# Phosphorus-31 Nuclear Magnetic Resonance Detection of Unexpected Phosphodiesters in Muscle†

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ABSTRACT: In the examination of intact muscles by $^{31}P$ nuclear magnetic resonance spectroscopy, a number of signals have been detected in the phosphodiester region (−0.5 to 0.5 ppm) of the spectrum which could not be correlated with the known common phosphates of muscle tissue. These signals arise from perchloric acid extractable compounds with several common chemical properties, one of which is a ready solubility in nearly anhydrous ethanol solutions. A component contributing to the major resonance has been identified as glycerol-3-phosphorylcholine. This characterization is based on both $^{31}P$ nuclear magnetic resonance and chromatographic data.

Technological advances have made it possible to apply the spectroscopic technique of $^{31}P$ nuclear magnetic resonance (NMR)[1] to the study of discrete molecular species, e.g., adenosine triphosphate, phosphocreatine, and inorganic orthophosphate, within living cells. Cellular systems to which this methodology has been successfully applied range from yeasts (Costello et al., 1975; Salhany et al., 1975) through intact mammalian muscles (Hoult et al., 1974; Bárány et al., 1975). In the analysis as usually performed, a quantitative profile of the tissue's phosphates is obtained and, by and large, the phosphates which give rise to measurable $^{31}P$ signals are also those which can be extracted from the tissue with perchloric acid (Burt et al., 1976). Signals from macromolecules and macromolecular complexes, such as the phospholipids of cell membranes, do not ordinarily give rise to $^{31}P$ signals.

Not surprisingly, examination of such phosphate profiles has led to the discovery of substantial amounts of phosphorus-containing molecules, giving rise to $^{31}P$ signals which could not be readily correlated with the usual phosphates anticipated for a given cellular system. This was the case for both Northern frog leg muscle (Bárány et al., 1975) and dystrophic chicken (Glonek et al., 1975). These unknown resonances at about zero parts per million (phosphodiester region) have been found to range in value to as much as 13 mM phosphorus in the toad gastrocnemius muscle (Burt et al., 1976). Chemical and spectroscopic data indicate that a family of related substances are responsible for these signals.

In this paper we present evidence that a major component which comes into resonance at 0.13 ppm is glycerol-3-phosphorylcholine. A preliminary report using rabbit soleus and beef heart muscles to identify GPC has appeared (Glonek et al., 1976). Independently, Seeley et al. (1976) observed in rabbit red semitendinosus high concentrations of a phosphodiester which was suggested to be a derivative of GPC.

## Experimental Section

*$^{31}P$ NMR Analysis.* For the $^{31}P$ NMR analysis, a Bruker HFX-5 spectrometer was used operating at 36.43 MHz for $^{31}P$ and equipped for all modes of heteronuclear $^{1}H$ decoupling and Fourier-transform spectroscopy (Henderson, et al., 1972). Technical details concerning the spectrometer, experimental conditions used to gather the spectra, and laboratory procedures used to mount living muscles in NMR tubes for analysis have been described in full elsewhere (Burt et al., 1976). Chemical shift data are reported relative to 85% orthophosphoric acid with positive chemical shifts associated with increasing magnetic field as has been the customary in $^{31}P$ NMR (Glonek and Van Wazer, 1974).

*Isolation of Ethanol-Soluble Phosphodiesters.* The material corresponding to a family of unidentified phosphodiester resonances in the $^{31}P$ NMR spectrum of several muscle types (frog gastrocnemius, rabbit soleus, beef heart, dystrophic chicken pectoralis, and toad gastrocnemius) was isolated from other muscle phosphates through use of the following procedure. Freshly excised muscle was minced at 4 °C, treated with cold 60% perchloric acid (0.1 ml/g muscle), centrifuged at 0 °C to give an extract which was neutralized with cold KOH, and decanted from the precipitated $KClO_4$. The extract was then lyophilized and exhaustively extracted with 1000 volumes of absolute ethanol. Alternatively, the neutral extract was concentrated by rotary evaporation at 24 °C and successively treated with increased quantities of absolute ethanol (removing the precipitate and then concentrating the supernatant solution after each addition) until the amount of water calculated to be remaining in the sample was less than 5%. With either procedure, the alcohol preparations were then concentrated to a syrup and taken up in a few milliliters of water (20% $D_2O$) for $^{31}P$ analysis.

