Table 1. Effect of fluorophenylalanine on flagellar morphology. The data for 37°C were obtained by scoring bacteria for the presence or absence of heteromorphic flagella. Bacteria grown in phenylalanine (phe) had only normal flagella. Of the bacteria grown in phenylalanine and then in fluorophenylalanine (flphe), 107 contained one or more distally curly flagella, whereas 319 contained no heteromorphic flagella but instead normal flagella, curly flagella, or both on the same bacterium.

Temperature (°C)	Incubation		Curly (No.)	Normal (No.)	Heteromorphic	
	First	Second			Curly distal	Curly proximal
37	phe	phe		500	100	0
37	phe	flphe			107	0
32	phe	phe	25	299	2	2
32	phe	flphe	292	25	37	17
32	flphe	phe	25	296	27	22
32	flphe	flphe	298	39	25	29

ultracentrifuge and then applied to carbon-coated Formvar grids. Samples were overlaid with a thin film of carbon prior to application of Ilford L-4 gel by the method of Caro (*12*). After exposure for 6 weeks, the gels were developed with Phenidon developer (*13*), stained for 1 hour with uranyl formate, and rinsed briefly with water. Samples were examined by electron microscopy and scored for the number of grains per wave. The presence of the hook structure defined the end of the flagellum that was attached to the bacterial membrane. Only those flagella with a hook at the basal end were counted (Fig. 1).

A total of 145 flagella were classified into the three categories: (i) the distally labeled class containing those labeled flagella which have no grains on the first wave near the hook and represent the labeling pattern expected if flagella grow by addition of subunits to the distal end, (ii) the proximally labeled class with grains on the first wave but not on all the other waves, and (iii) the totally labeled class containing those flagella which were completely synthesized during the second incubation. As can be seen in Fig. 2A, 43 (29 percent) of the flagella were totally labeled, 87 (60 percent) were distally labeled, and only 15 (10 percent) were proximally labeled. The proximally labeled flagella show the same average density of grains on the distal region suggesting that they are also labeled throughout. In addition, when the number of grains per wave in each class is averaged, the grain distribution is constant along the flagellum except in the distally labeled class where the number of grains per wave increases linearly with distance from the base until a plateau is reached (Fig. 2B). Since this plateau is the same as that for the totally labeled flagella, the most distal waves must have been synthesized completely during the second incubation and are maximally labeled.

Three flagella were not included in the above data. Of these, two contained two grains on a middle wave, and one exceptional flagellum contained eight grains on the first wave, none on the second, and one on the last. No other flagella with this distinct labeling pattern were detected in extensive scanning of the grids. This labeling pattern was interpreted as the result of a local inhomogeneity in the emulsion thickness. The data clearly demonstrate that flagellar elongation in vivo occurs by polymerization of subunits onto the distal tip of the filament.

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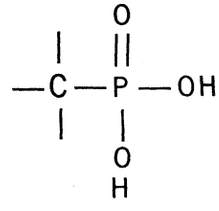
Biological Phosphonates: Determination by Phosphorus-31 Nuclear Magnetic Resonance

Abstract. *Advanced methods of phosphorus-31 nuclear magnetic resonance spectroscopy provided a method whereby biological phosphonates and phosphates can be determined on simple lipid fractions of biological origin. The spectra consist of two easily distinguished resonance bands; one corresponds to families of phosphonates, and the other corresponds to families of orthophosphates.*

Despite its obvious biological importance (*1*), no direct method for the determination of phosphonate-phosphorus of the alkylphosphonic acid grouping



has been available to the present time. Phosphorus of this type has been determined indirectly as the difference between total phosphorus and that released as orthophosphate upon prolonged acid hydrolysis.

