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Isotopic (^{18}O) shift in ^{31}P nuclear magnetic resonance applied to a study of enzyme-catalyzed phosphate–phosphate exchange and phosphate (oxygen)–water exchange reactions

(ADP–P$_i$ exchange/polynucleotide phosphorylase/site of bond cleavage/inorganic pyrophosphatase/oxygen scrambling)

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ABSTRACT An isotopic shift of the ^{31}P nuclear magnetic resonance due to ^{18}O bonded to phosphorus of 0.0206 ppm has been observed in inorganic orthophosphate and adenine nucleotides. Thus, the separation between the resonances of $^{31}P^{18}O_4$ and $^{31}P^{16}O_4$ at 145.7 MHz is 12 Hz and, in a randomized sample containing ~50% ^{18}O, all five ^{16}O–^{18}O species are resolved and separated from each other by 3 Hz. Not only does this yield the $^{18}O/^{16}O$ ratio of the phosphate but, more important, the ^{18}O-labeled phosphate in effect can serve as a double label in following phosphate reactions, for oxygen in all cases and for phosphorus, provided the oxygen does not exchange with solvent water. Thus, it becomes possible to follow labeled phosphorus or labeled oxygen continuously as reactions proceed. Rate studies involving (*i*) phosphorus and (*ii*) oxygen are illustrated by continuous monitoring of the exchange reactions between (*i*) the β phosphate of ADP and inorganic phosphate catalyzed by polynucleotide phosphorylase and (*ii*) inorganic orthophosphate and water catalyzed by yeast inorganic pyrophosphatase. In the ADP–P$_i$ exchange, the P$_i$ ($^{18}O_4$) yielded an α P($^{16}O_3{}^{18}O$) and a β P($^{18}O_4$), proving that bond cleavage occurs between the α P and the α-β bridge oxygen. Among the many additional potential uses of this labeling technique and its spectroscopic observation are: (*i*) different labeling of each phosphate group of ATP, (*ii*) to follow rate of transfer of ^{18}O from a nonphosphate compound such as a carboxylic acid to a phosphate compound, and (*iii*) to follow the rate of scrambling (for example, of the β-γ bridge oxygen of ATP to nonbridge β P positions) and simultaneously the rate of exchange of the γ P nonbridge oxygens with solvent water in various ATPase reactions.

The involvement of a large variety of phosphate compounds in the cell as structural units, metabolic intermediates, and regulatory factors has led to widespread use of radioactive ^{32}P as a tracer and, to a lesser extent, the stable isotope ^{18}O. Both ^{18}O and ^{32}P measurements require separation of individual components of the reaction mixture, and with neither mass spectrometer nor radioactivity measurements is it possible to follow rates of isotopic transfer or exchange continuously. Furthermore, analysis of [^{18}O]phosphate requires considerable chemical manipulation for mass spectrometric analysis (1) and even more complex and ingenious procedures for establishing ^{18}O scrambling in enzymatic reactions of phosphate compounds (2). In this communication, we describe a spectroscopic method for following the phosphate or oxygen of phosphate continuously. The method is based on our finding that substitution of ^{18}O for ^{16}O in phosphate induces a chemical shift in the ^{31}P nuclear magnetic resonance (NMR) spectrum for each O of the four Os substituted and in effect introduces a double label that simultaneously labels the phosphorus and oxygen of phosphates.

The usefulness of phosphate exchange and ^{18}O exchange reactions in elucidating mechanistic aspects of enzymatic reactions has been amply demonstrated (2–4). In particular, the site of bond cleavage has been determined, the existence of intermediates has been established, and rate-determining steps have been identified. Exchange rates of the order of milliseconds between enzyme-bound phosphate compounds at equilibrium have been measured by line broadening of ^{31}P resonances (5, 6), and exchange rates of the order of seconds have been measured by transfer of saturation in ^{31}P NMR (7). The method described in this communication is applicable to phosphate–phosphate and phosphate–oxygen exchanges occurring in minutes or hours.