*Isolation of Glycerol-3-phosphorylcholine.* The above described alcohol preparations were concentrated to a syrup by rotary evaporation at 20 °C. About 0.5 ml was taken and washed with water through a DEAE-cellulose column (2.5 × 60 cm) in the bicarbonate form. The washings (about 100 ml) were concentrated to 3 ml and fractionated on Bio-Gel P-2 (100–200 mesh; 2.5 × 60 cm). After a 90-ml void volume, three fractions, 45 ml each, were collected. The intermediate fraction, in which the only phosphate present was glycerol-3-phosphorylcholine, was concentrated on the rotary evaporator at 30 °C.

*Thin-Layer Chromatography.* Two thin-layer (Hanes and Isherwood, 1949) and one paper (Dittmar and Wells, 1969) chromatographic systems were employed. The thin-layer systems used silica gel H plates as the supporting medium; the developing solvents were: 1-propanol–concentrated

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[1] Abbreviation used: GPC, glycerol-3-phosphorylcholine; NMR, nuclear magnetic resonance; DEAE, diethylaminoethyl.

TABLE I: The Concentrations of Phosphodiesters in Several Muscle Types Determined by $^{31}$P Nuclear Magnetic Resonance.

| Muscle | Concn of Phosphodiesters (mM)[a] | | |
|---|---|---|---|
| | GPC | Other Major Component | All Other Minor Components |
| Meal worms | 5 | 3 | |
| Tortoise heart | 1 | 3 | 0.3 |
| Rabbit heart | 2 | 3 | 0.2 |
| Beef heart | 3 | 1 | 0.2 |
| Rabbit soleus | 3 | 0.1 | ND[b] |
| Female human pectoralis | 2 | 1 | |
| Rabbit extensor digitorum longus | ND | ND | ND |
| Rabbit psoas | ND | ND | ND |
| Normal chicken pectoralis | ND | ND | ND |
| Dystrophic chicken pectoralis | 1 | 2.5 | 0.2 |
| Summer frog gastrocnemius | ND | ND | ND |
| Winter frog gastrocnemius | 4 | 3 | ND |
| Winter toad gastrocnemius | 4 | 9 | 0.2 |

[a] Concentration in the whole muscle determined from spectra of whole muscles or perchloric acid extracts of these muscles. [b] ND, not detectable.

$NH_4OH$–$H_2O$ (3:3:1 and 6:3:1). The paper chromatographic system used Whatman No. 1 paper developed with phenol–water (4:1 w/w). The thin-layer systems were developed for phosphates, diols, and quaternary amines (Stahl, 1969); the paper system was only developed for phosphates. Authentic L-$\alpha$-glycerol-3-phosphorylcholine was obtained as the cadmium salt from Sigma Chemical Co. and converted to a sodium-containing preparation by ion exchange on acid Dowex 50 with subsequent titration to selected pH values.

## Results

*$^{31}$P Nuclear Magnetic Resonance.* Figure 1 shows $^{31}$P spectra obtained from several intact muscles. The principal signals are identified in the figure (see Burt et al., 1976 for the characterization of the major $^{31}$P signals from intact muscle). In this paper the subject for discussion is the group of signals centered at about 0.0 ppm, denoted by the arrows in the figure. These signals, which do not correspond to any of the common muscle phosphates, have several intriguing properties. They are present in all varieties of red and cardiac muscle thus far examined (see Table I). They are not present in white muscle, except in very small concentrations. They are not detectable in the leg muscles of Southern frogs but appear in these muscles of the Northern frogs. It is important that they are present in rather large amounts in the pectoralis muscle of chicken afflicted with hereditary muscular dystrophy.

