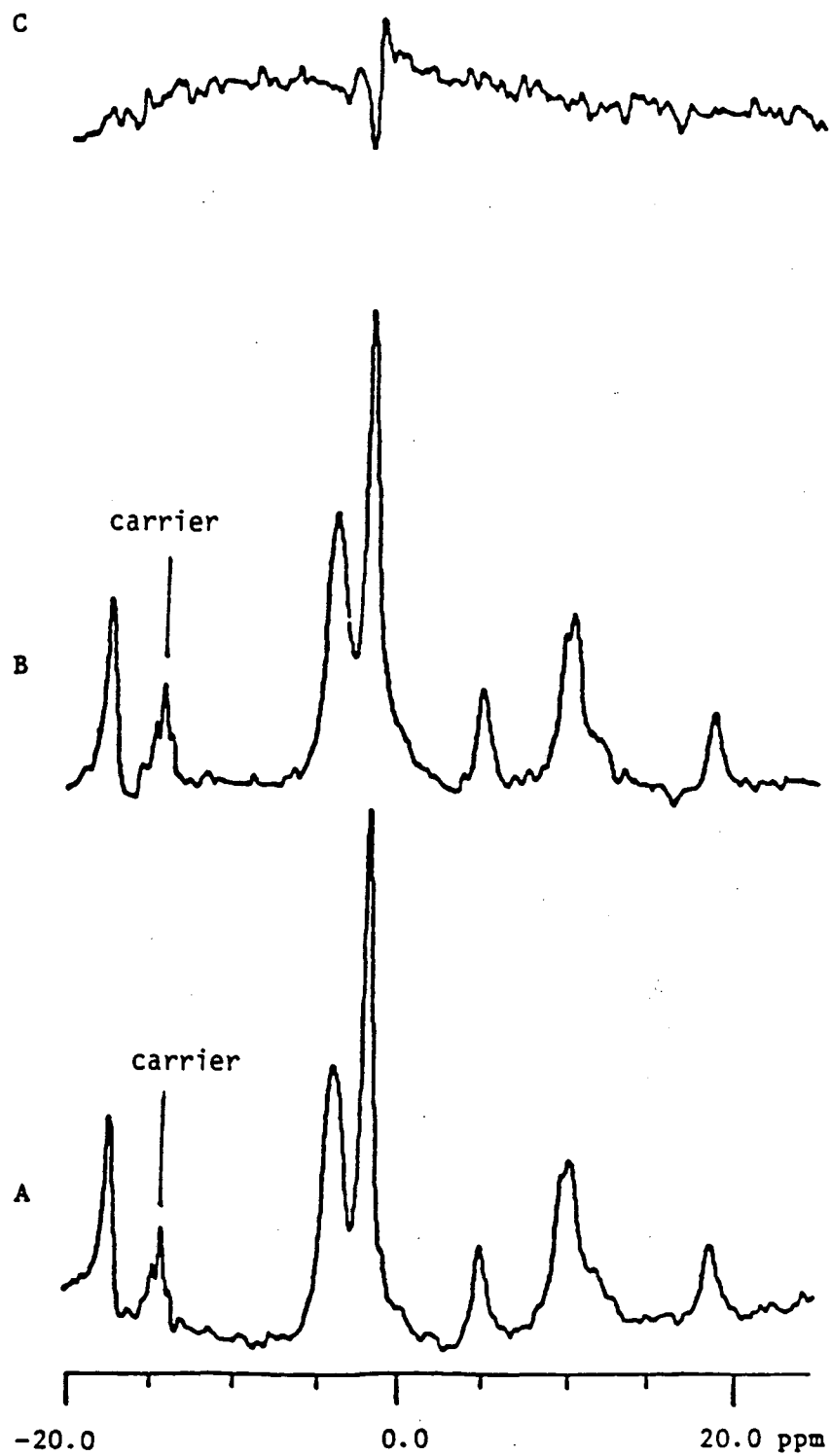
XBL 849-4014

FIGURE 8



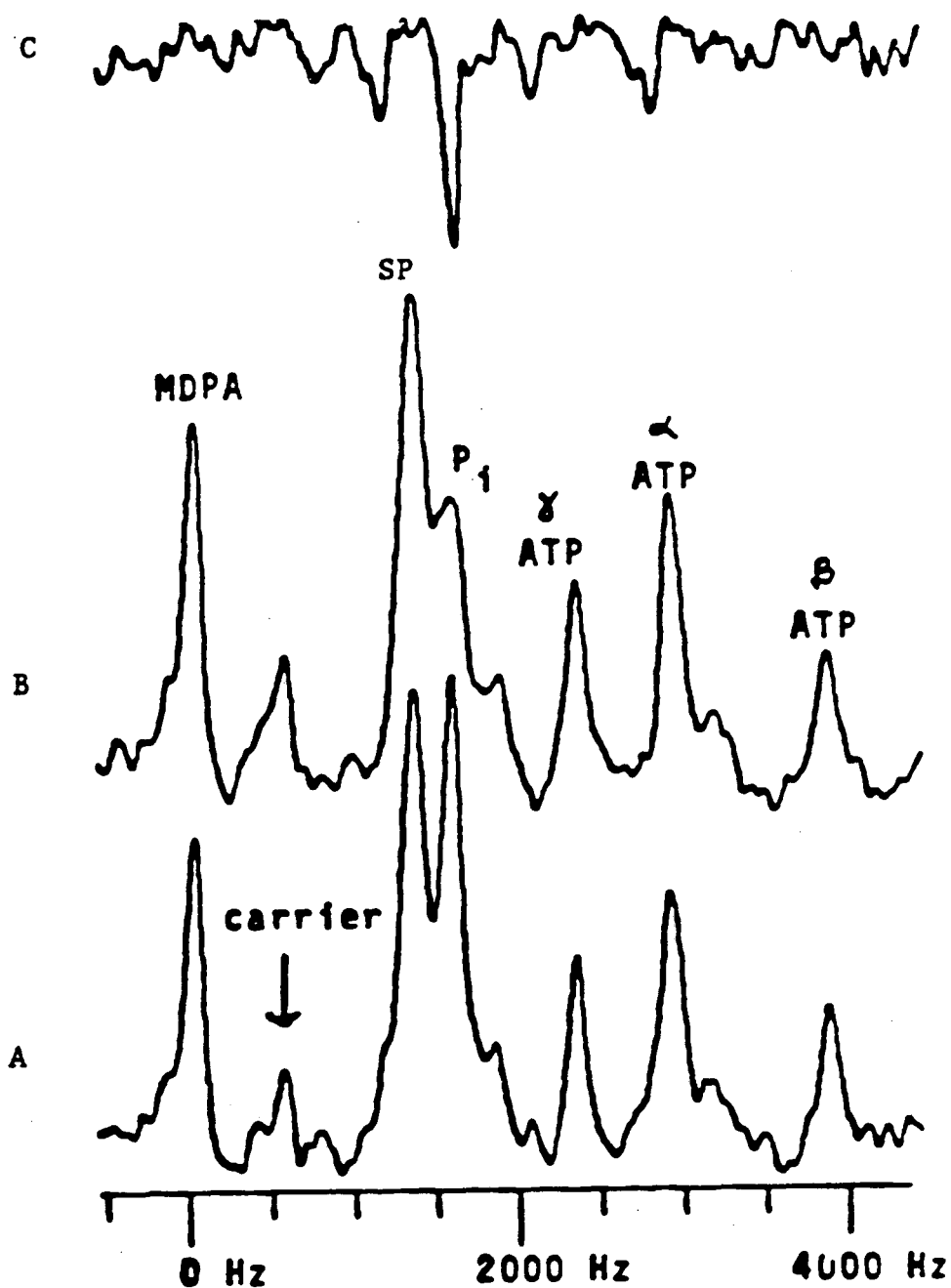
XBL 849-4013

FIGURE 9



XBL 849-4012

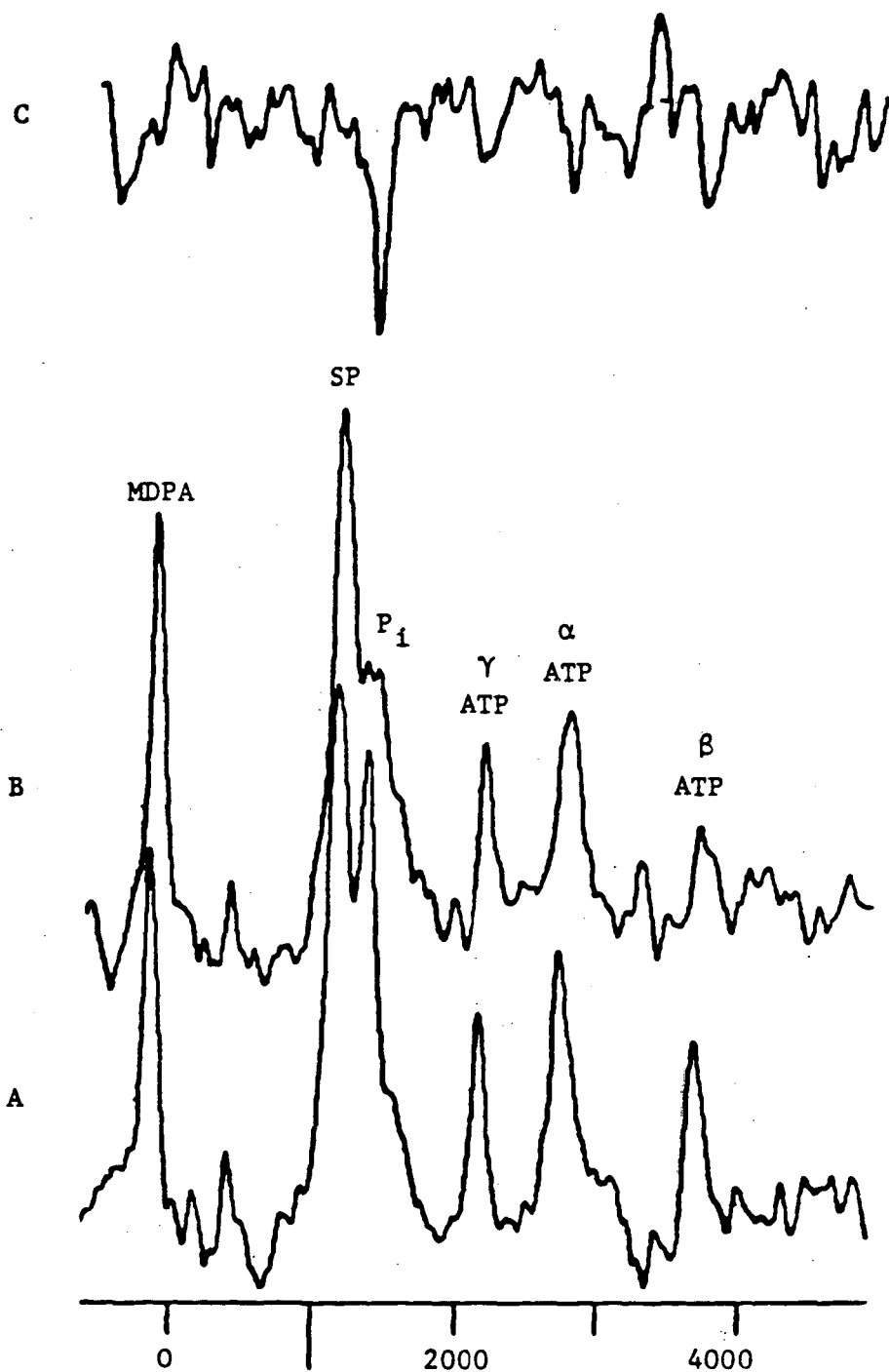
FIGURE 10



Effect of increased glucose concentration on transformed cells: Spectrum A is taken while cells are perfused with Medium 199 with 1 mg/ml glucose, Spectrum B is obtained after glucose concentration is increased to 40 mg/ml, and C is the difference B - A.