A technique which has the potential for the direct determination of biological phosphonate-phosphorus is that of phosphorus-31 nuclear magnetic resonance spectoscopy (^{31}P NMR). The chemical shift of phosphonate-phosphorus is considerably different from that of other known forms of biological phosphorus. The phosphonate-phosphorus of saturated monoalkylphosphonic acids of the general type known to occur in biological systems comes into resonance within the range of − 40 to − 15 ppm relative to the usual external standard of 85 percent orthophosphoric acid (*2*). The range of chemical shifts for other types of biological phosphorus lies from about − 12 ppm for phosphoramidates to about +20 ppm for middle phosphate groups of polyphosphate acompounds. Accordingly, it should be possible through ^{31}P NMR

to detect phosphonate-phosphorus in intact unhydrolyzed mixtures and to measure its amount relative to that of other forms present. The theoretical possibility of detecting biological phosphonates through ^{31}P NMR was mentioned (*3*), but its application was discounted due to limitations of the instrumentation available at that time. Technological advances have circumvented these limitations, and we now report the successful application of the method.

We used a Bruker HFX-5 NMR spectrometer with heteronuclear (^1H) field-frequency stabilization and operating at 90 Mhz for ^1H and 36.4 Mhz for ^{31}P. The single, oven-controlled crystal of a Bruker ND30M-B frequency-synthesizer served as a reference for all frequencies, and a Fabri-Tek 1064 was used for signal averaging. When the instrumentation was tested with a sample of trimethylphosphite, the resolution, measured with the 13-mm spinning sample tube used in this study, was 0.3 hz and the drift, measured over 72 hours, was 0.1 hz. The sensitivity was such that measurements could be taken readily at a total phosphorus concentration of 10^{-3} to $10^{-4}M$. This applied to biological as well as to purely chemical preparations.

Natural sources which were known to contain phosphonic acid derivatives were investigated first. These included two sea anemones, *Bunadosoma* sp. and *Metridium* sp., and the ciliate protozoan *Tetrahymena pyriformis*. Although the technique was used successfully to detect and estimate phosphonates in various fractions of these organisms, this report is limited to a discussion of their lipid fractions (*4*).

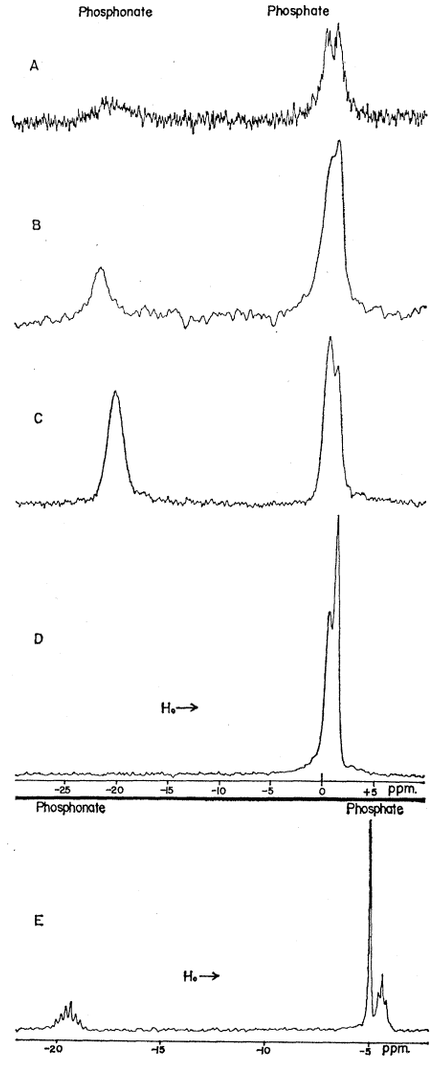
Bunadosoma sp. (Coral Sands Marine Supplies, Chicago) were homogenized in 0.1*M* KCl and lipids were extracted (*5*). The lipid phase was evaporated to dryness and dissolved in chloroform. This solution was used for ^{31}P NMR analysis (Fig. 1A).

Metridium sp. (Woods Hole Oceanographic Supply) were homogenized in distilled water. The homogenate was lyophilized, and the lipids were extracted from the powder with a 2 : 1 (v/v) mixture of chloroform and methanol (*6*). This solution was evaporated to dryness, and the residue was dissolved in benzene. The benzene solution was used for ^{31}P NMR analysis (Fig. 1B).