^{31}P NMR of phosphate (^{18}O) permits continuous monitoring of phosphate transfer from reactant to product or phosphate exchange between them at equilibrium, provided the oxygen is not exchanging with solvent water simultaneously, in which case the label is lost. The latter exchange does not occur spontaneously but is catalyzed by a number of enzymes (4). Phosphate–phosphate exchange rate measurement by ^{31}P NMR is exemplified in this report by the P$_i$ (^{18}O)–ADP (^{16}O) exchange catalyzed by polynucleotide phosphorylase (8). In the same experiment, the site of bond cleavage in ADP is simultaneously established. The resolution of the resonances arising from $P^{18}O_4$, $P^{18}O_3{}^{16}O$, $P^{18}O_2{}^{16}O_2$, $P^{18}O^{16}O_3$, and $P^{16}O_4$ also makes it possible to monitor continuously by ^{31}P NMR the rate of transfer of oxygen into phosphate or of exchange of the oxygen in the absence of net reaction between phosphates as well as scrambling of the oxygen label during reaction. An example is given by the phosphate (93.4% ^{18}O)–water (^{16}O) exchange reaction catalyzed by inorganic pyrophosphatase of yeast (9) and the concomitant scrambling of the four oxygens of inorganic phosphate.

MATERIALS AND METHODS

Potassium dihydrogen phosphate was exchanged with $H_2{}^{18}O$ as described (10) to yield a product containing 44.4% ^{18}O. Phosphoric acid containing 93.4% ^{18}O was a generous gift from I. A. Rose of the Institute of Cancer Research. $H_2{}^{18}O$ was purchased from Miles Chemical Co. Inorganic pyrophosphatase was a gift from B. Cooperman of the University of Pennsylvania, and polynucleotide phosphorylase, type 15 (*Micrococcus luteus*), was purchased from P-L Biochemicals. ADP and Tris were obtained from Sigma Chemical Co. All other reagents used were reagent grade.

NMR Measurements. The ^{31}P spectra were recorded either at 24.3 MHz on a Varian NV14 spectrometer modified to operate in the Fourier transform mode with quadrature detection

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Abbreviation: NMR, nuclear magnetic resonance.