XBL 849-4011

FIGURE 11



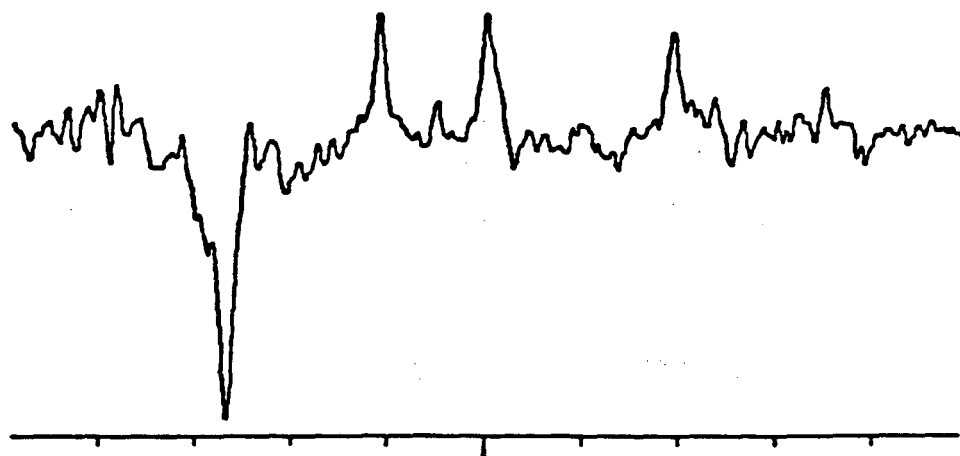
Effect of increased glucose concentration on transformed cells:
 Spectrum A is taken while cells are perfused with Medium 199
 containing 1 mg/ml glucose, Spectrum B is obtained after glucose
 concentration is increased to 10 mg/ml, and C is the difference
 B - A.

XBL 849-4010

FIGURE 12

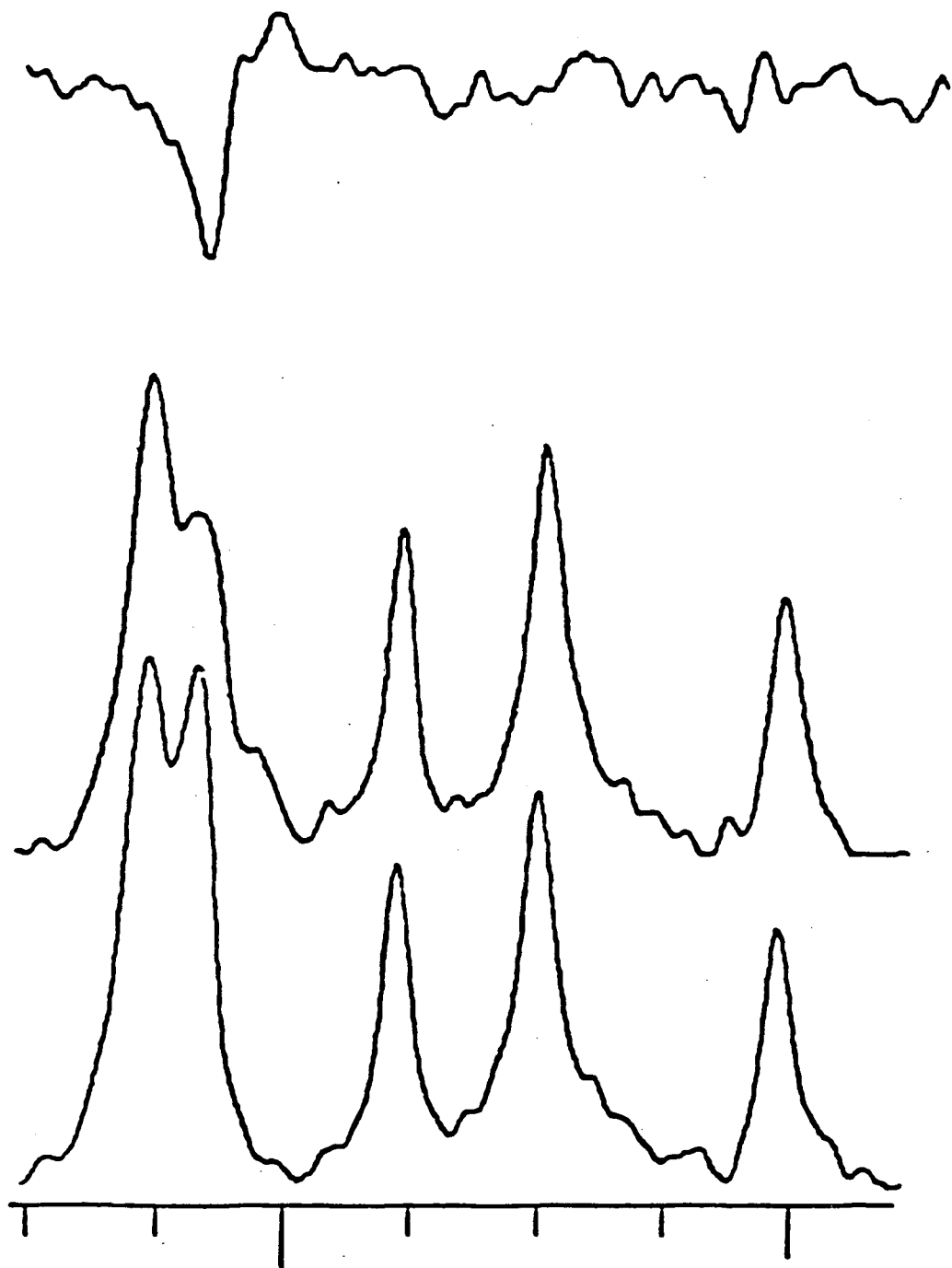
	P_i	α ATP	β ATP	γ ATP
INTEGRAL	100	37	41	32
CORRECTED INTEGRALS	289	67	74	58

INTEGRALS ARE CORRECTED USING T_1 (ATP) = 0.7 sec AND T_1 (P_i) = 2.0 sec. ABOUT 30% OF THE PHOSPHATE LOST FROM THE P_i POOL IS NOT ACCOUNTED FOR BY THE INCREASE IN ATP.



XBL 849-4009

FIGURE 13



XBL 849-4008

FIGURE 14

TOTAL PHOSPHATE AND ATP (CF111)

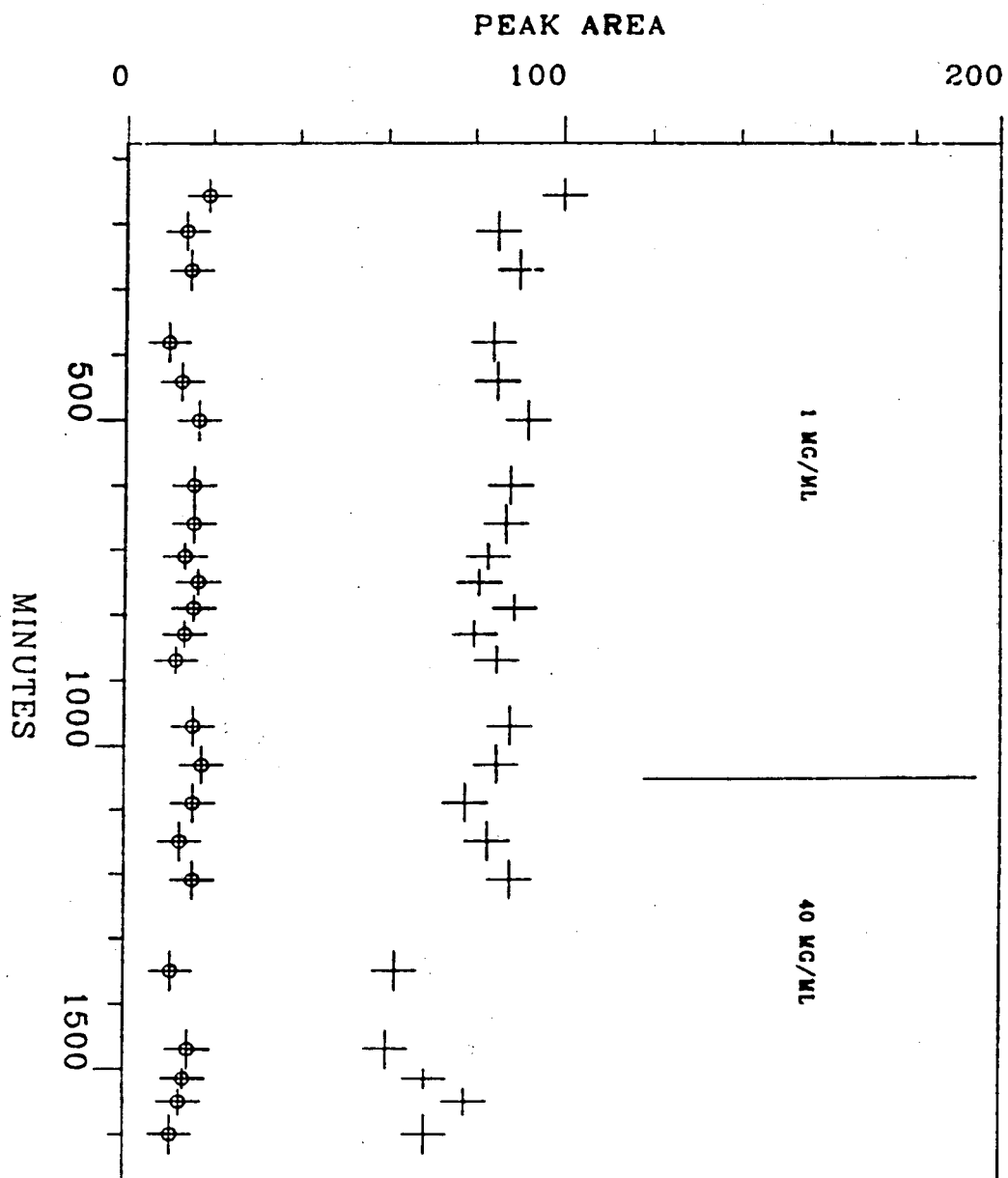


FIGURE 15A

TOTAL PHOSPHATE AND ATP (CH812)

