be stained by serums from patients with infectious mononucleosis, Burkitt's lymphoma, or nasopharyngeal carcinoma but they contained fewer, or none at all, that reacted with the positive donor reagents. Thus EBV may be associated with lymphoblastoid cells of carrier cultures in several ways: (i) in a few cells complete replicative cycles occur resulting in death of the cell; (ii) in additional cells, viral replication is arrested at the early antigen stage; (iii) in still other cells (as observed also in tumor biopsy cells) only EBV-induced cell membrane antigens are synthesized (*10*); and (iv) in the remaining cells the viral genome is present but wholly repressed, as indicated by the fact that clones derived from singly picked cells under conditions preventing external infection all were shown to harbor EBV (*12*).

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Bacterial Flagella: Polarity of Elongation

Abstract. *Newly synthesized regions of* Bacillus subtilis *flagella were labeled with fluorophenylalanine or [^{3}H]leucine. The flagella were then examined for altered gross morphology or by radioautography. Results of both experiments indicate that flagella elongate in vivo by polymerization of flagellin subunits onto the distal end of the filament.*

Bacterial flagella are primarily composed of a single protein, flagellin, which polymerizes in vitro and in vivo to form filaments with a characteristic wave. Asakura *et al.* (*1*) demonstrated that polymerization in vitro is polar and proceeds only at the distal end of seed fragments. Although subunits can be assembled onto flagella fragments in vitro, the fact that it has not been possible to polymerize subunits onto flagellar stubs in vivo (*2*) suggests that the mechanism of elongation may be different in vivo and in vitro. However, using the amino acid analog fluorophenylalanine, Iino performed experiments which implied that *Salmonella* flagellar growth in vivo also occurred at the distal end (*3*). Incorporation of the analog into flagellin resulted in a curly flagellum displaying half the normal wavelength (*4, 5*). If flagella were partially synthesized in medium containing phenylalanine, then completed in fluorophenylalanine, heteromorphic flagella containing both normal and curly waves on the same filament were produced. If subunits were assembled proximally, the basal region should have been curly, whereas if assembly were distal, the curly region should have been located at the tip. Iino's experiments with *S. typhimurium* showed that the heteromorphic flagella were curly at the tip. We have independently performed similar experiments with *Bacillus subtilis.*

Flagella were removed from cells requiring phenylalanine (*6*) by shearing in a blender. Short flagella were allowed to regenerate at 37°C in minimum salts medium containing 0.1 mg of phenylalanine per milliliter (*7*). After 15 minutes, the bacteria were washed and reincubated at 37°C in the same medium containing 0.1 mg of fluorophenylalanine per milliliter. Two hours later growth was terminated by addition of formaldehyde to 0.5 percent and the bacteria were stained with Leifson's flagella stain (*8*). These incubation periods were necessary for complete regeneration, since the rate of flagellar elongation appears to decrease with increasing flagellar length (*3*). Examination of the stained flagella by light microscopy demonstrated that the heteromorphic flagella were curly on the distal end, suggesting that flagellin is polymerized at the tip (Table 1).

A major criticism of this interpretation is that it assumes that fluorophenylalanine alters the morphology of the flagellum at the site of incorporation of the subunit. It is possible that subunits are added to the base but that the filament assumes the curly form at the free tip which might not be as conformationally restricted as the basal region. It is, therefore, important to do the reverse experiment. Flagella should first be regenerated in fluorophenylalanine, then in phenylalanine, and should contain the curly region at the base. This experiment was attempted repeatedly but the results were uninterpretable since the curly portion appeared at either end and heteromorphs were too rare to permit statistical evaluation. Furthermore, we found that the incorporation of fluorophenylalanine alone does not necessarily cause the formation of curly flagella. When flagella were completely regenerated at 32°C in either fluorophenylalanine or phenylalanine they had the normal wavelength. If the cells were fixed with formaldehyde and incubated at 4°C overnight, curly flagella appeared. Both samples contained some flagella that were normal, curly, or heteromorphous. The curly region can appear at either end of the flagellum (Table 1). The partial regeneration experiments were repeated at 32°C, and the flagella were scored after fixation and incubation at 4°C. Incubation with phenylalanine and then with fluorophenylalanine resulted in a nonrandom distribution of the curly region among the heteromorphs, confirming the initial results, but the reverse experiment led to a random distribution (Table 1). These data illustrate the difficulties in interpreting the results of analog incorporation. It is therefore necessary to test for the direction of flagellar growth by an independent method. Incorporation of isotopically labeled normal precursor into flagella can be measured directly by radioautography and should produce unambiguous results. If the cells are allowed

