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PHOSPHORUS MAGNETIC RESONANCE SPECTROSCOPY IN NUTRITIONAL RESEARCH

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CONTENTS

INTRODUCTION

Magnetic resonance spectroscopy enables investigators to study several biochemical reactions in normally functioning organs. This review focuses on the potential use of in vivo phosphorus magnetic resonance spectroscopy (^{31}P

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MRS) in studying nutritional problems. We briefly describe the basic methodology of ^{31}P MRS [for further information, see reference (13)]. Data on normal functioning organs are presented only if relevant to observations in the diseased state. We note that some observations made in our laboratory and described here for the first time are preliminary. Nevertheless, they suggest possible uses for ^{31}P MRS in nutrition studies. At the present time the use of ^{31}P MRS in the nutritional field is limited.

^{31}P MRS

Using magnetic field strength of 1.5–2.0 tesla, in vivo ^{31}P MRS can record only metabolites from muscle, heart, brain, and liver that are present in concentrations higher than about 0.5 mM. These spectra are relatively easy to interpret because only a small number of phosphorus-containing compounds are present in such concentrations. Most of the observations related to the field of nutrition have been ^{31}P MRS studies of muscle. The typical ^{31}P MRS spectrum of muscle at rest is shown in Figure 1*a*. There are five major peaks: three of the phosphate atoms in the ATP molecule (in positions alpha, beta, and gamma), one of phosphocreatine (PCr), and one of inorganic phosphates (P_i). The latter peak contains both $H_2PO_4^-$ and HPO_4^{--}. In brain and heart spectra, additional peaks are observed in the phosphomonoester (PME, to the left of the P_i peak in conventional presentation) and the phosphodiester (PDE, between the PCr and P_i peaks) regions (Figure 1*b* and *c*). These peaks probably represent several molecules in the different organs. For instance, muscle PME most likely is largely accounted for by glycolytic intermediates (sugar phosphates) while in the brain phosphoethanolamine is the major compound. Also, PCr is absent from the liver spectrum. The chemical shift (the spectral "distance") between the PCr and P_i peaks is determined by the intracellular pH. Thus, if properly calibrated the ^{31}P MRS spectrum can accurately determine the intracellular pH of functioning organs in vivo (30). The chemical shifts between the various ATP peaks are mainly determined by the amount of magnesium (Mg^{2+}) bound to the ATP molecule. Thus, ^{31}P MRS may be used to estimate changes in intracellular free Mg^{2+} (14).

The area under each spectral peak is related to the amount of each metabolite present in the sample recorded. The volume of tissue from which signals are recorded depends mainly on the size of the external coil used to record the MRS signals and the technique used for depth recording (depth recording may be required to eliminate contributions from nonrelevant superficial tissues). In studies of human muscle, the sample volume is generally a few cm^3 and the signals are averaged from the total volume. The volumes in many liver and brain studies were even higher. The tissue in the recorded volume is heterogeneous, and calibration to absolute amounts and concentrations requires an

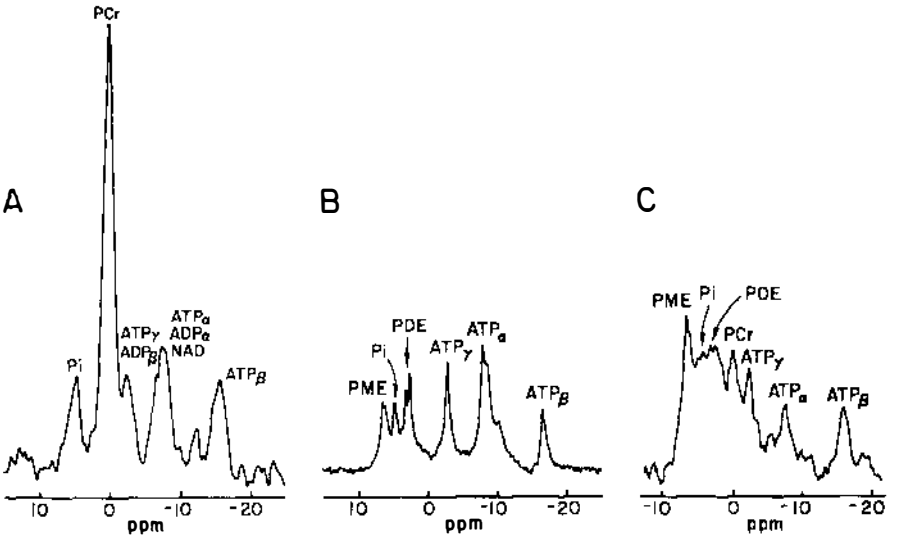

Figure 1 ^{31}P MRS spectra of (*a*) muscle at rest, (*b*) heart, and (*c*) brain with identification of the major peaks. PCr is phosphocreatine, P_i is inorganic phosphates, ATP is adenosine triphosphate, PME is phosphomonoesters, PDE is phosphodiesters. For the chemical shift for pH calculation, the position of the P_i peak is measured with respect to that of PCr at 0 ppm.