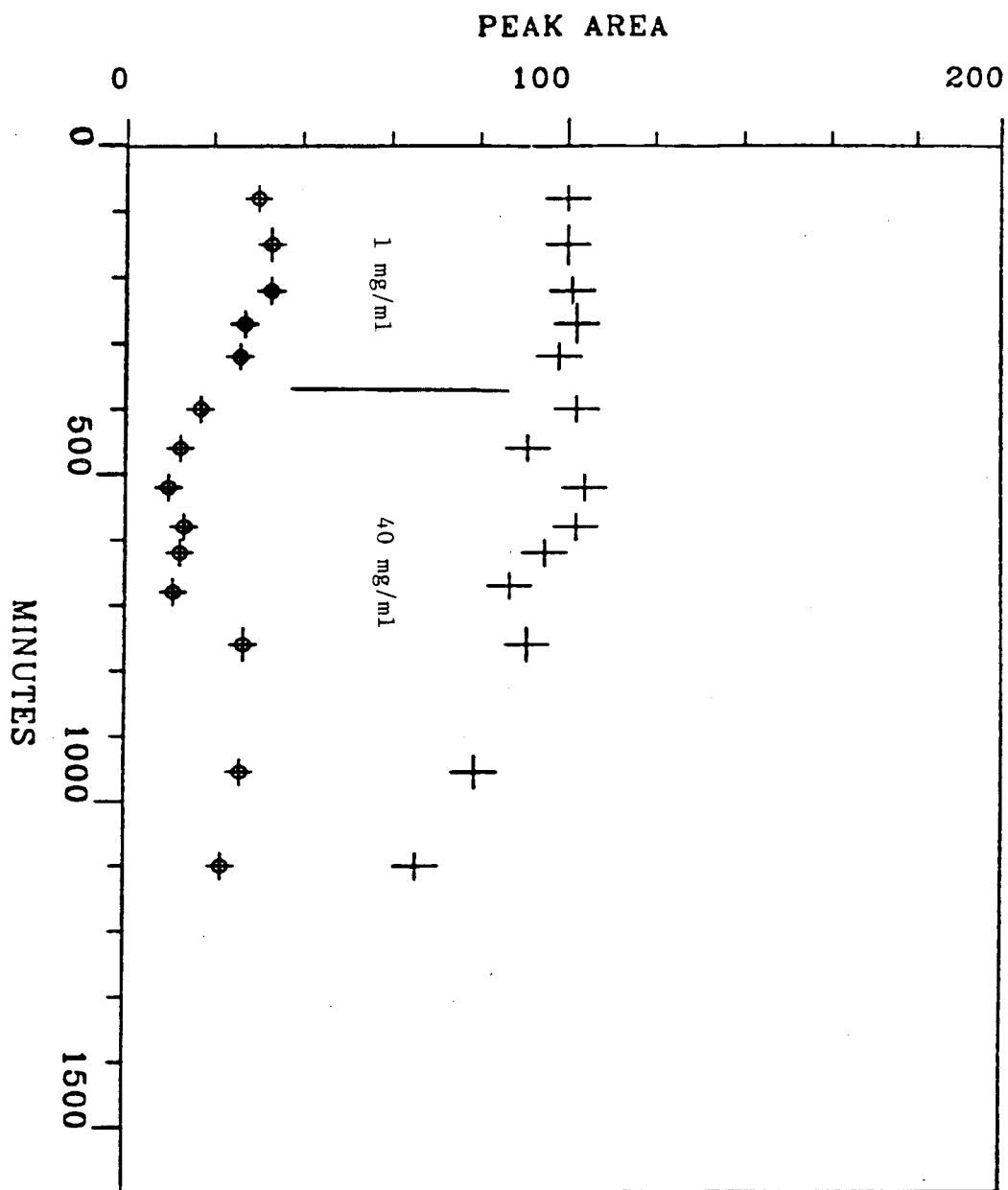


FIGURE 15B

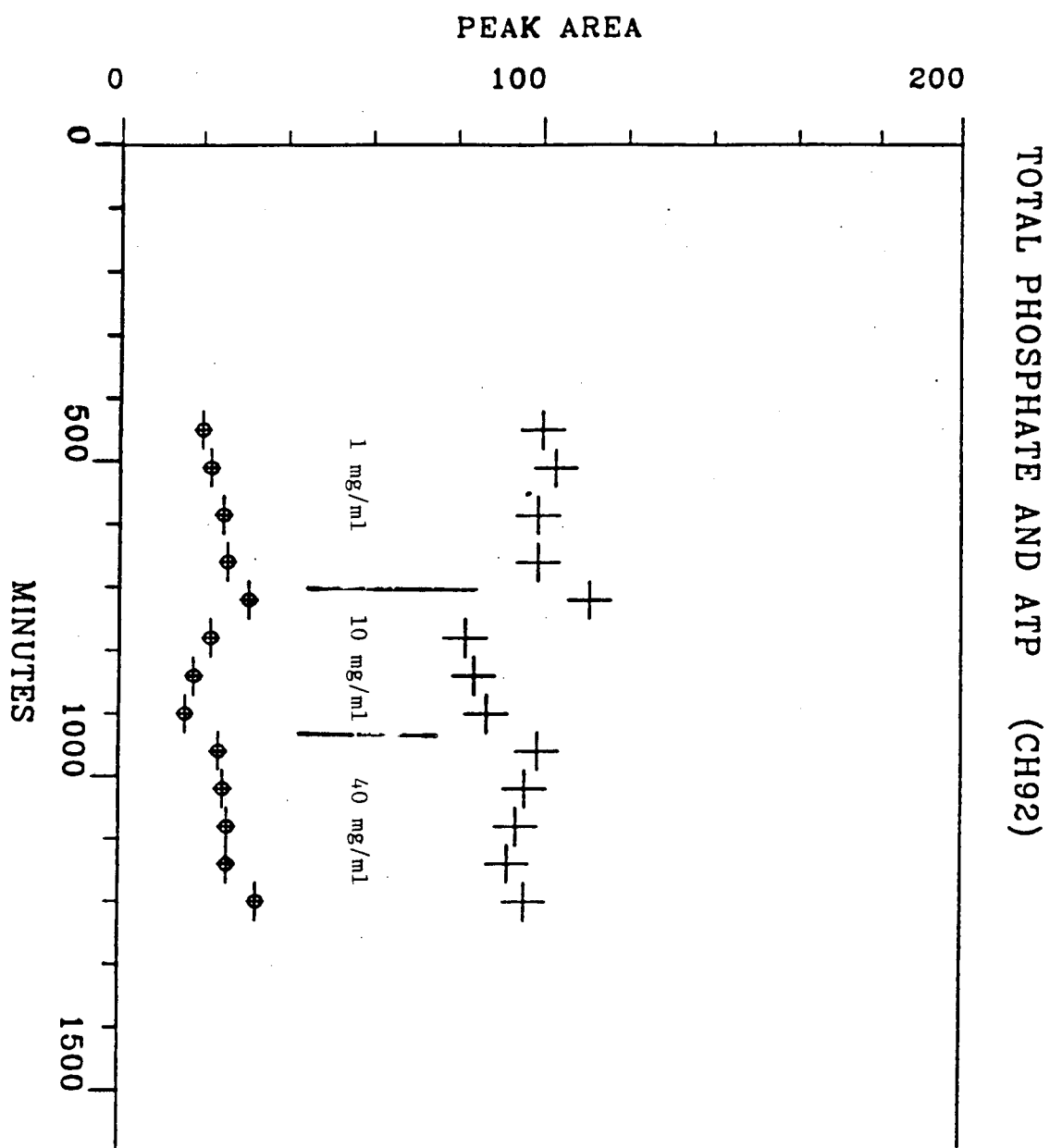


FIGURE 15C

TOTAL PHOSPHATE AND ATP (CTR84)