The compounds giving rise to these signals can be extracted from muscle with perchloric acid. The $^{31}$P spectra from such extracts show that a group of compounds is usually present. If the perchloric acid extracts are freeze-dried, a 90% ethanol extraction of the powder will dissolve them as a group; Figure 2, top spectrum, shows a typical $^{31}$P spectrum from such an ethanol extract. In this group of signals, the compound coming into resonance at 0.13 ppm, which is often seen to give rise to the major resonance, can be purified with the aid of ion exchange on DEAE-cellulose followed by gel filtration on Bio-Gel P-2 as described in the Experimental Section. The $^{31}$P spectrum of this substance after the P-2 fractionation step is shown in Figure 2 (bottom spectrum). Only a single sharp proton decoupled resonance is observed at 0.13 ppm.

When an equivalent quantity of commercial GPC was added to the sample of Figure 2, bottom spectrum, the resulting $^{31}$P

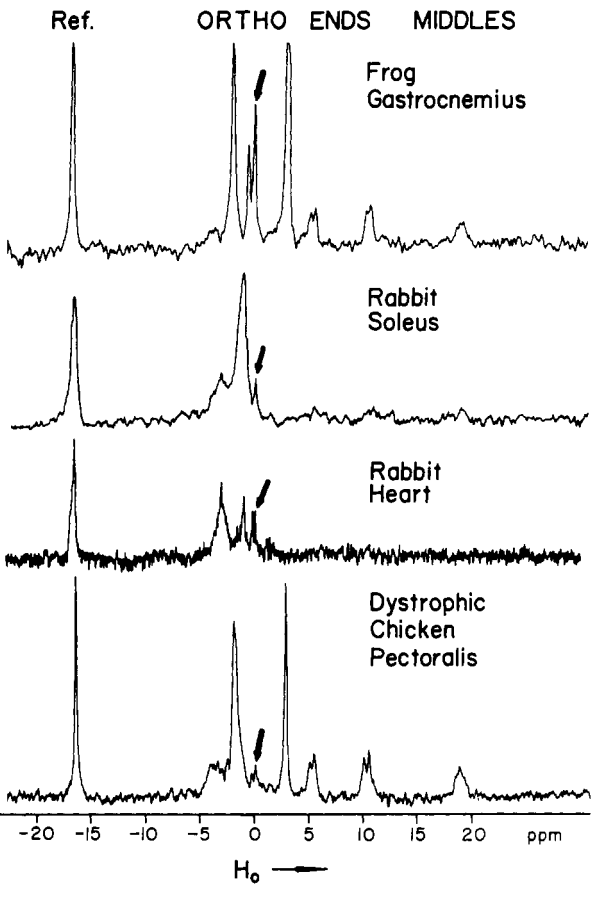


FIGURE 1: $^{31}$P NMR spectra from several intact muscles. The prominent resonance bands of the various spectra proceding from left to right in the figure are: The intensity reference, methylenediphosphonic acid, −16.3 ppm; the sugar phosphates and inosine monophosphate, −3 to −4 ppm; inorganic orthophosphate and other orthophosphate monoesters, −1 to −2 ppm; orthophosphate diesters, −0.5 to 0.5 ppm; creatine phosphate, 3 ppm; and the three phosphates of ATP, $\gamma$ at 5.6 ppm, $\alpha$ at 10.3 ppm, and $\beta$ at 19.5 ppm. The major divisions of the phosphate NMR spectrum are indicated in the figure; the arrows denote the resonances from the unidentified phosphodiesters which are the subject of this study. Chemical shifts are relative to the resonance position of 85% orthophosphoric acid, with increasing chemical shifts corresponding to increasing magnetic field intensities as is customary in $^{31}$P NMR.

