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^{31}P nuclear magnetic resonance studies of HeLa cells

(human astrocytoma/acid-soluble phosphates/metabolic inhibitors)

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ABSTRACT **A survey of phosphorus compounds present in HeLa cells and their acid extracts has been carried out by ^{31}P nuclear magnetic resonance spectroscopy at 40 MHz. The proton decoupled ^{31}P spectrum of the neutralized extract had resolution adequate to enable the identification of the main phosphate compounds. The spectral intensities were converted to concentrations. The lower detection limit with extensive signal averaging was 0.02 μmol for the extract. The composition, listed in order of decreasing concentration, was: inorganic phosphate, ATP, phosphorylcholine, creatine phosphate, UTP, NAD^+, glucose 6-phosphate, β-D-fructose 1,6-bisphosphate, α-D-fructose 1,6-bisphosphate, ADP, α-glycerophosphorylcholine, and α-glycerophosphorylethanolamine. UTP made up 1/5 of the total nucleotide triphosphate content. The composition was compared to the ^{31}P spectrum of an extract from a human astrocytoma grown in athymic mice. The signal from P-containing macromolecules such as nucleic acids was not detected in the intact HeLa cell spectrum because of broad lines. Effects of the glycolysis inhibitor iodoacetic acid could be clearly shown in spectra of both the intact cell and the extract as buildup of fructose 1,6-bisphosphate at the expense of ATP, UTP, and creatine phosphate.**

Advances in instrumental technology in nuclear magnetic resonance (NMR) spectroscopy have permitted enhanced observation of phosphorus nuclei in biological systems. The technique has recently been applied to the study of cells and tissues. Intracellular phosphorus compounds were first observed by NMR in erythrocytes (1, 2). This enabled the measurement of intracellular pH (1) as well as of concentration changes of several metabolites including 2,3-diphosphoglycerate and ATP (2). The technique has been applied to various tissues (3–10) and cells (11–13) and recently to Ehrlich ascites tumor cells (11). We report here the application of ^{31}P NMR at 40 MHz to the analysis of human cancer cells (HeLa) grown from tissue culture. Also included is a comparison with the ^{31}P NMR spectrum of a human astrocytoma. A survey of individual phosphorus compounds and their concentrations in tumor cells may yield clues to tumor metabolism. The effect of inhibition of glycolysis on the phosphorus profile in HeLa cells is also examined. Intracellular relaxation in HeLa cells will be described elsewhere.

MATERIALS AND METHODS

HeLa cells were grown in suspension culture according to standard procedure in Joklik modified minimum essential medium containing *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes) buffer (0.015 M), penicillin, streptomycin, ampicillin, and fetal calf serum (10%). The concentration of inorganic phosphate in the medium was 0.01 M. The cells were grown to a density just below confluency (2×10^6 cells per ml).

Cell extracts were prepared by addition of 5 ml of H_2O and 0.5 ml of 35% perchloric acid at 0° followed by sonication. The supernatant pH was adjusted to 7.4 within 30 min with 1 M KOH, and the $KClO_4$ precipitate was removed. The solution was lyophilized and dissolved in 5% EDTA at pH 7.4.

The NMR spectra were recorded at 40.29 MHz on a Jeol PS-100 NMR spectrometer using a deuterium lock and a standard phase sensitive detector and equipped with a Nicolet Technology Corporation 1080 series computer. Samples were placed in a 10-mm NMR tube containing a vortex plug, and the spectra were recorded with the sample spinning.

RESULTS AND DISCUSSION

The 40-MHz proton decoupled ^{31}P NMR spectrum of HeLa cells is presented in Fig. 1*a*. The spectrum contained seven broad peaks with some splitting present. The linewidths are approximately proportional to field strength.* Hence, the resolution does not appreciably improve at high field.* The chemical shifts of peaks I–VII span from −4 to +21 ppm, which is the characteristic range for biological phosphate compounds.

The resolution limitations of the intact cell spectrum (Fig. 1*a*) as well as the sensitivity of phosphate chemical shifts to the environment hamper direct identification of the compounds comprising the cell spectrum. Extracts provide an approach for identification (4).