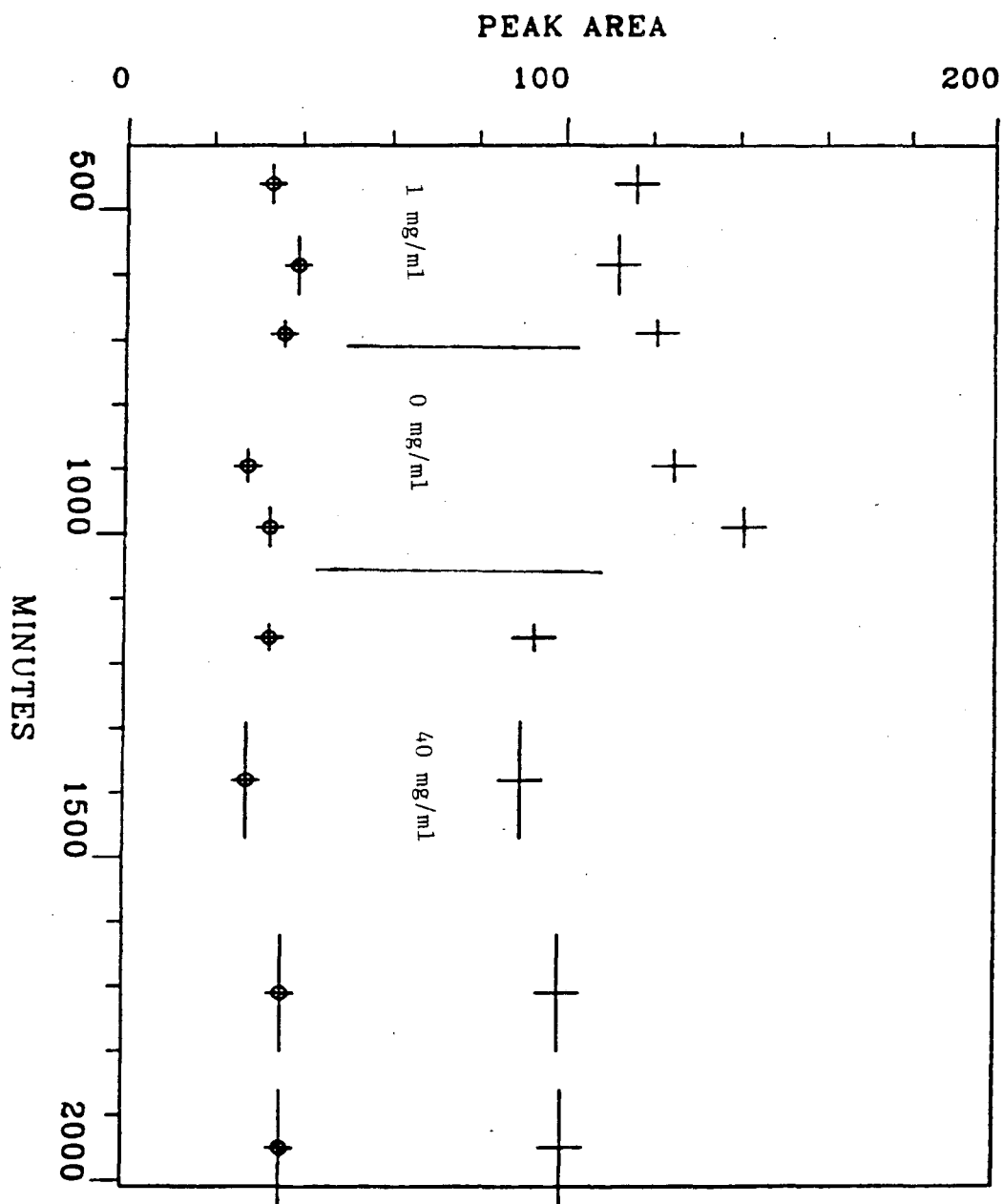


FIGURE 15D

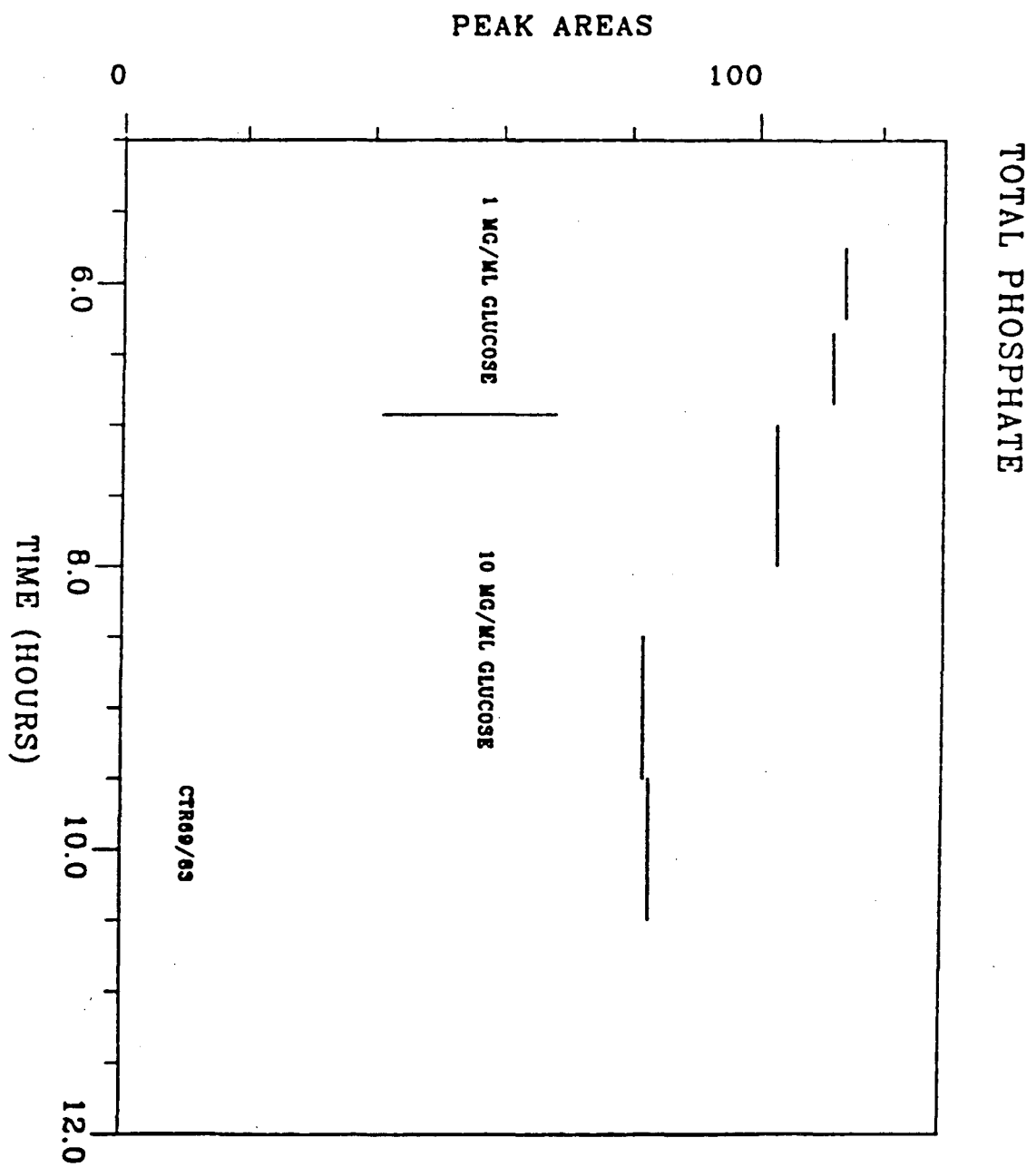


FIGURE 15E

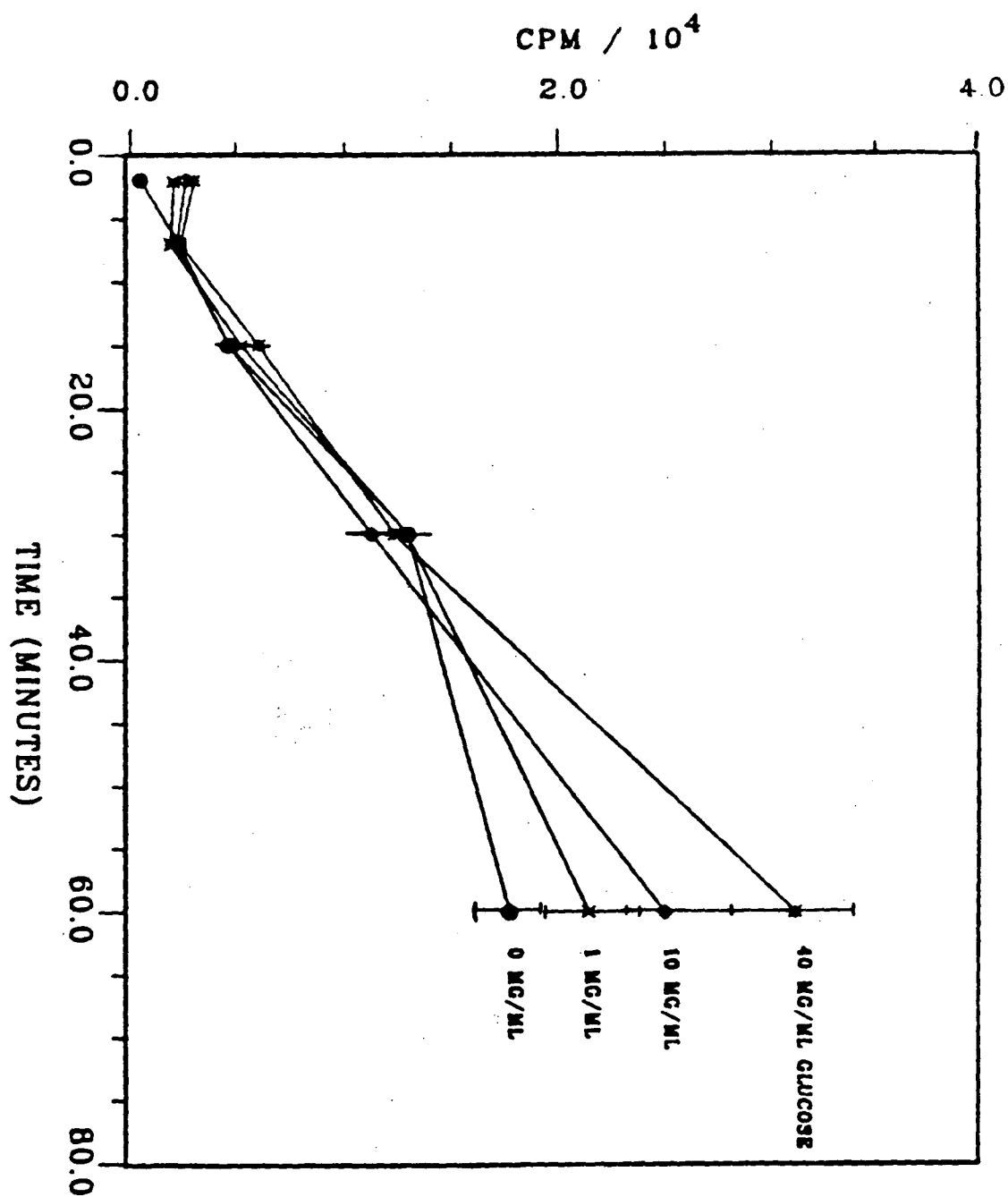
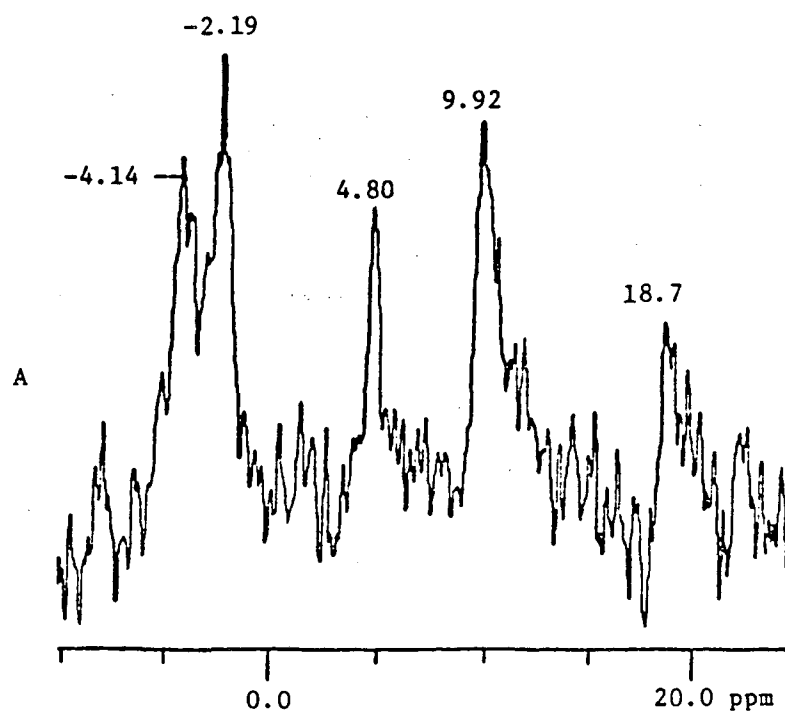
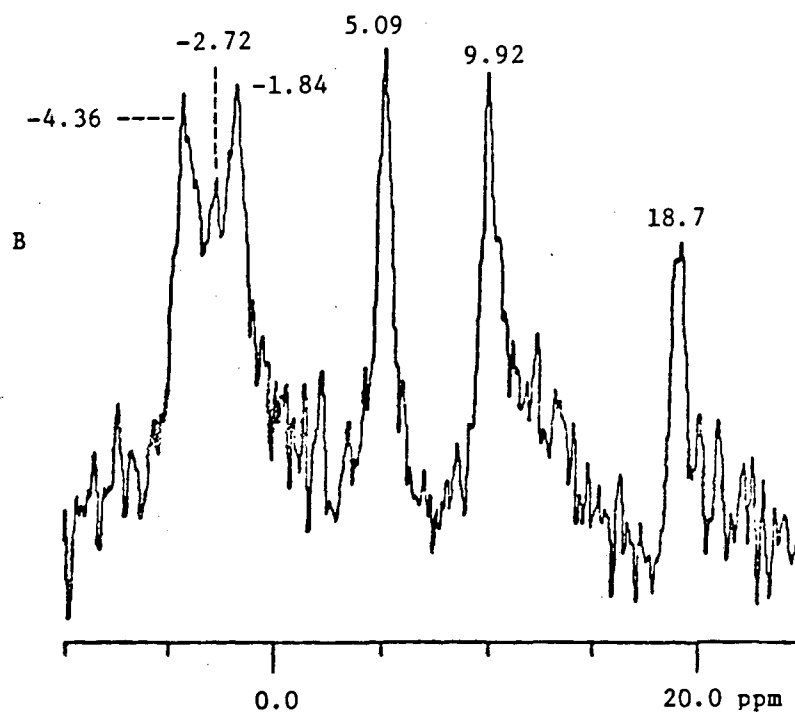


FIGURE 16



XBL 849-4005

FIGURE 17



XBL 849-4006

FIGURE 18

CHAPTER 6

CONCLUSIONS

The technique described in this thesis makes it possible to study isolated cells in suspension. Cells can be maintained at high densities in the bore of a superconducting magnet for up to forty eight hours. This technique can be used to study any cell which survives in suspension.

We have shown that two distinguishable pools of P_i exist. One is NMR detectable, and the other is not. The most reasonable interpretation of our data is that one of the pools is bound on some intracellular surface, or perhaps sequestered as a calcium salt, and thus has a long correlation time. As a result, its resonance is broadened out and disappears into the baseline. The other pool is dissolved in the cytosol and has a very short correlation time; thus it has a narrow signal and is detectable.

The exchange between these two pools correlates with the rate of glycolysis. At high rates of glycolysis the size of the bound pool increases, and when glycolysis is slowed down due to lack of glucose, the size of the free pool increases.

A change in the concentration of free cytosolic P_i may represent a change in the cytosolic phosphorylation potential. This in turn could effect the rate at which glucose can be metabolized.

Alternatively, the change in the amount of detectable phosphate may be a consequence of the change in the rate of glycolysis. It may be that at high rates of glycolysis significant concentrations of phosphates are bound to glycolytic enzymes and cannot be detected due to the long correlation times of the enzymes. The enzymes themselves may be bound to intracellular surfaces, and phosphorylated substrates may be sequestered near the enzymes so that catalysis is more efficient.

Although these results are potentially important, further experiments are needed. Cell fractionation experiments should be performed in order to determine where phosphates are sequestered. These experiments should be done on cells which have been 'quenched' at various rates of glycolysis so that the amount of bound phosphate which is measured using biochemical techniques can be compared with the amount of phosphate which is detected by NMR. It should be shown more clearly that the binding and release of phosphate is reversible. The data presented here are incomplete in this regard. The differences which were found between primary and cultured cells are interesting and should be explored further.

