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^{31}P NMR Studies of Isoprenoid Polyphosphate Acid-Base Equilibria.

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Isoprenoid polyphosphate intermediates of the mevalonate pathway were studied using ^{31}P NMR. Acid–base titrations of farnesyl and geranylgeranyl polyphosphates were conducted over the pH range 4–10, and ^{31}P chemical shifts were used to determine pK_a' values for the second ionization step of the terminal phosphate groups. Apparent pK_a values ranged from 6.16 to 7.07, which may have important implications for biological reactions involving these and similar molecules. In general, acid-catalyzed hydrolysis of the phosphomonoesters had little effect on the determination of pK_a' values during the course of this study. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Several isoprenoid diphosphates are key intermediates in the mevalonate pathway. This important biosynthetic route starts with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), and ultimately yields several products including isopentenyl adenine, dolichol, cholesterol and prenylated proteins.[1] Cholesterol is a major constituent of eucaryotic membranes and is also a precursor to steroid hormones, vitamin D and bile acids. Elevated levels of serum cholesterol are closely associated with increased risk of coronary heart disease. Accordingly, a major goal of drug design is to lower serum cholesterol, and some pharmaceuticals do so by targeting specific branches of the cholesterol biosynthetic pathway.

Farnesyl pyrophosphate (FPP), a primary substrate which occurs at a potential regulatory site in the mevalonate pathway, is a C_{15} isoprenoid diphosphate synthesized in either peroxisomes or the cytoplasm and used as a substrate in three primary reactions: the farnesylation of proteins, the formation of squalene en route to cholesterol and the formation of C_{20} geranylgeranyl pyrophosphate (GGPP), a precursor to C_{20} prenylated proteins.[2,3] Hypocholesterolemic drugs acting at the squalene synthesis step (e.g. squalestatin[4] and zaragozic acid[5]) bring about greatly increased FPP/GGPP ratios;[6] therefore, simultaneous monitoring of FPP, GGPP and other intermediates can provide an indication of drug performance.[7]

Recently, two reports have appeared,[8,9] one from our laboratories, on the capillary zone electrophoretic (CZE) separation of isoprenoid phosphates. In our own work, we observed that within a given isoprene species (e.g. farnesyl), observed electrophoretic mobilities of the mono-, di- and triphosphates were not in agreement with theoretical models.[10] In CZE, ion mobility depends upon net charge and friction coefficient in the separation buffer. To determine net charge on the isoprenoid phosphates, ^{31}P NMR studies of farnesyl and geranylgeranyl phosphate moieties were conducted.

Acid–base titrations of isoprenoid mono- and polyphosphates were performed with simultaneous monitoring of ^{31}P chemical shift values over the pH range 4–10. NMR pH titration data were used to calculate apparent pK_a values for the second ionization step of the terminal phosphate groups, and acid dissociation constants for five isoprenoid species are reported in this paper. Allylic pyrophosphates are known to undergo acid-catalyzed hydrolysis,[11,12] and the hydrolysis reaction was also monitored during the titration of farnesyl pyrophosphate.

EXPERIMENTAL

Isoprenoid mono-, di- and triphosphates were prepared as described previously.[13] Samples were originally present at *ca.* 0.01 M concentrations in 0.1 M ammonium hydrogencarbonate buffer (pH 8.4 ± 0.3) to minimize hydrolysis. An NMR lock signal was provided by adding 10% (v/v) D_2O (99.9 atom% D, Aldrich Chemical). The pH was adjusted for NMR titrations by adding concentrated NaOD or DCl solutions, and samples were diluted by up to 10% (v/v) over the course of the titrations. Sample pH was measured at 300 K using an Ingold 6030-02 combination electrode and a Corning 250 ion analyzer. The pH meter was calibrated by a two-point method using certified reference buffers

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(Fisher Chemical). No attempt was made to correct the measured pH values for the partially deuterated nature of the solvents.

One-dimensional NMR spectra were obtained at 8.5 T with a Bruker AMX-360 wide-bore Fourier transform NMR spectrometer, operating at a ^{31}P frequency of 145.78 MHz, using a 5 mm standard broadband probe. The temperature was controlled at 300 ± 0.5 K for all spectral acquisitions. ^{31}P chemical shift values were referenced to an external 85% H_3PO_4 signal at 0.00 ppm, with upfield shifts represented by negative values. An opposite sign convention was used when FPP NMR data were previously reported by other researchers.[14]

Qualitative ^{31}P spectra were acquired without ^{1}H decoupling over a spectral width of 200 ppm using a 1 s relaxation delay and a $\pi/2$ pulse. Typically 64K data points were sampled with a corresponding 1 s acquisition time. A variable number of transients were co-added depending on analyte concentrations and signal-to-noise (S/N) requirements. Data were not zero-filled, but a 5 Hz exponential line broadening function was applied prior to Fourier transformation. For quantitative data, 32K points were sampled over a spectral width of 100 ppm, and a 35 s relaxation delay was used. Data were zero filled to 64K total points and transformed after applying a 1.2 Hz line broadening function. The resultant S/N ratios gave integrated peak areas with accuracies to $\pm 5\%$.[15]

Apparent acid dissociation constants were calculated using SlideWrite Plus for Windows software. ^{31}P NMR chemical shift values were plotted *vs.* pH, and data were fitted to a non-linear least squares algorithm designed for pH titration curves based upon a monoprotic acid model. In each pK_a' determination 10–13 data points were used.