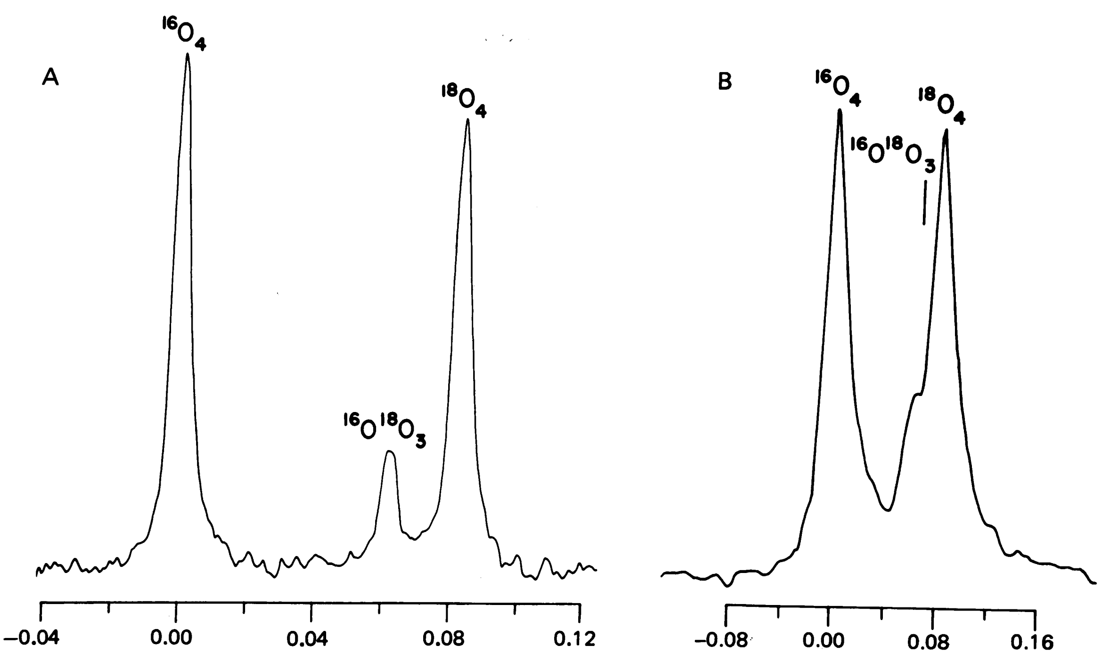


FIG. 1. ^{31}P NMR spectrum of a mixture of P_i (^{16}O) and P_i (^{18}O). (*A*) The solution contained 10 mM P_i (^{16}O), 10 mM P_i (93.4% ^{18}O), and 0.5 mM potassium EDTA in 2 ml of 50% D_2O, pH 8.2. NMR parameters: 145.7 MHz; two scans, acquisition time 13.6 sec; 45° pulse angle. (*B*) The solution contained 25 mM P_i (^{16}O), 25 mM P_i (93.4% ^{18}O), and 3.75 mM potassium EDTA in 0.8 ml of 50% D_2O, pH 8.5. NMR parameters: 24.3 MHz; 400 scans, acquisition time 6 sec; 60° pulse angle.

or at 145.7 MHz on a Bruker WH 360. Samples of 1 ml in 8-mm tubes, maintained at 29°, were used at 24.3 MHz; at 145.7 MHz, the sample volume was 2 ml in 10-mm tubes maintained at 22°.

RESULTS

Isotopic (^{18}O) Shift of ^{31}P Resonance. The ^{31}P spectra of a mixture of 10 mM P_i ($^{16}O_4$) and 10 mM P_i (93.4% ^{18}O) are shown in Fig. 1. The latter contained the following fractions by statistical distribution: 0.76 $^{18}O_4$, 0.215 $^{18}O_3{}^{16}O$, and 0.023 $^{18}O_2{}^{16}O_2$; other species were negligible. The separation between the $^{16}O_4$ species and the $^{18}O_4$ species at 145.7 MHz (Fig. 1*A*) was 12 Hz and all peaks were well resolved; at this signal-to-noise ratio, the $^{18}O_2{}^{16}O_2$ (0.023) species cannot be observed. The percentage ^{18}O calculated from the ratio of the intensities of the $^{18}O_3{}^{16}O$ and $^{18}O_4$ peaks from the binomial distribution is 93.9% compared to 93.4% obtained by mass spectrometric analysis. At 24.3 MHz (Fig. 1*B*), the separation between $P^{18}O_4$ and $P^{16}O_4$ peaks was only 2 Hz; the $P^{18}O_4$ and $P^{18}O_3{}^{16}O$ peaks, which were separated by 0.5 Hz, were only partially resolved. In Fig. 2, the spectrum of KH_2PO_4, prepared by equilibration with $H_2{}^{18}O$ (∼51% ^{18}O) shows all five possible species with the following fractional distribution of peak intensities: $^{16}O_4$ = 0.098, $^{16}O_3{}^{18}O$ = 0.302, $^{16}O_2{}^{18}O_2$ = 0.367, $^{16}O^{18}O_3$ = 0.194, and $^{18}O_4$ = 0.038. From this distribution, the $^{18}O/(^{18}O + {}^{16}O)$ ratio is calculated to be 0.444. On a statistical basis, the calculated values for the distribution of species in a sample of KH_2PO_4 containing 44.4% ^{18}O in the sample are 0.096, 0.305, 0.366, 0.195, and 0.039, respectively.

Exchange of Phosphate (^{18}O) with ADP (^{16}O). The exchange reaction $P_i \rightleftarrows$ ADP catalyzed by polynucleotide phosphorylase, a nucleotidyl transferring enzyme (8), usually measured with ^{32}P does not involve any ^{18}O exchange with the solvent water. Therefore, it should be possible to follow the exchange reaction between P_i (^{18}O) and the β phosphate of ADP (^{16}O). Initial, intermediate, and final time points of such an exchange experiment are shown in Fig. 3. The results show that, as P_i (93.4% ^{18}O) (see Fig. 1 for detailed spectrum of this species) exchanged with the β P of ADP (^{16}O), the $P^{16}O_4$ peak of P_i increase with time as the $P^{18}O_4$ and its accompanying $P^{18}O_3{}^{16}O$ decreased with time. The inverse growth pattern of ^{18}O species with time was observed for the β P of ADP. Because 15 mM ADP and 10 mM P_i were initially present, if no net reaction occurred at equilibrium, one would expect the transferred peak of P_i ($P^{16}O_4$) to be 1.5 times the original P_i peaks and an equivalent ratio of 2:3 for the new to the original peak of ADP. These quantitative relationships do not hold exactly because a small amount of oligo(A) was formed as observed in Fig. 3 at ∼3.7 ppm upfield from P_i.