internal or an external standard with known concentration. Most studies have used the ATP peak as an internal standard, because it changes very little during short-time physiological alterations. In human muscle, studies have assumed an unchanged average ATP concentration of about 8 mM (28). This concentration may be a "crude" average under normal conditions, but whether or not diseased muscle cells retain the same concentration of ATP has not yet been determined. External standards are technically difficult to apply because they are located outside the main recorded volume. Thus, for ^{31}P MRS quantitative measurements, ratios between compounds are used more frequently than single peak amounts. The ratios between some of these peaks have a metabolic significance (see Appendix).

A high-resolution muscle spectrum, such as that shown in Figure 1, is recorded in a few minutes. The time resolution for the other tissues is longer. Thus, ^{31}P MRS is very useful to monitor long-term changes, but acute alterations may be difficult to follow. Brain, heart, and liver spectra change very little during normal physiological function. However, in studying muscle metabolism one must also make measurements in the working state. The changes that occur in the concentrations of PCr and P_i in working muscle are very rapid, and a method with a time resolution of about 1 min (e.g.

muscle ^{31}P MRS) may fail to accurately represent the transient changes. To solve this problem, the steady-state type of exercise protocol was developed.

During exercise, the levels of PCr fall and those of P_i rise stoichiometrically (28) while the levels of ATP do not change (unless exercise is very intense) (Figure 2). The basic principle of the steady-state protocol was demonstrated by ^{31}P MRS study of isolated heart mitochondria in vitro (15). In that work, ADP and P_i were added to mitochondria to give a series of steady states. Each state had a distinct ATPase rate (corresponding to the ATP synthesis by oxidative metabolism), and ^{31}P MRS was used to measure the PCr/P_i value (corresponding to the intracellular ADP concentration). When the values of ATPase were plotted against the inverse ratios of PCr/P_i, a rectangular hyperbola was obtained. Such a profile is expected when all the oxidative metabolism is regarded as a single process that follows Michaelis-Menten kinetics of single enzyme systems (5, 7). The human and animal exercise protocols used in our laboratory follow a very similar approach. Graded levels of work rate were performed by the wrist flexors of the human arm, monitored by an ergometer. After a few minutes of a constant work performance, the PCr/P_i ratio was stabilized and measured and the subject increased the work rate until a new steady state was achieved. It should be noted that these were pseudo steady-states, since not all determinants remained constant (e.g. the

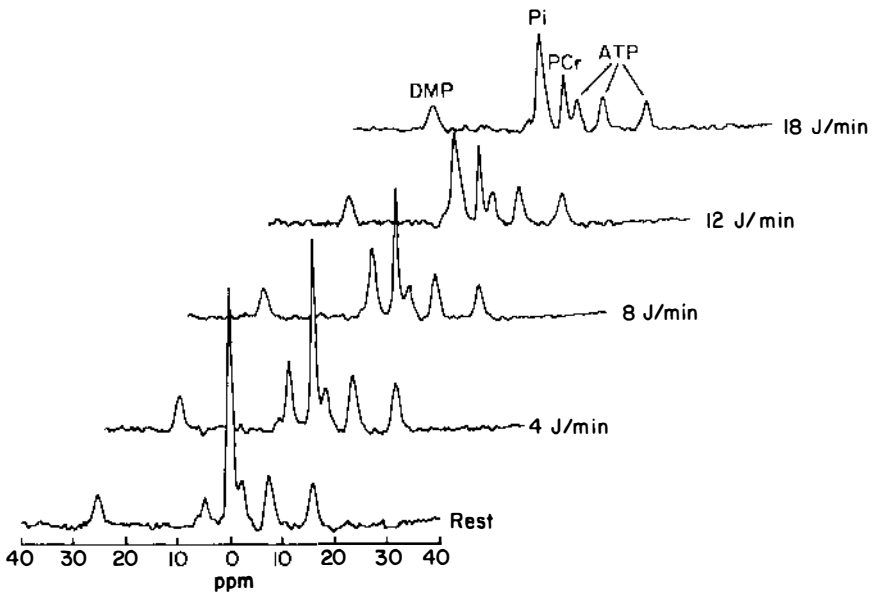

Figure 2 Changes in ^{31}P MRS during muscle work. Note the fall of PCr and rise of P_i as work load increases.