RESULTS AND DISCUSSION

^{31}P resonance assignments for isoprenoid polyphosphates follow the same trend as those for adenosine polyphosphates.[16] The terminal phosphate signal appears furthest downfield, followed by the phosphate adjacent to the R group, then the central phosphate as one moves upfield. The phosphate labeling scheme is illustrated for farnesyl and geranylgeranyl triphosphates in Fig. 1. Representative spectra are shown in Fig. 2. Coupling constants measured include $^{2}J_{PP} = 21$, 19 and 19 Hz for FPP, FTP and GGPP, respectively. $^{3}J_{C_1H_\alpha P} = 6$ Hz was resolvable for FPP.

Observed ^{31}P NMR chemical shift values as a function of pH were plotted as shown in Fig. 3 for FTP, and data were fitted using a non-linear least-squares routine. Observed and calculated chemical shifts for various species under acid (pH 4–5) and base (pH 10–11) conditions are listed along with the pK_a' values in Table 1.

For NMR-active nuclei, shielding constants depend upon hybridization and electronegativities of neighboring groups; therefore, in polyphosphates the ^{31}P resonance positions are strongly affected by ionization. Proton exchange rates for most ionizable groups in aqueous media are fast on the NMR time-scale, such that changes in pH result in concomitant changes in

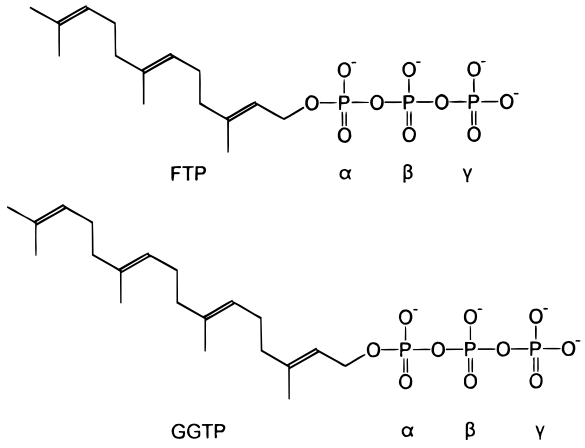

Figure 1. Structures and phosphate group labeling scheme for farnesyl and geranylgeranyl triphosphates.

Table 1. ^{31}P NMR and pK_a' data for inorganic phosphate, and terminal phosphate groups of farnesyl and geranylgeranyl polyphosphates, with all samples *ca.* 0.01 M in 0.1 M ammonium bicarbonate

	δP (ppm) Observed		δP (ppm) Calculated		Calculated	
Sample	Acid	Base	Acid	Base	pK_a'	Fit r^2
i-PO_4^{3-}	0.88	3.42	0.91 ± 0.02	3.39 ± 0.01	6.71 ± 0.01	0.999
FMP	0.81	4.61	0.98 ± 0.04	4.64 ± 0.05	7.07 ± 0.03	0.997
FPP α	−9.78	−9.28	−9.78 ± 0.01	−9.26 ± 0.01	6.50 ± 0.00	0.994
FPP β	−9.78	−5.26	−9.63 ± 0.10	−5.35 ± 0.07	6.46 ± 0.05	0.993
FTP α	−10.01	−9.64	−9.98 ± 0.01	−9.66 ± 0.01	6.61 ± 0.00	0.982
FTP β	−22.12	−20.73	−22.08 ± 0.03	−20.80 ± 0.03	6.58 ± 0.00	0.991
FTP γ	−9.90	−4.97	−9.75 ± 0.10	−5.12 ± 0.08	6.52 ± 0.05	0.993
GGPP α	−9.86	−8.73	−9.77 ± 0.03	−8.79 ± 0.03	6.92 ± 0.01	0.987
GGPP β	−9.21	−4.68	−9.11 ± 0.06	−4.83 ± 0.06	6.91 ± 0.04	0.997
GGTP α	−9.65	−9.18	−9.62 ± 0.01	−9.20 ± 0.01	6.16 ± 0.00	0.991
GGTP β	−20.92	−19.21	−20.88 ± 0.02	−19.24 ± 0.02	6.18 ± 0.00	0.998
GGTP γ	−9.29	−4.23	−9.20 ± 0.04	−4.31 ± 0.04	6.21 ± 0.03	0.999