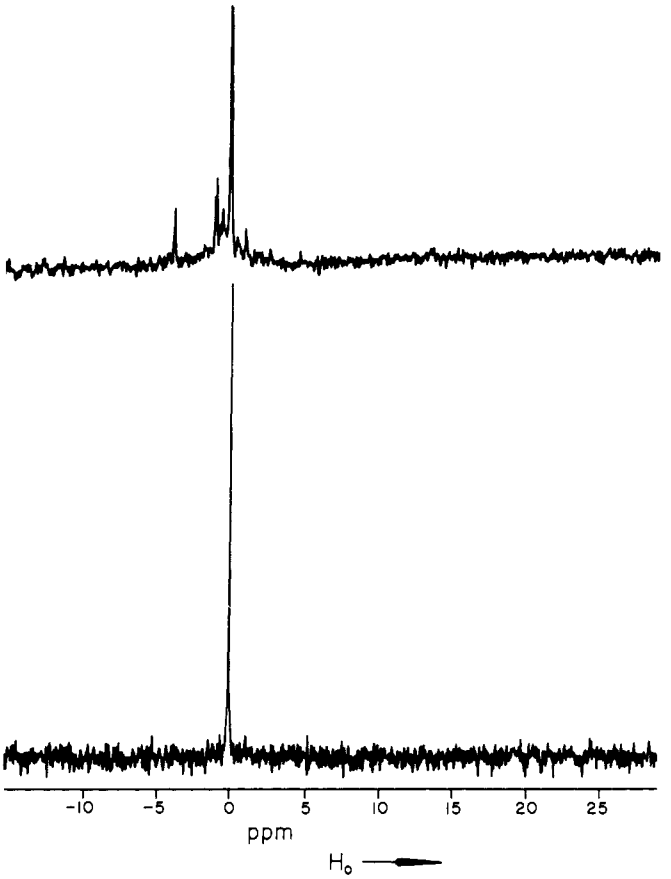


FIGURE 2: $^{31}P$ NMR spectra of typical phosphodiester preparations (example, beef heart). The top spectrum was obtained from the perchloric acid extracted material soluble in 90% ethanol. A number of resonances are observed which arise from several different compounds. The bottom spectrum shows the spectrum of the major component from this extract after the DEAE-cellulose and P-2 column treatment; only a single phosphate component is present.

signal was identical with that of the starting material. There was no indication of multiple peaks. To preclude a fortuitous overlap of signals, the resonance was shifted downfield to −0.18 ppm ($\Delta\delta$ = −0.31 ppm) by an ionic strength effect resulting from the dissolution of NaCl in the sample to a concentration of 5 M. (Similarly treated, diethyl phosphate also shifts downfield $\Delta\delta$ = −0.18 ppm (Costello et al., 1976).) The signal was then shifted upfield to 0.92 ppm by the addition of perchloric acid to 10% concentration. In each case only a single sharp signal was detected. The proton-coupled $^{31}P$ spectrum of the extract phosphate showed a complex higher order multiplet which was also identical with that from commercial GPC.

*Thin-Layer and Paper Chromatography.* With each chromatographic system and the stains employed (phosphate, quaternary nitrogen, and diols) and with each muscle extract, GPC and the extracted phosphate showed identical chromatographic and staining characteristics when either chromatographed separately or in cochromatographic combination. The measured $R_f$ values for the three systems were as follows: 1-propanol–concentrated $NH_4OH$–$H_2O$ (6:3:1, $R_f$ 0.22); 1-propanol–concentrated $NH_4OH$–$H_2O$ (3:3:1, $R_f$ 0.50); phenol–water (4:1, $R_f$ 0.85).

Typical relative amounts of GPC and other ethanol-soluble substances present in several muscle types are given in Table I. Note that when GPC is present its concentration is about 3 mM. The same is usually true for a second, and as yet unidentified, major component (see Figure 1). The summed concentrations of the minor components in Table I rarely exceed a few tenths mmolar.

## Discussion

It was over a quarter century ago that Schmidt et al. first reported GPC to be present in several tissues (Schmidt et al., 1945). However, subsequent work by the same group (Schmidt et al., 1952) has found only negligible amounts of GPC in beef muscle. Our NMR studies reveal high levels of GPC in intact slow twitch and heart muscles in contrast to fast twitch muscles which essentially lack this compound. These NMR findings have been verified by classical chemical identification of GPC.