The phosphorus compositions in the acid extract spectrum and in the cell spectrum were similar (Fig. 1), but some differences existed besides the resolution. Peak III of the cells does not appear to have a corresponding signal in the extract. The chemical shift of peak III is the same as phospholipids, which are not acid-soluble. Two inorganic phosphate peaks (peak II) arise from inorganic phosphate inside and outside the cell. The larger inorganic phosphate peak in the extract (Figs. 1*b* and 2) indicates that some phosphate hydrolysis occurred during the acid extraction. Differences in chemical shift between the cell and the extract result from the sensitivity of phosphorus chemical shifts to environment. For example, peak VII is somewhat downfield from the corresponding peak in the extract (Fig. 1). This is probably due at least in part to cation binding (5). Lastly, some signals are only detectable in the extract because of enhanced signal and resolution.

Cells contain more total phosphorus than do their acid extracts. In tumor tissues, 25–30% of the total cell phosphorus is acid-soluble (14). Most of the phosphorus (45%) is in nucleic acid (14). Nucleic acids, both DNA and RNA, have chemical shifts between 0 and 1 ppm. Obviously, the nucleic acids are undetected in the cell spectrum (Fig. 1*a*), being obscured by their large linewidths.*

Chemical shifts of known compounds were used to identify

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Abbreviation: NMR, nuclear magnetic resonance.

* To be reported elsewhere by F. F. Evans and N. O. Kaplan.

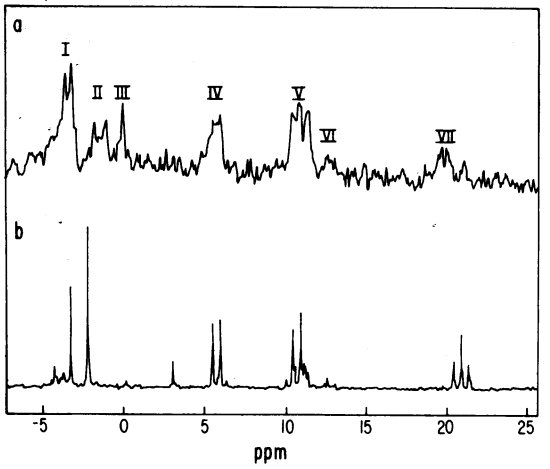

FIG. 1. (*a*) The 40 MHz ^{31}P NMR spectrum of a dense suspension of HeLa cells recorded at 4° with continuous broad band proton irradiation; 2400 70° pulses were applied at 2.8-sec intervals. The free induction decay was multiplied by an exponential function, resulting in 3-Hz line broadening. (*b*) ^{31}P NMR spectrum of acid extract of HeLa cells neutralized to pH 7.4 to facilitate comparisons with the intact cells. The spectrum was recorded at room temperature with proton irradiation applied only during data acquisition; 24,000 70° pulses were applied at 9.6-sec intervals (this required 60 hr). An 0.5-Hz line broadening function was used.

the main compounds in the extract. The uniqueness of a particular chemical shift, along with associated couplings, determines the reliability of this simple approach. The coupling constants present in the proton decoupled extract spectrum are of the ^{2}Jpp variety and these have magnitudes of about 20 Hz. The α, β, and γ chemical shifts of ATP are present in the extract, which supports the assignments of these resonances to ATP (Fig. 2). Adjacent to the α-P resonances of ATP are two narrowly separated peaks assigned to NAD (11 ppm) (Fig. 2). There are two individual phosphorus atoms of NAD, but they do not exhibit ^{2}Jpp because of their near magnetic equivalence at 40 MHz.

A noteworthy part of the extract spectrum is the presence, near the β resonances of ATP, of another triplet coupling pattern (20 ppm, Fig. 2). It must arise from another triphosphate

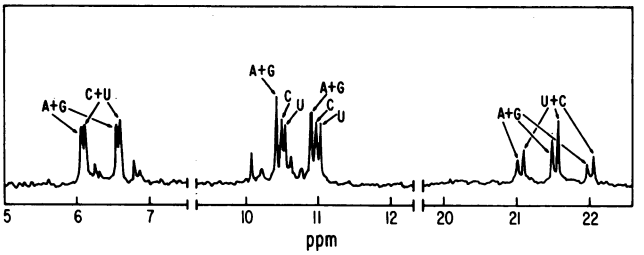

FIG. 3. ^{31}P NMR spectrum of a mixture of ATP (A), GTP (G), UTP (U), and CTP (C) at pH 7.4 to illustrate the extent of resolution at 40 MHz. The small unlabeled resonances are those of ADP and CDP.