pH was not always constant when high levels of work rates were achieved). However, when the work rate was plotted against P_i/PCr, a profile very reminiscent of a rectangular hyperbola was obtained (5, 7). The kinetic meanings of these findings are discussed elsewhere (7). If such a steady-state protocol can be followed, the initial slope of the graph (findings at mild to moderate work rates) will give a comparable value of the oxidative metabolism in different subjects and in the same subject under different conditions. In this review the slope of work rate to metabolic cost (P_i/PCr or ADP) is referred to as the "transfer function" (7). In animals, one may use a similar approach and stimulate the nerve (or the muscle directly) to achieve a constant work rate. Work can be estimated from the stimulation rate or more accurately by measuring the muscle twitch tension (4). In heart recording, the product of blood pressure times heart rate was used to monitor work, and pacing the heart could be used to alter the work rate. It is important to evaluate muscle ^{31}P MRS studies during work with respect to the method used for work measurements.

After exercise is stopped, the ^{31}P MRS spectrum returns to its resting state characteristics (PCr is high again and P_i is low) at a rate that is dependent upon the energetic deficiency established during exercise. In fact, the maximal metabolic rate of mitochondria may be assessed by this recovery rate. During this post-exercise recovery some loss of signal is noted and the whole spectrum is "noisier" with smaller peaks. These changes may be due to maximal increased blood volume and flow to muscle during this period. Thus, in order to evaluate recovery it is desirable, again, to look at ratios; we quantify the recovery by the change in PCr/P_i units per time. However, PCr/ATP (or PCr per standard) can also be used.

REPORT OF RELEVANT NUTRITIONAL ^{31}P MRS OBSERVATIONS IN MUSCLE AND LIVER

Muscle

HYPOCALORIC FEEDING Two volunteer subjects received no caloric intake (water only) for 72 h. Their respiratory quotients dropped from 0.85 to 0.7. In both subjects, the ^{31}P MRS at rest showed a fall in the P_i peak with increased PCr/P_i ratio after 72 h (Figure 3). No fall in ATP was detected. Human muscle at rest uses mainly free fatty acids (FFA) as its substrate for energy metabolism (12). The increased energy state may simply indicate that during fasting, human skeletal muscle has more FFA available and is operating at "preferred" conditions. This possibility is supported by another human ^{31}P MRS study on resting muscle during 1 g/Kg glucose load (31). A gradual reduction in PCr and elevation in the P_i peaks was followed by a maximal reduction in PCr/P_i after 1 h and a rise in the calculated ADP. Thus, glucose

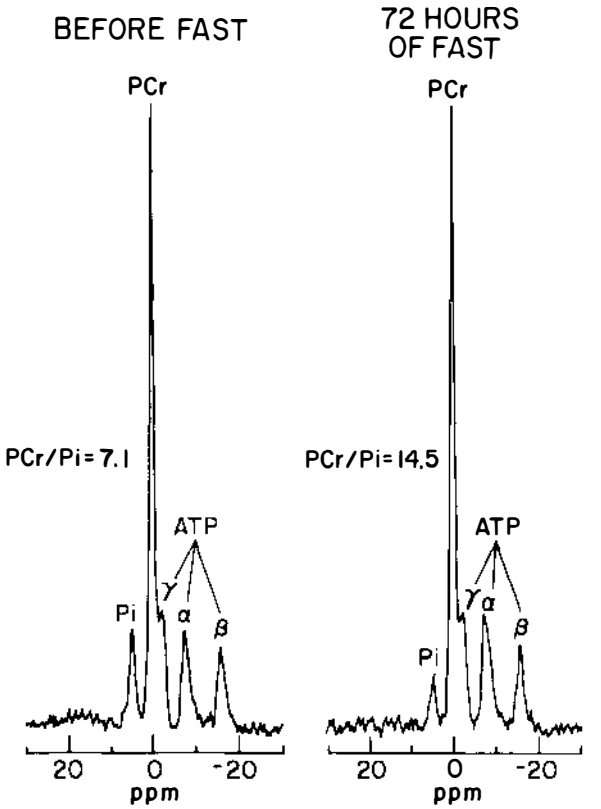

Figure 3 Changes in resting muscle spectrum before and during fasting. Note the fall of P_i and slight rise in PCr with the moderate rise in PCr/P_i.

load at rest may shift the energy metabolism to different and probably less efficient kinetics. Lunt et al (22) did not find changes in spectra of resting human muscle during 5 days of fasting.