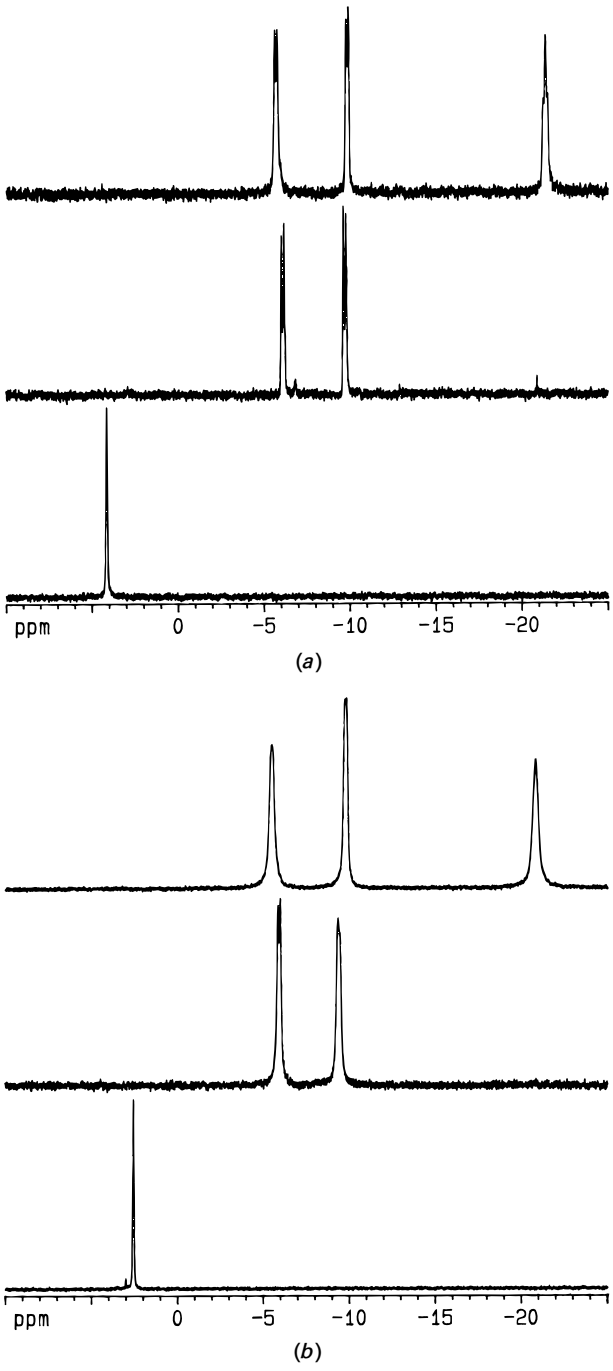

Figure 2. ^{31}P NMR spectra (top to bottom) of (*a*) farnesyl triphosphate (FTP), farnesyl pyrophosphate (FPP) and farnesyl monophosphate (FMP) and (*b*) geranylgeranyl triphosphate (GGTP), geranylgeranyl pyrophosphate (GGPP) and geranylgeranyl monophosphate (GGMP). Spectra were acquired as described in the Experimental section, except that a 1.2 Hz line broadening function was applied and data were zero filled to 128K total data points.

chemical shift, and the observed signal is a weighted average of the chemical shifts of the different forms:[17]

$$\delta_{obs} = f_{HA}\,\delta_{acid} + f_{A^-}\,\delta_{base}$$

where f_{HA} and f_{A^-} represent the mole fractions and δ_{acid} and δ_{base} represent the chemical shifts of the protonated and deprotonated species, respectively. At unit activity, the acid dissociation constant, K_a, may be determined

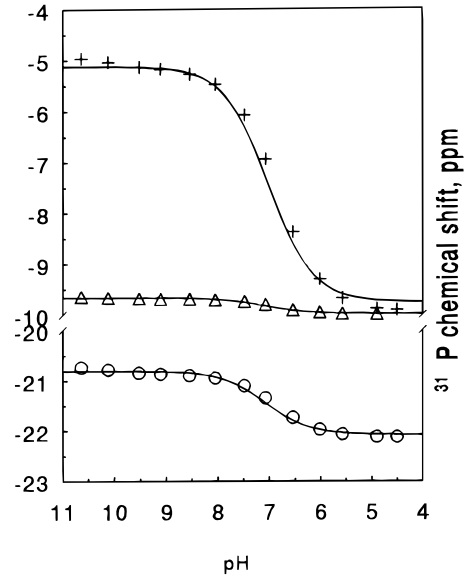

Figure 3. ^{31}P chemical shift *vs*. pH for farnesyl triphosphate. Phosphate groups are represented by (△) = α, (○) = β and (+) = γ phosphate.