linkage. To test the resolving power of nucleotide triphosphates observed at 40 MHz, the spectrum of a mixture of ATP, GTP, UTP, and CTP at pH 7.4 was recorded (Fig. 3). The purine triphosphates ATP and GTP were indistinguishable.† All three phosphorus atoms of the purine triphosphates could be resolved from the pyrimidine triphosphates UTP and CTP. The magnitude of the chemical shift separations between ATP and UTP can be computed from data in Table 1. The peak separations are in the order $\alpha > \beta > \gamma$ phosphorus. UTP and CTP were distinguishable only at the α position (Fig. 3). A correlation between ^{31}P chemical shift and backbone conformation has been observed in β-5′ mononucleotides (unpublished data). Each of the triphosphates was directly added to extracts, and the effect on the extract spectrum was examined. The UTP not only matched the extra triplet found at 20 ppm but also matched peaks with corresponding intensities in the α and γ parts of the spectrum (Fig. 4). It is probable that UTP is present at fairly high concentrations (20% that of ATP) in HeLa cells. CTP could not be detected. Small quantities of CTP would be overlooked due to resolution problems stemming from the nearby α resonances of ATP and UTP. The spectrum of dATP was also recorded. The α phosphorus chemical shift was 0.06 ppm *downfield* from that of ATP.

† The chemical shifts are sensitive to pH. ATP and GTP can be resolved at high or low pH at 40 MHz. However, at high pH, the peak separation is too small to be useful for the analysis of extracts. High field operation will resolve the two nucleotides. At low pH, line broadening from paramagnetic ions in the extract is a problem.

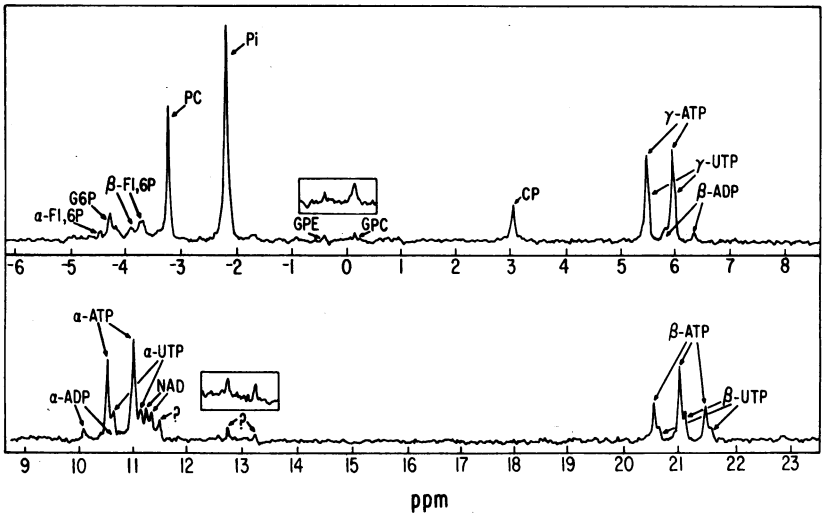

FIG. 2. Spectrum of extract, shown in Fig. 1*b*, split and presented in expanded form with assignments. (*Insets*) Obtained from different HeLa cell extracts recorded under more rapid pulsing conditions. Their purpose is to show that the corresponding small peaks in the main spectrum are real. Nonstandard abbreviations: F1,6P, fructose 1,6-bisphosphate; G6P, glucose 6-phosphate; PC, phosphorylcholine.

Table 1. Acid-soluble phosphates[a]

Compound	Chemical shift	μmol[b]
α-D-Fructose 1,6-bisphosphate[c]	−4.43	0.03
D-Glucose 6-phosphate[d]	−4.25	0.17
Unknown[e]	−4.15	0.06
β-D-Fructose 1,6-bisphosphate[c]	−3.88, 3.71	0.09
Unknown[f]	−3.65	0.11
Phosphorylcholine	−3.19	0.913
Inorganic phosphate	−2.12	1.92
α-Glycerophosphorylethanolamine	−0.36	0.02
α-Glycerophosphorylcholine	0.17	0.02
Creatine phosphate	3.09	0.33[g]
NAD^+[c]	11.25, 11.35	0.21
Unknown[c]	11.49	0.08
Unknown[c]	13.04	0.11
α-ADP	10.37	0.12
β-ADP	6.26	0.11
α-ATP	10.78	1.40[h]
β-ATP	21.25	1.26
γ-ATP	5.75	1.23
α-UTP	10.91	0.45[h]
β-UTP	21.33	0.31
γ-UTP	5.79	0.29
Total		9.77