Any attempt at an extension of this work would benefit if these results could be reproduced with cells other than CEFs. CEFs have the advantages that they can be obtained both as primaries and in culture, and that they can be transformed in culture. However there are two important problems with NMR experiments involving CEFs. They are difficult to obtain in quantities which are adequate for NMR experiments; the large number of experiments (and the large number of

cells per experiment) which will be necessary to answer the questions which are outlined above may not be feasible with CEFs. In addition the cultured normal cells are anchorage dependent and their metabolism is significantly affected when they are kept in suspension. It may be necessary to work with cells such as Hela cells, which can be grown in very large quantities and are not anchorage dependent, but which unfortunately are not available as primaries.

Appendix

Longitudinal Relaxation Times In Vivo

In order to properly design NMR experiments and to interpret some of the data, it was necessary to measure longitudinal relaxation times of the various intracellular phosphates. As was discussed in Chapter 2, the T_1 s determine the delay which is necessary between acquisitions in order to obtain a fully relaxed spectrum. At times, partially saturated spectra were acquired in order to improve the signal to noise ratio. In these cases, the formula given in Chapter 1 was used in order to calculate the true intensity of each resonance. The true intensities depend on the pulse angle, the delay time, and the T_1 . The purpose of this Appendix is to describe the way in which longitudinal relaxation times were measured.

These measurements have intrinsic value aside from their importance for the interpretation of spectra. The application of relaxation time measurements to the study of the intracellular environment is discussed below.

A.1 Why Study Relaxation?

The spin states of the nucleus are coupled very weakly to the lattice in which the nuclei exist. As a result relatively weak interactions between nuclei and their environments can contribute

significantly to the rates of relaxation. Thus measurements of T_1 and T_2 can yield information about the environment of the nucleus and the molecule of which it is a part. Unfortunately the analysis is very complicated because it involves a large number of variables. There are a number of different relaxation mechanisms; some of the more important ones are dipolar, quadrupolar, chemical shift anisotropy, and scalar relaxation. The characteristic correlation times for these processes affect the rates of relaxation. Some of the environmental factors which determine correlation times and the efficiency of relaxation are binding of the molecules to proteins and metal ions, chemical exchange, pH, and temperature. If all of these variables can be sorted out, a detailed picture of molecular motion and the surrounding environment may emerge. In cells and organs, such information is not available as yet.

Aside from the basic structural information which relaxation time measurements provide, they have great practical value. The longitudinal and transverse relaxation times of water protons are widely used in NMR imaging to distinguish between different types of tissue. The relaxation time of protons in many types of tumour tissue are found to be significantly longer than that of corresponding normal tissue. In 1971 Damadian reported that in liver tumours in rats, the T_1 for water was 0.83 seconds, and T_2 was 0.12 seconds, while in normal liver T_1 was 0.29 seconds, and T_2 was 0.05 seconds (Damadian, 1971) Subsequently, these findings have been confirmed in many other laboratories (for example, see Hazlewood, 1972).

Although there is no agreement as yet as to the details of the

mechanism for water proton relaxation, it is generally found that an increase in T_1 is associated with an increase in cell water content (Inch, 1975; Hazlewood, 1974). As a result T_1 and T_2 images cannot reliably distinguish between bruises and other inflamed tissues, and tumours. (Ling, G.R., 1982) A problem which affects the interpretation of measurements of T_1 s of water protons in vivo is that it is difficult to distinguish intracellular from extracellular water. This is not a problem with the measurement of phosphorous relaxation times.

Damadian was also the first to suggest that the T_1 of ^{31}P in inorganic phosphate was longer in tumours than in normal tissue (Damadian, 1975). He measured relaxation times of various tumours and normal tissues which were removed from rats and mice. The tissue was maintained on ice at 7 degrees during the NMR measurements (Damadian, 1975). In Novikoff hepatoma tissue ^{31}P T_1 s were 8.35 seconds, while in normal liver the T_1 s were 2.05 seconds. Similar results were obtained in other tumours as well. Damadian suggested that these differences might be due to the higher potassium content of transformed tissue. To date, these results have not been confirmed in other laboratories. If it is indeed true that ^{31}P T_1 s are longer in tumours than in normal tissue, imaging of tumours using ^{31}P NMR might provide an alternative to proton imaging which does not confuse cancer with other types of tissue pathology. At present, ^{31}P imaging is only of experimental interest. The very low sensitivity of phosphorous NMR, relative to that of proton NMR makes phosphorous imaging extremely impractical.

To date experiments confirming Damadian's work have not been reported. Here we describe experiments on normal, transformed and primary Chicken Embryo Fibroblasts. These three types of cells are very similar genetically, metabolically, and structurally, but there are a few well defined differences, which were discussed in Chapter I. Thus a comparison of longitudinal relaxation times in these cells constitutes a good test of the hypothesis that an intrinsic characteristic of the transformed state affects longitudinal relaxation times of phosphates.

In general, more attention has been given to phosphorous linewidths in vivo than to mechanisms of longitudinal relaxation. Our experiments have have been aimed primarily at measuring longitudinal relaxation times, but mechanisms of longitudinal and transverse relaxation in vivo are probably not independent. The discussion which follows is a brief review of literature which deals with both transverse and longitudinal relaxation.

To some extent relaxation of phosphorous and of water protons are related. Phosphates, particularly inorganic phosphate, can mimic water, and fit well into the hydrogen bonding structure of water. If there are pools of 'bound water' than one would expect that these pools also contain phosphorylated metabolites. If the size or the stability of pools of 'bound' water changes, phosphorylated metabolites should be affected as well. If metal ions are present which affect the relaxation times of water protons, these metal ions will also interact with phosphates. A great deal of work has been done on mechanisms of transverse and longitudinal relaxation of water

protons in vivo, primarily because of the importance of these parameters for NMR Imaging. In the discussion which follows, data on the relaxation of water protons will be used as a basis for speculation about relaxation of phosphorous nuclei. However, the analogy between phosphates and water is only of value if we keep in mind some important differences. First, phosphates will have a charge at physiological pHs while water has almost none. Second, diffusion rates for protons in aqueous solution are extraordinarily high; protons are so small that they can tunnel through solution, while phosphate ions are relatively bulky and immobile. Third protons can interact with neighboring protons while ^{31}P is so dilute that there are no neighboring phosphorous nuclei. Despite these differences, studies of the relaxation of water protons and phosphates may prove to be complimentary.

A.2 Studies of Relaxation Mechanisms In Vivo

Evans et al (Evans, 1978) have reported that the T_2 s for inorganic phosphate and sugar phosphate signals are longer than the apparent T_2 s (T_2^*) in Hela cells, and attributes this difference to pH heterogeneity in the cells. Fabry et al (Fabry, 1983) find that linewidth of phosphate resonances in red blood cells is largely due to magnetic susceptibility gradients across the cell membrane. These gradients exist primarily because of the high susceptibility of the high spin iron of deoxy-hemoglobin which exists in large quantities inside red cells.

Koretsky et al (Koretsky, 1984) have reported that ^{31}P linewidths increase with field in rat kidneys. Evans and Shulman have made a similar observations, but find that ^{13}C linewidths are independent of field, and that proton line widths, with the exception of water resonances are independent of field. These differences between linewidths of ^{31}P resonances, and those of other nuclei suggest that field dependent T_2 s in vivo may be due to interactions of phosphates with metal ions. Phosphorous nuclei, which, in living systems are always present as phosphates and phosphate esters, should interact much more strongly with metal ions than most protons and carbons. Water protons, and ketone, aldehyde and carboxylic acid carbons are exceptions; they interact strongly with metal ions.

There is no doubt that in some cells paramagnetic metal ions cause transverse relaxation. Nicolay et al report that due to the presence of high concentrations of paramagnetic metal ions in Rhodopseudomonas Sphaeroides $^{31}\text{P}_1$ spectra can only be obtained after the cells have been washed several times with a buffer containing EDTA (Nicolay, 1981). The β ATP phosphorous has a high affinity for divalent metal ions; over 90% of the cell's cytosolic ATP is bound to magnesium (Roberts, 1981)), but some Mn^{+2} is bound as well. The unusually large linewidth of the β phosphorous in virtually all in vivo spectra is due to the binding of Mn^{+2} . The mechanisms via which metal ions affect T_1 and T_2 are discussed below.

T_1 s in vivo are much shorter than in preparations of phosphorylated metabolites in very pure water. The reasons for the more rapid rate of longitudinal relaxation in vivo are not

understood, in fact mechanisms of longitudinal relaxation of ^{31}P nuclei in vivo have been given very little attention. Evans has attempted to assess the dipolar contribution to relaxation in Hela cells. He finds that there is some transfer of polarization (NOE) from protons to inorganic phosphate and to the alpha phosphorous of ATP. This implies that there is longitudinal relaxation due to the dipolar interaction. However, he points out that the NOE for phosphorylated metabolites inside cells (20% for α ATP) is much smaller than in cell free preparations (80% for α ATP). In cells it is likely that other relaxation pathways are more important. The importance of the dipolar interaction for longitudinal relaxation will be discussed in greater detail below.

It is generally agreed that proton relaxation times become longer when the water content of a tissue is increased (Inch, 1975; Hazlewood, 1974). It has been suggested that an important mechanism of relaxation involves the binding of water molecules to cell proteins. This results in a change in the rotational correlation times of water molecules, and also a close association of water protons with protons on the macromolecule. Both of these factors may result in more efficient relaxation of the nuclear spin state. Alternatively, Eisinger et al (Eisinger, 1962) have suggested that proteins can mediate the relaxation of water protons by binding paramagnetic metal ions. The correlation time for a magnetic interaction between a water proton and a metal ion can be unusually long if the ion is bound to a macromolecule, and a long correlation time can result in efficient relaxation of the proton.

A.3 Studies of the Mechanisms of Phosphorous Relaxation In Vitro

In deionized water solutions the dipolar coupling between ^{31}P nuclei and water protons is an important mechanism of longitudinal relaxation of inorganic phosphate. McCain et al (McCain, 1980) found that in 100% D_2O T_1 at 360 MHz for H_2PO_4 was 22.9 seconds (the rate of relaxation = $1/T_1 = .048 \text{ sec}^{-1}$) while in 20% D_2O (80% H_2O) it was 13.2 seconds ($1/T_1 = .077 \text{ sec}^{-1}$).

Assuming that under these conditions Chemical Shift Anisotropy (CSA) is the only field dependent mechanism for relaxation, and that it operates in the short correlation time limit ($\omega^2 \tau_c^2 \ll 1$), McCain and Markeley calculated that the rate of CSA mediated relaxation at 145 MHz was $.015 \text{ sec}^{-1}$. If chemical shift anisotropy were the dominant relaxation mechanism the T_1 would be about 70 seconds - much longer than the observed value. The CSA ($\Delta\sigma$) of H_2PO_4 has not been measured, but ab initio molecular orbital calculations predict that it is 163 ppm. Kohler (Kohler, S., 1975) has found that $\Delta\sigma$ for CaHPO_4 is on the order of 100 ppm. In order to account for the observed relaxation rate, the rotational correlation time for the phosphate ion must be approximately $1.0 \cdot 10^{-11} \text{ sec}$. Because of the strong hydrogen bonding interactions between the phosphate ion and surrounding water molecules, such a rotational correlation time is unreasonable. The authors propose that relaxation is caused by the breaking of a

hydrogen bond at one site on the orthophosphate molecule and a simultaneous formation of a new Hydrogen bond at a different site. This results in rotation of the shift tensor much more rapidly than would occur as a result of rotation of the inorganic phosphate molecule itself. The authors refer to this process as a 'pseudo rotation'.

Given typical values for $\Delta\sigma$ of phosphates and phosphate esters (on the order of 100 ppm) and the rotational correlation time for small molecules in solution, rates of transverse and longitudinal relaxation due to CSA ($.015 \text{ sec}^{-1}$) are much too slow to account for observed linewidths in vivo. It may be that correlation times for phosphates in vivo are much longer than they are in aqueous solution. This may occur because of the binding to macromolecules or unusually strong hydrogen bonding interactions in the cytosol. As τ_c approaches 10^{-8} seconds relaxation via CSA becomes more effective. The contribution of CSA to T_1 and T_2 would increase as the square of the field, but if CSA competes with other relaxation pathways the overall T_1 and T_2 need not increase so rapidly.