The origin of GPC in muscle is somewhat puzzling. The main biosynthetic pathway of muscle phosphoglycerides, including phosphatidylcholine, seems to be via cytidine diphospho bases (Schamgar and Collins, 1975). The idea that GPC is the degradation product of lecithin was tested by an extensive computer search for references to phospholipases in muscle. Of the over 200 citations only three seemed to have direct relevance. The combined occurrence of phospholipase $A_1$ and $A_2$ (a prerequisite for GPC formation) was reported only in the uterus (Bakesson and Gustavii, 1975), located in lysosomes. The activity of these enzymes in myometrial tissue was three to five times lower than that of the decidua. Phospholipase $A_1$ activity was found to be very low in heart and skeletal muscle (Gallai-Hatchard and Thompson, 1965). For subsequent conversion of lysolecithin to GPC, lysophospholipases would be needed, but only low levels of this enzyme have been reported in muscle (Brockerhoff and Jensen, 1974). Even in the pioneer paper of Schmidt (Schmidt et al., 1945), only muscle showed no increase in GPC-like material during the course of the incubation of a homogenate at 37 °C. GPC formation through an hitherto unknown general diesterase action seems unlikely in our preliminary experiments with rabbit soleus, which contains exclusively GPC without any glycerolphosphorylserine or glycerolphorylethanolamine. These considerations make it unlikely that the breakdown of phospholipids would be the source of this metabolite.

The specific occurrence of GPC in the slow-type muscles would suggest its being involved in the oxidative, energy-producing, metabolic pathways. However, the exact role of GPC in muscle may remain unexplained, as it is in semen, which contains high levels (Riar et al., 1973).

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# Phosphorus-31 Fourier Transform Nuclear Magnetic Resonance Study of Mononucleotides and Dinucleotides. 1. Chemical Shifts†

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ABSTRACT: A phosphorus-31 nuclear magnetic resonance (NMR) study of adenine, uracil, and thymine mononucleotides, their cyclic analogues, and the corresponding dinucleotides is reported. From the pH dependence of phosphate chemical shifts, p$K_a$ values of 6.25–6.30 are found for all 5′-mononucleotides secondary phosphate ionization, independently from the nature of the base and the presence of a hydroxyl group at the 2′ position. Conversely, substitution of a hydrogen atom for a 2′-OH lowers the p$K_a$ of 3′-monoribonucleotides from 6.25 down to 5.71-5.85. This indication of a strong influence of the 2′-hydroxyl group on the 3′-phosphate is confirmed by the existence of a 0.4 to 0.5 ppm downfield shift induced by the 2′-OH on the phosphate resonance of 3′-monoribonucleotides, and 3′,5′-cyclic nucleotides and dinucleotides with repect to the deoxyribosyl analogues. Phosphate chemical shifts and titration curves are affected by the ionization and the type of the base. Typically, deviations from the theoretical Henderson-Hasselbalch plots are observed upon base titration. In addition, purine displays a more deshielding influence than pyrimidine on the phosphate groups of most of the mononucleotides (0.10 to 0.25 ppm downfield shift) with a reverse situation for dinucleotides. These effects together with the importance of stereochemical arrangement (furanose ring pucker, furanose-phosphate backbone conformation, O-P-O bond angle) on the phosphate chemical shifts are discussed.

Many important biological molecules (nucleic acids, membrane phospholipids, some proteins and coenzymes, etc) are phosphorus-containing compounds. Their structures and interactions have been extensively documented using proton nuclear magnetic resonance over the past 15 years and, more recently, carbon-13 magnetic resonance. However, and quite surprisingly, only a limited attention has been given to the phosphorus magnetic resonance study of these molecules.

Recent instrumental improvements have rendered phosphorus NMR[1] more attractive despite the low sensitivity of this nucleus (about 7% of proton value at constant magnetic field) and the combined use of Fourier transform techniques, together with larger magnetic fields, larger sample tubes, proton noise decoupling and sensitivity enhancement devices (quadrature detection and single side band filters), allows one to carry out *routine* studies in the $10^{-4}$ M range of concentrations.

Phosphorus generally occurs as a phosphate group in biological molecules. The phosphate groups resonances appear in a wide range of chemical shifts (about 30 ppm) and their high sensitivity to chemical environment (pH, metal ions, etc) makes the study of biological phosphates a very informative

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; ATP, AMP, adenosine tri- and monophosphates; UMP, TMP, uridine and thymidine monophosphates.