Tetrahymena pyriformis strain WH14 (obtained from J. D. Smith, Univ. of Chicago) were grown in the medium described by Smith (*7*). The cells were collected by centrifugation, washed, and lyophilized. The lipids were extracted and prepared for ^{31}P NMR analysis in the same manner as were those of *Metridium* sp. (Fig. 1C).

We also examined the lipid found in the flesh of a Lake Michigan carp, *Cyprinus carpio* L. Fillets were homogenized in a 2 : 1 (v/v) mixture of chloroform and methanol, the solvent was removed, and the lipid dissolved in chloroform; this solution was used for ^{31}P NMR analysis (Fig. 1D).

A sample of dried lipid from *Bunadosoma* sp. was suspended in a 1 : 1 (v/v) solution of concentrated HCl and methanol. The solution was autoclaved in a capped tube for 16 hours at 121°C. The tube was then placed in an oven at 105°C for 48 hours. The hydrolysate was extracted with hexane. The aqueous phase was evaporated to dryness, redissolved in aqueous tetramethylammonium hydroxide (*p*H 10), and this solution was used for ^{31}P NMR analysis (Fig. 1E).



Spectra of lipid fractions of *Bunadosoma, Metridium*, and *T. pyriformis* show two major absorption bands (Fig. 1, A–C). One occurs at −3 to +3 ppm which corresponds to the absorption region of orthophosphoric acid and its esters (orthophosphate region). The second occurs at −18 to −24 ppm which is within the chemical shift range expected for alkylphosphonic acids and their esters (phosphonate region). The spectrum of the carp lipids with no phosphonates is given for comparison (Fig. 1D). Within each spectrum the relative amounts of phosphonate and phosphate, determined from the respective areas of their absorption bands, were 1 : 7, 1 : 4, and 1 : 1.5 for spectra of *Bunadosoma, Metridium*, and *T. pyriformis*, respectively. These ratios were reproducible to a precision of 10 percent.

Additional information can be obtained from an examination of the widths of the individual bands as well

Fig. 1. The ^{31}P NMR spectra of lipid extracts taken from: (A) *Bunadosoma* sp.; (B) *Metridium* sp.; (C) *Tetrahymena pyriformis*; (D) *Cyprinus carpio* L.; and (E) hydrolyzed lipid of *Bunadosoma* sp. All spectra were obtained by signal-averaging. Spectra B–E required from 5 to 17 hours of accumulation time each. Spectrum A required 72 hours of accumulation time which approached the practical limit for this instrumentation. Spectra A–D: sweep rate, 30 hz/sec; time constant, about 1 sec; total sweep width, 2400 hz; phosphorus concentration, 0.2 to 2.0 mmole/3 ml total volume. Spectrum E: sweep rate, 7.5 hz/sec; time constant, about 1 sec; total sweep width, 1200 hz; phosphorus concentration, 1.0 mmole/3 ml total volume. The ppm scale given was determined relative to an external standard of 85 percent orthophosphoric acid according to the following procedure. During signal-averaging the spectrometer was stabilized with reference to the strongest proton signal of the solvent (internal reference). After signal-averaging, a sealed capillary of 85 percent phosphoric acid was inserted into the sample. The spectrometer was then restabilized on the same proton reference signal, and the position of the phosphoric acid was determined. The differences in the chemical shifts of the orthophosphates in spectrum E relative to those of spectra A–D reflect the change in solvent and not the use of a different internal reference signal (*2*).