using the relationship[17]

$$\delta_{obs} = ([H^+]\delta_{acid} + K_a\,\delta_{base})/([H^+] + K_a)$$

Provided that the dissociation constants are significantly different, the pH behavior of multiprotic species can be analyzed in a stepwise fashion (monoprotic acid model) by using the Henderson–Hasselbach equation

$$pH = pK_a' + \log[A^-]/[HA]$$

where pK_a' is the apparent acid dissociation constant in terms of the molar concentrations of base $[A^-]$ and conjugate acid [HA]. The 'primary' ionization of phosphate groups occurs at low pH with typical pK_a values in the range of 0.8–2.1, similar to that for phosphoric acid, and the 'secondary' ionization of the terminal phosphate in phosphoesters occurs near neutral pH.[18] Thus, stepwise dissociation is a valid assumption for isoprenoid polyphosphates over the pH range analyzed.

Over the measured pH range, chemical shift differences of 3.8–5.1 ppm (terminal), 1.4–1.7 ppm (central) and 0.4–1.1 ppm (α) were observed for each type of ^{31}P signal. The results are interpreted to suggest that only the terminal phosphate undergoes deprotonation in each case, as evidenced by only minor changes in non-terminal ^{31}P signals and the diminishing pH dependence of chemical shifts with increasing distance from the titration site.[19] pK_a' values in the range of 6.16–7.07 were calculated, and for each different compound the values were essentially the same, within experimental error, regardless of the phosphate signal monitored.

Apparent pK_a values are concentration dependent and vary in different solvents and at different ionic strengths, as shown in the ^{31}P NMR study by Robitaille *et al.*[20] Salt effects on equilibrium constants are well known, and thermodynamic equilibrium constants at unit activity under standard conditions and at infinite dilution are smaller than experimentally determined values, i.e. $K_a(eq) < K_a'$, so $pK_a(eq) > pK_a'$. For example, pK_a values of 7.05 and 6.17 for inorganic

phosphate in doubly distilled and artificial sea water, respectively, have been reported.[20]

In this study, high ionic strength conditions (samples in 0.1 M ammonium hydrogencarbonate buffer), the addition of concentrated NaOD or DCl for pH titrations and the partially deuterated nature of the solvent are expected to play a large role.

In order to estimate the effects of the high ionic strength buffer used, a sample of 0.01 M phosphoric acid in 0.1 M ammonium hydrogencarbonate was titrated and a pK_a' of 6.71 was determined. This value is 0.34 units below that for inorganic phosphate in doubly distilled water.[20] Therefore, we believe our experimentally determined pK_a' values may be as much as 0.3–0.5 units lower than the equilibrium pK_a values for the polyphosphates. Owing to the limited amounts of sample available, no attempt was made here to measure K_a' at various ionic strengths and extrapolate the experimental values to obtain the limiting value of K_a(eq) at infinite dilution.

Pyrophosphate hydrolysis

The pyrophosphate groups of various isoprenoids undergo acid catalyzed hydrolysis, primarily below pH 5.[11] In allylic systems, hydrolysis proceeds by cleavage of the C—O bond yielding pyrophosphate, and in non-allylic systems bond cleavage occurs at the P—O position to give inorganic orthophosphate and the corresponding isoprenoid monophosphate.[12] During the various titrations performed in this study small signals corresponding to inorganic phosphate and/or pyrophosphate species were observed; however, because these signals could be resolved from the resonances of interest, hydrolysis did not interfere with the determination of pK_a' values. Over the course of a 9 h NMR titration carried out quantitatively from high (*ca.* 10) to low (*ca.* 4) pH, hydrolysis products represented less than 10% of the initial pyrophosphate present, and did not measurably affect the pH values. Thus, the hydrolysis reaction was considered inconsequential.

CONCLUSION

^{31}P NMR pH titrations were used to determine apparent pK_a values for the 'secondary' ionization of the terminal phosphate groups of five isoprenoid mono- and polyphosphates. ^{31}P chemical shift values were plotted *vs.* pH and pK_a' values ranging from 6.16 to 7.07 were calculated using a non-linear least squares algorithm. These pK_a' values are near physiological pH, and this may have important implications in biological pathways involving these molecules. Owing to the high ionic strengths of the sample solutions, reported pK_a' values may be as much as 0.5 units lower than equilibrium pK_a values. The acid-catalyzed hydrolysis of the pyrophosphate group of FPP was also monitored quantitatively during an NMR pH titration; the data indicate that less than 10% of the initial pyrophosphate present was hydrolyzed during the titration, but this did not interfere with the determination of pK_a' values.

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