The ^{31}P spectrum of the final equilibrium mixture at 145.7 MHz is shown in Fig. 4 with the P_i, α P, and β P resonances of

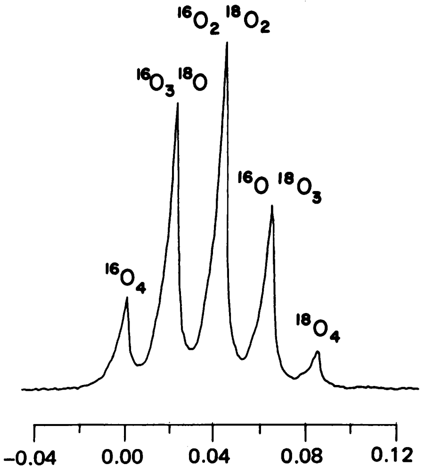


FIG. 2. ^{31}P NMR spectrum of a randomized sample of P_i (^{16}O, ^{18}O). The solution contained 50 mM P_i (∼44% ^{18}O) and 0.5 mM potassium EDTA in 2 ml of 50% D_2O, pH 8.2. NMR parameters: 145.7 MHz; 900 scans, acquisition time 8.2 sec.; 45° pulse angle.

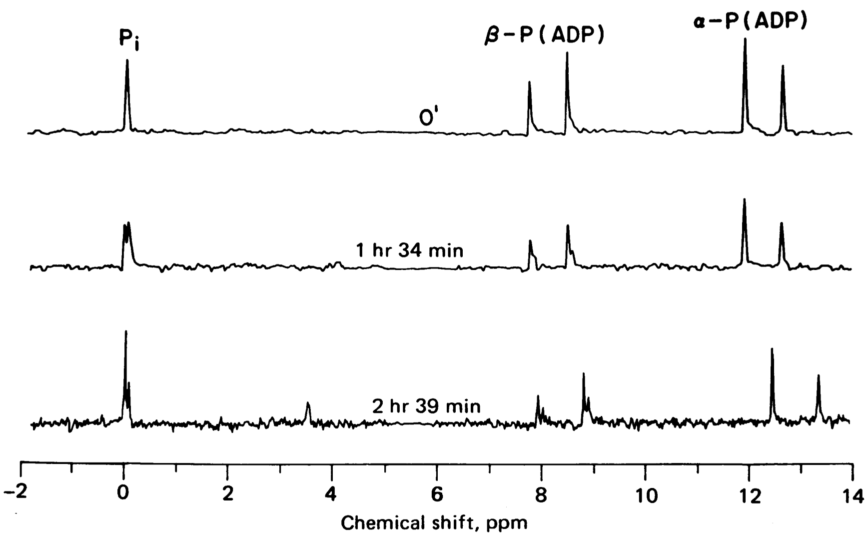
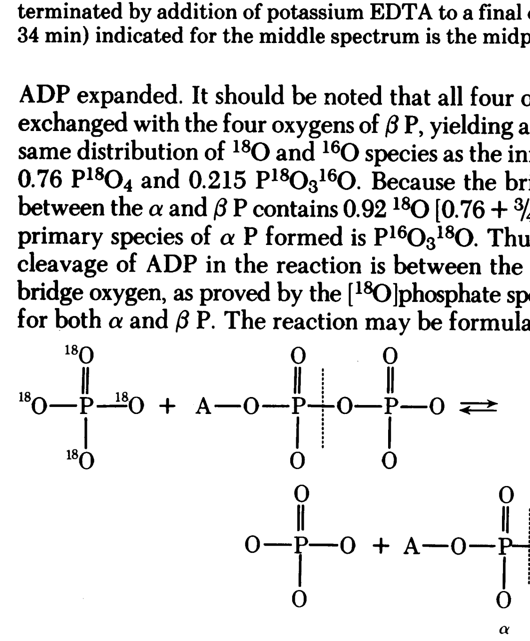


FIG. 3. ^{31}P NMR spectrum of the ADP-P_i exchange reaction catalyzed by polynucleotide phosphorylase at 29°. Initial conditions: 15 mM ADP (^{16}O), 10 mM P_i (93.4% ^{18}O), 20 mM $MgCl_2$, 1 mM potassium EDTA, and 0.1 M Tris-HCl buffer, pH 8.5, in 1 ml of 50% D_2O. After the top spectrum was recorded, the reaction was initiated by addition of 5 units of enzyme. The bottom spectrum was recorded after the reaction was terminated by addition of potassium EDTA to a final concentration of 50 mM. Each spectrum was accumulated for 18 min, and the time (1 hr 34 min) indicated for the middle spectrum is the midpoint of the accumulation period.