as the presence or absence of fine structure in these bands. The widths of the bands in Fig. 1, A–D, and the absence of extensive fine structure are consistent with the interpretation that each band arises from a large number of phosphorus atoms in slightly different chemical environments. Thus, in the spectra in Fig. 1, A–C, the widths of the phosphonate absorption bands are about 200 hz, whereas the width of the entire spectrum of 2-aminoethylphosphonic acid would be only about 60 hz (*4*, *8*). In the orthophosphate region two broad absorption bands are resolved for each sample, which reflects the presence of at least two kinds of orthophosphate. The total bandwidth in this region is approximately 180 hz, whereas that of a simple monoester of orthophosphoric acid would be about 15 hz. In contrast, both the phosphonate and phosphate regions of the spectrum of hydrolyzed lipid (Fig. 1E) have been altered as a result of hydrolysis. The spectrum in the phosphonate region appears to be essentially that of 2-aminoethylphosphonate; however, upon expansion of this region the presence of at least two other compounds was detected (*4*). The complexity apparent in Fig. 1A has been reduced, which enables the resolution of the comparatively few remaining resonance signals. Similarly, the orthophosphate region has been simplified to that of the signal of inorganic orthophosphate (−5 ppm) and a second upfield signal which may arise from an alkyl monoester of orthophosphate. Small amounts of other compounds were observed upon expansion of this region. The increased ratio of signal to noise in the spectrum in Fig. 1E as compared to that in Fig. 1A indicates that the large number of compounds in the unhydrolyzed lipid has been reduced, thereby significantly decreasing the number of unique chemical environments for the phosphorus atoms and concentrating the absorptive power of the sample into a few resonance bands.

In summary, ^{31}P NMR can be used for the detection of biological phosphate and phosphonate and for the determination of the relative amounts of each type. From the details of the respective resonance bands, the complexity of the phosphorus-containing mixtures can be established, and, with favorable conditions, specific structures can be identified. Further, this work has shown that problems exist in the classical colorimetric procedure for the determination of phosphonate-phosphorus (see Fig. 1E) (*4*). The technique is nondestructive, and therefore may be of value in the study of the mode of binding of phosphonic acid derivatives in intact proteins and lipids.

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Homozygous Hb J Tongariki: Evidence for Only One Alpha Chain Structural Locus in Melanesians

Abstract. *A high frequency of Hb J Tongariki (α 115 Ala→ Asp) was found in a Kilenge village in New Britain. Heterozygotes had 45 to 50 percent of the Hb J component (determined by cellulose acetate electrophoresis). Two homozygotes for Hb J had no Hb A, suggesting that in this family only one Hb_α structural locus is present.*

Many animal species possess more than one structural locus for the α chain of hemoglobin (*1*). Schroeder *et al.* (*2*) have demonstrated fractional ratios of glycine and alanine at residue 136 of the human fetal γ chain, which they interpret as evidence for the presence of two or more loci encoding for the γ chain.

While critical genetic data have been lacking for man, Lehmann and Carrell (*3*) have suggested that there may be two $Hb\alpha$ loci in this species on the basis of the observation that many heterozygotes for α chain variants have only 20 percent of the abnormal component, half the amount found in most heterozygotes for an abnormal Hb_β gene. Thus, only one of four Hb_α genes is presumed to have undergone mutation. We now, however, present genetic and biochemical evidence that in a Melanesian population there is a single Hb_α locus.

An Hb J migrating more rapidly than Hb A at *p*H 8.6 on starch-gel electrophoresis (*4*) has been discovered in the Kilenge, a group of natives who reside west of Cape Gloucester and north of Sacsac in New Britain (*5*). Samples from 67 natives in three villages were available for study as part of a malarial survey. Twenty-two individuals were heterozygous for Hb J (Fig. 1). Fourteen persons were direct descendants of I-2, of whom nine were heterozygotes along with I-2. In addition, two individuals, a father (II-3), now deceased, and his son (III-2), had only Hb J as the major component, and completely lacked Hb A. Although one parent of each apparent homozygote was not available for study, of the six spouses marrying into this family who were studied, four were heterozygotes for the abnormal hemoglobin. Thus, it is likely that the missing parents were also heterozygotes.

On a second visit to the area, we obtained hematologic data from the relatives (Table 1). Total hemoglobin concentration and hematocrits were measured in New Guinea within 5 hours of collection on seven samples. The hemoglobin concentration of all samples was measured upon receipt several days later in Ann Arbor. He-