Jacobs et al showed that in starved rats, ATP in muscle at rest was maintained at the expense of reduced PCr (18). Pichard et al (27) performed a more extensive in vivo ^{31}P MRS study, combined with in vitro biochemical assay analysis, on the effects of fasting on rat muscle at rest. They found that in both in vivo and in vitro studies of animals fasting for two days and hypocalorically fed rats, the following changes occurred: PCr levels fell, leading to lower PCr/P_i and PCr/ATP ratios. These changes were associated with a rise in ADP and lower free energy of ATP hydrolysis. In the hypocaloric fed animals, pH fell and an additional peak at the PDE region rose (the nature of this phosphodiester was not established). The authors concluded that, even at rest, ATP production in muscle is altered during a fast and that ATP levels are maintained at the cost of lower PCr levels.

In our preliminary observations on the two above mentioned volunteers, no clear effect of fasting on exercise kinetics could be observed. Lunt et al (22) followed the end-exercise state before and after 5 days of starvation. They noted that the exercise-induced changes in pH (reduction) and P_i/PCr (elevation) were "inhibited" by the fasting state. This study illustrates the problems that may arise when studying a single point of exercise, without relating it to the amount of work performed, via an analysis similar to our transfer function evaluation. Their data could be interpreted simply as showing that the starving subjects perform less work and thus have less changes in their end-exercise state. In experimental animal models, such a problem is partially avoided by nerve stimulation that induces muscle work involuntarily. Thus, the effects of starvation on muscle at work were studied by us in rats that were fed fluids only and that had a work protocol consisting of 40 min of nerve stimulation at 4 frequencies and continuous recording by ^{31}P MRS of relevant muscles on day 3 and day 6 (19). A clear fall in the transfer function that relates the stimulation frequency (a measure of work) to the P_i/PCr became significant at day 6 (Figure 4). These findings support the viewpoint that muscle oxidative

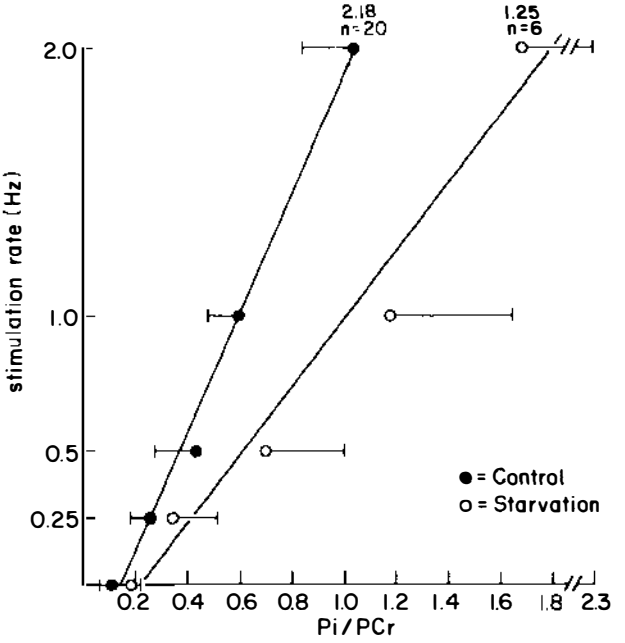

Figure 4 Changes in work-cost relation in starving rats. The stimulation rate serves as a measure of work load while the P_i/PCr indicates the bioenergetic state of the tissue. Note the fall in slope, indicating a "less efficient" oxidative metabolism.

phosphorylation becomes less efficient during starvation. Thus, even short-term starvation can change muscle bioenergetics, as recorded by ^{31}P MRS.

In another study, a protocol of 72-h fast (with water ad libitum) was used on a patient with carnitine palmitoyl transferase (CPT) deficiency. Such patients cannot utilize long-chain fatty acids for their energy metabolism. Clinically, such patients are known to be more prone to muscle pain and even myoglobinuria if they "miss" regular meals and especially if they fast (10). The metabolic basis for that feature was shown to be an increased failure of the CPT mutant enzyme when its substrate concentration (FFA) increases. During exercise the tested patient had a reduced slope of the transfer function after 65 h when compared to his prefast testing (Figure 5*a*). Furthermore, during prolonged exercise a failure to generate force was observed. When asked on day 3 to increase work rate the patient did so for a very brief period (1–2 min) and then complained of sudden weakness despite maximal effort. The ^{31}P MRS spectra returned to normal PCr/P_i despite the patient's attempt to produce work with an associated reduction in the ATP signal (Figure 5*b*). These findings show the vulnerability of patients with biochemical defects to a specific metabolic stress such as fasting. These preliminary observations also demonstrated the ability of ^{31}P MRS of muscle to detect acute changes in energy metabolism induced by nutritional changes in both normal and metabolically impaired persons. These considerations are of special importance in evaluating persons on restricted diets such as those proposed for cancer prevention.