[a] Total integrated intensities from the ^{31}P NMR spectrum (Fig. 2) were calibrated by analytical phosphate analysis to μmol of phosphorus. See legend of Fig. 1*b* for data-acquiring conditions.
[b] From 1.2×10^9 HeLa cells (Fig. 2).
[c] Two mol of phosphorus per mol of compound.
[d] α and β forms not resolved at 40 MHz.
[e] The chemical shift of glyceraldehyde 3-phosphate, fructose 1-phosphate, and glycerophosphate is in this region.
[f] The chemical shift of dihydroxyacetone phosphate, fructose 6-phosphate, serine phosphate, and AMP is in this region. Resolution and signal-to-noise limitations hamper assignment.
[g] Creatine phosphate is acid-labile (15). This value is probably low even though the acid extract was neutralized within 30 min.
[h] The α phosphorus of the nucleotide triphosphates appears to be in higher concentrations due to peak overlap problems in the α region (see *text*).

The peaks in the range 10–12 ppm (Fig. 2) arise from compounds in the P—O—P type linkages. The extract peaks come from γ-ADP, γ-ATP, γ-UTP, and NAD (Fig. 2). The doublet at 13 ppm is interesting in that it is set aside by about 2 ppm from the mainstream of peaks, and it has not yet been identified. A number of compounds including coenzyme A, FAD, uridine diphosphoglucose, cytidine diphosphocholine $NADP^+$, NADPH, and common nucleotide di- and triphosphates have been added without positive results. In principle, it should be possible to gain clues to the nature of this compound from its ^{31}P NMR parameters. The proton phosphorus coupling(s) would be useful, but we have not obtained a proton coupled spectrum with adequate signal for this weak resonance.* However, the ^{31}P chemical shifts alone are useful. The doublet at 13 ppm is separated by 20 Hz which is typical of ^2Jpp coupling. Furthermore, the intensities are skewed in the way expected for an "AB" coupling pattern. The complementary doublet is hidden by the other more intense peaks in the 10- to 12-ppm region. Thus, it is very likely the unknown compound has a P—O—P type linkage also. The 2-ppm upfield shift of one of the two phosphorus atoms remains to be considered. A nearby nitrogen atom can cause an upfield shift as exemplified by phosphorylcholine and creatine phosphate (Table 1). Cytidine diphosphocholine was checked, but its chemical shifts were not particularly close to the unknown resonance at 13 ppm. Another factor that can cause upfield shifts is proximity to an anomeric carbon—for example, glucose 1-phosphate resides 1.97 ppm upfield from glucose 6-phosphate. Added uridine diphosphoglucose had a chemical shift very close but not identical to that of the unknown. We suggest that the unknown may be a nucleoside diphosphosugar.

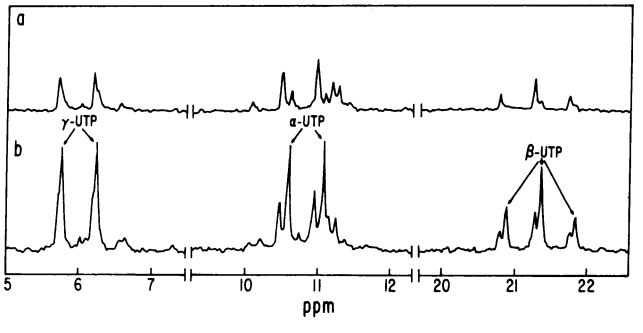

FIG. 4. (*a*) Spectrum of the triphosphate regions of a HeLa cell extract at pH 7.4. (*b*) Spectrum of the same extract with UTP added to it enables the identification of the UTP peaks in *a*.

The extract peaks at 6 ppm arise mainly from γ-ATP, γ-UTP, and β-ADP (Fig. 2). The remaining peaks downfield of 6 ppm are singlets, which indicates monophosphate linkages. The prominent peak at 3 ppm originates from creatine phosphate (Fig. 2). Measurement of creatine phosphate was not quantitative because of hydrolysis in acid (15). Further downfield were two small resonances of glycero-3-phosphorylethanolamine and glycero-3-phosphorylcholine. The two intense downfield resonances are inorganic phosphate and phosphorylcholine. Present at much lower concentration are fructose 1,6-bisphosphate and glucose 6-phosphate (Fig. 2). There is a resolution problem in identifying the remaining peaks in this region. Because of the number of glycolysis intermediates and sugar phosphates, several compounds have indistinguishable chemical shifts at 40 MHz. Several possible assignments for this region are given in Table 1. In such cases, comparison with concentration data provided by independent analytical techniques and observation at a higher field are desirable.