ADP expanded. It should be noted that all four oxygens of P_i exchanged with the four oxygens of β P, yielding a β P with the same distribution of ^{18}O and ^{16}O species as the initial P_i—i.e., 0.76 $P^{18}O_4$ and 0.215 $P^{18}O_3{}^{16}O$. Because the bridge oxygen between the α and β P contains 0.92 ^{18}O [0.76 + ¾(0.215)], the primary species of α P formed is $P^{16}O_3{}^{18}O$. Thus, the site of cleavage of ADP in the reaction is between the α P and the bridge oxygen, as proved by the [^{18}O]phosphate species formed for both α and β P. The reaction may be formulated as:



Exchange of Phosphate (^{18}O) with $H_2{}^{16}O$. The oxygen of inorganic orthophosphate does not exchange with water spontaneously at a measurable rate at room temperature, but a number of enzymes catalyze this reaction (4). The ^{31}P spectra of orthophosphate (93.4% ^{18}O) in Fig. 5 taken at different times after initiation of the reaction by the addition of inorganic pyrophosphatase shows the rise and fall of each intermediate between $^{18}O_4$ and $^{16}O_4$ as the exchange reaction proceeds. Computer simulations of such a process have been presented by Eargle *et al.* (11). No attempt has been made to determine the actual rate in this case because the peak heights are not a true measure of the concentration of each species, due to overlap of the resonances at 24.3 MHz. Although these spectra could be analyzed for concentration of each species, the spectra at 145.7 MHz (see Fig. 2) give the concentrations from peak heights directly, and future experiments should be done at the higher frequency.

DISCUSSION

Isotope effects of ^2H on hydrogen (12) and fluorine (13) chemical shifts have been observed and have been ascribed to differences in zero-point vibrational functions (14). The existence of an observable chemical shift of the ^{31}P NMR resonance due to ^{18}O substitution in the phosphate group opens up many potential uses of this phenomenon for the study of biochemical reactions. A few have been demonstrated in the experiments reported, including (*i*) a nondestructive method for the determination of high concentrations of ^{18}O (~5–95%) from the distribution of $^{16}O^{18}O$ species (Figs. 1 and 2); (*ii*) continuous monitoring of phosphate (oxygen)–water exchange as illustrated in inorganic pyrophosphatase-catalyzed reaction; (*iii*) continuous monitoring of $PO_4 \rightleftarrows XOPO_3$ exchange as illustrated by polynucleotide phosphorylase catalyzed exchange; and (*iv*) the site of bond cleavage in such reactions.

Bond cleavage of ATP between the β-γ bridge oxygen and the γ P (15) in the 3-phosphoglycerate kinase-catalyzed reaction has been demonstrated by following the ^{31}P NMR of the P_i–ATP exchange catalyzed by the coupled reactions of glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase.* In this set of reactions, the γ phosphate formed from $P^{18}O_4$ of P_i is $P^{18}O_3{}^{16}O$, in contrast to the $P^{18}O_4$ species that appears in the β P of ADP in the polynucleotide phosphorylase reaction. (The details of these experiments will be published elsewhere.) Obviously, rates of phosphate–phosphate exchange measurements can be generalized to any reactant–product pair such as ATP-PP_i. To follow rates of phosphate exchange, sufficient resolution is available at low frequency, and a 60-MHz instrument (24.3 MHz for ^{31}P) suffices. For oxygen exchange, a 360-MHz instrument (145.7 MHz for ^{31}P) is necessary to obtain accurate rate measurements.

The synthesis of ATP with each phosphate labeled differently with ^{18}O would permit the fate of each phosphate moiety to be followed: for example, in the type of reaction of phospho*enol*pyruvate synthetase in which it is not obvious whether the source of the phosphate of phospho*enol*pyruvate is the β or γ P of ATP. Thus, the advantages of this method of labeling phosphate groups over the use of ^{32}P are the possibility of multiple distinguishable labels, the removal of the need to separate reaction components, and the ability to monitor con-

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* M. Cohn and A. Hu, unpublished data.