In order to obtain the results described above, McCain and Markley had to go to great lengths to remove metal ions from their system. Even 1.0 ppm ferric ion affected T_1 values, especially below pH 7. At higher concentrations of metal ions, relaxation was completely dominated by interaction of phosphorous nuclei with metal ions. Since metal ions are present in the cytoplasm in significant concentrations, this may be the dominant factor in determining relaxation times in vivo.

Granot, Elgavish, and Cohen have investigated the effect of transition metal ions on the longitudinal relaxation time of inorganic phosphate (Granot, 1979). They find that in the presence of Cu^{+2} the T_1 of inorganic phosphate is pH dependent. Two minima occur in the plot of T_1 versus pH, at approximately the pKs of inorganic phosphate (2.2 and 6.8). They observe the same effect with AMP, ATP and TMP. This work, as well as earlier reports (Wasylishen, 1977; Morgan, 1975), indicates that the plot of T_1 v.s. pH for small molecules in solution with metal ions has minima near the pKas. These authors, like McCain and Markeley, find that this effect is not seen when solutions are treated with chelex and EDTA. They suggest that the T_1 minima are explained by a "pH dependent changeover" in the rate of ligand exchange from free to metal-bound.

A species such as $\text{Cu}^{+2} \text{PO}_4^{-3}$ may have a relatively long lifetime at pHs above 6.8. At pHs near 6.8, the lifetime of this species becomes shorter as a Cu^{+2} is displaced by H^+ . Exchange of copper on and off of the phosphate can modulate any of the magnetic interactions between metal ion and phosphorous nucleus. The resulting changes in the energies of nuclear spin states can lead to longitudinal relaxation if $\Delta\omega$, the populations of metal binding and metal-free phosphates, and the lifetime of the metal-binding form are all of the right order. Qualitatively speaking, the lifetime of the metal-binding species must be short enough so that the change in energy caused by the association with copper has spectral components at the Larmor frequency. At the same time the population of the species must be large enough, and its turnover rate must be great enough so that it

contributes significantly to the measured T_1 . At pHs well below 6.8, the lifetime of the $\text{Cu}_2^{+2}\text{PO}_4^{-3}$ is even shorter, but the population of the species is so small that it has no significant effect on the relaxation of the bulk of the P_i . At pH 2.2 the predominant metal ion - binding species, probably $\text{H}^+\text{Cu}^{+2}\text{PO}_4^{-3}$, goes through a similar transition. The chemical shift of the phosphorous nucleus is again modulated, and this may again result in longitudinal relaxation. Unfortunately, the lifetimes, and stoichiometries of the metal-phosphate complexes are not known at present.

A.4 Studies of the Interactions Between Paramagnetic Metal Ions and Water Protons.

A large body of work exists on the effect of paramagnetic ions on relaxation of water protons and oxygen (^{17}O labeled) nuclei. Because interactions between phosphate and metal ions are analogous to those of water and paramagnetic ions these experiments merit some attention here.

Interactions between water and paramagnetic metal ions have been studied as a function of field strength and temperature (Swift, 1963; Bloembergen, 1960; Morgan, 1959). It is found that there are two important ways in which paramagnetic metal ions can relax the nuclear spin states of the water molecules which chelate them. The first is through the dipolar interaction between the electronic and nuclear magnetic moments. The second is via the spin exchange or fermi contact interaction. The dipolar interaction is normally much stronger than

the hyperfine interaction. For a solution of Mn(II) in water Navon (Navon, 1970) calculates that the dipolar coupling constant is $2.0 \cdot 10^{15} \text{ sec}^{-2}$, while the hyperfine constant is only $6.2 \cdot 10^5 \text{ sec}^{-2}$. Both interactions can induce transverse relaxation, but they have different correlation times. The strength of the dipolar interaction is dependent on the orientation of the phosphate-metal ion complex with respect to the static magnetic field. The hyperfine interaction depends only on the spin state of the electron and the nuclear spins and on the lifetime of the interaction between the phosphate and the metal ion. Swift and Connick (Swift, 1963) find that for the interaction between water and Mn^{+2} the lifetime of the metal ion-water complex is $0.3 \cdot 10^{-7} \text{ sec}$, the lifetime of the electronic spin state (the longitudinal relaxation time) is $3.8 \cdot 10^{-9} \text{ sec}$, and T_1 for ^{17}O is on the order of a second. Thus the T_1 for the electronic spin state determines the correlation time for the scalar interaction.

Because of its very long correlation time, the hyperfine interaction may have very little spectral density at the Larmor frequency, and as a result, have little effect on longitudinal relaxation. By the same token, it may have relatively large spectral density at $\omega=0$, and thus constitute an important pathway for transverse relaxation. Bloembergen and Morgan (Bloembergen, 1960) find that in aqueous solutions of Mn^{+2} between 10^{-4} and 10^{-2} M longitudinal relaxation of water protons is caused primarily by the dipolar interaction with the metal ion, while the contact interaction is primarily responsible for transverse relaxation. Swift and Connick (Swift, 1963) found that in aqueous solutions of ^{17}O labeled water and

Mn^{+2} , the contact interaction is relatively strong ($2.7 \cdot 10^6$ cps) while the dipolar interaction is relatively weak because of the low gyromagnetic ratio of the Oxygen. Thus the scalar interaction determines the rates of both transverse and longitudinal relaxation. This may not be the case for hydrogen and phosphorous nuclei, which have larger gyromagnetic ratios, and which may have weaker covalent interactions with the metal. The results of Granot et al which were discussed earlier show that longitudinal relaxation rates of phosphates are extremely dependent on metal ion concentrations although there is no evidence that a dipolar interaction is involved.

When the electronic T_1 affects the nuclear T_2 through its effect on the hyperfine interaction linewidths of nuclear spin resonances increase with field. This occurs because as the field increases, the electronic T_1 becomes longer, and as a result the correlation time for the contact interaction becomes longer. As the spectral density of the contact interaction at $\omega=0$ increases, the rate of transverse relaxation increases. Swift and Connick reported that the electronic spin states of the metal were relaxed by modulation of the zero field splitting of the manganese ion due to collisions with solvent molecules. If the correlation time for this process (τ_v) is long, so that

$$\tau_v^2 \omega_s^2 \gg 1$$

(where ω is the Larmor frequency of the electrons)

then the electronic T_1 increases in proportion to the square of the static field. In this case, when the contact interaction is the most

important agent of transverse relaxation, T_2 of the nuclear spin state is expected to decrease in proportion to the square of the field. However, given typical values for τ_v , and typical fields at which NMR experiments are done, the low field approximation is not valid. A rough calculation shows that between 20 KG and 80 KG, the rate of transverse relaxation of the Oxygen should increase approximately linearly with field.

In the experiments discussed above, the dominant mechanism for longitudinal relaxation of water protons is the dipolar interaction between the electronic and nuclear spins. At the relatively low fields employed by these authors (14 Mc to 60 Mc), the field dependence of the T_1 was not significant.

It would be extremely difficult to apply the principles discussed above in order to obtain a quantitative picture of the interaction of phosphates with metal ions. However, it is reasonable to make some qualitative conclusions. Phosphates and phosphate esters have a higher affinity for metal ions than does water and the lifetimes of phosphate-metal ion complexes are longer than those of water-metal ion complexes. In crystals of tRNA, Holbrook et al have shown that Mg^{+2} bound to the sugar phosphates are within 3.1 Å of the phosphorous atom. Distances between a paramagnetic metal ion such as Mn^{+2} and a phosphorous nucleus in solution should be roughly the same. This is a short enough distance so that the magnetic field of the unpaired electron can interact strongly with the nuclear spin of the phosphorous. It is difficult to judge whether there is a strong contact interaction between the phosphorous and the metal ion; this

will depend on the electron density of the metal at the phosphorous nucleus. From the work of Swift and Connick, we know that there is a contact interaction between the water oxygen and Mn^{+2} , but the interaction between the phosphorous and the metal in a metal-oxygen-phosphorous bond is less direct. If scalar interactions determine the transverse relaxation times of phosphorous nuclei in solutions of phosphates with Mn^{+2} , a field dependent linewidth may be observed. The dipolar interaction would be the dominant pathway for longitudinal relaxation.

Clearly we are at present very far from a theoretical model for relaxation processes in vivo. However the preceeding discussion does provide a framework for consideration of the data which will be presented in this chapter.

A.5 Measurements of Longitudinal Relaxation Times in CEFS

Normal, transformed, and primary chicken embryo fibroblasts were prepared and maintained as described in Chapters I and II. During T_1 measurements the cells were perfused with phosphate free medium 199 containing 1 mg/ml glucose. Temperature was maintained at 25 °C. Under these conditions phosphorous spectra of the three types of cells are very similar, and the intracellular pH, measured from the chemical shift of inorganic phosphate was 7.0. In order to determine T_1 , the rate of recovery of the equilibrium magnetization following saturation was measured. This technique has the advantage that it is insensitive to inhomogeneities in the H_1 field. The spins were saturated using 30 $\pi/2$ pulses separated by 10 msec (reference). At various times after saturation, the equilibrium magnetization was measured. The recovery of equilibrium magnetization follows time course described by the equation:

$$M(t) = M_0 \cdot [1 - \exp(-t/T_1)]$$

where $M(t)$ is the magnetization at time t and M_0 is the equilibrium magnetization. Nicolet software was used to fit data to this equation to obtain T_1 . Typically, seven different delay times were used in each experiment, and the length of the experiments were approximately 3.5 hours. Two different experiments were performed on each batch of cells, and the T_1 s obtained from the two experiments were the same to within $\pm 5\%$. Measurements were made using both peak heights, integrals

of peaks, and fits of spectra using NTCCAP (reference Nicolet Manual); all of these techniques gave results which agreed to within 5%.

The Nicolet software was modified so that different numbers of acquisitions could be performed for each delay time. The software changes which were made are shown in Table 1. Typical acquisition parameters are shown in Table 2. The signal acquired per unit time for each delay time depends on the ratio

$$(M_0/t) \cdot [1 - \exp(-t/T_1)]$$

and the signal to noise ratio (S/N) per unit time is:

$$S/N(t) = (M_0/t)^{1/2} [1 - \exp(-t/T_1)]$$

$S/N(t)$ is at a maximum at $t = 1.27 \cdot T_1$ (Becker, 1979). For longer or shorter delay times the signal to noise ratio decreases. The number of acquisitions for each delay time are chosen so that S/N will be roughly the same for all seven data files. In order to insure that changes in the levels of metabolites and in other conditions do not affect the results, data at the seven different delay times are collected in parallel. For example, in the experiment described in Table 2, 200 acquisitions were performed with a delay time of 0.5 seconds, and the data were stored in a file, then 176 acquisitions with a delay time of 1.0 seconds were collected, etc. After data were collected at the last delay time (32 acquisitions with a delay time of 9.0 seconds) and stored, the cycle was repeated. Each cycle required

TABLE 1

MODIFICATION OF NTCFTB:

Changes program so that the number of acquisitions to be performed (NA) is stored with the rest of the parameter list for any experiment

	Location	Old contents	New contents
OVL PAR:	13716	440	763
OVL FIL:	13635	1216440	1216763
Main Program:	5053	1216440	1216763
Main Program:	5072	1222440	1222763
Main program:	5076	1232440	1232763

TABLE 2

Measurement of T_1 by Recovery from Saturation

Delay (sec)	NA	P_i	P_i^*	PME	PME^*
0.2	400	51	13	56	14
0.4	320	66	21	87	27
0.8	220	72	33	64	30
1.5	132	69	52	74	56
3.0	72	46	64	58	80
4.5	40	31	78	36	90
7.0	40	33	<u>82</u>	35	<u>88</u>

T_1 s calculated
from these data:

$$T_1 = 1.5 \pm 0.1$$

$$T_1 = 1.6 \pm 0.1$$

Shown above is a typical data set from a T_1 measurement. 'Delay' refers to the time between saturation and data acquisition. 'NA' is the number of acquisitions in each file per cycle. P_i is the amplitude of the P_i resonance, and PME is the amplitude of the PE and PC resonances at a given delay time. P_i^* and PME^* are the amplitudes normalized to 100 acquisitions/cycle.

approximately 30 minutes, and 7 cycles were executed.