Peak intensities in an NMR experiment can be converted to concentrations by consideration of the relaxation parameters and use of a calibrating standard. The data-acquiring conditions given in Fig. 1*b* were selected so as to minimize differences in relative intensities due to differences between relaxation parameters. The intensities of the compounds in Fig. 2 were determined and the sum was calibrated to the total phosphate present as determined by analytical total phosphorus analysis (15,16). The results are presented in Table 1. There are some small variations. For example, the α, β, and γ phosphorus atoms of ATP must be present in the same amount, yet in Table 1 the quantities are 1.40, 1.26, and 1.23. The discrepancy here is caused by overlapping of peaks. The β and γ peaks lie in fairly well isolated regions of the spectrum and are similar in their calculated molar quantities. The α P value probably is higher because other peaks reside underneath it. The same trend is seen with the α, β, and γ phosphorus atoms of UTP. In fact, the doublet of unknown origin at 13 ppm has a complementary doublet in the 10–12 ppm region. This complementary doublet is hidden under the α-ATP or α-UTP peak and contributes to the extra apparent intensity. After subtraction of the 0.11-μmol contribution of the unknown compound, the α phosphorus becomes much closer to the values of the β and γ phosphorus atoms. Still, however, there is a 0.19-μmol difference to be ac-

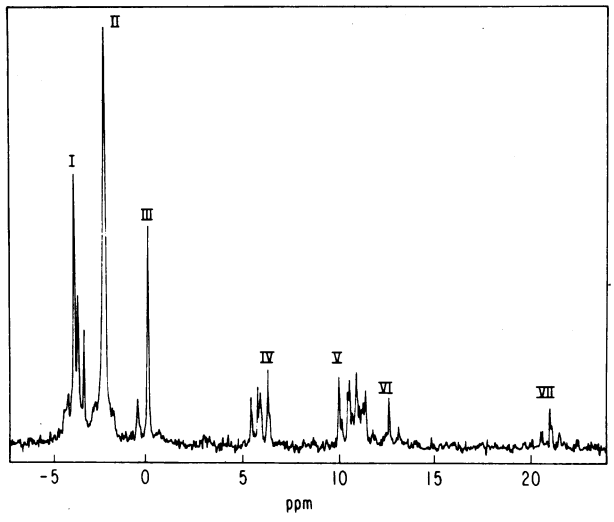

FIG. 5. Spectrum of an extract from a human astrocytoma.

counted for. This difference may be due, at least in part, to the presence of still other compounds at low concentration such as NADH, NADPH, and coenzyme A. Differences between relaxation parameters may contribute to intensity differences. Another source of error in the intensity measurements is the signal-to-noise limitations of individual peaks.

The list of acid-soluble phosphates (Table 1) shows several compounds at high levels. ATP is present at 1.24, phosphorylcholine at 0.93, creatine phosphate at 0.33, and UTP at 0.30 μmol. Approximately 7% of the phosphorus remains unidentified at present. The high level of UTP in HeLa cells is interesting in that this has not been detected in any other tissue or cell lines, including Ehrlich ascites cells (11), by ^{31}P NMR.

It is of interest to compare the phosphorus composition of HeLa tumor cells with that of other tumor cell lines and tissues as well as with that of nontumor cells. In Fig. 5 is illustrated the ^{31}P spectrum obtained from an extract of an astrocytoma human tumor grown in athymic mice. This P profile shows three overall differences compared to HeLa cells (Fig. 1). The sugar phosphate region (Fig. 5, peak I) is more complex in the astrocytoma. The ratio of ATP to ADP (peak IV) is lower in astrocytoma. The relative concentrations of α-glycero-3-phosphorylcholine and α-glycero-3-phosphorylethanolamine (peak III) are substantially higher in the astrocytoma. In human breast tumors grown in athymic mice, we have found that α-glycero-3-phosphorylcholine is present at higher concentration than inorganic phosphate (unpublished data). Preliminary inspection of the β phosphorus region of triphosphates (peak VII) shows the presence of UTP in the astrocytoma. The phosphorus composition in a number of different human tumors will be presented elsewhere.