SPECIFIC NUTRITIONAL DEFICIENCIES ^{31}P MRS can be used to estimate the free Mg^{2+}. In vivo tests in muscles of rats fed a magnesium-deficient diet showed a correlation between the ^{31}P MRS data and the rat state (20). Also, reduced serum phosphorus (renal hypophosphatemia) may be detected by muscle ^{31}P MRS (17), but the relationship between dietary, plasma and intracellular P_i remains to be established.

NUTRITIONAL SUPPLEMENT A supplement of substrates to muscles with enzyme defects can also be observed with ^{31}P MRS. The administration of glucose to normal subjects after overnight fast does not change the bioenergetic profile of working muscle (Figure 6*a*). This finding may be due to two reasons: either normal muscle does not use the extra blood-delivered glucose and is primarily dependent on intramuscular glycogen and FFA during such exercise or the blood-borne glucose does not modify the oxidative phosphorylation. In contrast, patients with myophosphorylase deficiency (McArdle's disease) cannot metabolize intramuscular glycogen, but blood-borne glucose was shown to increase their exercise capacity (9). This observation was clearly demonstrated by ^{31}P MRS in such patients. Lewis et al (21)

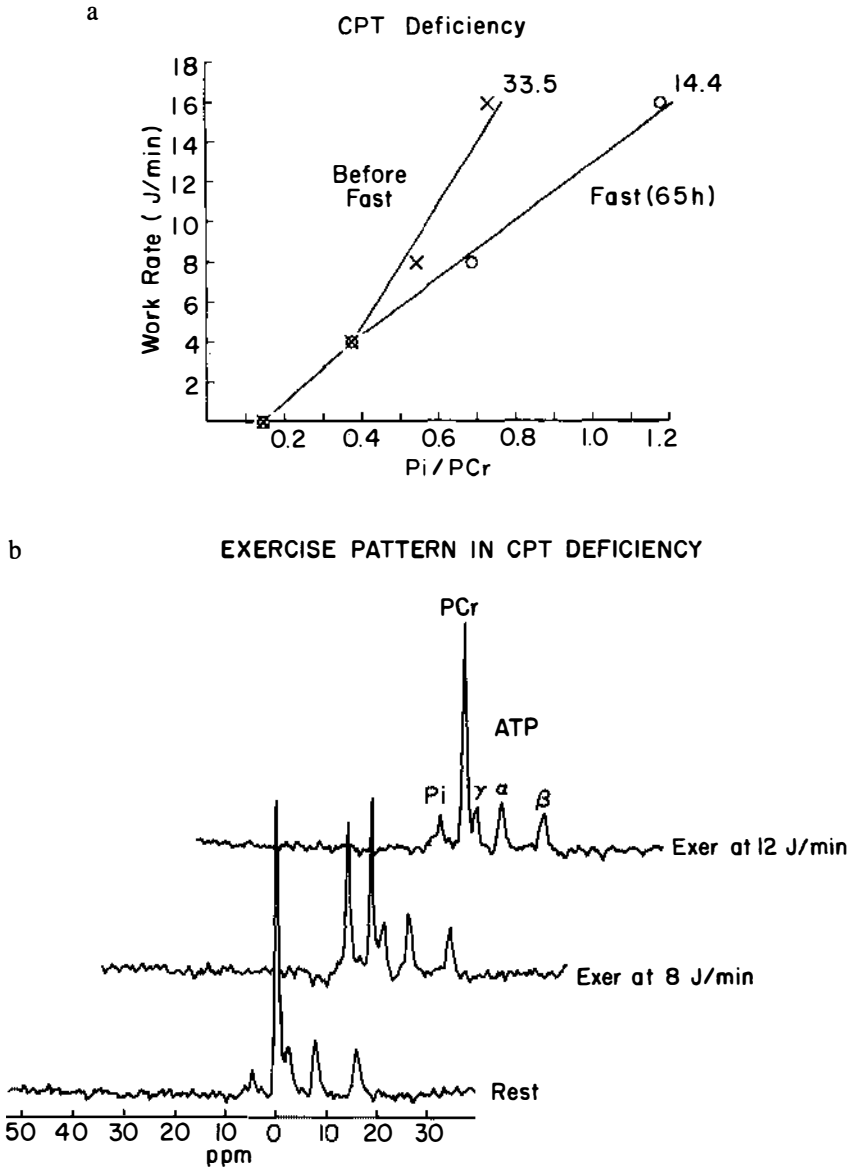

Figure 5 (*a*) Changes in transfer function during fasting of 65 h in human CPT deficiency. The slope fell from 33.5 to 14.4 J/min per P_i per PCr.

(*b*) Metabolic fatigue and return of ^{31}P MRS to rest state despite maximal effort in the same CPT-deficient patient after 72 h of fasting.