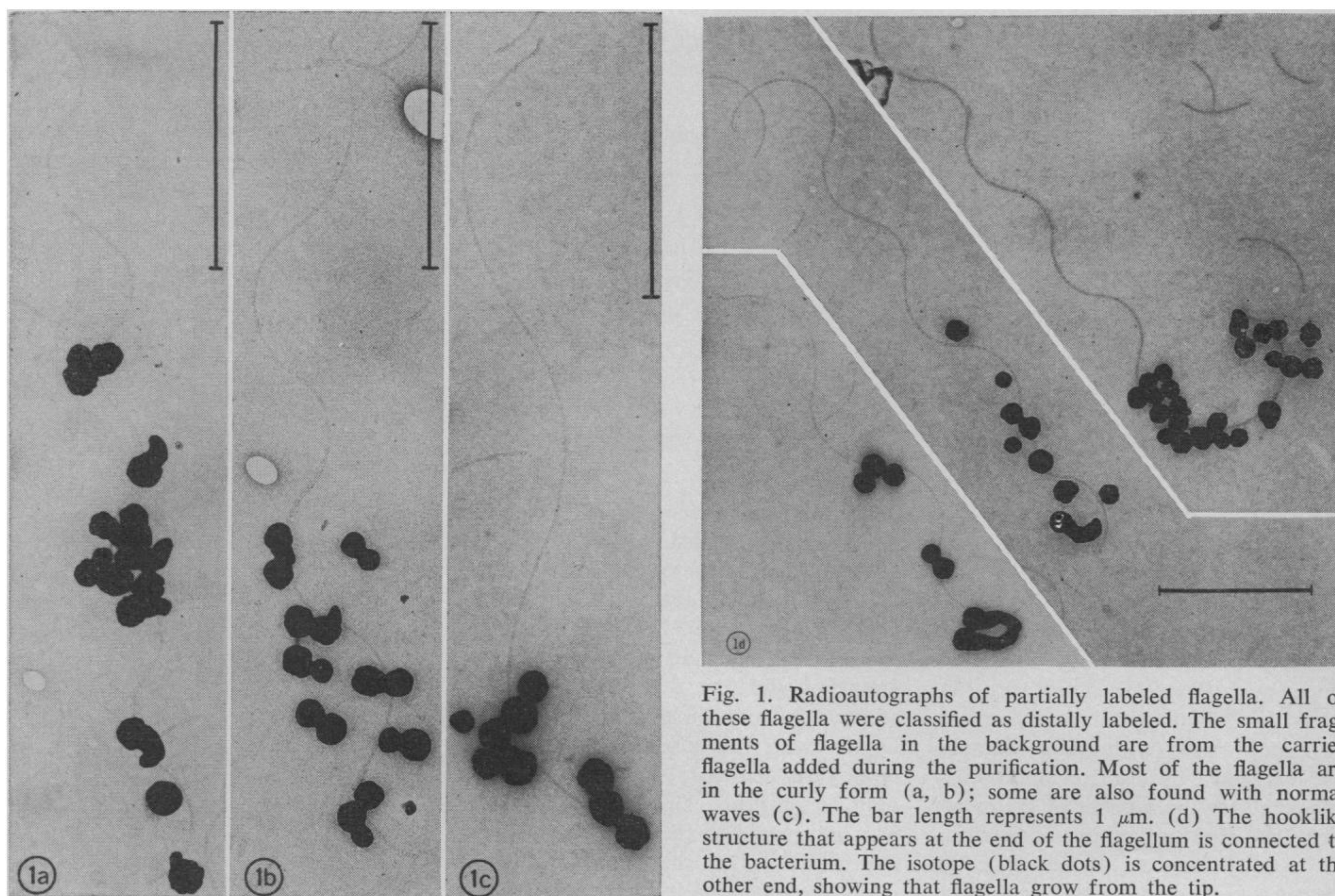

Fig. 1. Radioautographs of partially labeled flagella. All of these flagella were classified as distally labeled. The small fragments of flagella in the background are from the carrier flagella added during the purification. Most of the flagella are in the curly form (a, b); some are also found with normal waves (c). The bar length represents 1 μm. (d) The hooklike structure that appears at the end of the flagellum is connected to the bacterium. The isotope (black dots) is concentrated at the other end, showing that flagella grow from the tip.

to partially regenerate flagella and are then incubated with a tritiated amino acid, after radioautography only the newly synthesized region will show exposed silver grains.