Reports from other laboratories have at present only appeared on Ehrlich ascites mouse cells (11). Because concentrations have not yet been reported, it is only possible to compare the actual NMR spectra of the Ehrlich ascites cells and the HeLa cells qualitatively. HeLa cells are like Ehrlich ascites cells in possessing relatively high levels of phsophorylcholine. ATP and NAD^+ are also prominent in both. Low concentrations of glycero-3-phosphorylcholine and glycero-3-phosphorylethanolamine (11) are present in both cell lines. Differences lie in the absence of UTP and creatine phosphate resonances in the Ehrlich ascites cells. These compounds appear to be in high concentration in HeLa cells. At lower concentration is the unknown HeLa cell compound at 12 ppm. This is undetected in

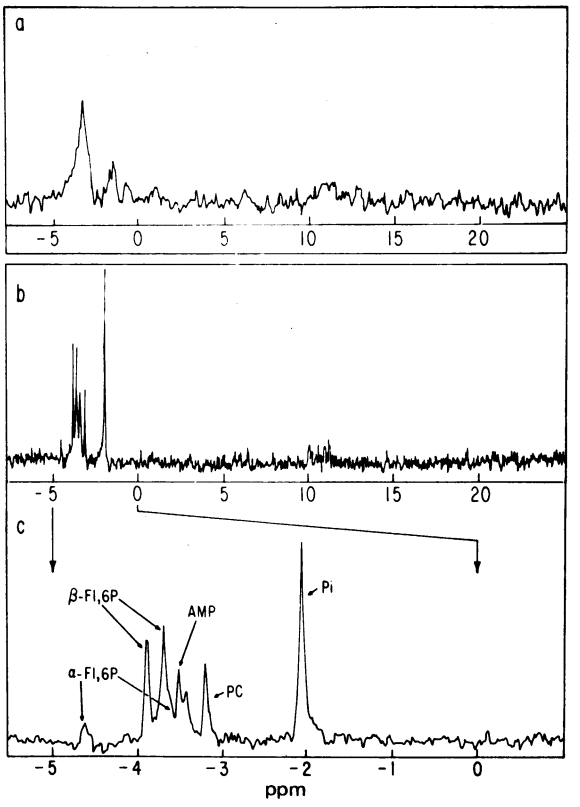

FIG. 6. (*a*) Spectrum of intact HeLa cells after incubation in the presence of iodoacetic acid, showing redistribution of phosphates. (*b*) Extract of the above cells. (*c*) Region between −5 and 0 ppm of *b* is expanded and the resonances labeled. The unlabeled resonance upfield of AMP has a chemical shift the same as that of UMP.

the Ehrlich ascites cells also (11). In the Ehrlich ascites cells, both β-D- and α-D-fructose 1,6-bisphosphate are present at a higher concentration. Other cell lines need to be surveyed for further comparison. Differences between the HeLa cells and Ehrlich ascites cells (11) may arise partly from differences in cell growth and handling procedures.

In addition to comparisons between cell lines or tissues, it is of interest to survey changes in the phosphate composition caused by various agents. Because glucose is a main source of energy for the HeLa cells, inhibition of glycolysis, as with added iodoacetic acid, should drastically change the phosphorus composition. The NMR spectrum of HeLa cells preincubated in the presence of iodoacetic acid is shown in Fig. 6. There was considerable alteration from the normal spectrum (Fig. 1) and the differences are consistent with the known inhibition of the glyceraldehyde-3-phosphate dehydrogenase reaction by iodoacetic acid. The resonances of highest intensity are those of β-D-fructose 1, 6-bisphosphate. This is the classical result expected. ATP is used up in initiating phosphorylations at the early steps of glycolysis. The blockage in glycolysis therefore causes a buildup in the sugar phosphates and prevents replenishment of ATP. The UTP and creatine phosphate supply has also been exhausted. The effect of the uncoupler dinitrophenol on the phosphorus composition also was tested. As expected, the ATP levels were decreased, but there was no longer a buildup in sugar phosphates.

These results demonstrate the feasibility of using ^{31}P NMR to monitor phosphate composition changes induced by enzyme inhibitors. It follows that details of the effects of other agents, including anticancer drugs, on various cell lines can also be examined by this technique.

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