We found that there was a great deal of variability in the T_1 s of the ATP resonances. The T_1 for the β ATP phosphate varied between 400 msec and 700 msec. The α and γ resonances had T_1 s of between 0.5 and 0.7 seconds. Given the limited number of experiments we were able to do, we could not determine the reason for this variability; it may have been due simply to the relatively poor S/N for ATP. Comparisons of T_1 in the 3 different types of cells were made only for the phosphoryl ethanolamine, phosphorylcholine, and inorganic phosphate resonances. A compilation of relaxation times is shown in Table 3. A typical data set, and a fit of the data are shown in Figures 1 and 2.

Relaxation times obtained for primary and transformed cells were very reproducible. There is somewhat more scatter in the data for normal secondaries. However, it is clear that there are no significant differences between normal and transformed cells. The T_1 s for both inorganic phosphate and the phosphate monoesters are significantly shorter in 1^0 cells than in cultured cells.

After the data shown in Figure 1a were acquired, the level of glucose in the perfusate was increased to 10 mg/ml. Within 3 hours, the intracellular pH had dropped from approximately 7.2 to below 6.7. At this point another T_1 measurement was begun; the data from this experiment are shown in Figure 1b. Within experimental error there was no change in the T_1 s of PE, PC, or P_i . Thus in the physiological pH range there is no effect of pH on the relaxation times of monophosphates.

TABLE 3

Relaxation Data

I EMBRYOS (Primary Cells)

Experiment	T_1 for P_i	T_1 for PME's
CEM520	1.5 ± 0.1	2.0 ± 0.1
CEM525	1.7 ± 0.2	1.9 ± 0.1
CEM527	1.5 ± 0.01	1.6 ± 0.1
AVERAGE	1.6 ± 0.1	1.9 ± 0.2

II NORMAL SECONDARY CELLS

CH317	2.7 ± 0.2	4.2 ± 0.2
CH55	2.4 ± 0.3	3.2 ± 0.4
CH512	2.2 ± 0.2	4.0 ± 0.4
CH812	3.2 ± 0.1	3.6 ± 0.3
AVERAGE	2.6 ± 0.4	3.8 ± 0.4

III TRANSFORMED SECONDARY CELLS

CTR218	2.7 ± 0.3	3.8 ± 0.2
CTR623	2.8 ± 0.1	2.8 ± 0.2
CTR69	3.3 ± 0.4	3.7 ± 0.4
CTR84	2.5 ± 0.1	3.5 ± 0.1
AVERAGE	2.8 ± 0.3	3.2 ± 0.2

We considered the possibility that there might be unusually high concentrations of a paramagnetic metal ion in embryonic cells, and that this might cause more rapid relaxation of phosphorous nuclei. If this were true, this metal ion would probably be present in cell extracts (unless it were bound very tightly to a protein even after denaturation). To test this possibility, relaxation times of phosphates in extracts were measured. The T_1 for phosphoryl ethanolamine was 3.4 ± 0.06 seconds, for phosphoryl choline, 3.4 ± 0.2 seconds, and for inorganic phosphate 2.9 ± 0.2 seconds. These relaxation times are much longer than those measured in embryonic cells in vivo, and are very similar to those measured in cultured cells in vivo. If paramagnetic metal ions dissolved in the cytosol were completely responsible for the relatively short T_1 s in vivo, then we would expect that T_1 in the extracts would be no longer than they are in vivo. Clearly this is not the case. Unfortunately T_1 s in extracts of cultured cells could not be measured due to lack of material.

In fact there is an indication that paramagnetic metal ions are present in the extract in higher concentrations than they are in vivo. T_1 s of ATP resonances in extracts of cultured cells were much shorter than those obtained in vivo: .18 seconds for the α phosphate, and 0.10 seconds for the β and γ phosphates. This may indicate that there are more metal ions available to bind ATP, resulting in more efficient longitudinal relaxation of the ATP phosphates. Other phosphates and phosphate esters are less sensitive to the presence of these ions because they bind metals much less strongly than ATP.

As was discussed earlier in this chapter, it has been suggested that higher protein content (or alternatively lower water content: gm protein/ml cell water) in normal tissue than in transformed tissue may account for the more efficient relaxation of water protons in normal tissue. It is possible that phosphorous T_1 s have a similar dependence. Thus it was of interest to determine the protein contents of the cells which were used in these experiments. The protein contents of three different types of cells were measured; cells taken directly from 10 day old embryos, secondary cultures of normal cells, and secondary cultures of transformed cells. Cell populations were determined using a Coulter counter, and the protein content per cell was determined using a standard Lowry assay. Within experimental error all cells contained the same amount of protein (see Table 4). These results are consistent with those of Bissell et al (Bissell, 1977), which showed that the intracellular volumes of normal and transformed cells, as measured by [^3H]-3-O-methylglucose uptake, are the same. Interpretation of these results is complicated by the fact that a population of embryonic cells is distributed randomly over the cell cycle. Some of the cells are growing rapidly, others have reached full size, and some are in the process of dividing. The cellular volumes of these cells vary over a wide range. Although we have measured the amount of protein per cell, we have not measured the amount of protein per ml of intracellular volume. An additional problem is that in order to dissociate embryonic cells, 5X trypsin must be used, while only 1X trypsin is used to remove cultured cells from dishes. The high concentration of trypsin may degrade cellular

TABLE 4

PROTEIN CONTENTS OF CULTURED AND PRIMARY CELLS

The errors given are standard deviations based on 3 measurements.

EMBRYOS: 120 ± 15 μgm protein/ 10^6 cells

SECONDARY CEFS: 103 ± 12 μgm protein/ 10^6 cells

SECONDARY TRANSFORMED CELLS: 101 ± 14 μgm protein/ 10^6 cells

proteins (perhaps primarily extracellular proteins) which are later replaced when the cells grow in culture.

A.6 Experiments on Red Blood Cells

As was discussed above, it is currently believed that the T_1 s of water protons are strongly influenced by the water content of cells. In the previous section data were presented which show that there is no correlation between the differences in relaxation times of phosphates in cultured and embryonic cells, and the protein content of the cells. Nonetheless we thought that it would be worthwhile to investigate more directly the interaction between the nuclear spins of water protons, and the ^{31}P nuclear spin states.

According to the data of Evans et al dipolar coupling of phosphorous nuclei to protons can produce some longitudinal relaxation in Hela Cells in vivo at 4 degrees. It would be interesting to know whether this is true in CEFs as well, but we were not able to do these experiments due primarily to lack of sufficient material. We were able to do some pilot experiments using Red Blood Cells.

Blood was drawn from a graduate student and centrifuged at 100G for 3 minutes. The supernatant was discarded, and cells were washed with physiological saline (Tris diluent) made up in either H_2O or D_2O . This process was repeated three times, to insure that all of the original plasma was removed. The longitudinal relaxation times were measured as described previously. Results of the T_1 measurements are

shown in Table 5. To study the effect of changes in cell water content on phosphorus T_1 s cells were washed twice with Tris which had been diluted by 30%. The result of this experiment is also shown in Table 5. The T_1 of 2,3 DPG in cells from which plasma had not been removed is shown as well. In all four cases, the T_1 s were very close to 2.8 seconds.

Interpretation of data obtained from red blood cells is complicated by the fact that when oxygen tension in the cells becomes low the heme irons are in the high spin form. The coupling of phosphorous nuclei to paramagnetic Fe(II) has a significant effect on phosphorous T_1 s. In fact, when cells were deoxygenated, by gentle bubbling with nitrogen gas, T_1 s of the 2,3 DPG phosphates decreased (see table).

A.7 Line Widths

As noted in Chapter II, linewidths (T_2^*) of phosphorous resonances in extracts of cells are the same as the linewidths in vivo. Linewidths of phosphorylcholine, phosphorylethanolamine, and the α and γ phosphates of ATP are approximately 50 Hz. The linewidth of the P_i resonance varied with pH, but was never less than 50 Hz. The line width of the β ATP phosphate was 50 Hz. After treatment with chelex and EDTA, linewidths decreased to as little as 10 Hz. Measurements of T_2 were not made, but the measurements of apparent linewidth support the qualitative conclusion that T_2 measured in vivo is primarily due to interaction of phosphates with metals. These

TABLE 5

LONGTITUDINAL RELAXATION TIMES FOR RED BLOOD CELLS

RBCs IN PLASMA:

$$T_1 \text{ (2,3 DIPHOSPHOGLYCERATE)} = 2.8 \pm 0.4 \text{ seconds}$$

RBCs IN PHYSIOLOGICAL SALINE:

$$T_1 \text{ (2,3 DPG)} = 2.7 \pm 0.2 \text{ seconds}$$

RBCs IN PHYSIOLOGICAL SALINE MADE UP IN D₂O:

$$T_1 \text{ (2,3 DPG)} = 2.8 \pm 0.2 \text{ seconds}$$

OSMOTICALLY SWOLLEN RBCs:

$$T_1 \text{ (2,3 DPG)} = 2.8 \pm 0.2 \text{ seconds}$$

EFFECT OF D₂O ON RELAXATION TIME OF H₃PO₄ AT pH 7.4

$$T_1 \text{ IN H}_2\text{O} = 3.65 \pm .04 \text{ seconds}$$

$$T_1 \text{ IN 50\% D}_2\text{O} = 6.4 \pm .07 \text{ seconds}$$

$$T_1 \text{ IN 90\% D}_2\text{O} = 8.7 \pm 0.09 \text{ seconds}$$

$$T_1 \text{ IN PHYSIOLOGICAL SALINE IN D}_2\text{O} = 7.1 \pm .2 \text{ seconds}$$

metals would be present in similar concentrations in extracts; thus extract spectra would look much like in vivo spectra.

X ray fluorescence analysis of cell extracts was used to look for metal ions in extracts of CEFs. No metals were detected, but the limit of sensitivity of this technique is at least 0.5 ppm. We plan to use Atomic Absorption Spectroscopy to look for trace amounts metals in our extracts. It is known, from extensive analysis of the trace metal content of various tissues (Bowen, 1966), that there are significant amounts of manganese, in addition to iron and zinc. In heart, for example there are 0.8 ppm manganese (based on dried tissue). Data on trace metal content of pure fibroblasts is not readily available. If Mn(II) is present in the cytoplasm at levels similar to those in heart, and if iron and zinc are present at even a fraction of their concentrations in heart and liver (Bowen) it is probable that both transverse and longitudinal rates of relaxation of phosphorous nuclei would be affected.

Two other observations provide support for this idea. Proton resonances in extracts are very narrow. In Figure 5, Chapter 3, the width of the lactic acid and choline resonances are approximately 10 Hz. In a natural abundance Carbon-13 spectrum of yeast in vivo the glycogen resonances are no more than 20 Hz broad. If metal ions are responsible for line broadening of phosphate resonances, which are relatively strong chelating agents, then it is expected that there would be less broadening of resonances from hydrogen and carbon moieties which will not interact strongly with metals (with the exception of water protons, ketone, aldehyde and carboxylic acid

carbons).

A.8 Conclusions

We have compared longitudinal relaxation times of inorganic phosphate, phosphorylethanolamine and phosphorylcholine in normal, transformed, and primary cells. This is the first such study done on isolated living cells.