showed that end-exercise PCr/P_i was higher in McArdle's patient muscle after a load of glucose. We further demonstrated that the transfer function was improved during the exercise protocol in such patients (2) (Figure 6*b*). A similar approach was used on a patient with CPT deficiency when medium chain triglycerides (which are metabolically available to muscle because they do not use the CPT system) were acutely administered. An improved bioenergetic profile in this patient was noted (Figure 7). Thus, muscle ^{31}P MRS can follow acute dietary supplements in metabolically impaired muscle.

Changes in metabolism can also be monitored by ^{31}P MRS. In a case of mitochondrial Complex III deficiency with severe exercise impairment and muscular weakness, vitamins K_3 and C were used as electron scavengers in high doses by mouth (11). A very prolonged follow-up with ^{31}P MRS showed clearly improved energy metabolism, which was maintained for 8 years and was associated with clinical improvement (1, 3). The use of other vitamins

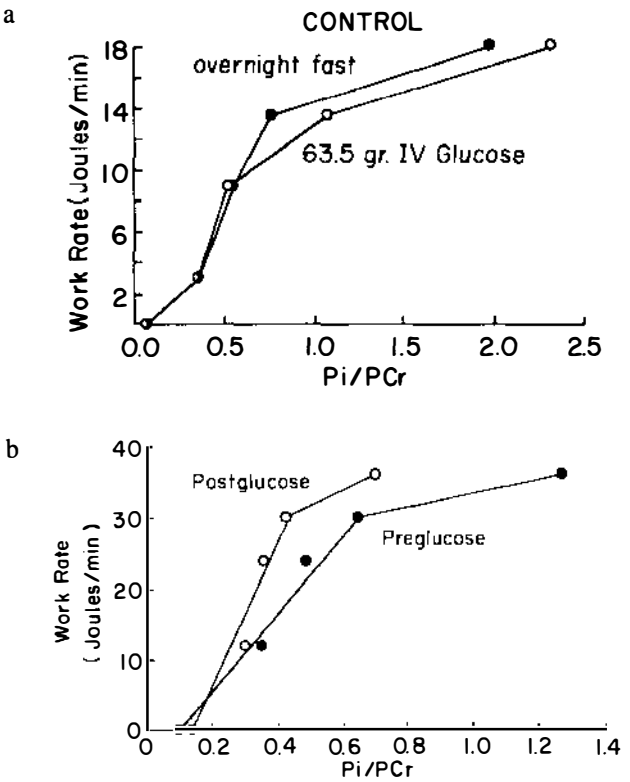

Figure 6 Effect of glucose administration after overnight fast on exercise kinetics in (*a*) normal and (*b*) myophosphorylase-deficient muscle (McArdle's disease). Note that, unlike control, the patient had a rise in the transfer function slope.

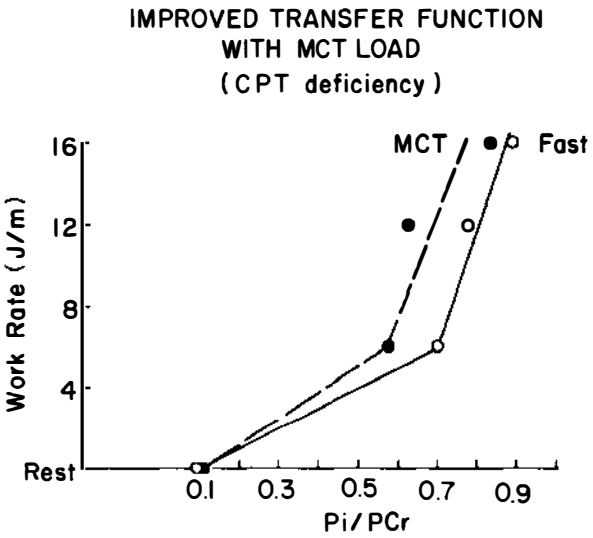

Figure 7 Changes in exercise kinetics in CPT deficiency after medium chain triglyceride load. A tendency for improvement is reflected in the slope.