A leucine requiring mutant, BR44 (*9*), was sheared and allowed to regenerate flagella in minimal medium containing 20 μg of leucine and 0.1 mg of all the other amino acids per milliliter. After 40 minutes at 37°C, the bacteria were washed and reincubated in 4 ml of the medium containing 1.65 μg of leucine per milliliter with 0.5 mc [^{3}H]-leucine per milliliter (*10*). One hour and 50 minutes later, the bacteria were washed with minimal salts medium containing 200 μg of leucine per milliliter. The flagella were freed by lysis (*11*) in the presence of 10 mg of bovine serum albumin, 0.5 mg of leucine, 5×10^{-6} mole of ethylenediaminetetraacetate, and 50 μg of chloramphenicol per milliliter. After treatment with deoxyribonuclease 100 μg of sheared thermolabile flagella (*11*) was added per milliliter as carrier, and the flagella were purified by precipitation with 15 percent ammonium sulfate. The precipitate was heated at 58°C for 15 minutes to disaggregate the carrier flagella and leave the more stable BR44 intact. The flagella were collected in the

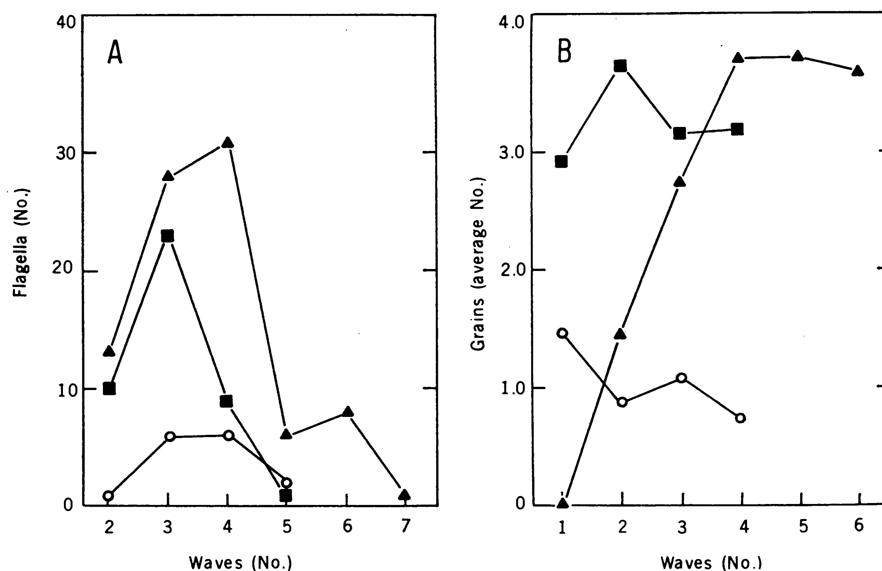

Fig. 2. (A) Distribution of flagella in each class. These data were combined with the grain counts used to calculate Fig. 2B and is included to show the variations in wave number and total flagella in each class. ▲——▲, Distally labeled flagella; ■——■, totally labeled flagella; ○——○, proximally labeled flagella. (B) Distribution of grains along the flagellum. Waves were numbered consecutively beginning with 1 at the hook end. The flagella were divided into three classes: (i) distally labeled flagella which contain no grains on the first wave, (ii) proximally labeled flagella which have grains on the first wave but not on all waves, and (iii) totally labeled flagella which have grains on all waves.

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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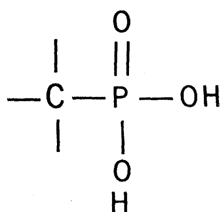
Biological Phosphonates: Determination by Phosphorus-31 Nuclear Magnetic Resonance

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