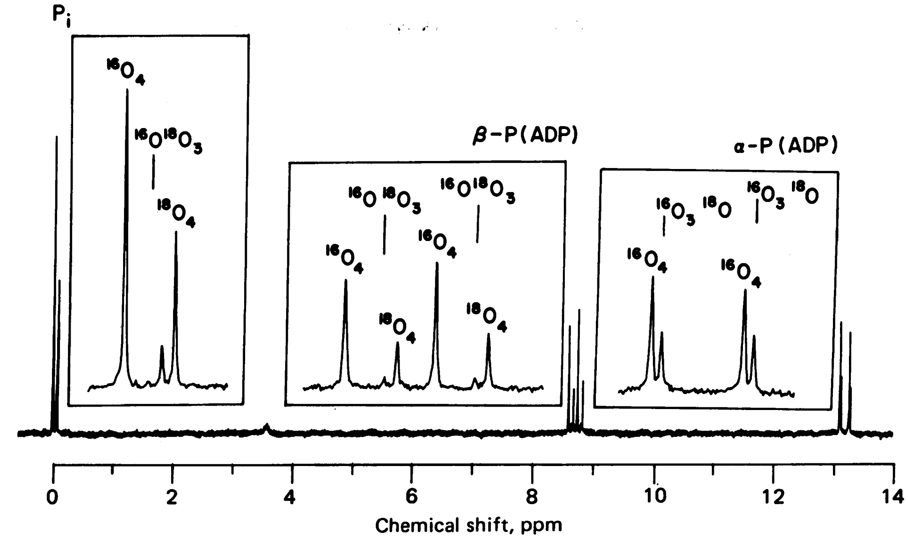

FIG. 4. ^{31}P NMR spectrum of equilibrated ADP–P$_i$ exchange reaction. The same reaction mixture shown in the bottom trace of Fig. 3 was recorded at 145.7 MHz and each ^{31}P is expanded to indicate the isotopic species.

tinuously all reaction components simultaneously. The serious disadvantages are the relatively large amounts of substrates that must be used, the high concentrations of ^{18}O, and the need for an NMR spectrometer (of high frequency for some applications). A less serious limitation is the loss of the label by ^{18}O exchange with solvent water, a reaction catalyzed by relatively few enzymes.

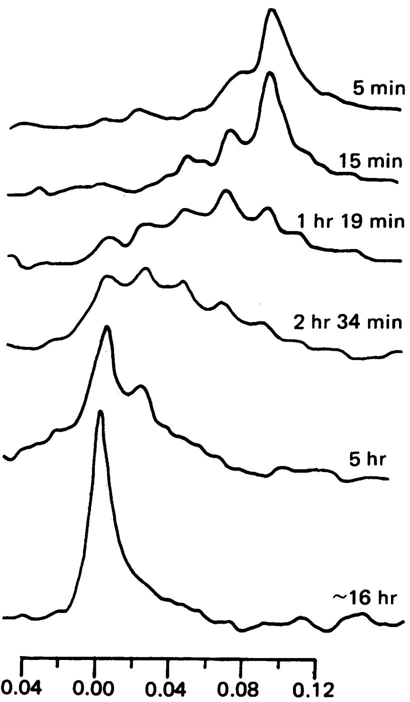

FIG. 5. Time course of P$_i$ (^{18}O)–water exchange catalyzed by yeast inorganic pyrophosphatase. The solution contained 20 mM P$_i$ (93.4% ^{18}O), 21 mM $MgCl_2$, 1 mM potassium EDTA, and 2.68 μg of enzyme in 1 ml of D_2O; the pH was 7.0. Each ^{31}P NMR spectrum was accumulated for 8 min at 24.3 MHz with 40 scans, an acquisition time of 6 sec, and a pulse angle of 60°.

For most reactions in which ^{18}O is to be followed with time, whether it is phosphate–water exchange (4) or the transfer of oxygen from a carboxyl group to a phosphate as in the succinyl-CoA synthetase reaction (16) or the rate of scrambling of the β-γ bridge oxygen of ATP in the glutamine synthetase reaction (2), ^{31}P NMR is the method of choice compared to mass spectrometric analysis.

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