and co-factors in mitochondrial cytopathies has also resulted in an improved clinical state associated with augmented ^{31}P MRS-measured energy state (23, 25). In a hypotonic infant with vitamin D deficiency, treatment with vitamin D induced a rise in the PCr/ATP that was in synchrony with the clinical improvement (24). All these reports demonstrate the possible use of ^{31}P MRS for monitoring and testing therapies and diet modifications.

Liver

ALCOHOLIC LIVER DISEASE The study of the effects of ethanol on the metabolism of hepatic phosphates serves here as a model for possible use of liver ^{31}P MRS in the study of other nutritional problems. The use of ^{31}P MRS for the study of this problem is not surprising, as liver mitochondria are known to be affected by chronic ethanol ingestion. Cunningham et al (8) studied the effects of acute ethanol administration on hepatic energy state by combining in vivo ^{31}P MRS and direct biochemical assay of freeze-clamped samples. They noted that alcohol administration increased the P_i/ATP ratio by 27%, as measured by ^{31}P MRS, in fed animals but decreased it by 30% in fasting rats. The trend of the ^{31}P MRS observations was confirmed by the biochemical assays, although an order of magnitude difference was detectable between the two methods (higher numbers in the latter). The calculated ADP/ATP and free magnesium were unaffected by ethanol. Helzberg et al (16) performed a similar study on the effects of both chronic and acute ethanol

administration (twice the dose used in the previous experiments) on hepatic energy state. The main finding was that ATP/ADP ratios decreased during chronic but not acute ethanol exposure, confirming the initial observations of Cunningham et al (8) regarding the trend of ATP/ADP changes. HPLC determinations of tissue samples suggested that the cause of this reduced ratio is a combination of a fall in ATP and a rise in ADP levels. The authors, however, noted that ATP/ADP calculated by ^{31}P MRS is higher than that derived from direct assays (contrary to Cunningham's data who had higher ratios on biochemical assays), but again the trend of the alcohol effect was similar in both methods. The reason for the discrepancy between ^{31}P MRS and biochemical assay of ATP/ADP is not clear and is unresolved in these papers; their methods were comparable, although the area under the curve determinations in the study by Helzberg et al appears more accurate. Since the MRS method records only free mobile phosphate compounds in cytoplasmic solution, and analytical assays measure all the cellular concentration (bound and unbound components), these differences will remain; but the encouraging finding is that the trends of both MRS and classical biochemical analysis are well correlated.

In a later study, Takahashi et al (29) studied the progression of alcoholic liver disease and also estimated the changes in pH. The pH determination by ^{31}P MRS in liver is difficult as there is no PCr signal and the calibration is derived from the chemical shift of the alpha phosphate of ATP to the P_i peak (probably assuming unchanged Mg^{2+}). They found that the P_i/ATP rose during the initial phase of alcoholic liver disease and then stabilized, largely as a result of the fluctuations in the P_i peak levels. Similarly, the PME/ATP rose but the pH did not change. The authors conclude that the P_i/ATP change is a direct result of the ethanol effect and is not secondary to liver cell disease, since the latter continues to deteriorate while the P_i/ATP rise stabilizes after the fifth week of exposure. Yet, the authors speculate that the change in energetic state contributes to the overall hepatic disease.

EFFECTS OF FASTING Although not the main focus of each of the above studies by Cunningham et al (8) and Helzberg et al (16), the ethanol data in their papers are compared to the effects of fasting. Cunningham et al (8) found that fasted livers had a 2.3 rise of P_i/ATP ratio, suggesting a lower energy state. The ADP/ATP levels were similar, although less accurately determined. Table 1 in Helzberg et al (16) suggests a fall in ATP/ADP levels due to 24-h starvation in both control group and chronic alcohol-fed animals. Their results were not tested for statistical significance, and a short fast may not induce a marked change.

LIVER METABOLIC CHALLENGE Patients and carriers with fructose intolerance were study by liver ^{31}P MRS during exposure to a dose of fructose

(26). In both patient and carriers fructose ingestion led to an increase in sugar phosphates (the PME peak) with concomitant reduction of the P_i. Such a response was not observed in controls, and this test is advocated as screening for heterozygotes for fructose intolerance in suspected families. The accumulation of hexose phosphates is not surprising, and the reduction of P_i may be due to trapping of inorganic phosphates in the nonmetabolized sugar phosphates, similar to the findings in muscle with phosphofructokinase deficiency (6).