Differences between normal and transformed cells which are grown in culture have no effect on the relaxation times of intracellular inorganic phosphate and phosphate monoesters. There are, however, differences between primary and cultured cells which affect relaxation times. It is possible that this result is of general significance. Normal cells in culture are not 'ideal' models for fibroblasts in vivo. Despite the fact that they perform their differentiated function of making an adhesive collagen/fibronectin matrix, they are in some other important respects abnormal. They are more glycolytic than fibroblasts in vivo, may contain relatively few mitochondria and high concentrations of glycolytic enzymes. Unlike their in vivo counterparts, they grow in monolayers. Furthermore, a confluent culture of normal fibroblasts is, for all practical purposes, synchronized in the G_0 phase of the cell cycle, and very few of the cells are dividing. Transformed cells however are much less affected by their environment than normal cells, and transformed cells in culture may be very good models for transformed cells in vivo. Thus,

the most valid comparison between transformed and normal cells may be between transformed cells either in culture or in vivo, and primary cells taken directly from the embryos. If this is the case then our results predict that normal and virally transformed cells can be distinguished on the basis of phosphorous relaxation times.

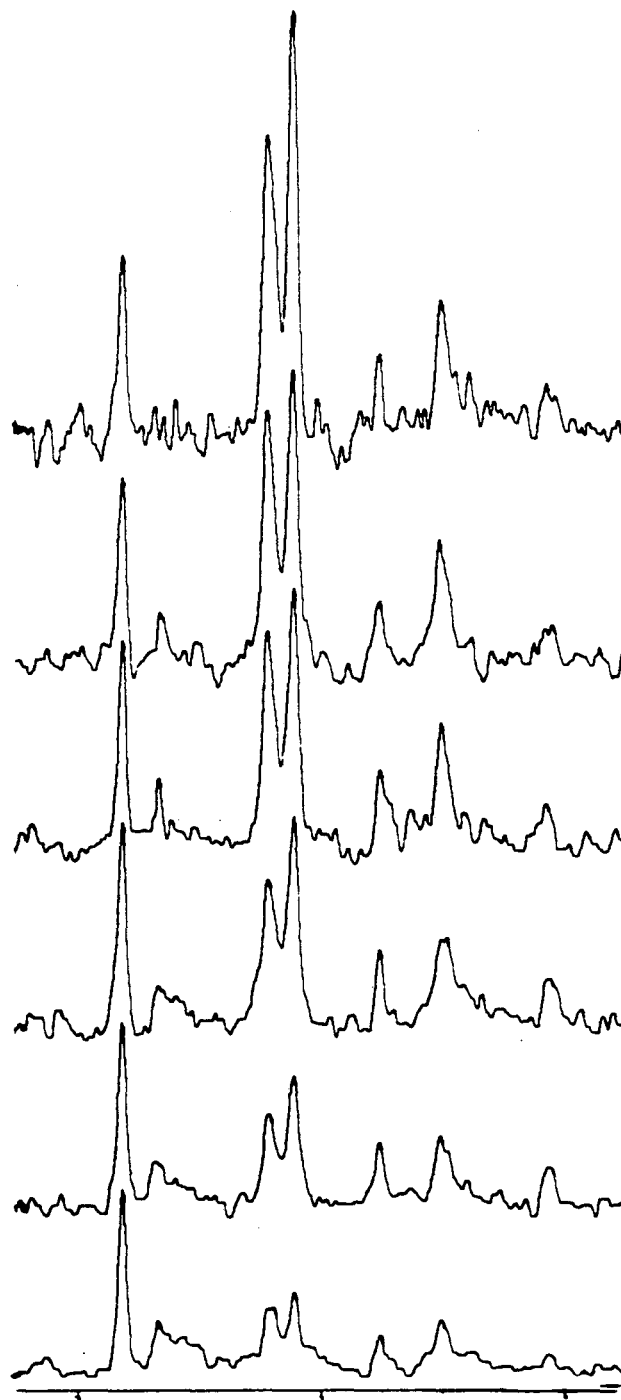
The pathways for the relaxation of ^{31}P nuclear spin states in vivo have not been studied systematically. The experiments presented here suggest that in red blood cells the interaction between water protons and ^{31}P nuclei is not an important relaxation mechanism. However these experiments should be repeated in other types of cells. The binding of paramagnetic metal ions to phosphates may cause relaxation in vivo. The transverse and longitudinal relaxation times of ^{31}P nuclei should be examined as a function of metal ion concentration and field strength. In addition the effect of macromolecules on the T_1 s of phosphorous nuclei should be investigated. Although a large number of measurements are required, the experiments are fairly simple, and the data would enable researchers to obtain specific information from in vivo measurements of longitudinal relaxation times.

Figure Legends for Appendix

1) Data points for T_1 determination. Each spectrum is labeled with its delay time. Spectra were taken while cells were perfused with Med 199 with 1 mg/ml glucose.

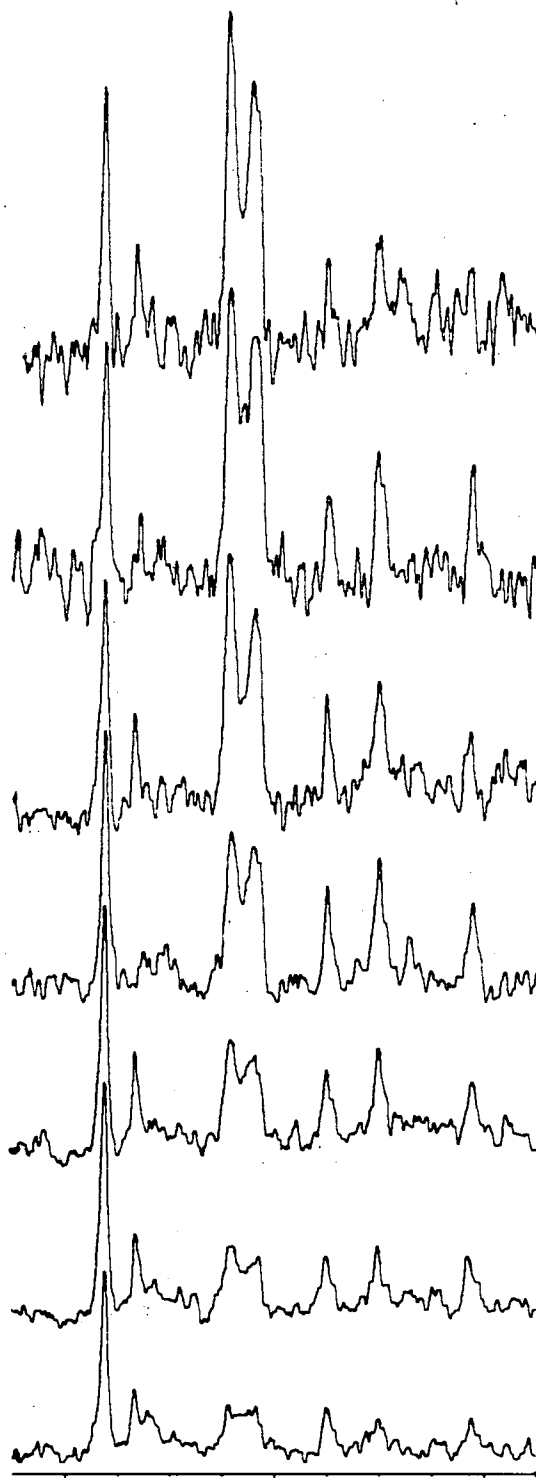
2) Data points for T_1 measurement done while cells are perfused with 10 mg/ml glucose.

3) Natural abundance ^{13}C spectrum of yeast in vivo. The signals come primarily from glycogen. Resonance positions and assignments are: 1) 93.0 ppm (reference is TMS), C_1 of glycogen 2) 73.8 ppm, C_3 of glycogen 3) 72.5 ppm, C_2 of glycogen 4) 70.6 ppm, C_4 of glycogen 5) 61.8 ppm, C_6 of glycogen. EM = 25.0 Hz



XBL 847-3157

FIGURE 1



XBL 847-3158

FIGURE 2

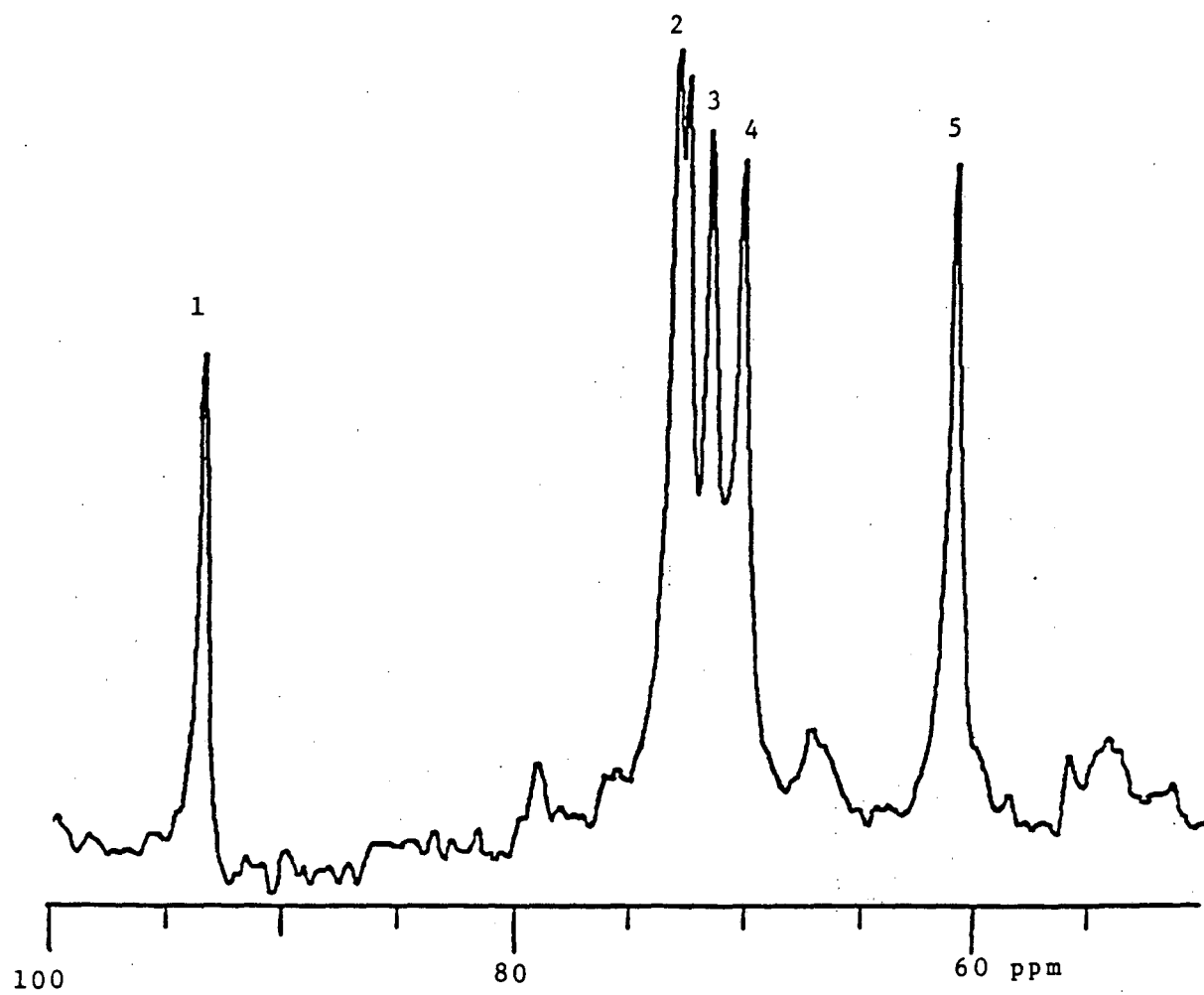


FIGURE 3

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