SUMMARY

In conclusion, muscle and liver ^{31}P MRS can be used to assess energy state in various nutritional conditions and to follow different metabolic changes induced by modification of substrate availability. ^{31}P MRS studies of humans and animals suggest that fasting and low caloric intake reduce the oxidative metabolism "efficiency" of muscle during exercise and produce a better bioenergetic state at rest. Liver studies during fasting also indicate a lower energy state. Acute supplements of substrate to working muscle that is affected by a metabolic disease can improve its energy metabolism as observed by ^{31}P MRS. Modification of diets in patients with a metabolic disease can also be followed by this technique, thereby aiding the clinical evaluation of therapies. The acute and chronic effects of alcohol, which reduce the energy metabolism of liver, are another example of nutritional problems studied by ^{31}P MRS both in humans and animal models. A valuable aid in nutritional research, especially in humans, ^{31}P MRS is noninvasive and can be repeated without harmful effects.

Appendix: The Bioenergetic Basis for the ^{31}P MRS Studies

Cytosolic phosphorylation potential (PP) is characteristic of the energy balance between ATP utilization and production and thus represents the energy state of cells and their ability to perform work at any given time. This ratio also plays a direct role in the control of mitochondrial respiration. It is defined as

$$PP = ATP/ADP \times P_i. \quad 1.$$

The creatine kinase (CK) reaction, which rapidly controls the cytosolic ATP concentration and its availability to mitochondria, is

$$PCr + ADP + H^+ \rightleftarrows ATP + Cr \quad \text{(Cr is free creatine).} \quad 2.$$

This reaction is normally at or near the equilibrium. The CK reaction equilibrium constant (K) is

$$K = [ATP] \times [Cr]/[PCr] \times [ADP] \times [H^+].$$

Derived from above is

$$[ADP] = [ATP] \times [Cr] / K \times [PCr] \times [H^+], \quad 3.$$

which when substituted in the PP equation gives:

$$PP = [PCr]/[P_i] \times K \times [H^+] \times 1/[Cr]. \quad 4.$$

From Equation 4, it can be seen that the tissue energetic state is directly proportional to the ^{31}P MRS measurable ratio of PCr/P_i. Thus, PCr/P_i is extensively used in vivo in metabolic observation.

In many studies of human muscle exercise, Chance finds that

$$[ADP] \rightleftharpoons [ATP][P_i]/[PCr] \times K \times [H^+] \quad 5.$$

and for neutral pH and muscle [ATP] and $K = 160$

$$ADP = 33\ P_i/PCr. \quad 6.$$

The rationale for this simple equation is that free Cr equals free P_i, from the equation

$$PCr = Cr + P_i. \quad 7.$$

When substituted in Equation 3, it gives Equation 5. An alternative calculation follows in Equations 8 and 9.

If the tissue concentration of total creatine and ATP is known, the ADP can be directly calculated by in vivo ^{31}P MRS of muscle. This calculation is obtained by further evaluation of Equation 3. K for the CK reaction has a known value. Free creatine [Cr] is determined from the tissue analysis as

$$[Cr] = [Cr]\ total - [PCr]. \quad 8.$$

[PCr] is calculated from PCr/ATP × [ATP]. Thus, the ADP equation is now

$$ATP \times [Cr_{total} - PCr]/K \times [PCr] \times [H^+]. \quad 9.$$

ATP/PCr is calculated from the ^{31}P MRS spectrum and $[H^+]$ is also directly determined from the ^{31}P MRS spectrum, since intracellular pH is measurable.

However, to make these calculations, fully relaxed spectra have to be used because the ratios of PCr/ATP and less PCr/P_i are affected by the mode of ^{31}P MRS spectral collection (i.e. differential saturation).

The above calculations are accurate only when a homogeneous tissue or cell collection is studied by ^{31}P MRS. The estimation of PP and ADP becomes more complicated when an inhomogeneous tissue is studied. In animal muscle Meyer et al (9) have shown that slow twitch, oxidative red muscles have significantly lower PCr/P_i values than fast twitch, glycolytic white muscles. Furthermore, different cells in the recorded sample may react differently to the studied nutritional change. Thus, during all the studies reported here one must remember that we are recording an average value of probably several PCr/P_i ratios.

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